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Sensitivity of nested-PCR for plasmodium detection in pooled whole blood samples and its usefulness to blood donor screening in endemic areas

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ABSTRACT

Transfusion-transmitted malaria is a severe disease with high fatality rate. Most Brazilian blood banks in the Amazon region perform malaria screening using microscopic examination (thick smears). Since low parasite concentrations are expected in asymptomatic blood donors a high sensitivity test should be used for donor screening. This study determined the sensitivity of a nested-PCR for plasmodium detection in pooled samples. We performed a one-stage criterion validation study with 21 positive samples pooled with samples from ten negative volunteer until three different concentrations were reached (0.33; 0.25; 0.20 parasites/μL – p/μL). Nested PCR was performed as described by Snounou et al. (1993). Sensitivities (and confidence intervals) were determined by stratum of final parasite concentration on the pooled samples. All samples with parasitemia values of 0.33 and 0.25 p/μL had 100% sensitivity (95%CI = 86.3–100). One negative result was obtained from a sample with 0.20 p/μL sensitivity = 95.2% (95%CI = 76.2–99.9). Compared to parasitemia detectable under ideal conditions of thick smear, this nested-PCR in pooled sample was able to detect 40 times more parasites per microliter. Nested-PCR in pooled samples should be considered as a high sensitive alternative to thick smear for donor screening in blood banks at endemic regions. Local authorities need to assess cost:benefit advantages of this method compared to alternatives.

1. Introduction

Malaria causes nearly 230 million clinical episodes every year across the world [1]. In the Brazilian Amazon region there are nearly 300 thousand episodes per year. Although rare compared to natural infection, transfusion-transmitted malaria (TTM) is a severe disease with a high fatality rate [2,3]. In Brazil, according to National Blood transfusion Surveillance System, there were four notified cases of TTM (*Plasmodium vivax*) since 2005, all fatal (personal communication).

Three important factors affect TTM: (1) malaria prevalence in blood donors, (2) donor selection by questionnaires (clinical sign/symptoms and epidemiological exposures) and (3) laboratory screening. In the Brazilian Amazon region endemic for malaria, the algorithm to select blood donors similarly uses questions about signs, symptoms and exposure history. After questionnaire screening parasitological laboratory test are mandatory for malaria screening. Although laboratory screening procedures with high sensitivity are desirable in blood

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banks, malaria detection remains a challenging task. Almost all blood banks in the Brazilian Amazon region rely on the microscopic examination of thick smears for malaria screening.

It is well known that examination of thick smears has several limitations when applied to screening donors for blood banking: (a) the expected low parasite counts in asymptomatic donors (incubating, asymptomatic plasmodium carriers, plasmodium species variations) can result in much lower sensitivity of thick smear examinations compared to nucleic acid test (NAT) [4]; (b) thick smear tests are very dependent on a technicians manual preparation and reading of slides, often subject to wide variations; (c) there is no consensus of how many fields of a malaria slide should be examined to call it "negative". The Brazilian Guidelines for Malaria Diagnostic Procedures recommend at least 500 fields be examined for a negative determination. Other studies recommend the examination of 100–500 fields [5–7].

Asymptomatic plasmodium carrier (APC) have low parasitemias [8,9] and are the source of most cases of TTM [10,11]. Worldwide, the use of the NAT to detect low and sub-microscopic parasite density cases of APC is widespread for research purposes. The use of the NAT as a malaria screening test in blood bank settings of endemic areas, should be evaluated as an alternative. Compared to thick smears, NAT is known to have higher sensitivity [4,12,13] and less dependency on human error. On the other hand, costs are higher than those of conventional screening tests. The use of pooled samples may overcome this limitation [14]. The screening of blood donors for malaria has a few key issues which must be addressed from both a practical and a scientific standpoint. This paper begins to looks at the practicality of pooling blood samples to defray costs.

It has been previously described that the nested polymerase chain reaction (nested-PCR), as described by Snounou et al. [15], is able to detect from 20.0 to 1.0 parasites/μL (p/μL), depending on protocols of DNA purification and sample type [16–19]; We present here a sensitivity analysis of the nested-PCR previously described [15], using pooled whole blood samples, and we discuss the usefulness and limitations of this procedure in blood banks to reduce costs and to improve safety of blood transfusions.

2. Materials and methods

2.1. Type of study

This is a one-stage criterion validation study for the assessment of sensitivity. The study was carried out in the context of a validation study for a pooled-sample PCR test, a potentially useful tool to screen for malaria-positive donors in blood bank settings. This study used slide confirmed malaria samples for *P. vivax* and *Plasmodium falciparum* from 20 patients and one *in vitro* culture of *P. falciparum*. Slide confirmed negative samples were obtained from 10 subjects who were not from a malaria endemic area.

2.2. Preparation of pooled samples

The parasite concentrations were determined on all malaria patients by thick smear. We then diluted these positive samples using whole blood from the negative samples until concentration of 1.0 p/μL (in different volumes) was reached. This lower limit was based on the assumption that 1.0 p/μL would be easily missed diagnosed as negative by routine thick smear screening of blood bank donors and occurs often enough to be dangerous among APC in populations of endemic areas. This first step represents the low parasitemias of patients presenting for blood donation who have passed clinical and epidemiologic screening procedures. In the next step, representing further dilutions by "pooling" of blood samples, we added different volumes of negative whole blood samples to reach a volume of 3.0 mL – attaining different final parasite concentrations, as follows: 0.33, 0.25 and 0.20 p/μL. The negative samples were not pooled before added in the positive samples. Once sensitivity dropped below 100% no further dilutions were done.

2.3. Microscopy

Thick smears were fixed in methanol and stained with 3% Giemsa for 30 min at room temperature. Parasite concentrations were determined by one experienced microscopists from the Julio Müller Hospital (a teaching hospital affiliated to the Mato Grosso Federal University) examining 200 fields.

2.4. Nested-PCR

i. *DNA extraction*. About 3.0 mL of whole blood was used for DNA extraction for PCR. The extraction was performed using the extraction kit Wizard® Genomic (DNA Purification Kit) from Promega®, as instructed by the manufacturer. After rehydration step, DNA was used for amplification immediately or storage at 2–8 °C.

ii. *Amplification and detection*. The nested-PCR method was previously described by Snounou et al. [15], with small changes. This strategy targets sequences for amplification of the 18S ribosomal subunit genes of three prevalent malaria parasites in Amazon region, *P. vivax*, *P. falciparum* and *Plasmodium malariae*. The genus amplification used the rPLU5 and rPLU6 primers. The specie-specific amplification used the rVIV1 and rVIV2 for *P. vivax*, rFAL1 and rFAL2 for *P. falciparum* and rMAL1 and rMAL2 for *P. malariae*. To reduce the risk of contamination and pipetting error during preparation of the reagent mixture (Mix), the PCR Master Mix, of Promega® was used. The final volume reaction was 25.0 μL including: 20.0 μL of PCR Master Mix (0.6 units of Taq DNA Polymerase, 400 μM of each DNTP, 3 mM of MgCl2 and Tris buffer pH 8.5), 1.0 μL of each primer at 6.25 μM, and 3.0 μL of DNA sample. All primers were purchased from IDT® (Integrated DNA Technologies, Inc., Coralville, Iowa, USA). The samples were amplified using a thermocycler (Mastercycler® Pro Eppendorf Scientific Inc., Westbury, NY, USA). 25 and 30 cycles for amplification were used in the genus and specie-specific amplification reaction, respectively. To visualize the

products of the nested PCR, an agarose gel electrophoresis containing 1.5% ethidium bromide was performed. Electrophoresis visualization was carried out using a photo documentation system. In all samples, a 1 kb marker (Invitrogen, Inc., USA) for further measurement of the molecular weight was used.

One negative control and one positive control for each *P. falciparum* and *P. vivax* were included alongside each run of DNA extraction, amplification and detection.

2.5. Data analysis

The gold standard for this study was the use of spiked, known positive samples (albeit to lower dilutions). The sensitivity was determined for each stratum of parasite concentration among the pooled samples. Sensitivity was stratified by dilution (parasite concentration) for pools. Pools with a positive result in the nested-PCR were considered true-positive cases. A binomial 95% confidence interval (95%CI) was calculated for each stratum.

2.6. Ethical approval

This study was approved by the Ethical Committee of Hospital Universitário Júlio Müller at Universidade Federal do Mato Grosso (Register #640/09) and by the Ethical Committee of Medicine School at Universidade de Brasília (Register #028/2009).

3. Results

There were 21 different positive samples with initial parasitemias ranging from 25 p/μL to 20,537 p/μL. These isolates were from patients contracting their infections from 12 different regions across the Brazilian Amazon. Eighteen samples were identified as *P. vivax* and three as *P. falciparum*, including one from *in vitro* culture. Negative samples were obtained from 10 different subjects from the city of Cuiaba, Mato Grosso state, Brazil. Each of the positive samples were diluted to 1.0 p/μL, then further diluted to 2:1, 3:1 and 4:1 (representing pools of 3, 4 and 5 samples) resulting in final parasite concentration of 0.33 p/μL, 0.25 p/μL and 0.20 p/μL, respectively. A total of 63 pooled samples (3 of each positive sample and of each parasite concentration) were tested. All pools with parasitemia values of 0.33 p/μL and 0.25 p/μL had a positive result in the nested-PCR. One negative result was obtained from pooled samples with 0.20 p/μL (*P. vivax*).

The sensitivities for the pooled samples are shown in Table 1. The sensitivity was 100% (95%CI = 86.3–100) for each of the 1:2 and 1:3 dilution pools. A slightly lower sensitivity result was obtained in the 1:4 dilution pool (sensitivity = 95.2%; 95%CI = 76.2–99.9).

Table 1
Sensitivity results for nested-PCR in the pooled samples using three dilution ratios ($n = 63$) from 21 positive samples for *Plasmodium* sp.

Pool	p/μL	+/total	Pool sensitivity (%)	95%CI[a]
1:2	0.33	21/21	100.0	86.3–100
1:3	0.25	21/21	100.0	86.3–100
1:4	0.20	20/21	95.2	76.2–99.9

[a] Binomial 95% confidence intervals.

4. Discussion

The use of NAT technology to screen blood bank donors for infectious agents is becoming more feasible through technological advances. There has been little done to study its practical application for malaria screening in real life settings, with the high cost of NAT a major deterring factor. The theoretical high sensitivity of NAT suggests that pooling of samples might be a way to reduce costs while maintaining test validity. Nested-PCR, as described by Snounou et al. [15], has shown high sensitivity during epidemiologic research when spot samples are obtained on filter paper [20].

However, PCR screening of pooled whole blood samples for blood bank should consider four issues which might affect sensitivity. 1. Degradation of target DNA during collection, storage or processing of samples. 2. Known ubiquitous inhibitors of PCR presenting in all donors (hemoglobin (Hb), immunoglobulin G (IgG), and lactoferrin [21]. 3. Unknown inhibitors (present in some of donors but not in others). 4. Dilution of target DNA (and inhibitors). This study looks at all but the third issue.

Mimicking a 1:3 or 1:4 pooling scenario for blood bank screening, we showed that dilution of positive blood sample with negative blood samples of three different negative donors do not affect the expected sensitivity of a standard nested-PCR [22]. The level of detection achieved in our experiment was compatible with other studies [20,22,23]. However, since the initiation of this study, Mahajan et al. [24] describes a newer method of plasmodium DNA purification from whole blood, which includes saponin incubation, that achieved a sensitivity of 0.002 p/μL, about a thousand fold increase above our observed sensitivity. This impressive sensitivity was reached in the context of a cultivated *P. falciparum* added in whole blood and with a different set of primers. In our protocol, adding saponin was not performed since Promega extraction kit Instructions do not included this step. But even with the potential for Mahajan's new methods to greatly increase the sensitivity (implying reduced cost by adding more donors to the pools and/or detecting even lower levels of parasitemias which still could cause TTM) this methods must be studied under conditions mimicking blood bank conditions because of the potential for inhibitor issues (item 3, above).

The inhibitory effect of hemoglobin (Hb) and other inhibitory factors on PCR limits the volume of blood that can be used in the PCR-based detection of intraerythrocytic plasmodium parasites [25] and, consequently, the pool size. For the known inhibitors it was assumed that all donors in our study have more or less the same quantity and quality of these types of inhibitors, so pooling samples would represent all donors in this respect. There may be other, unidentified PCR inhibitors in whole blood (in both malaria positive as well as malaria negative samples) only detectable by screening large numbers of donors. Our source of only 10 negative donors was too small to guarantee a low prevalence of these types of rarer, individual

inhibiting factors [21]. This issue should be resolved in a future study incorporating a much larger number of negative samples. In theory, as the PCR test is modified to increase its sensitivity to dilution, any attempts to increase the pool size to reduce costs could increase the chance of these types of inhibitors, resulting in false negative tests. Until this research is done, perhaps a positive control could be used to detect false negatives, but this would double the material cost of testing.

It is also necessary to consider the costs and benefits for using lower volume of blood in each pool to reduce inhibitor effects. This is because a PCR result could be seen as a "competition" between target DNA (usually very low) and inhibitors. On one hand, the probability to included plasmodium infected red cells tends to increase as a higher blood volume is tested. On the other hand, based on Mahajan et al. [24] results, it is possible that the limiting effect of inhibitors will tend to decrease if lower blood volume is tested. Therefore, we may question if lower blood volume in each pool could enhance the PCR sensitivity and overcome the low parasitemia limitation.

Under ideal conditions, thick smear examinations are able to detect between 10 and 50 p/μL [26,27]. In practice one can only detect parasites at 10 times these concentrations (100–500 p/μL) [28,29]. Furthermore, in areas where slides are not routinely read (imported malaria), the lack of expertise among microscopists has been a major problem [7,12,23]. This same argument can be applied to those who screen blood donors by slide examinations in blood banks. They may see very few cases because the clinical/epidemiological screening excludes all cases except for those with nearly undetectable, though infectious, parasitemias. In our study nested-PCR in pools of four samples using whole blood was able to detect 0.25 p/μL with 100% sensitivity (1:3 dilution). This parasite concentration is 40 times less than ideal conditions for thick smear test or 400 less than practical conditions. Furthermore, this increased sensitivity is under the conditions of pooled samples which not only reflects dilutional factors but also the relative increased concentration of the known inhibitors present in all whole blood samples (i.e. had we diluted to final volume with only serum the inhibitors would not be present). Since the continued use of thick smear for blood bank screening has a high risk of false-negative results, some authors have discussed the importance of the NAT to better understand the epidemiology of malaria as it relates to TTM [17,30,31]. Fujikaha et al. [32] using nested-PCR, found up to 3% prevalence in blood donors from Amazon region. Another study using Real-Time PCR found a prevalence of 1.3% *P. vivax* infections in blood bank donors of an endemic region (State of Para) [33]. These authors concluded that a more sensitive method of diagnosing malaria is necessary in blood banks in endemic regions. However, the high cost of NAT compared to thick smear is considered an obstacle to its implementation. Even though it is economically attractive to consider only a genus screen as the first step, the poor sensitivity reported by others, when a single genus-specific PCR amplifications is performed (set of primers rPLU 1–rPLU 5; rPLU 3–rPLU 4; rPLU 5–rPLU 6), has shown this to be too inaccurate to use as a screening test [15,18].

Under the limitation of our study (small number of negative subjects used for pooling) nested-PCR proved to be a good alternative laboratory technique for blood bank screening compared to standard examination of thick smears. As sensitivity to detect even more dilute DNA improves [24] enabling larger numbers in pools, larger number of blood donors need to be studied to assess whether or not individuals carry unidentified PCR inhibitors, and if so, in concentrations high enough to result in false negatives of pooled samples. It would also be necessary to conduct cost-effectiveness analysis comparing to standard malaria screening algorithms.

In Brazil, the directives of the National Health Surveillance Agency (ANVISA) and Ministry of Health have made malarial screening of blood bank donors mandatory in endemic regions. The directives require that tests need to have high sensitivity and that the NAT can be used for single or pooled samples. However, in practice Brazilian blood banks usually base malaria screening on questionnaire data and thick smear tests. Our study shows the promise of a better laboratory test, which someday may be cheaper through pooling protocols. This study has some subtle limitations. The tested samples are not fully independent. Although each pool (of a stratum) has a different positive source – the negatives come from a smaller pool and are replicated across all the positive samples. This may have artificially decreased the variance of the data and the 95%CI may have been underestimated. Furthermore, test results were not read blindly. However, unlike reading of slides, this is a minor problem since the technique's outcomes are very objective/automated with little influenced of the outcome by observers. We did not include *P. malariae* in our samples since these are very rare parasites causing malaria infection in Brazil.

In conclusion, nested-PCR in pooled samples is a promising alternative to thick smear for screening for malaria in blood banks in endemic regions. Blood bank managers and regulatory authorities should consider the level of sensitivity, number of samples to be tested in each pool and expected parasite concentrations to ensure the minimum risk for TTM. Finally, cost:benefit considerations, specific to each endemic area, should determine the relative advantages among different laboratory procedures screening blood bank donors for malaria.

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References

[1] WHO: World malaria report: 2010. WHO; 2010. p. 204.
[2] Garraud O, Relave J, Flori P, Perraut R. Post-transfusion malaria: is the risk irreconcilable with biological silence? Transfus Clin Biol 2004;11:87–94.
[3] Mungai M, Tegtmeier G, Chamberland M, Parise M. Transfusion-transmitted malaria in the United States from 1963 through 1999. New Engl J Med 2001;344:1973–8.
[4] Alves F, Durlacher R, Menezes M, Krieger H, Silva L, Camargo E. High prevalence of asymptomatic *Plasmodium vivax* and *Plasmodium*

falciparum infections in native Amazonian populations. Am J Trop Med Hyg 2002;66:641–8.
[5] Steenkeste N, Incardona S, Chy S, Duval L, Ekala MT, Lim P, et al. Towards high-throughput molecular detection of Plasmodium: new approaches and molecular markers. Malar J 2009;8:86.
[6] Stauffer WM, Cartwright CP, Olson DA, Juni BA, Taylor CM, Bowers SH, et al. Diagnostic performance of rapid diagnostic tests versus blood smears for malaria in US clinical practice. Clin Infect Dis 2009;49:908–13.
[7] Nkrumah B, Agyekum A, Acquah SE, May J, Tannich E, Brattig N, et al. Comparison of the novel Partec rapid malaria test to the conventional Giemsa stain and the gold standard real-time PCR. J Clin Microbiol 2010;48:2925–8.
[8] Camargo EP, Alves F, Pereira da Silva LH. Symptomless *Plasmodium vivax* infections in native Amazonians. Lancet 1999;53:1415–6.
[9] Oner YA, Okutan SE, Artinyan E, Kocazeybek B. Malaria problem in Afghanistan: malaria scanning results of the Turkish medical aid group after the war. Transfus Apher Sci 2005;32:133–7.
[10] Kitchen AD, Barbara JA, Hewitt PE. Documented cases of post-transfusion malaria occurring in England: a review in relation to current and proposed donor-selection guidelines. Vox Sang 2005;89:77–80.
[11] Slinger R, Giulivi A, Bodie-Collins M, Hindieh F, John RS, Sher G, et al. Transfusion-transmitted malaria in Canada. CMAJ 2001;164:377–9.
[12] Rosanas-Urgell A, Mueller D, Betuela I, Barnadas C, Iga J, Zimmerman P, et al. Comparison of diagnostic methods for the detection and quantification of the four sympatric Plasmodium species in field samples from Papua New Guinea. Malar J 2010;9:361.
[13] Rantala A, Taylor S, Trottman P, Luntamo M, Mbewe B, Maleta K, et al. Comparison of real-time PCR and microscopy for malaria parasite detection in Malawian pregnant women. Malar J 2010;9:269.
[14] Taylor S, Juliano J, Trottman P, Griffin J, Landis S, Kitsa P, et al. High-throughput pooling and real-time PCR-based strategy for malaria detection. J Clin Microbiol 2010;48:512–9.
[15] Snounou G, Viriyakosol S, Zhu XP, Jarra W, Pinheiro L, do Rosario VE, et al. High sensitivity of detection of human malaria parasites by the use of nested polymerase chain reaction. Mol Biochem Parasitol 1993;61:315–20.
[16] Andrade BB, Reis-Filho A, Barros AM, Souza-Neto SM, Nogueira LL, Fukutani KF, et al. Towards a precise test for malaria diagnosis in the Brazilian Amazon: comparison among field microscopy, a rapid diagnostic test, nested PCR, and a computational expert system based on artificial neural networks. Malar J 2010;9:117.
[17] Proux S, Suwanarusk R, Barends M, Zwang J, Price RN, Leimanis M, et al. Considerations on the use of nucleic acid-based amplification for malaria parasite detection. Malar J 2011;10:323.
[18] Singh B, Bobogare A, Cox-Singh J, Snounou G, Abdullah MS, Rahman HA. A genus- and species-specific nested polymerase chain reaction malaria detection assay for epidemiologic studies. Am J Trop Med Hyg 1999;60:687–92.
[19] Fuehrer HP, Fally MA, Habler VE, Starzengruber P, Swoboda P, Noedl H. Novel nested direct PCR technique for malaria diagnosis using filter paper samples. J Clin Microbiol 2011;49:1628–30.
[20] Hsiang MS, Lin M, Dokomajilar C, Kemere J, Pilcher CD, Dorsey G, et al. PCR-based pooling of dried blood spots for detection of malaria parasites: optimization and application to a cohort of Ugandan children. J Clin Microbiol 2010;48:3539–43.
[21] Kermekchiev MB, Kirilova LI, Vail EE, Barnes WM. Mutants of Taq DNA polymerase resistant to PCR inhibitors allow DNA amplification from whole blood and crude soil samples. Nucl Acids Res 2009;37:5.
[22] Hwang J, Jaroensuk J, Leimanis ML, Russell B, McGready R, Day N, et al. Long-term storage limits PCR-based analyses of malaria parasites in archival dried blood spots. Malar J 2012;11:339.
[23] Ndao M, Bandyayera E, Kokoskin E, Gyorkos TW, MacLean JD, Ward BJ. Comparison of blood smear, antigen detection, and nested-PCR methods for screening refugees from regions where malaria is endemic after a malaria outbreak in Quebec, Canada. J Clin Microbiol 2004;42:2694–700.
[24] Mahajan B, Zheng H, Pham PT, Sedegah MY, Majam VF, Akolkar N, et al. Polymerase chain reaction-based tests for pan-species and species-specific detection of human Plasmodium parasites. Transfusion 2012;52:1949–56.
[25] Al-Soud WA, Radstrom P. Purification and characterization of PCR-inhibitory components in blood cells. J Clin Microbiol 2001;39:485–93.
[26] Suárez-Mutis MC, Coura JR. Evaluation of the thick smear in a field condition in a malaria endemic area in the Middle Region of Rio Negro, Amazon. Rev Soc Bras Med Trop 2006;39:495–7.
[27] Moody A. Rapid diagnostic tests for malaria parasites. Clin Microbiol Rev 2002;15:66–78.
[28] Milne LM, Kyi MS, Chiodini PL, Warhurst DC. Accuracy of routine laboratory diagnosis of malaria in the United Kingdom. Clin Pathol 1994;47:740–2.
[29] Guerin PJ, Olliaro P, Nosten F, Druilhe P, Laxminarayan R, Binka F, et al. Malaria: current status of control, diagnosis, treatment, and a proposed agenda for research and development. Lancet Infect Dis 2002;2:564–73.
[30] Shehata N, Kohli M, Detsky A. The cost-effectiveness of screening blood donors for malaria by PCR. Transfusion 2004;44:217–28.
[31] Ali MS, Yousif AG, Mustafa MS, Ibrahim MH. Evaluation of malaria parasite screening procedures among Sudanese blood donors. Clin Lab Sci 2005;18:69–73.
[32] Fugikaha E, Fornazari PA, Penhalbel RSR, Lorenzetti A, Maroso RD, Amoras JT, et al. Molecular screening of *Plasmodium* sp. asymptomatic carriers among transfusion centers from Brazilian Amazon region. Rev Inst Med Trop Sao Paulo 2007;49:1–4.
[33] Batista-Dos-Santos S, Raiol M, Santos S, Cunha MG, Ribeiro-Dos-Santos A. Real-time PCR diagnosis of *Plasmodium vivax* among blood donors. Malar J 2012;11:345.