

Improved detection of mixed *P. falciparum*-*P. vivax* infection at a rural health centre in Ethiopia using PCR

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Received 18 April 2016; received in revised form 1 September 2016; accepted 5 September 2016

Abstract. This study aims to compare the co-infection *Plasmodium falciparum* + *Plasmodium vivax* and compare the detection of cases of mixed-species malaria using light microscopy versus semi-nested multiplex PCR (sPCR). Investigators collected 3060 samples at a rural health centre in Ethiopia from December 2010 to October 2011. Two capillary blood specimens were taken from each patient, one for diagnosis of *Plasmodium* infection by light microscopy and the other for sPCR-based diagnosis. LM detected 627 positive cases; these samples, together with 582 negatives by LM, were also subjected to sPCR testing. Of the 627 positive samples by LM, 68.4% were positive for *P. vivax*, 30.5% for *P. falciparum*, and 1.1% for *P. falciparum* + *P. vivax* co-infection. Using the sPCR technique, we identified 788 samples positive for *Plasmodium*: 33.0% for *P. vivax*, 26.5% for *P. falciparum*, 3.7% for *P. falciparum* + *P. vivax* co-infection, 2.0% for *P. ovale* and 0.8% for *P. vivax* + *P. ovale* co-infection. In the case of *P. falciparum* + *P. vivax* co-infection, light microscopy diagnosis showed a sensitivity of 11.1%, a specificity of 99.8%, a positive predictive value of 71.4% and a negative predictive value of 96.6%. The concordance rate for identifying *P. falciparum* + *P. vivax* co-infection (kappa statistic) with microscopy and sPCR was 0.184. The LM approach has low sensitivity for the detection of mixed-species infections, while sPCR is more useful.

BACKGROUND

Approximately 52 million people in Ethiopia are considered to be at risk of malaria (Federal Democratic Republic of Ethiopia, 2004). While the country's health facilities report 4 to 6 million clinical malaria cases annually, the real number is estimated to be as high as 10 to 15 million. Epidemic malaria is frequent (Zhou *et al.*, 2004), particularly in the highlands (1000-2000 m/3200-6500 ft above sea level), where the population lacks immunity to the disease. The main *Plasmodium* species causing malaria in Ethiopia include *P. falciparum* (about 60% of cases) and *P. vivax* (about 40% of cases) (Abeku *et al.*, 2003; Jima 2004; Ramos *et al.*, 2005). This ratio may change during the dry season, when most relapses occur due to

infection by *P. vivax* (Seboxa *et al.*, 1997; Ramos *et al.*, 2005). Furthermore, these species' prevalence rates vary due to climatic and seasonal factors and in different geographical locations (Checchi *et al.*, 2006).

The most common method used to identify the *Plasmodium* species responsible for malaria infection is microscopic observation of thick and thin Giemsa-stained blood slides. Different molecular detection methods, including polymerase chain reaction (PCR) techniques, have been developed to diagnose *Plasmodium* sp. These methods outperform microscopy in measurements of *Plasmodium* parasites, detecting submicroscopic or low level parasites that may not be detected by conventional light microscopy (LM)

(Snounou *et al.*, 1993; Ohrt *et al.*, 2002; Ehtesham *et al.*, 2015). Molecular diagnosis through real-time PCR is also more reliable than microscopy, particularly when parasitaemia levels are low and in the case of mixed infections (Snounou *et al.*, 1993; Ohrt *et al.*, 2002; Ehtesham *et al.*, 2015). This is important because the coexistence of two *Plasmodium* species in a single host (mixed-species infection) can disrupt the diagnosis and treatment of malaria (Ehtesham *et al.*, 2015). Several studies performed in South America (Rodulfo *et al.*, 2007), the Middle East (Bin Dajem 2015; Ehtesham *et al.*, 2015) and South Asia (Gupta *et al.*, 2010; Alam *et al.*, 2011; Shahzadi *et al.*, 2013) have revealed a higher prevalence of mixed-species infections using PCR compared to light microscopy. Few studies in Africa have been published (Tajebe *et al.*, 2014).

The purpose of the current study was to re-evaluate the level of mixed infection of *P. falciparum* + *P. vivax* using semi-nested multiplex PCR (sPCR).

METHODS

Survey site and sample collection

The survey was conducted in a neighbourhood (*kebele*) of Bulbula (N 7° 42' 52" and E 38° 39' 7"), a town in West Arsi Province, Oromia Region (Ethiopia), with a population of 7364. It is situated in the Rift Valley at an altitude of 1700 m (5577 feet) above sea level, 184 kilometres south of the country's capital, Addis Ababa.

The study included all patients who attended the Bulbula Health Centre (BHC) from December 2010 to October 2011, presenting with fever and suspected of having uncomplicated malaria. We collected a total of 3060 samples from two capillary blood specimens taken from each patient, one for diagnosis of *Plasmodium* infection by microscopic observation of Giemsa-stained thin and thick blood film by health workers, and the other on filter paper (Whatman® 3MM) for diagnostic confirmation by sPCR at the National Centre for Tropical Medicine (NCTM), Carlos III Institute of Health, Spain. Blood samples were stored at 4°C and

transported to Spain in double zip lock plastic bags at room temperature.

Microscopy

Thin films were fixed with methanol for five minutes and allowed to air dry. Subsequently, thick and thin films were stained with 5% Giemsa solution for 20 minutes, whereafter BHC microscopists examined them according to diagnostic guidelines for health workers in Ethiopia (Federal Democratic Republic of Ethiopia, 2011). In keeping with the routine practices of health centres in rural Ethiopia, we did not evaluate parasite density, as these centres generally only check for the presence of parasites.

Treatment of infected patients

Patients testing positive for *Plasmodium* by LM were treated according to national guidelines: participants with *P. falciparum* infection or *P. falciparum*-*P. vivax* co-infection received treatment with artemether/lumefantrine (AL) (CoArtem®), while those diagnosed with *P. vivax* received chloroquine (CQ). Pregnant women were treated with clinic-based quinine therapy. (Federal Democratic Republic of Ethiopia, 2004; Federal Democratic Republic of Ethiopia, 2006; Federal Democratic Republic of Ethiopia, 2011)

sPCR amplification

We analysed all LM positive samples, along with an initial 15% of negative ones, by sPCR. Since some of the negative microscopy samples tested positive for sPCR, we examined an additional 10%, bringing the total number of negative samples analysed to 582.

We performed DNA extraction on filter paper samples using commercial kits (Speedtools tissue DNA Extraction Kit, Biotools) and sPCR amplification using the methods described by Rubio *et al.* (1999). We then performed ultraviolet transillumination with electrophoresis on the sPCR product, using 2% agarose gels stained with ethidium bromide.

Of the 3060 individuals with suspected malaria, 736 were diagnosed as having *Plasmodium* sp. infection by LM. We

excluded 109 due to lack of filter paper, errors in numbering or contamination of the filter paper by droplets of blood. We took 25% of the 2324 LM-negative samples collected as control LM-negative samples (582 negative samples). A flowchart of the sample selection process is presented in a previous report (Diaz *et al.*, 2005).

Non-concordant results

In the event of any disagreement between the sPCR and LM results, we repeated the sPCR procedure to confirm the sPCR results.

Data analysis

We carried out a descriptive analysis, obtaining prevalence estimates and their 95% confidence intervals (CIs) for both LM and PCR diagnoses. We calculated sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) for LM, along with their respective CIs, using sPCR as the gold standard. We used the concordance test kappa with Epidat 3.1 software to compare LM with sPCR.

Ethical considerations

Ethical approval was granted by the Rural General Hospital of Gambo (West Arsi Province, Oromia Region), which is dependent on the Health Centre of Bulbula. Personal data was anonymised, participation was voluntary, and all patients or their parent/legal guardian provided informed consent. Involvement in the study did not influence treatment at the health centre.

RESULTS

Of 627 samples testing positive by LM, 429 were positive for *P. vivax* (68.4%; 95% CI 64.7–71.9), 191 for *P. falciparum* (30.5%; 95% CI 27–34.2), and 7 for *P. falciparum* + *P. vivax* co-infection (1.1%; 95% CI 0.5–2.3). Of 1209 samples analysed (627 LM-positive and 582 LM-negative) by sPCR (Table 1), 788 were positive for *Plasmodium* species: 398 for *P. vivax* (33%; 95% CI 30.3–35.6), 321 for *P. falciparum* (26.5%; 95% CI 24.0–28.9), 45 for *P. falciparum* + *P. vivax* co-infection (3.7%; 95% CI 2.6–4.8), 23 for *P. ovale* (2%; 95% CI 1.2–2.8), and 1 for *P. vivax* + *P. ovale* co-infection (0.08%; 95% CI -0.08–0.24) (Table 1).

Using LM, investigators correctly diagnosed five cases of co-infection (true positive), but in two other cases, LM diagnosis for *P. falciparum* + *P. vivax* co-infection was disproven by PCR, which produced diagnoses for *P. falciparum* infection (false positive). Likewise, investigators obtained 40 false negatives for *P. falciparum* + *P. vivax* co-infection by LM when using PCR as the reference standard: 38 cases were incorrectly diagnosed as *P. vivax* and 2 as *P. falciparum*. The other 1162 cases analysed by LM were true negatives for co-infection (Table 2).

Sensitivity of LM for diagnosis of co-infection was 11.1% (95% CI 4.2–24.8); specificity was 99.8% (95% CI 99.3–99.7), PPV was 71.4% (95% CI 99.3–99.7), NPV was 96.6% (95% CI 95.5–97.6), and the positive

Table 1. Number, percentage of *Plasmodium* species diagnoses by light microscopy and semi-nested multiplex PCR (sPCR)

	Light microscopy (n=627)		sPCR (n=788)	
	N	% (95% CI)	N	% (95% CI)
<i>P. falciparum</i>	191	30.5 (27 – 34.2)	321	26.5 (24.0 – 28.9)
<i>P. vivax</i>	429	68.4 (64.7 – 71.9)	398	33.0 (30.3 – 35.6)
<i>P. falciparum</i> + <i>P. vivax</i>	7	1.1 (0.05 – 2.3)	45	3.7 (2.6 – 4.7)
<i>P. ovale</i>	0	–	23	2.0 (1.2 – 2.8)
<i>P. vivax</i> + <i>P. ovale</i>	0	–	1	0.08 (-0.08 – 0.24)

Note: CI: confidence interval

Table 2. Results of light microscopy and semi-nested multiplex PCR (sPCR) for diagnoses of *P. falciparum* + *P. vivax*

		sPCR		Total
		Positive	Negative	
Light microscopy	Positive	5	2*	7
	Negative	40**	1162	1202
	Total	45	1164	1209

*Two samples with light microscopy positive for *P. falciparum* + *P. vivax* were diagnosed by sPCR as *P. falciparum*.

**Thirty-eight samples diagnosed by light microscopy as *P. vivax*, and two samples diagnosed as *P. falciparum*, tested positive for *P. falciparum* + *P. vivax* by sPCR.

Table 3. Validity of light microscopy compared with semi-nested multiplex PCR (sPCR) for diagnosing *P. falciparum* + *P. vivax*

		sPCR		
		Positive	Negative	
Light microscopy	Positive	TP: 0.6% (0.3%–1.2%)	FP: 28.6% (5.1%–69.9%)	PPV: 71.4% (30.2%–94.9%)
	Negative	FN: 3.3% (2.4%–4.5%)	TN: 99.4% (98.7%–99.7%)	NPV: 96.7% (95.4%–97.6%)
		Sn: 11.1% (4.2%–24.8%)	Sp: 99.8% (99.3%–99.7%)	

Note: 95% confidence intervals are presented in brackets.

Positive likelihood ratio (sensitivity/1-specificity): 64.9 (95% CI: 12.9%–32.4%).

Sn: sensitivity, Sp: specificity, PPV positive predictive value; NPV: negative predictive value; TP: True positive; TN: True negative; FP: false positive; FN: false negative.

likelihood ratio was 64.9% (95% CI 12.9–32.4) (Table 3).

The *P. falciparum* + *P. vivax* coinfection concordance rate (kappa statistic) of LM with nested PCR (reference test) was 0.184 (0.95% CI 0.043–0.325).

DISCUSSION

In an area where *P. falciparum* and *P. vivax* malaria infections are both present, the diagnosis of mixed-species malaria infections by LM is associated with limited sensitivity and difficulties in correctly identifying both species (Ebrahimzadeh *et*

al., 2007; Gupta *et al.*, 2010; Zakeri *et al.*, 2010; Alam *et al.*, 2011; Shahzadi *et al.*, 2014; Ehtesham *et al.*, 2015). In our study, sensitivity was just 11.1%.

The molecular detection method is effective in detecting mixed-species malaria infection. In our study, *P. falciparum* + *P. vivax* co-infection by LM represented 1.1% of all positive plasmodium infections, compared to the 5.7% of the co-infections detected by sPCR. One cause of the low sensitivity obtained for LM with regard to the *P. falciparum* + *P. vivax* co-infection in this rural area of Ethiopia could be the low levels of parasitaemia.

Reports on the prevalence of *P. falciparum* + *P. vivax* co-infection show different results. In a study performed in northwest Ethiopia using real time PCR, the prevalence of this mixed infection was 12.5%, considerably higher than in our study (Tajebe *et al.*, 2014). However, in another study in Bangladesh, the co-infection rate was 2.5% (Alam *et al.*, 2011), and in southwest Saudi Arabia, investigators did not find any cases of mixed malaria infections by microscopy and just 1.9% using PCR analysis (Bin Dajem, 2015).

In our study, the 38 sPCR diagnoses for *P. falciparum* + *P. vivax* co-infection had appeared to be *P. vivax* infections using LM. In line with national guidelines for their diagnoses (Federal Democratic Republic of Ethiopia, 2004; Federal Democratic Republic of Ethiopia, 2006; Federal Democratic Republic of Ethiopia, 2011), participants were treated with chloroquine, which is effective against *P. vivax* but not against *P. falciparum* (WHO, 2015). We did not follow the patients in this cross-sectional study, so we do not know how their condition evolved; however, this misdiagnosis of two mixed-species infections highlights the clinical/therapeutic relevance of the PCR diagnostic technique. Shahzadi *et al.* (2014) argued that the detection of mixed infection may be of additional clinical importance because interactions between different species in the same individual could significantly change the course of the infection and disease. All of this points to PCR as a helpful method in the differential treatment of malaria (Shahzadi *et al.*, 2014; Ehtesham *et al.*, 2015). Because molecular techniques require advanced instruments, their applicability has traditionally been limited in low-resource settings (Roper *et al.*, 1996; Ehtesham *et al.*, 2015). However, new molecular techniques such as loop-mediated isothermal amplification (LAMP) may be able to overcome some of these obstacles (Cook *et al.*, 2015). The use of rapid diagnostic tests (RDTs) in rural areas will allow improved diagnosis of mixed parasitic infections (WHO, 2015). The proper use of blood and tissue schizonticidal drugs is necessary for

the correct treatment of *P. vivax* and *P. falciparum* in cases of mixed infections not diagnosed by LM. Indeed, the incorrect use of sulfadoxine-pyrimethamine in the past has caused *P. vivax* resistance to these drugs in Ethiopia (Mula *et al.*, 2011).

The diagnosis of 23 cases of *P. ovale* in our study is also noteworthy. While some cases of *P. ovale* have been reported in Northern Ethiopia (Alemu *et al.*, 2013), our study was performed in southeastern Ethiopia, in Bulbula, a village in the Rift Valley.

One limitation of our study was that we did not use RDT kits to detect mixed-species malaria infections. Several studies have shown that these kits have a higher sensitivity to mixed-species infections in comparison with microscopy (Ehtesham *et al.*, 2015). A second limitation was that we did not quantify the parasitaemia counts. The two mixed-species infections detected by LM probably exceeded 2000 parasites/ μ L, (Alam *et al.*, 2011; Ehtesham *et al.*, 2015). Without analysing parasite density, we could not correlate the low detection of mixed *Plasmodium* infection with the low parasitaemia count. However, it is likely that *P. falciparum* counts were low in these participants, as LM did detect *P. vivax* infection in 38 of the 47 cases of co-infection.

Our findings suggest that the molecular method is useful to distinguish mixed-species infections, but it needs special facilities and trained personnel. The LM approach has low sensitivity for the detection of mixed-species infections and also requires skilled personnel and additional time for detecting parasites. Moreover, it may not always be available in rural and semi-rural areas. Larger clinical studies of *P. falciparum* + *P. vivax* co-infections in endemic regions such as Ethiopia and Asia will shed more light on the prevalence of *P. falciparum* + *P. vivax* co-infection.

Competing interests

The authors declare that they have no competing interests.

Acknowledgements. We would like to thank the laboratory staff of the Bulbula Health Centre for their assistance in collecting the data, the nursing staff for their care of the patients and the Network of Tropical Diseases Research Centres (*Red de Investigación Cooperativa en Enfermedades Tropicales/RICET* – RD06/0021/0000). This study (Project Reference TRPY 1400/09) was funded by the Carlos III Institute of Health.

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