Am. J. Trop. Med. Hyg., 93(1), 2015, pp. 181–185
doi:10.4269/ajtmh.14-0650

Detection of Mixed-Species Infections of *Plasmodium falciparum* and *Plasmodium vivax* by Nested PCR and Rapid Diagnostic Tests in Southeastern Iran

Reyhaneh Ehtesham, Asghar Fazaeli, Ahmad Raeisi, Hossein Keshavarz, and Aliehsan Heidari*

Department of Parasitology, School of Medicine, Zanjan University of Medical Sciences, Zanjan, Iran; Department of Medical Entomology and Vector Control, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran; Department of Medical Parasitology and Mycology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran; Department of Parasitology, School of Medicine, Alborz University of Medical Sciences, Karaj, Iran

Abstract. Coexistence of two species of *Plasmodium* in a single host has disrupted the diagnosis and treatment of malaria. This study was designed to evaluate the ability of rapid diagnostic test (RDT) kits for the diagnosis of mixed-species malaria infections in southeastern Iran. A total of 100 malaria patients were included in the study out of 164 randomly suspected symptomatic malaria patients from May to November 2012. Nested polymerase chain reaction (PCR) was also used to judge the ability of microscopy versus RDT kits for detecting mixed species. The sensitivity of light microscopy for the detection of mixed-species malaria infections was 16.6% (95% confidence interval [CI] = 3–49.1). Nested PCR revealed 12 patients with mixed-species infection. The CareStart Pv/Pf Combo kit detected 58% of the mixed-species infections, which were determined by nested PCR (sensitivity = 58.3%; 95% CI = 28.5–83.5). For identifying *P. falciparum*, *P. vivax*, and mixed-species infections, the concordance rates (kappa statistics) of microscopy and CareStart Pv/Pf Combo kit with nested PCR were 0.76 and 0.79, respectively ($P = 0.001$). This study underlines the effectiveness of RDT kits to improve the differentiation of mixed-species malaria infections in endemic areas where the prevalence of chloroquine resistance is high.

INTRODUCTION

Malaria remains an important parasitic disease in the world and is endemic in 104 countries, with approximately 219 million cases in 2010.[1] Globally, *Plasmodium falciparum* is the type most responsible for malaria-related mortality, whereas *P. vivax* is the most common cause of benign malaria in Asia and Central and South America.[2] Coexistence of two species of the *Plasmodium* genus in a single host (mixed-species infection) has disrupted the diagnosis and treatment of malaria. Furthermore, a delay or failure in detecting *P. falciparum* leads to infection aggravation and rise of the mortality rate, particularly in low or nonimmune people.[3] For more precise epidemiological decisions, an accurate identification of mixed-species infections is necessary.

The most common method used for the identification of the species of *Plasmodium* agent of malaria infection is microscopic observation of thick and thin Giemsa-stained blood slides. The thick blood smear technique is 10–30 times more sensitive than the thin-smear technique, with a detection threshold of approximately 5–15 parasites/mL.[4–6] Although this method is simple and inexpensive, its sensitivity decreases in low parasitemia and mixed-species infections. Furthermore, it is not applicable in border areas and places without adequate facilities.[7] Meanwhile, the detection threshold is highly dependent on the microscopist's experience.[8]

In recent years, several molecular techniques have been used to detect malaria parasites, with polymerase chain reaction (PCR) being the most common method using ribosomal DNA (18s-rRNA).[3] Several studies in various parts of the world have revealed the high prevalence of mixed-species infections using PCR.[9,10] Even in very low parasitemia, *P. vivax* and *P. falciparum* have been detected using this method.[11] The main advantage of molecular methods is their ability for the detection of trace cases of malaria that are not scrutable by other methods, particularly microscopy.[12–14] However, it requires some equipment, such as a thermal cycler, gel electrophoresis apparatus, and trained personnel.

Rapid diagnostic tests (RDT) have also become practical as the most convenient method. Various malaria RDT kits are now commercially available to detect antigens derived from malaria parasites. In these tests, the nitrocellulose strips containing monoclonal antibody (IgM) are used to detect parasite antigens in blood, including dedicated *P. falciparum* antigens *horseradish peroxidase-2* (HRP-2) and lactate dehydrogenase (LDH). Also, three-band RDT are designed to diagnose other agents of malaria and mixed-species infections using aldolase or specific lactate dehydrogenase (pLDH). This technique is precisely equivalent to the cost of one individual test in Iran, is feasible in poor technical conditions by nonspecialized personnel and saves time for rendering the results to the medical staff. There is no information about the evaluation of RDT kits in malaria-endemic areas of Iran. Approximately 16% of the Iranian population is at the risk of malaria.[15] Located in the eastern Mediterranean region, Iran is generally defined as a low transmission area. Sistan and Baluchestan province is located in southeastern Iran, close to the Pakistan and Afghanistan borders. The highest prevalence of malaria infections has been reported in this province during the past decade.[16] Both autochthonous and imported malaria have been reported in this area. The National Malaria Control Program reported that more than 90% of malaria cases are caused by *P. vivax* and the rest are caused by *P. falciparum*. At present, *P. falciparum* is resistant to chloroquine and is treated with artemisinin-based combination therapy. Chloroquine and primaquine drugs are administered for the treatment of *P. vivax* malaria in the country. Therapeutic decisions on *P. falciparum* and *P. vivax* species are different regarding their duration and type of applied drugs; therefore, misdiagnosis of mixed species of malaria infection may also lead to severe malaria because of failure in the treatment of *P. falciparum* malaria with chloroquine.

*Address correspondence to Aliehsan Heidari, School of Medicine, Alborz University of Medical Sciences, Baghestan, Golestan 1, Karaj, Iran. E-mail: aliehsan2001@yahoo.com

The accurate detection of mixed-species infections of malaria by more sensitive and specific methods is very critical for successful control programs in Iran because of 1) existence of both *P. vivax* and *P. falciparum* species in endemic areas of Iran, 2) entering of immigrants with malaria from Afghanistan and Pakistan (countries with high prevalence of malaria), and 3) insecticide resistance[17] and the presence of drug-resistant strains in the region.

Previous studies have shown a significant difference between the rate of mixed-species infections reported by PCR and microscopy[18,19]; however, because of inapplicability of PCR technique, the microscopy method is still used in the field. Therefore, this study was designed to evaluate the ability of RDT kits for the diagnosis of mixed-species malaria infections in southeastern Iran. Nested PCR was also used to judge the ability of microscopy versus three RDT kits for detecting mixed-species malaria infections.

MATERIALS AND METHODS

Specimen collection. Malaria transmission in Iran occurs mostly during May–June and October–November. Although malaria cases have decreased in the country in recent years, local malaria transmission continues due to specific reasons, including less socioeconomic development and cross-border population movements. Study sampling was performed between May–November 2012. A total of 100 malaria patients were included out of 164 randomly suspected symptomatic malaria patients using microscopy. Blood samples were obtained from individuals who were clinically suspected for malaria (having symptoms, such as chills, fever, and sweating) and attended health-care centers in Pishin (5 km from Pakistan borders) and Yahocalat in Sarbaz and Chabahar districts in Sistan and Baluchestan province. Thick and thin blood smears were stained with 3% Giemsa and examined microscopically by two blinded independent examiners. At least 200 thick and thin blood fields at 1,000 × magnification were examined for *Plasmodium* detection and species differentiation, respectively. Parasite density in thick blood films was calculated as numbers of asexual parasites per 200 white blood cells. Whole blood samples were kept in ethylenediaminetetraacetic acid (EDTA)–coated tubes; one blood drop was used for each RDT kit immediately after the microscopy assay (maximum of 3 hours after sampling; in this time, the sample was stored at 4°C) and the rest were frozen for later PCR analysis.

In this study, three sets of kits were applied according to the manufacturers' instructions: 1) First Response® Malaria Antigen pLDH/HRP2 Combo (Premier Medical Corporation, Daman, India), 2) CareStart™ Malaria RAPYDTEST HRP2/pLDH (Pf/PAN) Combo (Access: DiaSys, Wokingham, Berkshire, England) (CareStart1), and 3) CareStart Pv/Pf Combo (Access Bio Inc., Somerset, NJ). The diagnostic ability of three RDT kits was evaluated; the results were compared with the findings of microscopy and nested PCR. In general, First Response and CareStart1 kits have one (HRP-2) or two (HRP-2 and pLDH) bands for *P. falciparum* infection alone. Usually there is one (pLDH) band for *P. vivax* infection alone and two (HRP-2 and pLDH) bands for mixed-species infection. Because these RDT kits contained HRP-2 and pLDH (pan) antigens, it is likely that these antigens were positive in *P. falciparum* alone and mixed-species infections. Therefore, it was a limitation of these RDT kits. In CareStart Pv/Pf Combo kit, there is HRP-2 for the diagnosis of *P. falciparum* and a pLDH-specific band for *P. vivax*; therefore, both bands appear in mixed-species malaria infection.

Nested PCR. DNA was derived from 200 μL blood samples using a QIAquick PCR purification kit (Qiagen, Hilden, Germany), according to the manufacturer's instruction. DNA template was eluted in 200 μL double-distilled water, and approximately 100 ng DNA template was used in each PCR assay. Nested PCR was performed using the *Plasmodium* 18 subunit ribosomal ribonucleic (Ssr RNA) genes to detect the mixed-strain infections and to identify the malaria parasites in the samples with low parasite (< 10 parasites/μL). Primers and detailed nested PCR amplifying methods have been described previously in a study.[20] For the sake of certainty, both positive and negative controls were used for nested PCR. The amplified products were run in 2% agarose gel electrophoresis and stained by DNA Safe stain for visual detection of bands using ultraviolet transillumination. Findings of microscopy and three RDT kits were verified by our reference diagnostic technique, PCR.

Statistical analysis. Sensitivity was determined as the proportion of positive test findings identified among patients containing mixed-malaria infection detected by nested PCR. A 95% confidence interval (CI) was calculated for each proportion. McNemar test was applied to compare paired proportions. *t* test was used to compare the parasitemia in the correct versus incorrect mixed-species infection using microscopy and CareStart Pv/Pf Combo kit. P values < 0.05 were considered statistically significant. The concordance test kappa was used to compare each individual technique with nested PCR as the reference test.

Ethical considerations. Informed, written consent was obtained from each patient. This study was approved by the Research Ethical Review Committee of Zanjan University of Medical Sciences, Zanjan, Iran.

RESULTS

Patients' ages ranged from 4 to 58 years, with an average of 31 years. Of the patients, 85% were males. Parasitemia ranged from 120 to 96,000 parasites/μL blood, with a mean of 9,500 parasites/μL. The burden of parasitemia in 10 patients was below 1,000 parasites/μL. The findings of nested PCR, microscopy, CareStart1, First Response, and Pv/Pf Combo kits are presented in Table 1. Twelve patients indicated mixed-species infections in nested PCR assays (Table 2). The sensitivity of light microscopy for the detection of mixed-species malaria infections was 16.6% (95% CI = 3–49.1). There was a significant difference between the sensitivity of microscopy and nested PCR in identifying mixed-species malaria infections ($P = 0.0009$). Specifically, the CareStart Pv/Pf Combo kit could detect 58% of mixed-species infections that were determined by nested PCR (sensitivity = 58.3%; 95% CI = 28.5–83.5).

For identifying *P. falciparum*, *P. vivax*, and mixed-species infections, concordance rates (kappa statistics) of microscopy and Pv/Pf Combo kit with nested PCR (reference test) were 0.76 and 0.79, respectively ($P = 0.001$). The diagnosis of *P. vivax* malaria was confirmed in 61/69, 63/69, and 64/69 patients in CareStart1, First Response, and CareStart Pv/Pf Combo kits, respectively. The diagnosis of *P. falciparum* malaria was confirmed in 18/19 patients in CareStart1 kit,

TABLE 1
The results of malaria species detection in patients with clinical malaria according to three methods in southeastern Iran

		Nested PCR			
		Plasmodium falciparum	*P. vivax*	Mixed *Pf*/*Pv*	Total
		N = 19	*N* = 69	*N* = 12	*N* = 100
Microscopy	*P. falciparum*	19	0	1	20
	P. vivax	0	69	9	78
	Mixed *Pf*/*Pv*	0	0	2	2
	Negative	0	0	0	0
First Response® Malaria Combo	HRP-2 alone	12	–	–	12
	Pan-pLDH alone	–	63	2	65
	Both antigens	7	1	6	14
	Negative	–	5	4	9
CareStart™ Malaria Combo	HRP-2 alone	12	–	3	15
	Pan-pLDH alone	–	61	2	63
	Both antigens	6	–	6	12
	Negative	1	8	1	10
CareStart™ Pv/Pf Combo	HRP-2 alone	19	–	2	21
	pLDH alone	–	64	2	66
	Both antigens	–	–	7	7
	Negative	–	5	1	6

HRP = *horseradish peroxidase*; LDH = lactate dehydrogenase; PCR = polymerase chain reaction; *Pf*/*Pv* = *P. falciparum*/*P. vivax*.

but all of the *P. falciparum* infection were detected by First Response and CareStart Pv/Pf Combo kits. Two diagnosed mixed-species infections by microscopy were identified by PCR and the three kits. In comparison with microscopy, CareStart Pv/Pf Combo kit diagnosed more samples with mixed-species infection ($P = 0.03$). The CareStart and First Response kits also could rectify diagnosis of four of *P. falciparum* in mixed-species malaria infections that were missed by microscopy.

In all patients infected by *P. vivax*, there was no HRP-2 band in RDT kits, whereas the pLDH band was observed in these patients, except false-negative cases.

DISCUSSION

In an area where *P. falciparum* and *P. vivax* malaria infections are both present, the diagnosis of mixed-species malaria infections by microscopy is associated with limited sensitivity and difficulty in correct identification of both species. PCR with high sensitivity can detect mixed-species malaria infection but requires advanced instruments and is not field applicable. Accordingly, because of the limitations of using microscopy and PCR in this study, detection of mixed-species malaria infections was investigated using three low-cost RDT kits. Because of the higher sensitivity of CareStart Pv/Pf Combo kit in our study, more cases of mixed-species infections were diagnosed in comparison with microscopy ($P = 0.03$). These results about the sensitivity of CareStart kit and light microscopy in identifying mixed-species malaria infections are consistent with findings of a study conducted in Ethiopia.[7] In this study, CareStart Pv/Pf Combo kit demonstrated better agreements with nested PCR than microscopy in detecting mixed-species infections. The sensitivity of microscopy was low in comparison with PCR for identifying mixed-species malaria infections; this finding is similar to two previous studies conducted in Iran.[18,19] It seems in mixed-species infections, one species often dominates the other numerically.[21] Therefore, identifying the two species in such infections could be complicated by conventional light microscopy because of notable decreases in the number of parasites of one species.

TABLE 2
Comparison of microscopy and three RDT kits for detection of 12 PCR detectable mixed-species malaria infections in southeastern Iran

Methods	Mixed *Pv*/*Pf* infection	*Plasmodium falciparum*	*P. vivax*	Negative	Total
Microscopy	2	1	9	0	12
First Response® Malaria Combo	6	0	2	4	12
CareStart™ Malaria Combo	6	3	2	1	12
CareStart™ Pv/Pf Combo	7	2	2	1	12

PCR = polymerase chain reaction; *Pf*/*Pv* = *P. falciparum*/*P. vivax*; RDT = rapid diagnostic test.

Corresponding to the parasitemia rate in mixed-species malaria infections, two mixed-species infections detected by microscopy were above 2,000 parasites/μL, and microscopy and the three RDT kits missed the mixed infections more frequently at lower parasitemia; thus, the sensitivity of RDT kits decreased with declining parasitemia ($P = 0.03$). Other studies have reported false-negative cases in the evaluation of RDT kits.[22,23] Houzé (2013) also reported the low sensitivity of RDT tests in the diagnosis of *Plasmodium* species at very low parasitemia; the majority of tests have high detection rate at densities more than 2,000 parasites/μL.[24]

The misdiagnosis of two mixed-species infections that were detected by microscopy was not observed using CareStart Pv/Pf Combo kit. This is consistent with a study conducted in Pakistan in which all mixed-species infections in microscopy were also detected by rapid antigen testing.[25] However, CareStart1 and First Response kits could not clearly differentiate mixed-species malaria infection from single *P. falciparum* infection because they had pan LDH. Because most malaria infections in Iran are caused by *P. vivax* and it is possible coinfection with *P. falciparum* is missed by microscopy, application of these kinds of kits could rectify the diagnosis of *P. falciparum* species. In this study, among nine cases of *P. falciparum* infections missed by microscopy because of a mixed infection with *P. vivax* species, the diagnosis was rectified by both kits in four cases (44%).

According to our findings, the PCR method is useful to distinguish mixed-species infections, but it needs some facilities

and trained personnel. The microscopic approach has low sensitivity for the detection of mixed-species infections, requires skillful personnel and additional time for detecting parasites and may not always be available in rural and borderline areas[26,27]; therefore, the use of RDT kits as quick techniques in such places is essential, especially for the detection of mixed-species infections. Despite the superiority of diagnostic kits for the detection of mixed-species infections, they were incapable of diagnosing all *P. vivax* and *P. falciparum* malaria infections. Therefore, it is recommended to use the microscopic method as soon as possible after the kit is used to overcome the false-negative percentages. It is also concluded that mixed-species malaria infection is almost common in the region where both species coexist and their detection is only based on traditional microscopy; this may underestimate their importance. The Pv/Pf Combo kit used in this study could improve the mixed-species malaria diagnosis by microscopy in the endemic area where resistance to chloroquine is increasing. Nevertheless, CareStart1 and First Response kits are usually not applied for identifying mixed-species malaria infection, but they could rectify the diagnosis of *P. falciparum* in mixed-species malaria infection that were missed by Giemsa-stained microscopy in southeast Iran. Therefore, using RDT kits can facilitate a better diagnosis of mixed-species infections and correct treatment of malaria patients, especially in regions without enough diagnostic facilities.

Received October 17, 2014. Accepted for publication April 3, 2015.

Published online May 11, 2015.

Acknowledgments: We are grateful to Sakeni and Gorgige for their contribution to sampling and also Shojaee and Salimi from the Department of Medical Parasitology and Mycology, School of Public Health Tehran University of Medical Sciences. We also thank those individuals from the malaria endemic region of Iran who kindly contributed to this study.

Financial support: This study was financially supported by Zanjan University of Medical Sciences, Zanjan, Iran.

Authors' addresses: Reyhaneh Ehtesham, Aliehsan Heidari, and Asghar Fazaeli, Department of Parasitology, School of Medicine, Zanjan University of Medical Sciences, Zanjan, Iran, E-mails: reyhane.ehtesham@hotmail.com, aliehsan2001@yahoo.com, and fazaeli@zums.ac.ir. Ahmad Raeisi, Department of Medical Entomology and Vector Control, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran, E-mail: raeisia@tums.ac.ir. Hossein Keshavarz, Department of Medical Parasitology and Mycology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran, E-mail: hkeshavarz@tums.ac.ir.

REFERENCES

1. World Health Organization, 2012. *World Malaria Report 2012*. Geneva, Switzerland: World Health Organization. Available at: http://www.who.int/malaria.
2. White NJ, Imwong M, 2012. Relapse. *Adv Parasitol 80:* 113–150.
3. Mixson-Hayden T, Lucchi NW, Udhayakumar V, 2010. Evaluation of three PCR-based diagnostic assays for detecting mixed *Plasmodium* infection. *BMC Res Notes 3:* 88.
4. Gay F, Traore B, Zanoni J, Danis M, Gentilini M, 1994. Evaluation du système QBC pour le diagnostic du paludisme. *Sante 4:* 289–297.
5. Moody AH, Chiodini PL, 2000. Methods for the detection of blood parasites. *Clin Lab Haematol 22:* 189–202.
6. Trape JF, 1985. Rapid evaluation of malaria parasite density and standardization of thick smear examination for epidemiological investigations. *Trans R Soc Trop Med Hyg 79:* 181–184.
7. Moges B, Amare B, Belyhun Y, Belyhun Y, Tekeste Z, Gizachew M, Workineh M, Gebrehiwot A, Woldeyohannes D, Mulu A, Kassu A, 2012. Comparison of CareStart™ HRP2/pLDH COMBO rapid malaria test with light microscopy in northwest Ethiopia. *Malar J 11:* 234.
8. Thellier M, Datry A, Alfa Cissé O, San C, Biligui S, Silvie O, Danis M, 2002. Diagnosis of malaria using thick blood smears: definition and evaluation of a faster protocol with improved readability. *Ann Trop Med Parasitol 96:* 115–124.
9. Rodulfo H, De Donato M, Mora R, González L, Contreras CE, 2007. Comparison of the diagnosis of malaria by microscopy, immune chromatography and PCR in endemic areas of Venezuela. *Braz J Med Biol Res 40:* 535–543.
10. Snounou G, Viriyakosol S, Jarra W, Thaithong S, Brown KN, 1993. Identification of the four human malaria parasite species in field samples by the polymerase chain reaction and detection of a high prevalence of mixed infections. *Mol Biochem Parasitol 58:* 283–292.
11. Brown AE, Kain KC, Pipithkul J, Webster HK, 1992. Demonstration by the polymerase chain reaction of mixed *Plasmodium falciparum* and *Plasmodium vivax* infections undetected by conventional microscopy. *Trans R Soc Trop Med Hyg 86:* 609–612.
12. Genc A, Eroglu F, Soner Koltas I, 2010. Detection of *Plasmodium vivax* by nested PCR and real-time PCR. *Korean J Parasitol 48:* 99–103.
13. Khairnar K, Martin D, Lau R, Ralevski F, Pillai RD, 2009. Multiplex real-time quantitative PCR, microscopy and rapid diagnostic immuno-chromatographic tests for the detection of *Plasmodium* spp.: performance, limit of detection analysis and quality assurance. *Malar J 8:* 284.
14. Steenkeste N, Incardona S, Chykala MT, Lim P, Hewitt S, Sochantha T, 2009. Towards high-throughput molecular detection of *Plasmodium*: new approaches and molecular markers. *Malar J 8:* 86.
15. World Health Organization, 2012. *Profile of Iran (Islamic Republic of) 2012*. Available at: http://www.who.int/malaria/publications/country-profiles/2012/en/.
16. Heidari A, Keshavarz H, Shojaee S, Raeisi A, Dittrich S, 2012. In vivo susceptibility of *Plasmodium vivax* to chloroquine in southeastern Iran. *Iran J Parasitol 7:* 8–14.
17. Vatandoost H, Mashayekhi M, Abaie MR, Aflatoonian MR, Hanafi-Bojd AA, Sharifi I, 2005. Monitoring of insecticides resistance in main malaria vectors in a malarious area of Kahnooj district, Kerman province, southeastern Iran. *J Vector Borne Dis 42:* 100–108.
18. Ebrahimzadeh A, Fouladi B, Fazaeli A, 2007. High rate of detection of mixed infections of *Plasmodium vivax* and *Plasmodium falciparum* in south-east of Iran, using nested PCR. *Parasitol Int 56:* 61–64.
19. Zakeri S, Kakar Q, Ghasemi F, Raeisi A, Butt W, Safi N, Afsharpad M, Memon MS, Gholizadeh S, 2010. Detection of mixed *Plasmodium falciparum* and *P. vivax* infections by nested-PCR in Pakistan, Iran and Afghanistan. *Indian J Med Res 132:* 31–35.
20. Snounou G, Viriyakosol S, Zhu XP, Jarra W, Pinheiro L, do Rosario VE, Thaithong S, Brown KN, 1993. High sensitivity of detection of human malaria parasites by the use of nested polymerase chain reaction. *Mol Biochem Parasitol 61:* 315–320.
21. Snounou G, White NJ, 2004. The co-existence of *Plasmodium*: sidelights from falciparum and vivax malaria in Thailand. *Trends Parasitol 20:* 333–339.
22. Eibach D, Traore B, Bouchrik M, Coulibaly B, Coulibaly N, Siby F, Bonnot G, Bienvenu AL, Picot S, 2013. Evaluation of the malaria rapid diagnostic test VIKIA malaria Ag Pf/Pan™ in endemic and non-endemic settings. *Malar J 12:* 188.
23. Koita OA, Doumbo OK, Ouattara A, Tall LK, Konaré A, Diakité M, Diallo M, Sagara I, Masinde GL, Doumbo SN, Dolo A, Tounkara A, Traoré I, Krogstad DJ, 2012. False-negative rapid diagnostic tests for malaria and deletion of the histidine-rich repeat region of the *hrp2* gene. *Am J Trop Med Hyg 86:* 194–198.
24. Houzé S, Boutron I, Marmorat A, Dalichampt M, Choquet C, Poilane I, Godineau N, Le Guern AS, Thellier M,

Broutier H, Fenneteau O, Millet P, Dulucq S, Hubert V, Houzé P, Tubach F, Le Bras J, Matheron S, 2013. Performance of rapid diagnostic tests for imported malaria in clinical practice: results of a national multicenter study. *PLoS One 30:* e75486.

25. Beg MA, Ali SS, Haqqee R, Khan MA, Qasim Z, Hussain R, Smego RA Jr., 2005. Rapid immunochromatography-based detection of mixed-species malaria infection in Pakistan. *Southeast Asian J Trop Med Public Health 36:* 562–564.
26. Ansah EK, Epokor M, Whitty CJ, Yeung S, Hansen KS, 2013. Cost-effectiveness analysis of introducing RDTs for malaria diagnosis as compared to microscopy and presumptive diagnosis in central and peripheral public health facilities in Ghana. *Am J Trop Med Hyg 89:* 724–736.
27. Hailu T, Kebede T, 2014. Assessing the performance of CareStart Malaria Pf/Pv Combo test against thick blood film in the diagnosis of malaria in northwest Ethiopia. *Am J Trop Med Hyg 90:* 1109–1112.