ORIGINAL RESEARCH ARTICLE

EXPLORING THE SUBSTANTIAL ROLE OF MULTIPLEX PCR TO DIAGNOSE MALARIA IN EASTERN NEPAL

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ABSTRACT

Background: Malaria is still a major health problem in Nepal as 43.3% of population are at risk of infection. However, the national priorities have been given to achieve malaria elimination in Nepal, the question of diagnostic accuracy could jeopardize the elimination target. Among others, multiplex PCR could be the reliable alternative tool to detect the low number of parasitemia and circulating asymptomatic infection in low endemic countries like Nepal. Hence, this paper aims to analyse the diagnostic precision among microscopy, rapid diagnostic test (RDT) and multiplex real time PCR assay.

Methods: A total of 63 archived blood samples obtained from patient with clinical suspicion of malaria were subjected to multiplex PCR and their results were compared with microscopy and rapid diagnostic test. The Latent Class Analysis (LCA) approach was used to compare the diagnostic precision among three different assays.

Results: Our study documented that PCR had 97.4% sensitivity and 83.9% specificity. Among the negative microscopy results, 10 (15.9%) were false negatives whereas 4 (6.3%) RDT results were false negatives based on PCR results.

Conclusions: PCR could be the useful alternative tool to detect the low number of parasite in low endemic settings which could be crucial to rule out the false diagnosis determined by microscopy or RDT.

INTRODUCTION

Malaria is the infection of humans caused by protozoan parasites belonging to *Plasmodium* genus and transmitted through the bites of an infected female *Anopheles* mosquito. It has recently been reported that, worldwide 219 million cases in 2017, an increase from 217 million cases in 2016. The number of death due to malaria is estimated to have decreased from 445,000 in 2016 to 435,000 in 2017.[1] The five *Plasmodium* species which cause malaria in human are *P. falciparum, P. vivax, P. malariae, P. ovale* and *P. knowlesi.*[1-3] The first three species are distributed across Africa, Asia and South-Central America, whereas *P. ovale* is mainly found in Africa and *P. knowlesi* in Asia.[4,5] The global malaria elimination programme has planned to reduce the malaria incidence and mortality rates by at least 90% by 2030.[1] In Nepal, national programme has aim of malaria-free by 2026. The main strategies of malaria elimination programme include the early and accurate diagnosis and correct treatment of malaria cases.[6] Despite the availability of the microscopy and rapid tests, the accurate malaria diagnosis has still been

challenged by several other factors, such as sensitivity and specificity of the diagnostic tool, age, and transmission intensity in low endemic settings.[7-10]

The most common diagnostic tools currently available for the identification of *Plasmodium* infection include microscopy, immunochromatographic assay (also known as rapid diagnostic test, RDT), fluorescence microscopy and molecular tool such as polymerase chain reaction (PCR).[11] The microscopy has poor sensitivity in low transmission setup and in asymptomatic individuals, resulting in underestimation of malaria prevalence as compared to the highly sensitive DNA detection by PCR.[12,13] Likewise, RDT assay also shares similar limitations with microscopy,[14] and have been shown to be insufficient sensitivity for screening low parasitemia in case of asymptomatic carrier.[15] Hence, diagnostic tools with better analytical sensitivity are always required to detect the low level of parasite in order to achieve the goal of global malaria elimination programme. In this context, different format of the multiplex PCR are widely used not only to increase the sensitivity of the diagnosis but also for the correct speciation of the circulating parasites but these tests suffer from cost and demand technical expertise for its use in resource-limited setting like ours.[16]

In this study, PCR results are compared with microscopy and RDT and the explicit role of PCR has also been explored by using latent class model. The development of classical frequentist and bayesian statistical approaches for evaluation of the diagnostic tests without considering the gold standard test has been now increasingly applied to many tropical diseases.[17] Thus, Latent class models with two latent classes are widely used to estimate the sensitivities and specificities of test in the absence of a gold standard. In case of malaria diagnosis, microscopy has been considered as the gold standard. However, admitting microscopy as a reference technique impairs the sensitivity.[18] To the best of our knowledge, no publication regarding the exploration of latent class models for malaria diagnosis in Nepal so far exists in the literature. Therefore, this work was undertaken to assess the performance and explore the accuracy of three diagnostic tests for malaria, using Bayesian approach.

METHODS

Study design

This is the experimental design in order to explore the analytical performance of multiplex PCR for the diagnosis of malaria. Archived samples collected from the routine diagnosis of malaria were re-tested by PCR. All these samples were obtained from the patients with acute febrile illness with the clinical suspicion of malaria. All samples were obtained from the archival storage at -80ºC in the department of microbiology at B P Koirala Institute of Health Sciences (BPKIHS), Dharan, a tertiary care centre located in Eastern Nepal. Due to the storage condition, the integrity of the samples was well maintained. Altogether, 63 blood samples in EDTA containing vials were recovered from the storage which were collected from January 2009 to August 2010.

Blood film Microscopy

According to the clinical requisition, all samples were subjected for blood film microscopy. Thick and thin smear of blood were prepared by spreading 20 µl of blood in a clean slide, which were dried and stained with 5% Giemsa stain for 30 minutes. Smears were then viewed at 1000X magnification with oil immersion. Smears were considered negative if no parasite was seen in 200 consecutive fields in a thick blood smear. Slides were stored in a secure slide box and were again reconfirmed by microscopic investigation in order to avoid the mismatch of the samples for PCR analysis.

RDT

All samples were tested with the rapid test. This test utilizes a dipstick coated with monoclonal antibodies against the intracellular metabolic enzyme, pLDH, produced by viable malarial parasites. Differentiation of malaria species is based on antigenic differences among the pLDH isoforms. The assay was done by following the manufacturer's instruction. In short, one drop of whole blood was mixed with 2 drops of reagent A, which disrupts the erythrocytes and releases the pLDH, and the specimen was allowed to migrate to the top of the test strip. After 8 min, the rapid test strip was cleared by adding 2 drops of reagent B. The appearance of a dark band on the strip indicates a positive reaction for any one

of the four major malaria. The monoclonal antibody at this site is one against an enzyme common to the four target *Plasmodium* species. If *P. falciparum* was present in the test sample, a second band appeared on the strip. The monoclonal antibody at this site is specific for *P. falciparum* only. A mixed infection with other species is indicated when both genus- and species-specific bands appear and genus-specific band is much darker and more intense than the species-specific band. A test control band appears at the top of the strip as an indicator that the test is valid and working correctly.

Polymerase chain reaction

DNA was extracted from human blood using the QiaAmp DNA mini kit (Qiagen, www.qiagen.com). Briefly, 200 μL blood was taken for DNA extraction, the product after proteinase K digestion was filtered through QIAmp column and washed with AW1 and AW2 buffer. DNA was eluted in 200 μL AE buffer. DNA concentration and purity was verified by spectrophotometric measurement with the NanoDrop 2000 (Thermo Scientific, Waltham, MA, USA). Real-time PCR was performed in Rotor Gene Q (Qiagen) machine by using commercially available abTES Malaria 5 qPCR II kit (AITbitech, Singapore). The reaction was performed with a final volume of 25 μL containing, 6μL of Reaction mix, 2μL of Primer/Probe mix, 12μL of nuclease free water and 5 μL of DNA template. The PCR thermocyclic program was performed as taq activation 95°C for 2 min, followed by 45 cycle of 95°C for 5 sec, and annealing at 60°C for 20 sec. Five Plasmodium species specific fluorescent hydrolysis probes, FAM for *P. falciparum*, ROX for *P. vivax*, HEX for *P. malariae*, Cy5 for *P. ovale* and Q705 for *P. knowlesi* were read to determine the Cq value of each sample. The positive reference control and PCR water as a no-template control are used to validate the each batch of PCR.

Data analysis

The statistical analysis of three diagnostic tests: microscopy, RDT diagnosis and PCR (binary variable taking the value: 1 = positive versus 0 = negative) were taken into account a unique dataset of samples with respective frequency as indicated in Table 1. We used Latent Class Analysis (LCA) to assess diagnostic test accuracy.[19] In contrast to the gold standard approach, LCA hypothesize the existence of one or more unobserved categorical variables to explain in relationship between a set of observed categorical variables. We assume the latent variable corresponds to multiple positive diagnoses as the unobserved true disease status of the patients.[20] Sensitivity and specificity of the individual tests were estimated from the LCA approach and also compared with the gold standard approach. The sensitivity is the probability of the respective test to identify the subject correctly as infected under the condition that the subject is infected. On the other hand, specificity is the probability of correctly identifying a subject as not infected. Since, the true infection status is unknown the latent class method constructs a hypothetical standard, and assesses the individual test's performance with respect to this hypothetical standard.[21] The 95% confidence limits were calculated using bootstrap methods.[22] All analysis were performed with statistical software R 3.3.2[23] in support of packages randomLCA and BayesLCA.[24]

RESULTS

Table 1: Samples frequencies of each pattern of test results (N=63).

Microscopy	RDT	PCR	Frequency
0	0	0	19
0	0	1	4
0	1	1	6
1	0	0	2
1	1	0	1
1	1	1	31

Note: 1= Positive test result, 0= Negative test result

A total 63 blood samples were tested by three malaria diagnostic methods and the results were shown in Table 1. Microscopy was positive in 34 (53.97%) whereas 38 (60.32%) RDT tested positive. PCR documented infection in 41 (65.08%). The diagnostic agreement was found higher between PCR and RDT (kappa = 0.83, p-value <0.0001) followed by microscopy and RDT (kappa = 0.742, p-value<0.0001), PCR and microscopy (kappa = 0.577, p-value <0.0001). Prior to the Bayesian analysis, the disagreement

among three tests were also analysed with McNemar test, but none of them were significant (p-value > 0.05).

The posterior estimation of three diagnostic assays using Bayesian latent class analysis were presented in Table 2. In Bayesian approach, the sensitivity of diagnostic test was were highest with RDT 99.9% (95% CI, 99.7% to 99.9%) followed by PCR 97.4% (95% CI, 83.5% to 99.6%) and microscopy 84.2% (95% CI, 53.0% to 95.0%). The specificity of RDT 99.9% (95% CI, 99.6% to 99.9%) was highest followed by Microscopy 91.9% (95% CI, 91.9% to 97.9%) and PCR 83.9% (95% CI, 64.3% to 93.9%).

In this study, two *Plasmodium* species (*P. falciparum* and *P. vivax*) were determined by PCR assay and four cases were infected with both species. Among different age groups, 20 to 40 years (24/31) were mostly found positive by PCR. Out of 4 mixed infections, three cases were found in age groups of 20 to 40.
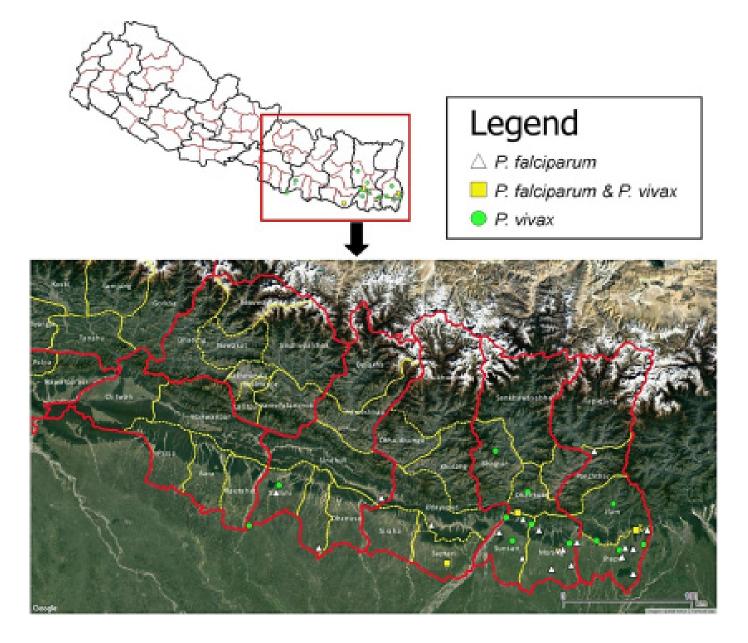

Figure 1: Spatial distribution of *Plasmodium* species in Eastern Nepal.

Table 2: Analysis of three assays for the diagnosis of malaria.

Diagnostic test	Test Positive (n/N)	Contigency analysis*		Latent class analysis	
		Sensitivity % (95% CI)	Specificity % (95% CI)	Sensitivity % (95% CI)	Specificity % (95% CI)
Microscopy	34/63	91 (76-98)	66 (46-82)	84.2 (53.0-95.0)	91.9 (73.1-97.9)
RDT	38/63	97 (86-100)	84 (64-95)	99.9 (99.7-99.9)	99.9 (99.6-99.9)
PCR	41/63	NA	NA	97.4 (83.5-99.6)	83.9 (64.3-93.9)

*Note: *PCR is used as a gold standard reference test*

Regarding geographical origin, patients were from 11 different districts of Nepal (Bhojpur, Dhankuta, Ilam, Jhapa, Mahottari, Morang, Panchthar, Saptari, Sarlahi, Sunsari, and Udayapur) and one malaria negative patient was from India. *P. falciparum* was detected in patients from Jhapa, Mahottari, Morang, Panchthar, Sarlahi, Sunsari and Udaypur districts. *P. vivax* was identified in patients from seven districts: Bhojpur, Dhankuta, Ilam, Jhapa, Morang, Sarlahi and Sunsari. Mixed cases of *P. falciparum* and *P. vivax* belonged to four districts: Jhapa, Morang, Saptari and Sunsari. Overall spatial distribution of *Plasmodium* species were depicted in Figure 1.

DISCUSSION

Malaria parasitological diagnosis unconditionally defines malaria burden and the epidemiological dynamics. Early identification also supports the management of non-malarial febrile illnesses. Although microscopy is considered as gold standard for malaria diagnosis, its precision to remain so has been criticized due to lower sensitivity.[25,26] Hence, alternative Bayesian approaches has been currently used to analyse the performance of diagnostic assays without assumption of any diagnostic test as a perfect gold standard.[18] In this study, the diagnostic performance of microscopy was also comparatively weak and found consistent with reports from elsewhere.[27,28] The sensitivity might be influenced by experience of the microscopist as skilled person can detect malaria parasite at densities as low as 4-20 parasites/μL of blood.[29] Additionally, factors such as quality of blood smear[30] and sampling from hypoen-demic region[31] could also affect the diagnostic performance.

The sensitivity and specificity of RDT diagnostic assay employed was also comparable with other studies in Nepal[32,33] and could be considered as more useful diagnostic tool.[34] Indeed, RDT can achieve better results in cases with high parasitemia, as demonstrated by various studies. Increased performance ranging above 95% when parasitemia exceeds 1000 parasites/μL and decreased sensitivity when the parasite density is below 100 parasites/μL.[35,36] However, false-positive RDT results could also over-estimate the detection limit of RDT, since antigenic persistence has been reported even after the two weeks of treatment.[37,38]

In recent days, the molecular diagnostic test using PCR is generally considered as a gold standard in diagnostic efficiency[39,30] as theoretical lowest limit of detection of PCR is ranged between 0.02 to 1 parasite/μL.[40] Hence, this study documented with the higher PCR sensitivity (97.4%) but comparatively low specificity (83.9%) which were similar to another study performed in India[41] and Equatorial Guinea[42] from central Africa. In order to rule out the false negative RDT cases, PCR could be the important tool. PCR is highly useful in low parasitemia which is undetectable to microscopy and also to overcome the dilemma in the identification *Plasmodium* species. Our study found *Plasmodium vivax* and *Plasmodium falciparum* together with the mix infection in some of the cases which were geographically plotted (Figure 1) to generate the baseline information of parasite circulation in Eastern Nepal.

However, due to the resource constrains, PCR facility is not affordable in every laboratory setup. Present study being the hospital based study, sample biasness cannot be ruled out. Bayesian analysis demonstrates the RDT having the better diagnostic efficiency. RDT is comparatively simple, rapid, and easy to perform in resource limited setup. But in turn, PCR could be crucial to diagnose malaria in low parasite density during the end of malaria elimination phase.

CONCLUSION

Our study concludes that the complimentary application of RDT and PCR is recommended for accurate diagnosis of malaria in case of acute febrile illness. These results should be further corroborated with the epidemiological situation of malaria which also avoids the false diagnosis and to support the malaria elimination programme in the world.

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