Tropical Biomedicine 30(3): 459–466 (2013)

Comparison of two nested PCR methods for the detection of human malaria

Anthony, C.[1], Mahmud, R.[1], Lau, Y.L.[1*], Syedomar, S.F.[2] and Sri La Sri Ponnampalavanar, S.[2]
[1]Department of Parasitology, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia
[2]Department of Medicine, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia
[*]Corresponding author's e-mail: lauyeeling@um.edu.my
Received 27 December 2012; received in revised form 29 March 2013; accepted 13 May 2013

Abstract. Battling malaria will be a persistent struggle without the proper means to diagnose the parasitic infection. However, the inherent limitations of microscopy, the conventional method of diagnosing malaria, affect the accuracy of diagnosis. The present study aimed to compare the accuracy of two different set of primers targeting the small subunit ribosomal RNA (ssRNA) and the dihydrofolate reductase-thymidylate synthase linker region (dhfr-ts) in detecting species specific malaria infections by nested PCR. The sensitivity and specificity of nested PCR assay using the two primers were calculated with reference to microscopy as the 'gold standard'. The results show that 18S rRNA primers had 91.9% sensitivity and 100% specificity in detecting human *Plasmodium* species as opposed to dhfr-ts primers which had 51.4% sensitivity and 100% specificity. The higher sensitivity of 18S rRNA primers suggests that it may be a better diagnostic tool for detecting human malaria.

INTRODUCTION

The five species known to cause malaria in humans are *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae*, and the most recent *Plasmodium knowlesi*. Despite various control efforts, malaria still remains a severe global health problem (Stratton *et al*., 2008). The incidences of malaria cases that occur annually vary from one report to another and this by itself reflects the lack of precision in malaria statistics (Wongsrichanalai *et al*., 2007).

Accurate diagnosis is imperative in the effective management of malaria (Tangpukdee *et al*., 2009). While microscopy has been and still remains the mainstay in malaria diagnosis, its limitations has often led to the misdiagnosis of *Plasmodium* species and ultimately the mistreatment of malaria. Furthermore, the lack of skilled microscopists has massively hampered the efficacy of this traditional technique. It is also important to note that low-level parasitemia which is below the detectable limit of blood smears is a norm under conditions of host acquired immunity or exposure to antimalarials (Taylor *et al*., 2010).

Rapid immunochromatographic tests were developed to aid better conduct of diagnosis. The basis of these tests is the detection of antigens in the blood of malaria patients (Di Santi *et al*., 2004). Monoclonal antibodies have been developed to target the conserved element of Plasmodium lactate dehydrogenase (PLDH) or specific regions which are unique to *P. falciparum* or *P. vivax* (Murray *et al*., 2008). Unfortunately, in cases of low parasitemia, this method is somewhat insensitive. Another flaw of this method is the possibility of false positive results due to the persistence of antigenemia weeks beyond the actual infection (Mangold *et al*., 2005).

Molecular methods have brought about much headway in further refining existing diagnostic methods. The polymerase chain

reaction (PCR) was first applied to malaria diagnostics in the early 1990s (Erdman & Kain, 2008) and it was not long before this method was extensively used. In terms of sensitivity and specificity, comparative studies have consistently demonstrated the superiority of PCR-based diagnosis over microscopy-based diagnosis of malaria (Putaporntip *et al.*, 2009). Apart from diagnosis, this DNA-based molecular method has also been commonly used in areas involving epidemiological studies and assessment of drug efficacy (Harris *et al.*, 2010).

Nested PCR, multiplex PCR and real-time PCR are PCR based assays that have been developed to overcome the limitations and discrepancy of traditional diagnostic methods. Nested and multiplex PCR are able to shed light on species determination and differentiation when cumbersome morphological problems arise during attempts to identify parasites at species level (Chavalitshewinkoon-Petmitr, 2010). Real-time PCR assays have proven its ability in identifying mixed infections, detecting low parasitemia levels and also in the differentiation of *Plasmodium* species based on melting curve analysis (Mangold *et al.*, 2005). The common misdiagnosis of *P. knowlesi* as *P. malariae* and *P. falciparum* via microscopy can now be circumvented with the aid of these PCR based assays. The aim of this study was to compare the specificity and sensitivity of two nested-PCR methods using two different sets of primers for the molecular diagnosis of human malaria.

MATERIALS AND METHODS

Patient samples

A total of 57 whole blood samples and 17 blood smears were obtained from the General Medical Ward, University Malaya Medical Centre (UMMC). The presence of malarial parasites in the specimens was determined by both microscopy and PCR. Twelve blood samples taken from healthy individuals with no history of malaria infection were used as negative controls in this study. Approval for the use of these samples was obtained from the University of Malaya Medical Centre Ethics Committee (Reference no: 817.18).

Microscopy

Giemsa (5%) stained blood smears, both thick and thin were prepared and examined under light microscope. Examination of these slides was carried out by skilled and experienced microscopists who were able to identify and differentiate the malaria parasites. At least 200 microscopic fields were examined before concluding the slide was negative for malaria parasites. Parasitaemia was calculated as number of infected red blood cells per 1000 red blood cells counted in the thin film.

DNA extraction from whole blood

The template DNA required to carry out the nested PCR assay was prepared using the DNeasy Blood & Tissue Kit (QIAGEN, Valencia, CA). Parasite DNA was extracted from 100 µl of whole blood according to the manufacturer's protocol. Purified DNA was eluted from the column with 50 µl elution buffer and this DNA was stored at -20°C for further use.

DNA extraction from slides

Prior to the extraction of DNA, the slides were initially cleaned to remove oil residues. Approximately 50 µl of Tris-EDTA (TE) buffer was then pipetted onto the thin film. Whatman filter paper was cut into strips and placed on the slide to absorb the buffer. The smear was wiped off from the slide and placed in a 1.5 ml centrifuge tube using sterile forceps. The DNA from the filter paper was extracted using the DNeasy Blood & Tissue Kit (QIAGEN, Valencia, CA).

Nested PCR assay

The malaria species in the samples were determined via nested PCR assay. Two different set of primers as previously mentioned was used. The first nested PCR was performed based on 18S ribosomal RNA genes (18S rRNA) developed by Singh *et al.* (1999). A total nest 1 reaction mixture of 25 µl contained 4 µl of DNA template, 1.0 µl of

genus-specific primers (rPLU1: 5'-TCA AAG ATT AAG CCA TGC AAG TGA-3'and rPLU5: 5'-CCT GTT GTT GCC TTA AAC TCC-3'), 2.5 µl 10X Buffer, 2.0 µl of 0.25 M dNTP and 0.2 µl of 1 u *Taq* polymerase and 15.3 µl of nuclease free water. The nest 1 amplification conditions were as follows: 1) Initial denaturation at 94°C for 4 minutes, 2) 35 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 1 minute and extension at 72°C for 1 minute, 3) Final extension at 72°C for 10 minutes and a hold temperature of 4°C. Each of the nest 2 amplification mixture of 25 µl contained 4 µl of the nest 1 product and same amounts of buffer, dNTP, *Taq* polymerase and nuclease free water as in nest 1. The nest 2 amplification conditions were identical to that of nest 1 except the annealing temperature of 58°C for the species-specific primers.

The second nested PCR targeted at dihydrofolate reductase-thymidylate synthase linker region (dhfr-ts) was developed by Tanomsing *et al*. (2010). The amount of 10X buffer, dNTP, *Taq* polymerase and DNA template used were the same as the first set of nested PCR. Cycling conditions for both primary and secondary amplifications were followed as stated in Tanomsing *et al*. (2010). The amplified products were visualized through gel electrophoresis using a 2% agarose gel stained with Sybr Safe. A 100bp molecular marker (Fermentas) was used.

Analysis

The sensitivity and specificity of the 2 different primers were calculated with regards to microscopy as the reference. Percentage of sensitivity and specificity (%) were calculated as follows:

% Sensitivity = (number of true positives/ number of true positives + number of false negatives) x 100

% Specificity = (number of true negatives/ number of true negatives + number of false positives) x 100

RESULTS

Microscopy analysis

In total, 74 patients were identified as being infected with malaria via blood film analysis. Out of this, 33 were identified as having *P. falciparum* infection, 23 were found to be *P. vivax* infection, 2 samples were found to have *P. ovale* infection and 16 were identified as either *P. malariae* or *P. knowlesi* infection. The samples were then subjected to nested PCR using the 18S rRNA and dhfr-ts primers. Nested PCR was also carried out on 12 non malaria samples to determine specificity of the two different set of primers. The different species identified by microscopy and PCR are illustrated in Table 2.

Nested PCR

The comparison between microscopy and nested PCR assays using the 18S rRNA and dhfr-ts primes are shown in Table 1. The PCR analysis using the 18S rRNA primers detected 22 cases of *P. falciparum*, 21 cases of *P. vivax*, 2 cases of *P. ovale*, 14 cases of *P. knowlesi* and 9 cases of mixed infections. There were 6 samples which were positive for *P. falciparum* via microscopy but produced negative results with PCR. Another 6 were found to have a different *Plasmodium* species picked up by PCR when compared to microscopy.

The results obtained with the dhfr-ts primers, however, was not satisfying. Of the total 74 samples, only 7 cases were positive for *P. falciparum*, 11 for *P. vivax*, 1 for *P. ovale*, 17 for *P. knowlesi* and 2 mixed infection cases. A total of 36 samples which were positive for *Plasmodium* when viewed under the microscope were negative for all of the 5 human *Plasmodium* species when tested with nested PCR using dhfr-ts primers. Two samples were found to be diagnosed as *P. knowlesi* by PCR but observed to be *P. falciparum* by means of microscopy. There were no *P. malariae* samples identified via nested PCR assay with either sets of primers. Results remained discordant even after re-examination of slides and the repeating of nested PCR.

Table 1. Nested PCR primers targeting 18S rRNA and dhft-ts genes in malaria parasites

Species	Primer	Primer Sequence (5'–3')	Expected size (bp)
18S rRNA			
P. falciparum	rFAL 1	TTAAACTGGTTTGGGAAAACCAAATATATT	205
	rFAL 2	ACACAATGAACTCAATCATGACTACCCGTC	
P. vivax	rVIV 1	CGCTTCTAGCTTAATCCACATAACTGATAC	120
	rVIV 2	ACTTCCAAGCCGAAGCAAAGAAAGTCCTTA	
P. ovale	rOVA 1	ATCTCTTTTGCTATTTTTTAGTATTGGAGA	800
	rOVA 2	GGAAAAGGACACATTAATTGTATCCTAGTG	
P. malariae	rMAL 1	ATAACATAGTTGTACGTTAAGAATAACCGC	144
	rMAL 2	AAAATTCCCATGCATAAAAAATTATACAAA	
P. knowlesi	Pmk8	GTTAGCGAGAGCCACAAAAAAGCGAAT	153
	Pmk9r	ACTCAAAGTAACAAAATCTTCCGT	
DHFR-TS			
Plasmodium spp.	Pla DHFR F	ATGGARSAMSTYTSMGABGTWTTYGA	1000
	Pla TS R	AAATATTGRTAYCTGGRTG	
P. falciparum	PF Lin F	AAAAGGAGAAGAAAAAAATAA	160
	PF Lin R	AAAATAAACAAAATCATC	
P. vivax	PV Lin F	CGGGAGCACTGCGGACAGCG	144
	PV Lin R	CACGGGCACGCGGCGGGGC	
P. ovale	PO Lin F	GACACACAAAATGATGGGGA	177
	PO Lin R	ATTGTCCTTTCCTTGACTCG	
P. malariae	PM Lin F	GACCCAAGAATCCCTCCC	177
	PM Lin R	CCCATGAAGTTATATTCC	
P. knowlesi	PK Lin F	CGATGGATATGGATAGTGG	144
	PK Lin R	CGCGGGAGAGCATTTCCTC	

Table 2. Number of samples with various malaria species detected by microscopy and PCR

Method/Species	Number of Samples								
	Pf	Pv	Po	Pk	Pm	Pm/Pk	Mixed	Neg	Total
Microscopy	33	23	2	0	0	16	0	12	86
Nested PCR (18 ssRNA)	22	21	2	14	0	0	9	18	86
Nested PCR (dhfr-ts)	7	11	1	17	0	0	2	48	86

Note: Pf = *P. falciparum*; Pv = *P. vivax*; Po = *P. ovale*; Pk = *P. knowlesi*; Mixed = Pv+Pk or Pv+Pf or Pk+Pf; Neg = Negative

Sensitivity and specificity

The sensitivity and specificity of nested PCR using the two sets of primers compared to microscopy are shown in Table 3. Of the 74 malaria positive samples confirmed by means of microscopy, 68 were confirmed to be positive by nested PCR with 18S rRNA primers and 38 by nested PCR with dhfr-ts. Overall, nested PCR with 18S rRNA primers showed 91.9% sensitivity and 100%

Table 3. Sensitivity and specificity of 18S rRNA and dhfr-ts primers compared to microscopy

Microscopy (N)	Nested PCR			
	18S rRNA		Dhfr-ts	
	Positive N (%)	Negative N (%)	Positive N (%)	Negative N (%)
Positive (74)	68 (91.9)	6 (8.1)	38 (51.4)	36 (48.6)
Negative (12)	0 (0)	12 (100).	0 (0)	12 (100)

Note: N = Number of samples

specificity. Nested PCR with dhfr-ts primers on the other hand, showed only 51.47% sensitivity and 100% specificity.

DISCUSSION

In malaria studies, PCR is known to be a promising method especially in the identification of parasites in areas where four *Plasmodium* species occur simultaneously (Snounou *et al*., 1993). However, it has been recognized that the success of the technique is dependable on the quality of DNA. Previous studies have shown that intrinsic (DNA amount or content of human DNA or haemoglobin) and extrinsic (use of heparin or inadequate conditions of blood collection, storage and amplification of samples) factors can inhibit the PCR assay (Barker *et al*., 1992; Snounou *et al*., 1993).

In general, the ribosomal ribonucleic acid (rRNA) has proven to be of benefit in molecular studies due to their cocktail of regions which have evolved at different rates and thus enabling them to be utilized at various taxonomic levels (Rubio *et al*., 2002). The decreased susceptibility and possible resistance to antifolate antimalarial drugs have led to the thorough investigation of sequence variations in the *Plasmodium* dhfr domain (Tanomsing *et al*., 2010).

While nested PCR assay using either the 18S rRNA or dhfr-ts primers were unable to pick up all the microscopically positive *Plasmodium* samples, this assay was able to detect several mixed infections. The ability of the PCR assay to identify 2 and 9 mixed infections using dhfr-ts and 18S rRNA primers respectively exhibits the greater sensitivity of the PCR assay over microscopy. With regards to mixed infections, it is possible for microscopic misdiagnosis to occur due to the domination of one species over the other (Ebrahimzadeh *et al*., 2007). Limitations of the light microscope could also serve as a reason in the misdiagnosis of mixed infections as a single infection (Ohrt *et al*., 2002).

The present study has demonstrated that nested PCR assay using the 18S rRNA primers are more sensitive and specific as opposed to carrying out the same assay with the dhfr-ts primers. While the 18S rRNA primers were able to identify the species of 68 (57 whole blood and 11 blood smears) samples, there were 6 *P. falciparum* positive blood smears that were not detected by PCR (18S rRNA). This may be an effect of a reduced number of parasites present in the sample, as some could have been lost during the process of scraping the slides. It is also known that factors pertaining to slide preparation for microscopic examination may contribute to the stability of the DNA template (Scopel *et al*., 2004). However, using this as reasons to justify 36 (20 whole blood and 16 blood smears) negative results obtained with the dhfr-ts primers would be farfetched. Tanomsing *et al.* (2010) reported that these newly developed primers are effective at detecting low level parasitemia, particularly *P. falciparum*. Interestingly, such was not the case in this study. Only 7 samples of 33 which were identified as *P. falciparum* gave the same results when tested by nested PCR with dhfr-ts primers. In fact these set of primers appear to be better at identifying *P. knowlesi*

samples and to a lesser extent, *P. vivax*. Relative to 9 samples which were identified as mixed infections with 18S rRNA primers, the dhfr-ts primers were only able to detect 2 samples with mixed infections.

Of the 17 blood smears tested, the 18S rRNA primers were able to identify 11 malaria species while the dhfr-ts primers were able to identify only one infection. This is an interesting finding as the previous study by Tanomsing *et al.* (2010) did not test these primers with DNA extracted from blood smears. We also found that when the percentage parasitaemia of infection was above 1%, nested PCR methods using the dhfr-ts primers was able to identify the species of the malaria parasite. However, when the percentage parasitaemia of infection was below 1%, we observed that there were inconsistent results with this particular primer set. While nested PCR using the 18S rRNA primers was able to identify malaria species (68/74) with a parasitaemia as low as 0.03%, such was not the case with the dhfr-ts primers. There were instances where the dhfr-ts primers was able to detect an infection with a parasitaemia of 0.31% but was not able to identify malaria species with a parasitaemia of 0.63%.

There were 3 cases whereby the 18S rRNA primers detected both *P. vivax* and *P. knowlesi* but only one species (either *P. vivax* or *P. knowlesi*) was detected by the dhfr-ts primers. While this may appear as though the 18s rRNA primers are more sensitive towards detecting mixed infections, a study carried out by Imwong *et al.* (2009) demonstrated otherwise. False positive amplification was observed when *P. vivax* genomic DNA was used implicating stochastic cross reaction with the Pmk8-Pmkr9 primers. In this case, it is possible that the dhfr-ts primers are better at discriminating between *P. vivax* and *P. knowlesi* infections.

There were 6 blood smears which were positive for *P. falciparum* but negative when subjected to PCR with 18S rRNA primers. These results remained discordant upon re-examination of smears and repetition of PCR. It is important to note that presence of artifacts in blood smears can lead to false positive readings. This may be due to the similar resemblance between artifacts and malaria parasites and as such would be a possibility why the PCR assay was not able to detect the parasite (Ohrt *et al.*, 2002). The reasons could also be as what was explained by Scopel *et al.* (2004). The mis-identification of *P. vivax* as *P. falciparum* could be attributed to the differences in smear preparation and also quality of the stain.

Based on this study, we found the 18S rRNA primers to be more effective at identifying the different species of human malaria. We also found that the dhfr-ts primers may not be as effective at identifying *P. falciparum* as initially proposed.

Acknowledgement. Special thanks to PARASEAD, University of Malaya for providing part of the blood samples for analysis. The authors would also like to thank the Ministry of Science, Technology and Innovation, E-Science Fund (Grant no: 12-02-03-2086) and UM Postgraduate Research Fund (PV044/2012A) for supporting this study.

REFERENCES

Barker, R.H.J., Banchongaksorn, T., Courval, J.M., Suwonkerd, W., Rimwungtragoon, K. & Wirth, D.F. (1992). A simple method to detect *Plasmodium falciparum* directly from blood samples using the polymerase chain reaction. *American Journal of Tropical Medicine and Hygiene* **46**: 416-426.

Chavalitshewinkoon-Petmitr P. (2010). Laboratory diagnosis of malaria. *Siriraj Medical Journal* **62**: 98-102.

Di Santi, S.M., Kirchgatter, K., Brunialti, K.C., Oliveira, A.M., Ferreira, S.R. & Boulos, M. (2004). PCR-based diagnosis to evaluate the performance of malaria reference centers. *Revista do Instituto de Medicina Tropical de Sao Paulo* **46**: 183-187.

Ebrahimzadeh, A., Fouladi, B. & Fazaeli, A. (2007). High rate of detection of mixed infections of *Plasmodium vivax* and *Plasmodium falciparum* in South-East Iran, using nested PCR. *Parasitoly International* **56**: 61-64.

Erdman L.K. & Cain, K.C. (2008). Molecular diagnostic and surveillance tools for global malaria control. *Travel Medicine and Infectious Disease* **6**: 82-99.

Harris, I., Sharrock, W.W., Bain, L.M., Gray, K-A., Bobogare, A., Boaz, L., Lilley, K., Krause, D., Vallely, A., Johnson, M-L., Gatton, M.L., Shanks, G.D. & Cheng, Q. (2010). A large proportion of asymptomatic *Plasmodium* infections with low and sub-microscopic parasite densities in the low transmission setting of Temotu Province, Solomon Islands: challenges for malaria diagnostics in an elimination setting. *Malaria Journal* **9**: 254.

Imwong, M., Tanomsing, N., Pukrittayakamee, S., Day, N.P.J., White, N.J. & Snounou, G. (2009). Spurious amplification of a *Plasmodium vivax* small-subunit RNA gene by use of primers currently used to detect *P. knowlesi*. *Journal of Clinical Microbioly* **47**: 4173-4175.

Mangold, K.A., Manson, R.U., Koay, E.S.C., Stephens, L., Regner, M., Thomson, R.B. Jr., Peterson, L.R. & Kaul, K.L. (2005). Real-time PCR for detetction and identification of Plasmodium spp. *Journal of Clinical Microbiol*ogy **43**: 2435-2440.

Murray, C.K., Gasser, R.A. Jr., Magill, A.J. & Miller, R.S. (2008). Update on rapid diagnostic testing for malaria. *Clinical Microbiology Reviews* **21**: 97-110.

Ohrt, C., Purnomo, Sutamihardja, M,A., Tang, D. & Kain, K.C. (2002). Impact of microscopy error on estimates of protective efficacy in malaria-prevention trials. *Journal of Infectious Diseases* **186**: 540-546.

Putaporntip, C., Hongsrimuang, T., Seethamchai, S., Kobasa, T., Limkittikul, K., Cui, L. & Jongwutiwes, S. (2009). Differential prevalence of *Plasmodium* infections and cryptic *Plasmodium knowlesi* malaria in humans in Thailand. *Journal of Infectious Diseases* **199**: 1143-1150.

Rubio, J.M., Post, R.J., Docters van Leeuwen, W.M., Henry, MC., Lindergard, G. & Hommel, M. (2002). Alternative polymerase chain reaction method to identify *Plasmodium* species in human blood samples: the semi-nested multiplex malaria PCR (SnM-PCR). *Transactions of the Royal Society of Tropical Medicine and Hygiene* **96**(Suppl 1): 199-204

Scopel, K.K.G., Fontes, C.J.F., Nunes, A.C., de Fatima Horta, M. & Braga, E.M. (2004). Low sensitivity of nested PCR using *Plasmodium* DNA extracted from stained thick blood smears: An epidemiological retrospective study among subjects with low parasitaemia in an endemic area of the Brazilian Amazon region. *Malaria Journal* **3**: 8.

Singh, B., Bobogare, A., Cox-Singh, J., Snounou, G., Abdullah, M.S. & Rahman, H.A. (1993). A genus- and species-specific nested polymerase chain reaction malaria detection assay for epidemiologic studies. *American Journal of Tropical Medicine and Hygiene* **60**: 687-692.

Snounou, G., Viriyakasol, S., Jarra, W., Thaithong, S. & Brown, K.N. (1993). Identification of the four human malaria parasite species in field samples by the polymerase chain reaction and detection of a high prevalence of mixed infections. *Molecular and Biochemical Parasitology* **58**: 283-289.

Stratton, L., O'Neill, M.S., Kruk, M.E. & Bell, M.L. (2008). The persistent problem of malaria: addressing the fundamental causes of a global killer. *Social Science and Medicine* **67**: 854-862.

Tangpukdee, N., Duangdee, C., Wilairatana, P. & Krudsood, S. (2009). Malaria diagnosis: a brief review. *Korean Journal of Parasitol*ogy **47**: 93-102.

Tanomsing, N., Imwong, M., Theppabutr, S., Pukrittayakamee, S., Day, N.P., White, N.J. & Snounou, G. (2010). Accurate and sensitive detection of *Plasmodium* species in humans by use of the dihydrofolate reductase-thymidylate synthase linker region. *Journal of Clinical Microbiology* **48**: 3735-3737.

Taylor, S.M., Juliano, J.J., Trottman, P.A., Griffin, J.B., Landis, S.H., Kitsa, P., Tshefu, A.K. & Meshnick, S.R. (2010). High-throuput pooling and real-time PCR-based strategy for malaria detection. *Journal of Clinical Microbiology* **48**: 512-519.

Wongsrichanalai, C., Barcus, M,J., Muth, S., Sutamihardja, A. & Wernsdorfer, W.H. (2007). A Review of malaria diagnostic tools: microscopy and rapid diagnostic test (RDT). *American Journal of Tropical Medicine and Hygiene* **77**(Suppl 6): 687-692.