Original article

European Journal of Microbiology and Immunology 5 (2015) 2, pp. 159–163
DOI: 10.1556/1886.2015.00006

COMPARISON OF TWO REAL-TIME PCR ASSAYS FOR THE DETECTION OF MALARIA PARASITES FROM HEMOLYTIC BLOOD SAMPLES – SHORT COMMUNICATION

Ralf Matthias Hagen[1], Rebecca Hinz[1], Egbert Tannich[2] and Hagen Frickmann[1,3,*]

[1]Department of Tropical Medicine at the Bernhard Nocht Institute, German Armed Forces Hospital of Hamburg, Germany
[2]Bernhard Nocht Institute for Tropical Medicine Hamburg, Germany
[3] Institute for Microbiology, Virology and Hygiene, University Medicine Rostock, Germany

Received: February 21, 2015; Accepted: February 28, 2015

We compared the performance of an in-house and a commercial malaria polymerase chain reaction (PCR) assay using freeze–thawed hemolytic blood samples.

A total of 116 freeze–thawed ethylenediamine tetraacetic acid (EDTA) blood samples of patients with suspicion of malaria were analyzed by an in-house as well as by a commercially available real-time PCR.

Concordant malaria negative PCR results were reported for 39 samples and malaria-positive PCR results for 67 samples. The in-house assay further detected one case of *Plasmodium falciparum* infection, which was negative in the commercial assay as well as five cases of *P. falciparum* malaria and three cases of *Plasmodium vivax* malaria, which showed sample inhibition in the commercial assay. The commercial malaria assay was positive in spite of a negative in-house PCR result in one case. In all concordant results, cycle threshold values of *P. falciparum*-positive samples were lower in the commercial PCR than in the in-house assay.

Although Ct values of the commercial PCR kit suggest higher sensitivity in case of concordant results, it is prone to inhibition if it is applied to hemolytic freeze–thawed blood samples. The number of misidentifications was, however, identical for both real-time PCR assays.

Keywords: malaria, PCR, test comparison, difficult sample matrix, freeze–thawed blood, molecular diagnostics, in-house, real-time

Introduction

Malaria, travel-associated diarrhea as well as skin and sexually transmitted diseases are major health concerns on deployments in subtropical and tropical endemic settings [1]. Rapid diagnosis in case of suspected malaria is mandatory. Traditional microscopy of Giemsa-stained thin and thick blood smears remains the diagnostic golden standard. However, this cheap and easy-to-perform procedure requires expert knowledge and substantial skills in parasite microscopy, which is not easy to maintain in non-endemic areas, where malaria is rare or constitutes a travel-associated disease.

In resource-limited deployment settings, alternative, less investigator-dependent diagnostic approaches are desirable. Rapid diagnostic tests (RDTs) are a frequently chosen option. Such tests are usually based on lateral-flow immunochromatography, and parasites are detected by respective antibodies. In detail, type 2 RDTs are based on anti-HRP-(histidine rich protein)-2-antibodies for *Plasmodium falciparum* and anti-aldolase-antibodies for all species; type 3 RDTs on anti-HRP-2-antibodies for *P. falciparum* and anti-pLDH-(plasmodium lactate dehydrogenase)-antibodies for all species; and type 4 RDTs on anti-pLDH-antibodies for *P. falciparum* and anti-pLDH-antibodies for all species. In a recent Cochrane meta-analysis, average sensitivities and specificities were 78% and 99% for type 2 and 3 tests, and 89% and 98% for type 4 tests [2]. Thus, sensitivity of RDTs is still a matter of concern. Moreover, in the presence of heterophilic IgM antibodies or in cases of exceedingly high parasitemia, RDTs may even produce false negative results [3, 4].

Molecular DNA-based assays have been proven as alternative approaches, with high sensitivity and specificity for malaria diagnostics, which can be standardized and require less experienced skills than microscopy. If the re-

* Corresponding author: Hagen Frickmann; Department of Tropical Medicine at the Bernhard Nocht Institute, German Armed Forces Hospital of Hamburg, Bernhard Nocht street 74, D-20359 Hamburg, Germany; Phone: 004940/694728743; Fax: 004940/694728709; E-mail: Frickmann@bni-hamburg.de

ISSN 2062-509X / $ 20.00

spective infrastructural prerequisites are in place in a tropical field setting, polymerase chain reaction (PCR) might substitute for microscopy or RTDs. However, PCR can be limited by sample inhibition, e.g., due to hemolysis of blood samples [5]. This is a relevant concern, as adequate sample storage and transport cannot always be guaranteed under tropical deployment conditions. To assess the dimension of this problem, we evaluated a commercial and an in-house real-time PCR assay using repeatedly freeze–thawed, hemolytic blood of patients with suspected malaria.

Methods

Samples

A total of 116 freeze–thawed ethylenediamine tetraacetic acid (EDTA) blood samples of patients with suspicion of malaria were analyzed by traditional thick and thin blood smear microscopy by experienced technical assistants at the Bernhard Nocht Institute for Tropical Medicine Hamburg, the German National Reference Center for Tropical Diseases, which is also responsible for malaria slide reading proficiency testing of diagnostic German laboratories. Parasitemia was determined according to WHO standards and recorded as parasites per microliter.

Residual materials of EDTA blood were subjected to repeated freeze–thawing to ensure progressive hemolysis prior to further processing.

Nucleic acid extraction

From the hemolytic EDTA blood samples, DNA was extracted and enriched using the EZ1 DNA Blood 200 µl Kit (Qiagen, Hilden, Germany) on the automated EZ1 system (Qiagen) exactly as described by the manufacturer. EZ1-based extraction has recently been shown to be equally efficient than extraction by the column-based QIAamp DNA Blood Mini Kit (Qiagen) for such specimens [6].

PCR analyses

All samples were subjected to real-time in-house one-tube SybrGreen malaria PCR and commercial altona Diagnostics Realstar Malaria PCR, a Taqman approach. In detail, the in-house SybrGreen melting curve analysis-based approach targeting *Plasmodium (P.) falciparum*, *P. malariae*, *P. ovale*, and *P. vivax* was performed as previously described [7]. The *Plasmodium* spp.-specific real-time PCR RealStar Malaria PCR Kit 1.0 (altona Diagnostics, Hamburg, Germany) was applied exactly as described by the manufacturer.

Analysis

If concordant positive PCR results in both PCR approaches were observed, achieved cycle threshold (Ct) values were assessed including calculation of median and mean values as well as standard deviations (SDs). Commercial RealStar Malaria PCR included an inhibition control, which allowed monitoring of PCR inhibition as well. Of note, negative inhibition control PCR in PCR-positive samples is caused by competitive amplification in this assay and not a sign of procedural failure.

Matched pairs of recorded Ct values were compared using Wilcoxon matched-pairs signed ranks testing with the software GraphPad InStat, version 3.06 (GraphPad Software Inc., La Jolla, CA, USA). Significance was accepted in case of a two-tailed P value ≤ 0.05. The nonparametric Spearman correlation coefficient was calculated (GraphPad Software Inc., La Jolla, CA, USA) to confirm effective pairing.

Results

Microscopic assessment

Of the 116 samples analyzed, 76 were microscopically positive for plasmodia. These comprised 61 cases of mono-infections with *P. falciparum*, seven cases with *P. vivax*, two cases with *P. ovale*, and one case with *P. malariae*, respectively. Parasite densities are shown in *Table 1*. Of note, parasitemia was not determined in one case of *P. vivax* infection and there were two cases in which microscopic discrimination between *P. vivax* and *P. ovale* was uncertain (1240/µl and 8720/µl). There was one case of *P. vivax* infection with a possible coinfection with *P. malariae* (100,000/µl), one case with a double infection of *P. falciparum* and *P. malariae* (500,000/µl), and one

Table 1. Parasite density of mono-infections with African *Plasmodium* spp. as assessed by thick and thin smear microscopy

	P. falciparum	*P. vivax*	*P. ovale*	*P. malariae*
Lowest observed density [/µl]	0.2	2400	714	1400
Highest observed density [/µl]	350,000	8040	5200	1400
Median [/µl]	7000	3040	3117	n.a.
Mean [/µl]	43,753	4120	3117	n.a.
Standard deviation [/µl]	81,990	2167	3398	n.a.

n.a. = Not applicable

Table 2. Characterization of samples with discordant PCR results

Species in microscopy	Parasite density in microscopy [/µl]	Species in in-house PCR	Ct value in in-house PCR	Ct value in commercial PCR
P. falciparum	0.4	*P. falciparum*	28	Negative
P. falciparum	800	*P. falciparum*	22	Inhibited
P. falciparum	96	*P. falciparum*	24	Inhibited
P. falciparum	0.2	*P. falciparum*	25	Inhibited
P. falciparum	37,600	*P. falciparum*	16	Inhibited
P. falciparum	50,000	*P. falciparum*	15	Inhibited
P. vivax/*ovale*	1240	*P. vivax*	20	Inhibited
P. vivax	2920	*P. vivax*	19	Inhibited
P. vivax	2920	*P. vivax*	17	Inhibited
Negative	Negative	Negative	Negative	31

case in which microscopic discrimination of *Pasmodium* spp. beyond the genus level failed (480/µl). In 40 cases, malaria microscopy was negative.

PCR results

In direct comparison of the two PCR assays regarding the overall detection of malaria, concordant results were recorded for 106 of the 116 samples of which 67 were parasite positive and 39 were parasite negative according to microscopy. The in-house assays further detected one case of a *P. falciparum* infection, which was negative by the commercial assay as well as five cases of *P. falciparum* and three cases of *P. vivax* infections, which showed sample inhibition in the commercial assay *(Table 2)*. All of the latter discordant nine samples were positive by microscopy. In one case, the commercial malaria assay was positive with a relatively high Ct value of 31, but was negative with the in-house PCR. Of note, microscopy was negative in this sample *(Table 2)*.

Inhibition control PCR of the commercial PCR approach remained negative for 46 of 116 samples. Negative inhibition control PCR was observed in 29 PCR-positive samples, in nine PCR-negative samples in concordance with microscopic results, and in eight PCR-negative samples in discordance with microscopic results. In total, 17 out of 116 (14.7%) PCR results of the commercial assay were non-interpretable, reducing the number of unambiguously false negative results of this approach with inconspicuous inhibition control PCR to one.

Of note, in-house PCR recorded *P. vivax* only in two microscopically uncertain cases of *P. vivax*/*P. ovale* infections and *P. falciparum* in a case in which microscopic differentiation beyond the genus level of *Plasmodium* spp. failed. In addition, in-house PCR recorded *P. falciparum* in a case of microscopic finding of *P. vivax* infection with possible coinfection with *P. malariae* and, in another case, in-house PCR recorded *P. falciparum* mono-infection, but microscopy suggested double infection with both *P. falciparum* and *P. malariae (Table 3)*.

Cycle threshold comparison of the two assessed PCR assays

In cases of concordant results, cycle threshold values of *P. falciparum*-positive samples were significantly lower with the commercial PCR than with the in-house assay. However, there were no differences in Ct values between the two assays for samples containing *P. vivax*. The number of samples containing *P. ovale* or *P. malariae* was too low for meaningful statistical analyses *(Table 4)*.

Table 3. Characterization of samples with discordant results in in-house PCR and microscopy

Species in in-house PCR	Ct value in in-house PCR	Ct value in commercial PCR	Species in microscopy	Parasite density in microscopy [/µl]
P. vivax	16	15	*P. vivax*/*ovale*	8720
P. vivax	20	Inhibited	*P. vivax*/*ovale*	1240
P. falciparum	16	12	*P. vivax* with possible coinfection with *P. malariae*	100,000
P. falciparum	19	16	*P. falciparum* with coinfection with *P. malariae*	500,000
P. falciparum	23	20	*Plasmodium* spp.	480

Table 4. Comparison of Ct values of commercial PCR and the in-house approach

	P. falciparum	*P. vivax*	*P. ovale*	*P. malariae*
Median Ct value in-house PCR	18	16.5	22.5	26
Median Ct value commercial PCR	15	16	21	20
Mean Ct value in-house PCR	18.6	16.3	22.5	26
Mean Ct value commercial PCR	15.7	15.8	21	20
Standard deviation in-house PCR	3.7	1.4	3.5	n.a.
Standard deviation commercial PCR	4.0	2.4	2.8	n.a.
P value in Wilcoxon matched-pairs signed ranks testing	$P < 0.0001$	$P = 0.4375$	n.a.	n.a.
P value of nonparametric Spearman correlation	$P < 0.0001$	$P = 0.0167$	n.a.	n.a.

n.a. = Not applicable

Discussion

The role of PCR for the diagnosis of malaria is still under ongoing debate. As PCR assays have been shown to be more sensitive and specific than other approaches, some authors argue that this technology should be regarded as the new golden standard for the diagnosis of malaria at least in non-endemic countries. These authors justify their claim with the fact that a negative PCR finding shows the highest reliability in excluding malaria infections, a matter of high importance in returning travelers with a low risk of acquiring malaria [8]. Diagnostic PCRs for, e.g., *P. falciparum*, typically target genes such as SSUrRNA, *pf155/resa*, or *cox1* [9]. Alternative diagnostic approaches such as flow cytometry and mass spectrometry that might allow for high-throughput screenings are still in experimental states [10].

More critical authors argue that several factors limit the use of PCR for the diagnosis of malaria at least in resource-limited high-endemicity settings. These factors comprise the time lag between sample collection, transportation and processing, and dissemination of results back to the physician in comparison to traditional microscopy next to limited financial resources, persistent subclinical parasitemia, and inadequate laboratory infrastructures in resource-limited, remote areas [11]. Such problems can be overcome in deployment situations, e.g., in medical field camps. Therefore, the developmental interest should be focused on potential technical limitations.

Indeed, the here described analysis showed good concordance between microscopic findings and PCR results in spite of a considerably high inhibition rate of the hemolytic samples as detected by the internal control approach of the commercial PCR. Regarding the overall detection of malaria, non-inhibited, truly discordant PCR results were observed in only two cases. Commercial PCR failed in one case without indicating inhibition. This sample contained a very low number of parasites. In contrast, commercial PCR was positive with a high cycle threshold value in a sample which was negative in in-house PCR as well as in microscopy. Of note, this sample was positive for hemozoin in matrix-assisted laser desorption–ionization time-of-flight mass spectrometry (MALDI-TOF-MS) [12, 13] (data not shown). This result makes a false-positive result, e.g., due to contamination, unlikely. Instead, it suggests either a case of extremely low parasitemia or a post-treatment case with parasite DNA and antigen remnants still circulating in the blood.

The high number of eight malaria cases that were missed due to inhibition of the commercial PCR is bothersome. Regarding this aspect, in-house PCR proved to be more inhibition-resistant than the commercial approach. In cases of concordant results between the two PCRs, however, cycle threshold values of the commercial PCR were lower, suggesting higher sensitivity. However, statistical significance of this phenomenon was observed for *P. falciparum* only, while detection of other *Plasmodium* ssp. was too rare for reliable statistical results.

Melting curve-based species identification of the in-house PCR approach allowed for the identification of one case of *P. falciparum* infection and two cases of *P. vivax* infections in which microscopy was not able to reliably discriminate the parasites on species level. This is in line with the widely accepted fact that PCR is more suitable for *Plasmodium* species identification than microscopy, in particular in case of low parasite counts, which make microscopic identification challenging [9].

However, discordant results of in-house PCR were observed for two cases of microscopically detected double infections. In case of a double infection with *P. falciparum* and *P. malariae*, only *P. falciparum* was identified by PCR. In the applied one-tube SybrGreen approach, the primers obviously reacted more readily with the quantitatively dominating DNA of *P. falciparum*, leading to a neglect of *P. malariae* coinfection. This is a known disadvantage of this procedure [7], although other molecular approaches were reported to be able to identify coinfections with different *Plasmodium* spp. even more reliable than microscopy [9]. In another instance, in-house PCR indicated *P. falciparum* although microscopy suggested *P. vivax* with a possible *P. malariae* coinfection. Obviously, schizonts of *P. vivax* and *P. malariae* had been observed in microscopy and the ring stages of *P. falciparum* had been attributed to one of these species. PCR, again, detected only DNA

of quantitatively dominating *P. falciparum* in this sample which contained two or maybe even three different *Plasmodium* spp. The disadvantage of the low discriminatory potential of the one-tube SybrGreen in-house approach in case of infections with more than one *Plasmodium* spp. has to be balanced against the obvious advantage of high robustness even with hemolytic samples as observed in this assessment.

The development of molecular approaches for the diagnosis of malaria is ongoing. The use of microfluidic and chip-based approaches has been suggested several years ago [14]. More recently, a review on currently introduced loop-mediated isothermal amplification (LAMP) schemes as robust molecular approaches for the detection of *Plasmodium* spp. has been published [15]. On the intermediate and long term, the targeted detection of drug resistance-related mutations to support targeted antimalarial therapy might become an option for the diagnostic routine setting in well-equipped laboratories [9].

Conclusions

The analysis presented here suggests that malaria PCR leads to reliable results even in cases of poor sample quality due to hemolysis of blood. The one-tube SybrGreen in-house approach proved to be more robust than the commercial assay, in particular with regard to inhibition tolerance, although the cycle threshold values were lower in the commercial PCR. If sample inhibition in the commercial assay and associated negative *Plasmodium* spp. specific PCRs are observed, other diagnostic approaches have to be applied in order to rule out malaria infections.

Acknowledgements

Simone Priesnitz, Christine Wegner, and Iris Zielke are greatly acknowledged for skillful technical assistance.

Declaration of interest

The authors declare that there are no conflicts of interest.

References

1. Frickmann H, Schwarz NG, Holtherm HU, Maaßen W, Vorderwülbecke F, Erkens K, Fischer M, Morwinsky T, Hagen RM: Compliance with antimalarial chemoprophylaxis in German soldiers: a 6-year survey. Infection 41, 311–320 (2013)
2. Abba K, Kirkham AJ, Olliaro PL, Deeks JJ, Donegan S, Garner P, Takwoingi Y: Rapid diagnostic tests for diagnosing uncomplicated non-falciparum or *Plasmodium vivax* malaria in endemic countries. Cochrane Database Syst Rev 12, CD011431 (2014)
3. Gillet P, Mori M, Van Esbroeck M, Van den Ende J, Jacobs J: Assessment of the prozone effect in malaria rapid diagnostic tests. Malar J 8, 271 (2009)
4. Gillet P, Scheirlinck A, Stokx J, De Weggheleire A, Chaúque HS, Canhanga OD, Tadeu BT, Mosse CD, Tiago A, Mabunda S, Bruggeman C, Bottieau E, Jacobs J: Prozone in malaria rapid diagnostics tests: how many cases are missed? Malar J 10, 166 (2011)
5. Mahajan B, Zheng H, Pham PT, Sedegah MY, Majam VF, Akolkar N, Rios M, Ankrah I, Madjitey P, Amoah G, Addison E, Quakyi IA, Kumar S: Polymerase chain reaction-based tests for pan-species and species-specific detection of human *Plasmodium* parasites. Transfusion 52, 1949–1956 (2012)
6. Frickmann H, Hinz R, Hagen RM: Comparison of an automated nucleic acid extraction system with the column-based procedure. Eur J Microbiol Immunol (Bp) 5, 94–102 (2015)
7. Mangold KA, Manson RU, Koay ES, Stephens L, Regner M, Thomson RB Jr, Peterson LR, Kaul KL: Real-time PCR for detection and identification of *Plasmodium* spp. J Clin Microbiol 43, 2435–2440 (2005)
8. Berry A, Benoit-Vical F, Fabre R, Cassaing S, Magnaval JF: PCR-based methods to the diagnosis of imported malaria. Parasite 15, 484–488 (2008)
9. Berry A, Fabre R, Benoit-Vical F, Cassaing S, Magnaval JF: Contribution of PCR-based methods to diagnosis and management of imported malaria. Med Trop (Mars) 65, 176–183 (2005)
10. Hawkes M, Kain KC: Advances in malaria diagnosis. Expert Rev Anti Infect Ther 5, 485–495 (2007)
11. Hänscheid T, Grobusch MP: How useful is PCR in the diagnosis of malaria? Trends Parasitol 18, 395–398 (2002)
12. Scholl PF, Kongkasuriyachai D, Demirev PA, Feldman AB, Lin JS, Sullivan DJ Jr, Kumar N: Rapid detection of malaria infection in vivo by laser desorption mass spectrometry. Am J Trop Med Hyg 71, 546–551 (2004)
13. Nyunt M, Pisciotta J, Feldman AB, Thuma P, Scholl PF, Demirev PA, Lin JS, Shi L, Kumar N, Sullivan DJ Jr: Detection of *Plasmodium falciparum* in pregnancy by laser desorption mass spectrometry. Am J Trop Med Hyg 73, 485–490 (2005)
14. Gascoyne P, Satayavivad J, Ruchirawat M: Microfluidic approaches to malaria detection. Acta Trop 89, 357–369 (2004)
15. Oriero EC, Jacobs J, Van Geertruyden JP, Nwakanma D, D'Alessandro U: Molecular-based isothermal tests for field diagnosis of malaria and their potential contribution to malaria elimination. J Antimicrob Chemother 70, 2–13 (2015)