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Soil-transmitted helminth infections among malaria patients determined by microscopy and real-time PCR methods at two district hospitals in Sarawak, Malaysia

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Abstract. Malaria is still endemic in Sarawak and Sabah. Numerous studies have indicated that patients with malaria are commonly co-infected with helminthes particularly in endemic regions. The aim of this study was to investigate the incidence of soil-transmitted helminth (STH) infection among malaria patients using microscopy and multiplex real-time PCR at two district hospitals in Sarawak. A total of 94 patients who were clinically-suspected to have malaria were confirmed to be infected by both microscopy and multiplex real-time PCR. By the molecular method, 23.4%, 74.5% and 2.1% of the samples were positive for *Plasmodium falciparum*, *P. vivax* and mixed *P. falciparum* and *P. vivax*, respectively. Among the malaria patients, 48.9% were found to be co-infected with STHs. In comparison, microscopic examinations showed that 6.4% of the malaria patients were infected with STHs. From the real-time PCR positive samples, 31.9% had single helminth infections while 17% had mixed infections. In conclusion, this study showed that almost half of the malaria patients at the two Sarawak hospitals were co-infected with helminth. Future studies should be specifically designed to determine if there is any correlation between the two infections in terms of incidence and intensity.

INTRODUCTION

Human malaria is caused by *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium ovale* and *Plasmodium knowlesi*. The endemic regions are mainly located in under-developed countries in sub-saharan Africa, Asia and America Latin (WHO, 2013). In year 2012 these parasites kill approximately 627 000 people worldwide; and in Malaysia there were 4725 confirmed cases (WHO, 2013). The endemic areas in Malaysia are located mainly in Sarawak and Sabah; and Pahang in the Malaysian Peninsular (WHO, 2013). General symptoms of uncomplicated malaria include a combination of fever, chill, headache, sweat, fatigue, nausea and vomiting, anemia and malaise. Meanwhile, severe malaria symptoms include neurological abnormalities, hemoglobinuria, severe anemia, respiratory distress, kidney failure, metabolic acidosis and hypoglycemia (Trampuz *et al.*, 2003).

Helminth infection was found to either ameliorate or exacerbate malaria severity (Nacher *et al.*, 2001, Spiegel *et al.*, 2003, Le Hesran *et al.*, 2004). In light of the potential significance of the malaria and helminth co-infection, we determined the prevalence of the helminth infections among malaria patients at two district hospitals in Sarawak, using both the traditional method (microscopy) and real-time multiplex PCR.

MATERIALS AND METHODS

Sample Collection

Ninety four (n=94) pairs of blood and faecal samples were obtained from patients exhibiting malaria symptoms such as fever, chill, headache, sweat, fatigue and nausea from two district hospitals in Sarawak i.e. Hospital Lundu, and Hospital Serian, Kuching, from November 2007 until April 2010. The hospitals were randomly chosen based on the criteria that they catered for rural residents and did not pose great logistical challenges. Prior approvals were obtained from the research ethics committees of Ministry of Health Malaysia and Universiti Sains Malaysia. Samples were processed for routine diagnosis at the hospitals within four hours after collection and one aliquot from each sample was stored at -20°C for DNA extraction.

Microscopy

Microscopic examinations were performed by laboratory technicians at both hospitals. Blood samples were collected by finger prick and were used to prepare Giemsa-stained thick and thin blood smears for malaria parasite identification and quantification. Meanwhile, direct faecal smear and Kato-Katz concentration technique (http://www.tropeduweb.ch/parasitology_methods_pdf/8_stool_kato-katz.pdf) using 41.7 mg plastic template were performed on the faecal samples to detect for the presence of soil-transmitted helminthes (STHs) ie. *Ascaris lumbricoides*, *Trichuris trichiura*, hookworm and *Strongyloides stercoralis*. The microscopic identifications of the diagnostic stages of these helminthes were based on standard morphological features (http://www.cdc.gov/dpdx/diagnosticProcedures/stool/morphcomp.html).

Real-time PCR

Real-time PCR assay has been widely used to detect parasites in clinical specimens due to its high sensitivity and specificity, as well as low risk of contamination (Verweij *et al.*, 2007; 2009; Othman *et al.*, 2010, Basuni *et al.*, 2011). In this study, for real-time PCR to detect *Plasmodium* spp., 5 ml venous blood in EDTA-coated tube was obtained from each patient. DNA extractions were then performed on venous blood and faecal samples using Qiagen kits (Wiria *et al.*, 2010; Basuni *et al.*, 2011). DNA from blood was used for malaria detection while DNA from faecal samples was used for STH detection. For malaria, two sets of PCR master mixes for multiplex real-time PCR were prepared for detection of four species of malarial parasites (*P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale*) using primers and probes that have been reported in previous studies (Wiria *et al.*, 2010). Although *P. ovale* is not endemic in this part of the world, it was included in this study because we were using parameters of the previously reported assay in order to minimize optimization of the assay. For the first set, master mix was prepared for detection of *P. falciparum* and an internal control (PhHV-1) while the second set was prepared for detection of *P. vivax*, *P. malariae* and *P. ovale*. The multiplex real-time PCR was run using Rotor-gene Q (Qiagen, Germany). The two step PCR was performed at 95°C for 15 min, 50 cycles of 95°C for 5 sec and 60°C for 60 sec.

Multiplex real-time PCR on the faecal DNA samples was performed for simultaneous detection of four helminths i.e. *A. lumbricoides*, *Necator americanus*, *Ancylostoma duodenale*, *S. stercoralis* and PhHV-1 (internal control). It was performed according to our previous report (Basuni *et al.*, 2011). Amplification reactions were repeated for samples with Ct values above 35. In addition, absence of inhibitory factors was monitored by checking that the Ct value of PhHV-1 internal control remained below 37. Real-time PCR assay was considered as negative if the Ct value was more than 40 or if no amplification curve was obtained. Preparing of the master mix, extraction of DNA, and handling of PCR products were performed in separate rooms using allocated pipettes and equipment to prevent contamination.

RESULTS

The blood samples from all of the 94 clinically-suspected malaria patients were positive for the presence of *Plasmodium* spp by microscopy, and the parasites count per thick blood smear ranged from 80 to 256 000. From the thin smears, 25, 68 and one of the samples were identified as having *P. falciparum*, *P. vivax* and mixed infection of *P. falciparum* and *P. vivax*, respectively. Using multiplex real-time PCR, *Plasmodium* spp. DNA were also detected in all the 94 blood samples, with the median Ct value of 23 (18<Ct< 35). In terms of species, the molecular method showed that 22, 70 and two of the samples contained DNA of *P. falciparum*, *P. vivax* and mixed *P. falciparum* and *P. vivax* respectively. Comparison of the results of these two detection methods showed that five samples identified as *P. falciparum* by microscopy were detected as *P. vivax* by multiplex real-time PCR; while two samples identified as *P. vivax* by microscopy were detected as *P. falciparum* by the molecular method. In addition, one sample identified by microscopy as mixed infection with the two species was found to have only *P. vivax* by the multiplex real-time PCR. The results are shown in Table 1.

The median Ct value for the helminth multiplex PCR was 34 (23<Ct<39). Chi-square test showed that the multiplex real-time PCR assay detected 7.7 fold more samples with helminthes than microscopic examination ($p<0.05$). By multiplex real-time PCR, 46 of 94 samples (48.9%) had single or mixed helminth infections. From the 46 patients, 31, 13 and two were infected with *P. vivax*, *P. falciparum* and mixed *P. vivax* and *P. falciparum* respectively. Meanwhile microscopic examination of faecal specimens showed only single helminth infections i.e. 6 of 94 (6.4%). There were 3 samples with *A. lumbricoides* ova, 1 with hookworm ova and 2 with *S. stercoralis* larvae. Kato-Katz results showed that the ova counts were 576, 1152 and 1728 for the *A. lumbricoides* samples and 14,400 for the hookworm sample. In comparison, multiplex real-time PCR assay detected single infections in two, 15, six and seven samples, i.e. with *A. lumbricoides*, *S. stercoralis*, *A. duodenale* and *N. americanus* respectively. Mixed infections by real-time PCR detected two helminthes species in one, eight, one and two samples i.e. *N. americanus* and *A. lumbricoides*; *N. americanus* and *S. stercoralis*; *A. duodenale* and *A. lumbricoides;* and *A. duodenale* and *S. stercoralis* respectively. Meanwhile mixed infections with three helminthes species were found in four samples; two comprised *N. americanus*, *S. stercoralis* and *A. lumbricoides*, and the other two comprised *A. duodenale*, *N. americanus* and *S. stercoralis*. The results are shown in Table 2.

Table 3 shows four categories of parasitemia in the malaria patients and the corresponding real-time PCR results of their faecal samples.

Table 1. Results of microscopic examination and real-time PCR on whole blood samples (n=94)

Technique	No. of positive samples (%)	Parasite count / Ct value
Microscopic		
P. falciparum	25 (26.0)	480 – 256 000
P. vivax	68 (72.4)	80 – 32 500
P. falciparum + *P. vivax*	1 (1.0)	6800 – 8200
Multiplex real-time PCR		
P. falciparum	22 (23.4)	(20<Ct<35)
P. vivax	70 (74.5)	(18<Ct<35)
P. falciparum + *P. vivax*	2 (2.1)	(26<Ct<30)

Ct : cycle threshold.

Table 2. Types of STH infection by microscopy or real-time PCR), and the corresponding results of the malaria molecular detection

	Microscopic Examination (helminthes)	Multiplex real-time PCR (helminthes)	Multiplex real-time PCR (*Plasmodium* species)		
	No. of positive samples (%)	No. of positive samples (%) [Ct value]	No. of positive samples (%) [Ct value]		
Organisms			*P. falciparum*	*P. vivax*	*P. falciparum* + *P. vivax*
A. lumbricoides	3 (3.2)	2 (2.1) [30;31]	0 (0)	2 (2.1) [31;34]	0 (0)
S. stercoralis	2 (2.1)	15 (16.0) [24<Ct<39]	3 (3.2) [21<Ct<29]	11 (11.7) [18<Ct<26]	1 (1.1) [29;30]
A. duodenale	1 (1.1)	6 (6.4) [25<Ct<35]	2 (22) [20;22]	4 (4.3) [20<Ct<25]	0 (0)
N. americanus	0 (0)	7 (7.4) [28<Ct<38]	0 (0)	7 (7.4) [21<Ct<25]	0 (0)
N. americanus + *A. lumbricoides*	0 (0)	1 (1.1) [29;30]	1 (1.1) [25]	0 (0)	0 (0)
N. americanus + *S. stercoralis*	0 (0)	8 (8.5) [29<Ct<39]	2 (2.2) [21;24]	5 (5.3) [19<Ct<23]	1 (1.1) [26;27]
A. duodenale + *A. lumbricoides*	0 (0)	1 (1.1) [26;34]	1 (1.1) [31]	0 (0)	0 (0)
A. duodenale + *S. stercoralis*	0 (0)	2 (2.1) [29<Ct<36]	2 (2.2) [20;24]	0 (0)	0 (0)
N. americanus + *S. stercoralis* + *A. lumbricoides*	0 (0)	2 (2.1) [23<Ct<38]	(0)	2(2.2) [21;30]	(0)
A. duodenale + *N. americanus* + *S. stercoralis*	0 (0)	2 (2.1) [31<Ct<37]	2 (2.2) [20;29]	(0)	(0)

Ct: Cycle threshold. The lower the Ct value, the greater the amount of target DNA in the sample. Amplification reactions were repeated for samples with Ct values >35. Ct value of internal control (PhHV-1) of < 37 indicated absence of inhibitory factors. Real-time PCR assay was considered as negative if the Ct value > 40 or if no amplification curve was obtained.

Table 3. Four categories of parasitemia in malaria patients and corresponding real-time PCR results of the faecal samples

Category	Parasitemia (parasite/µl blood)	No of faecal samples positive by real-time PCR for STH	No. of faecal samples negative by real-time PCR for STH
1	20 – 100	0	1
2	100 – 10,000	33	38
3	10,000 – 100,000	11	9
4	100,000 – 250,000	1	0
5	250,000 – 500,000	1	0
	Total	46	48

DISCUSSION

In this study, both microscopic examination and multiplex real-time PCR confirmed infection by *Plasmodium* spp in all patients clinically suspected of malaria. By both methods, only two species of *Plasmodium* were detected, i.e. *P. falciparum* and *P. vivax;* the latter recorded the highest number which is consistent with the previous report that *P. vivax* infection dominated the study area (WHO, 2013). When compared to real-time PCR results, microscopic examination at the two study sites showed that accurate *Plasmodium* species identification is a challenge since 8.5% of blood samples were wrongly identified, this may be due to human error during microscopic examination. In addition, microscopic examination did not detect mixed infection of *P. falciparum* and *P. vivax* in 2.1% of the samples, possibly due to the low sensitivity of the traditional method.

With regard to faecal samples, only 6.4% were positive for helminthes by microscopic technique, as compared to multiplex real-time PCR which detected 7.7 times more cases of helminth infections, thus highlighting the high sensitivity of the molecular diagnosis. Furthermore, the multiplex real-time PCR assay detected 17% mixed helminth infection and showed that the highest percentage of helminth infection was caused by *S. stercoralis*, followed by hookworms, either as single or mixed infections.

PCR detects the presence of the helminth DNA in the stool, thus it is considered as a direct detection method, despite non-visualization of the actual parasite. Even if some patients may have been treated for STH before being sampled in the present study, the treatment may not kill all species of the worms (Rodriguez-Perez *et al.*, 2011; Soukhathammavong *et al.*, 2012). In such cases, the reduced number of eggs post-treatment may not be detectable by microscopy (Vercruysse *et al.*, 2011). PCR would be a better method to detect light infection, especially those post-treatment (Phuphisut *et al.*, 2014). In this regard, a previous study has reported that PCR was able to detect STH infection in feacal samples of patients who were under post-antihelmintic drug treatment (Phuphisut *et al.*, 2014).

With regard to hookworm infection, a recent study showed that the Kato thick smear is as sensitive as PCR (Knoop *et al.*, 2014). However the study only used primers to amplify *N. americanus*, thus the presence of *A. duodenale* may not be detected. Furthermore Knoop *et al.* (2014) noted that a clear limitation in their study is the absence of an internal control to monitor the success of both DNA extraction and real-time PCR assay. Thus, they could not determine whether there was PCR-inhibitory substances in the stool samples. On the other hand in the present study, two set of primers were used to detect the two species of hookworm; and PhHV-1 virus was used as the internal control.

The results of the present study showed that 48.9 % of malaria patients were infected with helminthes. To the best of our knowledge, this is the first report of helminth infections among malaria patients in Malaysia. Previous similar studies among children in Nigeria and Cameroon showed lower levels of co-infections of malaria and helminth i.e. 24.7% and 4.3% respectively (Theresa *et al.*, 2006, Ojurongbe *et al.*, 2011). The lower percentages in those studies may be due to factors such as the use of only microscopy as the detection method; different study design and study population. In this study, only patients with clinical symptoms of malaria were selected, thus although there seemed to be similar number of malaria patients with (n=46) and without (n=48) STH infections, it cannot be ascertained whether there is any correlation between malaria and helminth infection. One limitation of this study was the omission of *P. knowlesi*, this was because reports on this species were still limited at the time the study was initiated. It is also noted the blood samples were microscopically identified as either *P. falciparum* or *P. vivax* and none was identified as *P. malariae* (which is the species morphologically similar to *P. knowlesi*). This seems to indicate that the populations that we studied may not have *P. knowlesi*

infection. Therefore despite the above limitation, most probably it did not significantly affect our results.

In addition, molecular diagnosis on the faecal samples did not include *T. trichiura* since during the study period, there was still no report on successful DNA detection for this helminth ova. In future studies, molecular detection of *Ancylostoma ceylanicum* may be included since a recent report from Malaysia by Ngui *et al.* (2012) showed human infection with *A. ceylanicum* among the aborigines; and a high prevalence of human infection with this species was recently reported in Cambodia (Inpankaew *et al.*, 2014).

Despite the above limitations, this study has demonstrated the high prevalence of helminth infections among malaria patients in Sarawak. Further studies using a much larger and a more appropriate population, as well as a robust and statistically sound study design may be performed to answer questions such as whether a significant correlation exist between malaria and helminth infections; and whether severity of malaria is influenced by helminth infection and vice versa. Such studies will provide valuable information on epidemiology, improve patient management and increase our understanding of the immunology and pathogenesis of malaria and helminth co-infections.

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