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Considerations on PCR-based methods for malaria diagnosis in China malaria diagnosis reference laboratory network

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Summary **Precise diagnosis is a key measurement for malaria control and elimination, traditional microscopy and rapid diagnostic tests cannot satisfy the requirements especially in the low transmission endemic areas or in the malaria elimination phase. Polymerase chain reaction (PCR) with high sensitivity and specificity can be considered as a diagnostic standard while no uniform PCR assay was established due to variations in their performance and lack of formal external quality assurance programs for validation for PCR assays in use. Here, 24 articles including 43 paired comparative evaluations limited to paired comparison of diagnostic performance between real-time PCR and conventional PCR to detect plasmodium in blood samples of human subjects from clinics or the field are systematically summarized. And according to the Landis and Koch classification, nineteen pairs showed almost perfect agreement, followed by 8 pairs of moderate agreement and 4 pairs of good agreement, while the kappa values of 12 pairs couldn't be examined. Moreover, the performance of 14 pairs were completely the same and 8 pairs had no differences, but 14 pairs were significant different including 8 pairs of real-time PCR with better performance than conventional PCR. Therefore, it is still an outstanding issue to choose PCR methods, and more work such as the standardization of materials and methods in use and their availability are needed to settle priority to better promote the role of malaria diagnosis reference laboratories.**

Keywords: *Plasmodium species*, real-time PCR, conventional PCR, reference laboratory, quality assurance

1. Introduction

One of the strategies to control and eliminate malaria is the precise laboratorial diagnosis in order to recognize infection promptly and treat positive cases appropriately. A variety of diagnostic methods are used for plasmodium parasites identification and speciation.

Malaria microscopy on Giemsa-stained thick and thin blood smears is still the gold standard method for malaria diagnosis, clinical trials efficacy evaluation and epidemiological surveys. However, it has many limitations such as low sensitivity detection limit, poor specificity due to morphological changes that are enhanced by the staining and the similarities between several parasites, and operator dependence that even highly qualified microscopists can make an incorrect or incomplete assessment of the *Plasmodium* species (*1*).

Alternatively, a variety of rapid diagnostic tests (RDT) for plasmodium antigen detection have been developed for use with sensitivity above 90% if > 100 parasites/μL, and a result can be obtained within half an hour by nonskilled technicians (*2*). But, there is low sensitivity if < 100 parasites/μL, and false positives particularly after treatment, and limitations for *Plasmodium* species speciation and case review.

Nucleic acid tests based on polymerase chain reactions (PCR) have high sensitivity and specificity to detect and identify pathogens. This is primarily useful for epidemiological investigations of malaria because the

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infections are frequently associated with asymptomatic and/or microscopically sub-patent parasite levels and various mixed infections. Numerous PCR assays have been developed for the laboratory diagnosis of malaria, including conventional and real-time PCR techniques, that allow the differentiation of all five species of human Plasmodium with as few as five parasites per microliter of blood and even probably as low as 0.002 parasites/μL (*3,4*). Moreover, the nested PCR developed by Snounou *et al*. (*5*) has been widely used and considered as the molecular gold standard for malaria parasites detection due to its good performance (*6*) and it also was recommended as the confirmatory test documented in the manual of China Malaria Diagnosis Reference Laboratory Network (*7*). Meanwhile, malaria case diagnosis according to the laboratorial detection based on nucleic acids of plasmodium parasites was firstly documented in the new version of 'Diagnosis of malaria (WS 259-2015)' in China (*8*), although no detailed protocol was mentioned. Additionally, the use of malaria real-time or quantitative PCR as a confirmatory endpoint assay in field research has increased exponentially. Several provincial malaria diagnosis reference laboratories recommended the application of real-time PCR for malaria cases confirmation and quality assurance of malaria diagnosis in the network due to its convenience and time saving. Therefore, the issue is not which PCR method should be adopted but whether PCR be the accepted gold standard, and it is necessary to compare the benefits of different PCR methods, optimize the protocol, and train staff in the reference laboratory network.

2. Comparison of Real-time PCR and Conventional PCR Assays for malaria diagnosis

A total of 24 articles about 14 clinical studies and 10 field studies related to pair comparison of real-time PCR and conventional PCR to detect samples of human subjects from clinics or the field rather than model organisms for malaria parasites were enrolled (Supplementary Table 1, *http://www.biosciencetrends.com/action/getSupplementalData.php?ID=29*) (*9-32*), and agreement of paired methods was determined by calculating Kappa Statistics. The kappa values (κ) were interpreted with the Landis and Koch classification (*33*): $\kappa < 0$, poor agreement; $\kappa = 0.01$-0.20, slight agreement; $\kappa = 0.21$-0.40, fair agreement; $\kappa = 0.41$-0.60, moderate agreement; $\kappa = 0.61$-0.80, good agreement; and $\kappa = 0.81$-1.00, almost perfect agreement, respectively. Moreover, the differences of the detection levels of compared PCR assays were analyzed by the χ^2 test or Fisher exact test, $p < 0.05$ was considered statistically significant.

2.1. *PCR assays for malaria parasites detection*

Six fluorescence-reporting systems (TaqMan [13/24], SYBR green [6/24], Photo-induced electron transfer fluorogenic primer (PET) [2/24], molecular beacon probes [1/24], LightCycler probes [1/24] and Fluorescence resonance energy transfer (FRET) [1/24]) were used. 14 different real-time PCR methods were used in the 14 studies, and another two methods were used in two studies each respectively.

In respect to conventional PCR assays, nested PCR was the most selected method (18/24) used. Moreover, five and six studies applied multiplex PCR and simple PCR respectively. In addition, two classical nested PCR assays developed by Snounou *et al*. (11/24) (*5*) and Singh *et al*. (3/24) (*34*) were used extensively.

2.2. *Target molecules*

Plasmodium 18S rRNA gene was used most frequently in both real-time PCR assays (21/24) and conventional PCR assays (21/24), and the *Plasmodium falciparum*-specific cytochrome c oxidase subunit 1 (Cox1) gene was used in two studies of real-time PCR, and the *Plasmodium* cytochrome B gene, *Plasmodium ovale*-specific reticulocyte-binding protein 2 (Porbp2) gene and mitochrondrial cytochrome C oxidase gene for *Plasmodium falciparum* and *Plasmodium vivax* were used in one paper each by real-time PCR (Supplementary Table 1, *http://www.biosciencetrends.com/action/getSupplementalData.php?ID=29*). Meanwhile, merozoite surface protein 2 (MSP2) gene, cytochrome B gene, *Plasmodium ovale* tryptophan-rich antigen (*Potra*) gene and mitochrondrial cytochrome C oxidase gene were selected in conventional PCR assays (Supplementary Table 1, *http://www.biosciencetrends.com/action/getSupplementalData.php?ID=29*).

2.3. *Agreement analysis*

IA total of 43 paired comparative evaluations performed in the 24 enrolled studies, and 19 pairs showed almost perfect agreement, followed by 8 pairs of moderate agreement and 4 pairs of good agreement, while the kappa values of 12 pairs couldn't be examined directly or calculated using the data provided, partly due to the fact that the identification of *Plasmodium* spp were not identical although the number of positive and negative samples were the same detected by both real-time PCR and conventional PCR assays.

2.4. *Differences in detection performance*

The results of 14 paired comparisons (14/43) were completely the same in respect to the composition of positive and negative samples, and 8 pairs were between real-time PCR and nested PCR. Moreover, there were 8 paired analyses that showed no differences in detection capacity between real-time PCR and conventional PCR (including 4 nested PCR, 2 simple

PCR and multiplex PCR each) ($p > 0.05$), but 14 pairs were significantly different including 8 pairs of real-time PCR with better performance than conventional PCR (including 4 nested PCR, 2 simple PCR and multiplex PCR each) ($p < 0.05$). In addition, detection capacity can't be compared in several studies because not all of the samples were tested by the comparative assays due to insufficient sample volumes.

3. Challenges and prospects

Precise diagnosis and confirmatory identification of malaria infection is crucially important, especially in low-density parasitaemia or sub-microscopic infections, which is an important reservoir of malaria parasites and a big challenge for malaria elimination and prevention of re-introduction. Quality assurance performed by reference laboratories is a key tool to guarantee and maintain the performances of diagnostic assays. PCR with much more sensitivity and specificity than microscopy and RDT is a useful tool for confirmatory identification of *Plasmodium* spp, and it could be used in a quality assurance scheme to provide an excellent quality control for results obtained by conventional microscopy and other diagnostic methods to ensure that results are reliable and comparable.

Unlike in routine clinical practice for malaria diagnosis, the time lag between sample collection, transportation and processing, and dissemination of results (*35*) is less important than the precise result in quality assurance which is a key role of the reference laboratory. Factors such as limited financial resources and inadequate laboratory infrastructures hindered the application of PCR assays previously has been improved significantly in the China Malaria Diagnosis Reference Laboratory Network. Additionally, nucleic acids detection based on PCR for malaria parasites has been recommended as the confirmatory test documented in the manual of China malaria diagnosis reference laboratory network and the diagnosis standard of 'Diagnosis of malaria (WS 259-2015)' in China. Nevertheless, a uniform PCR method considered as the gold standard to formalize and extend the exercise couldn't be determined due to a wide variation in the performance of the numerous PCR methods.

In view of the summary of agreement analysis and performances in detecting clinical and field samples from 43 comparative evaluations between real-time PCR and conventional PCR (including nested PCR, multiplex PCR and simple PCR) provided in the 24 articles above, it is still difficult to select the best PCR assay used as standard protocol for malaria diagnosis, although real-time PCR seems better than conventional PCR. Moreover, the standardization of PCR templates including positive as well as negative ones for controls in reference laboratories should be a priority. It will help researchers in different conditions to ensure that the nucleic acid amplification protocols they use provide the requisite level of sensitivity, and permit comparison between sites and the efficacy of various PCR protocols. Only *Plasmodium falciparum* DNA standard material was established, but it is not widely available, and no application was found in these 24 articles. Moreover, the standardization of materials and source material in use are also important to ensure the compatibility of tests, because of the differences in the target molecules, space, time, reagents or enzymes, the type of thermocycler used, and/or subtle variations, and even data interpretations, and much more, can cause variations.

Although no significant differences were found in the performance between real-time PCR and conventional PCR for malaria diagnosis above, and nested PCR represents the most appropriate techniques for detecting malaria parasites, but the advantages of quantification of parasite densities, a less laborious workflow, rapidity, lower contamination and more possibility for the diagnosis of low-level parasitaemia, contribute to real-time PCR to be an appropriate method used in reference laboratory and routine malaria diagnosis, especially for asymptomatic infections. Furthermore, very few quality control programs for PCR protocols about malaria diagnosis were published (*7,36,37*), and it is time to develop a formal external quality assurance scheme to provide validation for PCR assays in use, and ensure accurate diagnosis (*38*). In addition, differences in protocols of sample collection, storage, and DNA extraction etc. can influence the specificity and sensitivity of PCR amplification.

In conclusion, PCR assays should be considered to be the gold standard for malaria diagnosis, but more work should be needed to settle the issue of which PCR method is adopted. Moreover, the standardization of materials and methods in use and their availability must be given priority in the process of the development to better promote the role of malaria diagnosis reference laboratorys.

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