

Validation of micro-chip based PCR assays for diagnosis of both *Plasmodium falciparum* and *Plasmodium vivax*

Syed Shah Areeb Hussian¹, Vijay P. Ojha¹, Meenakshi Jeena¹, Manju Rahi², Anupkumar Anvikar¹, Amit Sharma¹, Ramesh C. Dhiman¹

¹ICMR-National Institute of Malaria Research, New Delhi, India; ²Indian Council of Medical Research, New Delhi, India

ABSTRACT

Background & objectives: Microscopy is considered as the gold standard for malaria diagnosis, however sub-microscopic infections can only be detected by Polymerase chain reaction, which demands high cost and elaborate laboratory setup. The Micro-chip PCR based Truenat Malaria Pv-Pf and Pf assay is a portable solution for detection of sub-microscopic/asymptomatic cases of malaria in the field, three lots of which were evaluated for *P. falciparum* and *P. vivax* malaria.

Methods: Three lots of Truenat[®] Malaria Pv-Pf and Pf assay (kits) were assessed using blood samples of *P. vivax* and *P. falciparum* as well as malaria negative blood samples. DNA was extracted from the blood samples using the Trueprep Auto v2 Universal Cartridge based sample prep device and real time qPCR was performed using Truelab DUO micro PCR Analyzer with three lots of Truenat[®] Malaria Pv-Pf and Pf Assays. Mean, Standard deviation and one-way analysis of variance (ANOVA) was used to assess the significance of inter-lot variability in Cycle threshold values.

Results: The Truenat[®] Malaria Pv-Pf and Pf assays identified the malaria parasites with 100% accuracy. Based on the test for variance (ANOVA) the inter-lot variability in cycle threshold values were not significant, indicating a high degree of precision.

Interpretation & conclusion: Based on high accuracy and precision between different lots, the Truenat[®] Malaria Pv-Pf and Pf assays were found to be suitable for the diagnosis of sub-microscopic infections in field conditions to provide support in elimination of malaria.

Key words Sub-microscopic infections, Malaria diagnosis, Truenat Malaria Assay, *P. falciparum*, *P. vivax*

INTRODUCTION

The burden of malaria is gradually reducing in India with 3,38,494 cases and 77 deaths reported in 2019. Owing to gradual reduction in cases in the last decade, the National Vector Borne Disease Control Programme (NVBDCP) has planned to eliminate malaria by 2030¹. Earlier studies by Alves *et al.* (2005)², Dal-Bianco *et al.* (2007)³ and Baliraine *et al.* (2009)⁴ have suggested the phenomenon of asymptomatic and sub-microscopic malaria in Brazil, Gabon and Kenya, respectively. It has been evident that asymptomatic carriers act as a source of infection through mosquito vectors². Asymptomatic cases may or may not be sub-microscopic. It has been found that sub-microscopic cases contribute for 20–50% of human to mosquito transmission⁵. In India, asymptomatic malaria has been reported from high as well as low endemic malarious areas ranging from 8.4 % in Purulia (West Bengal)⁶ to 70–80 % in Chennai⁷ and up to the tune of 80% in the North-eastern states⁸. The National Framework for Malaria Elimination (NFME) 2016¹ underscored the importance of detection of asymptomatic malaria cases for

elimination. To achieve the goal of malaria elimination, strengthened surveillance and even detection of reservoirs of malaria infection would be crucial⁹.

Microscopy is considered as the standard method used for surveillance by NVBDCP for diagnosis of malaria, and Rapid Diagnostic Tests (RDTs) are used for on the spot diagnosis where results of microscopy are not available within 24 hours. But sub-microscopic infections can only be detected by Polymerase chain reaction (PCR)^{3, 8, 10} which requires well equipped laboratory infrastructure. PCR is rarely used for malaria diagnosis due to its high cost and need for elaborate laboratory setup, making it unsuitable for surveillance and point-of-care applications. Recently Nair *et al.* (2016)¹¹ reported the differential diagnosis of *Plasmodium vivax* and *P. falciparum* malaria by a portable, real-time, Micro-chip based PCR device which can be used in field conditions. This device named as Truelab Uno[®]/DUO is capable of detecting <5 malaria parasites (*P. falciparum* and *P. vivax*) per microlitre¹¹.

In order to use the Truenat[®] in field conditions, Bigtec Labs, Goa (India) has developed three lots of assay (kits) for diagnosis of *P. falciparum* alone and in combination

with *P. vivax* (combo). The present study was undertaken to assess the consistency of results in three lots of Truenat[®] Malaria Pv-Pf and Truenat[®] Malaria Pf assays using the Truelab[®] DUO real-time Micro-chip PCR so that the device maybe used in field conditions for detection of asymptomatic/sub-microscopic cases of malaria for malaria elimination.

MATERIAL & METHODS

Diagnostics using PCR

The Truenat[®] Malaria assay is a probe-based micro-chip PCR malaria assay based on Taqman chemistry that offers accurate, quick and easy point-of-care diagnosis of malaria. There are two types of Truenat[®] Malaria assays: one specific to *P. falciparum* (Truenat[®] Malaria PF assay) and the other detects both *P. vivax* and *P. falciparum* (Truenat[®] Malaria PV-PF assay).

Collection of samples

Fever surveys were conducted in Nuh district of Haryana, India between November 2019 and May 2020. Patients were first tested for malaria using RDT after which smears were prepared from finger pricked blood. For diagnosis using the Truenat[®] Malaria assay, 3–4 drops (150–200 µl) of blood were collected by finger prick in heparin coated vials from each malaria patient. Blood samples with *P. vivax* as well as malaria negative samples were also collected in the same way from the field. Blood samples of *P. falciparum* were available in-house from known positive source and were used directly for the study.

Processing of samples

DNA extraction from blood samples was performed using the Trueprep[®] Auto v2 Universal Cartridge based sample prep device. Blood samples were first transferred into vials containing the lysis buffer and allowed to mix for 5 min, following which they were transferred to the Trueprep cartridge (beset with DNA extraction kit). This was then inserted into the Trueprep AUTO v2 and DNA extraction took about 20 minutes. After DNA was extracted from each blood sample, 6µl of the DNA sample was added to the microtube containing the master mix (provided in the Truenat[®] Malaria Assay) and allowed to stand for 30 sec. The micro-chip, also provided in the Truenat[®] Malaria Assay, was placed into the chip tray of the Truelab[®] DUO micro-PCR Analyzer and the DNA/master mix solution was transferred to the reaction well of the micro-chip. The micro-chip contains information regarding the test parameters as well as standard values

required for quantification. To initiate PCR, ‘Malaria Pf’ or ‘Malaria Pv-Pf’ test profile was selected from the Analyzer screen (based on the type of assay used for the sample. The reaction takes approximately 50 min to complete, and the amplification curve is displayed on the Analyzer screen in real time.

For the validation of different lots of Truenat[®] Malaria Pv-Pf Assay, 40 samples were tested (20 negative, 10 Pf positive and 10 Pv positive) using three lots (ML007, ML008 and ML009). Similarly, for validation of different lots of Truenat[®] Malaria Pf Assay, 30 samples were tested (20 negative, 10 Pf positive) using three lots (PF010, PF011 and PF012). Conventional nested PCR was also conducted on all the DNA samples thus obtained using previously identified primers¹³ in Biorad PCR machine.

Assessment of inter-lot variability

For this the Cycle threshold (Ct) value of each sample for different lots was compared. The range, mean and standard deviation of Ct values of all samples of three lots was evaluated for Truenat[®] Pv-Pf and Pf assays. For malaria negative samples, the Ct value of the control available in the Trueprep[®] AUTO Cartridge was compared. Ideally, the Ct value of the control sample should lie in the range of 25–35. The analysis of variance between three lots of both the assays was done by test of ANOVA.

Ethical statement

Informed consent was taken from each participant prior to administering the test, and all procedures followed for sample collection were in accordance with the ICMR’s ethical guidelines¹².

RESULTS

All the samples were correctly diagnosed using the Truenat[®] Malaria Assay. There was 100% agreement between the results of parasite detection using conventional nested PCR and Truenat[®] Malaria Pv-Pf (Table 1) and Pf Assays (Table 2). For the Truenat[®] Malaria Pv-Pf Assay, average standard deviation in Ct values between the three lots was 0.5 for *P. vivax* and *P. falciparum* positive samples, while it was 0.65 for the control in the negative samples (Table 3). Similarly, for the Truenat[®] Malaria Pf Assay, average standard deviation in Ct values between the three lots was 0.51 for *P. falciparum* positive samples and 0.91 for the control in negative samples (Table 3). The one-way analysis of variance (ANOVA) between three lots of both Truenat[®] Malaria Pv-Pf and Truenat[®] Malaria Pf assays was found insignificant (Table 4). Please see limitations section for more details.

Table 1: Results for diagnosis of *P. falciparum* and *P. vivax* using Rapid Diagnostic kits, Nested PCR and three lots of TrueNat® Pv/Pf Malaria Assay

Sample ID	RDT	Nested PCR	TrueNat® Lot 1	P/μl	TrueNat® Lot 2	P/μl	TrueNat® Lot 3	P/μl
IN2F1-A1	Pf+	Pf+	Pf+ (Ct=19.4)	5.2 x 10 ⁶	Pf+ (Ct=19.1)	8.6 x 10 ⁶	Pf+ (Ct=18.73)	1.1 x 10 ⁷
IN2F1-A2	Pf+	Pf+	Pf+ (Ct=19.2)	5.9 x 10 ⁶	Pf+ (Ct=19.25)	7.7 x 10 ⁶	Pf+ (Ct=19.38)	7.3 x 10 ⁶
IN2F1-B	Pf+	Pf+	Pf+ (Ct=23.1)	4.4 x 10 ⁵	Pf+ (Ct=21.19)	2.0 x 10 ⁶	Pf+ (Ct=22.27)	1.0 x 10 ⁶
IN2F2	Pf+	Pf+	Pf+ (Ct=19.14)	6.2 x 10 ⁶	Pf+ (Ct=18.5)	1.3 x 10 ⁷	Pf+ (Ct=18.75)	1.1 x 10 ⁷
IN2F6	Pf+	Pf+	Pf+ (Ct=19.67)	4.3 x 10 ⁶	Pf+ (Ct=19.8)	5.3 x 10 ⁶	Pf+ (Ct=19.7)	5.9 x 10 ⁶
IN3F1	Pf+	Pf+	Pf+ (Ct=21.3)	1.5 x 10 ⁶	Pf+ (Ct=20.06)	4.4 x 10 ⁶	Pf+ (Ct=19.75)	5.7 x 10 ⁶
IN3F2	Pf+	Pf+	Pf+ (Ct=21.11)	1.7 x 10 ⁶	Pf+ (Ct=21.82)	1.3 x 10 ⁶	Pf+ (Ct=20.29)	3.9 x 10 ⁶
IN3F4	Pf+	Pf+	Pf+ (Ct=20.33)	2.8 x 10 ⁶	Pf+ (Ct=20)	4.6 x 10 ⁶	Pf+ (Ct=19.18)	8.4 x 10 ⁶
IN3F6	Pf+	Pf+	Pf+ (Ct=20.8)	3.3 x 10 ⁶	Pf+ (Ct=19.71)	5.6 x 10 ⁶	Pf+ (Ct=23)	6.3 x 10 ⁵
IN4F2	Pf+	Pf+	Pf+ (Ct=18.2)	5.7 x 10 ⁶	Pf+ (Ct=19.19)	8.1 x 10 ⁶	Pf+ (Ct=18.67)	1.2 x 10 ⁷
BP2	Pv+	Pv+	Pv+ (Ct=21.67)	1.5 x 10 ⁶	Pv+ (Ct=21.33)	1.6 x 10 ⁶	Pv+ (Ct=21.43)	1.3 x 10 ⁶
BBP4	ND	Pv+	Pv+ (Ct=30)	4.3 x 10 ³	Pv+ (Ct=31.67)	1.1 x 10 ³	Pv+ (Ct=31.75)	1.0 x 10 ³
BBP11	Pv+	Pv+	Pv+ (Ct=18.6)	1.3 x 10 ⁷	Pv+ (Ct=18.2)	1.5 x 10 ⁷	Pv+ (Ct=18.2)	1.3 x 10 ⁷
DN2	ND	Pv+	Pv+ (Ct=21.8)	1.4 x 10 ⁶	Pv+ (Ct=21.4)	1.5 x 10 ⁶	Pv+ (Ct=23.05)	4.3 x 10 ⁵
N1	ND	Pv+	Pv+ (Ct=22.67)	7.3 x 10 ⁶	Pv+ (Ct=22.6)	6.5 x 10 ⁵	Pv+ (Ct=23)	4.5 x 10 ⁵
SP10	Pv+	Pv+	Pv+ (Ct=29.5)	6.2 x 10 ³	Pv+ (Ct=31)	1.7 x 10 ³	Pv+ (Ct=29.4)	5.2 x 10 ³
SP27	Pv+	Pv+	Pv+ (Ct=24)	2.9 x 10 ⁵	Pv+ (Ct=25.2)	1.0 x 10 ⁵	Pv+ (Ct=25.17)	9.9 x 10 ⁴
SP29	Pv+	Pv+	Pv+ (Ct=30.5)	3.1 x 10 ³	Pv+ (Ct=29.43)	5.2 x 10 ³	Pv+ (Ct=29.17)	6.1 x 10 ³
SP56	Pv+	Pv+	Pv+ (Ct=29)	8.8 x 10 ³	Pv+ (Ct=29.4)	5.3 x 10 ³	Pv+ (Ct=30)	3.4 x 10 ³
DH37	Pv+	Pv+	Pv+ (Ct=32.33)	9.4 x 10 ²	Pv+ (Ct=30.5)	2.5 x 10 ³	Pv+ (Ct=32)	8.6 x 10 ²
DN3	ND	ND	ND (Ctrl=29.5)	-	ND (Ctrl=29)	-	ND (Ctrl=30)	-
DN4	ND	ND	ND (Ctrl=29.29)	-	ND (Ctrl=32)	-	ND (Ctrl=30.5)	-
DN5	ND	ND	ND (Ctrl=30.29)	-	ND (Ctrl=29.8)	-	ND (Ctrl=32.71)	-
DN6	ND	ND	ND (Ctrl=32.23)	-	ND (Ctrl=30.6)	-	ND (Ctrl=34)	-
DN7	ND	ND	ND (Ctrl=29.33)	-	ND (Ctrl=28.83)	-	ND (Ctrl=28.17)	-
DN8	ND	ND	ND (Ctrl=31.4)	-	ND (Ctrl=29.4)	-	ND (Ctrl=31.43)	-
DN11	ND	ND	ND (Ctrl=30.43)	-	ND (Ctrl=29.57)	-	ND (Ctrl=32)	-
DN12	ND	ND	ND (Ctrl=30.33)	-	ND (Ctrl=30.4)	-	ND (Ctrl=29.67)	-
DN13	ND	ND	ND (Ctrl=29.6)	-	ND (Ctrl=31)	-	ND (Ctrl=31.29)	-
DN14	ND	ND	ND (Ctrl=30)	-	ND (Ctrl=32.33)	-	ND (Ctrl=31.43)	-
DN15	ND	ND	ND (Ctrl=30.13)	-	ND (Ctrl=30.25)	-	ND (Ctrl=30.17)	-
DN18	ND	ND	ND (Ctrl=29.75)	-	ND (Ctrl=30.14)	-	ND (Ctrl=29.17)	-
DN19	ND	ND	ND (Ctrl=29)	-	ND (Ctrl=28.57)	-	ND (Ctrl=28.13)	-
BBP1	ND	ND	ND (Ctrl=32)	-	ND (Ctrl=30.8)	-	ND (Ctrl=30.13)	-
BBP5	ND	ND	ND (Ctrl=27.29)	-	ND (Ctrl=27.33)	-	ND (Ctrl=27.33)	-
BBP6	ND	ND	ND (Ctrl=26.6)	-	ND (Ctrl=30.11)	-	ND (Ctrl=30.6)	-
BBP8	ND	ND	ND (Ctrl=30.8)	-	ND (Ctrl=30.17)	-	ND (Ctrl=30.29)	-
BBP9	ND	ND	ND (Ctrl=29.8)	-	ND (Ctrl=29.6)	-	ND (Ctrl=30)	-
STP1	ND	ND	ND (Ctrl=32.5)	-	ND (Ctrl=32.2)	-	ND (Ctrl=33.29)	-
STP4	ND	ND	ND (Ctrl=30.25)	-	ND (Ctrl=30)	-	ND (Ctrl=30.2)	-

ND = Not Detected

Table 2: Results for diagnosis of *P. falciparum* using Rapid Diagnostic kits, Nested PCR and three lots of TrueNat® Pf Malaria Assay

Sample ID	RDT	Nested PCR	TrueNat® Lot 1	P/μl	TrueNat® Lot 2	P/μl	TrueNat® Lot 3	P/μl
IN2F1-A1	Pf+	Pf+	Pf+ (Ct=17.17)	3.1 x 10 ⁷	Pf+ (Ct=18.38)	1.6 x 10 ⁷	Pf+ (Ct=18.1)	1.2 x 10 ⁷
IN2F1-A2	Pf+	Pf+	Pf+ (Ct=16.67)	4.3 x 10 ⁷	Pf+ (Ct=17.29)	3.3 x 10 ⁷	Pf+ (Ct=17.82)	1.4 x 10 ⁷
IN2F1-B	Pf+	Pf+	Pf+ (Ct=20)	4.7 x 10 ⁶	Pf+ (Ct=20.2)	4.6 x 10 ⁶	Pf+ (Ct=19.83)	2.8 x 10 ⁷
IN2F2	Pf+	Pf+	Pf+ (Ct=17.15)	3.1 x 10 ⁷	Pf+ (Ct=18.29)	2.5 x 10 ⁹	Pf+ (Ct=17.29)	2.0 x 10 ⁷
IN2F6	Pf+	Pf+	Pf+ (Ct=18.5)	1.3 x 10 ⁷	Pf+ (Ct=18)	2.0 x 10 ⁷	Pf+ (Ct=18.87)	7.4 x 10 ⁷
IN3F1	Pf+	Pf+	Pf+ (Ct=18.57)	1.2 x 10 ⁷	Pf+ (Ct=17.4)	3.1 x 10 ⁷	Pf+ (Ct=19.23)	5.7 x 10 ⁷
IN3F2	Pf+	Pf+	Pf+ (Ct=20.38)	3.6 x 10 ⁶	Pf+ (Ct=10.9)	1.7 x 10 ⁷	Pf+ (Ct=20)	3.4 x 10 ⁶
IN3F4	Pf+	Pf+	Pf+ (Ct=18.14)	1.6 x 10 ⁷	Pf+ (Ct=18.29)	1.6 x 10 ⁷	Pf+ (Ct=19.1)	6.2 x 10 ⁶
IN3F6	Pf+	Pf+	Pf+ (Ct=17.4)	2.6 x 10 ⁷	Pf+ (Ct=18.2)	1.8 x 10 ⁷	Pf+ (Ct=18.71)	8.0 x 10 ⁶
IN4F2	Pf+	Pf+	Pf+ (Ct=18.75)	1.1 x 10 ⁷	Pf+ (Ct=16.25)	6.7 x 10 ⁷	Pf+ (Ct=17)	2.5 x 10 ⁷
DN3	ND	ND	ND (Ctrl=30.25)	-	ND (Ctrl=30.8)	-	ND (Ctrl=28.6)	-
DN4	ND	ND	ND (Ctrl=29.8)	-	ND (Ctrl=30.33)	-	ND (Ctrl=31)	-
DN5	ND	ND	ND (Ctrl=29.43)	-	ND (Ctrl=30.4)	-	ND (Ctrl=28.75)	-
DN6	ND	ND	ND (Ctrl=29.2)	-	ND (Ctrl=31)	-	ND (Ctrl=29.14)	-
DN7	ND	ND	ND (Ctrl=28.33)	-	ND (Ctrl=27.75)	-	ND (Ctrl=27.75)	-
DN8	ND	ND	ND (Ctrl=30.6)	-	ND (Ctrl=30)	-	ND (Ctrl=29.4)	-
DN11	ND	ND	ND (Ctrl=29.29)	-	ND (Ctrl=29)	-	ND (Ctrl=29.43)	-
DN12	ND	ND	ND (Ctrl=28.2)	-	ND (Ctrl=30.6)	-	ND (Ctrl=29)	-
DN13	ND	ND	ND (Ctrl=32.5)	-	ND (Ctrl=28.8)	-	ND (Ctrl=30.4)	-
DN14	ND	ND	ND (Ctrl=30)	-	ND (Ctrl=31.33)	-	ND (Ctrl=29.75)	-
DN15	ND	ND	ND (Ctrl=30.14)	-	ND (Ctrl=29.17)	-	ND (Ctrl=29.5)	-
DN18	ND	ND	ND (Ctrl=34.67)	-	ND (Ctrl=29.5)	-	ND (Ctrl=29)	-
DN19	ND	ND	ND (Ctrl=34)	-	ND (Ctrl=29.33)	-	ND (Ctrl=29.8)	-
BBP1	ND	ND	ND (Ctrl=30.17)	-	ND (Ctrl=28.6)	-	ND (Ctrl=32.2)	-
BBP5	ND	ND	ND (Ctrl=27)	-	ND (Ctrl=29.2)	-	ND (Ctrl=26.75)	-
BBP6	ND	ND	ND (Ctrl=29.33)	-	ND (Ctrl=26.43)	-	ND (Ctrl=30.17)	-
BBP8	ND	ND	ND (Ctrl=30.25)	-	ND (Ctrl=30.75)	-	ND (Ctrl=31.8)	-
BBP9	ND	ND	ND (Ctrl=28.17)	-	ND (Ctrl=29.8)	-	ND (Ctrl=29)	-
STP1	ND	ND	ND (Ctrl=29)	-	ND (Ctrl=29.5)	-	ND (Ctrl=29.67)	-
STP4	ND	ND	ND (Ctrl=29)	-	ND (Ctrl=29)	-	ND (Ctrl=29.5)	-

ND = Not Detected

Table 3: Validation data

Truenat® Malaria Pv-Pf Assay			
	Lot 1	Lot 2	Lot 3
Pf Positive Samples (N = 10)			
Range (Ct)	18.20 – 23.10	18.50 – 21.82	18.67 – 23.00
Mean (Ct)	20.23	19.86	19.97
Standard Deviation (Ct)	0.50		
Pv Positive Samples (N = 10)			
Range (Ct)	18.60 – 32.33	18.20 – 31.67	18.20 – 32.00
Mean (Ct)	26.01	26.07	26.32
Standard Deviation (Ct)	0.51		

*Negative Samples (N = 20)

Range (Ct)	26.60 – 32.50	27.33 – 32.33	27.33 – 34.00
Mean (Ct)	30.03	30.11	30.53
Standard Deviation (Ct)	0.65		
Truenat® Malaria Pf Assay			
	Lot 1	Lot 2	Lot 3
Pf Positive Samples (N = 10)			
Range(Ct)	16.67 – 20.38	16.25 – 20.90	17.00 – 20.00
Mean (Ct)	18.27	18.32	18.60
Standard Deviation (Ct)	0.51		
*Negative Samples (N = 20)			
Range (Ct)	27.00 – 34.67	26.43 – 31.33	26.75 – 32.20
Mean (Ct)	29.97	29.56	29.53
Standard Deviation (Ct)	0.91		

*Negative samples do not show any Ct value. However, the given Ct values are of positive control in the Trueprep cartridge.

Table 4: One-way analysis of variance (ANOVA)

Sample	Significance (<i>p</i> value)
Truenat® Malaria Pv-Pf Assay	
Pf positive samples	0.821
Pv positive samples	0.989
Negative samples (comparison of Ct values of positive control)	0.514
Truenat® Malaria Pf Assay	
Pf positive samples	0.236
Negative samples (comparison of Ct of positive control)	0.581

The tests of variance between three lots show that there is no significant difference in the Ct values of malaria positive samples and positive control in negative samples as the *p*-value is much greater than 0.05. Further, the variance in the Ct values for Pf positive samples was least (*p* = 0.821) for Truenat® Malaria Pv-Pf assay as compared to the Truenat® Pf Assay (*p* = 0.236).

DISCUSSION

The results of the validation provide evidence that all the three lots of Truenat® Malaria assays developed by Bigtec Labscan diagnose *P. falciparum* and *P. vivax* infections with high accuracy and precision. They also indicate that differential diagnosis of Pv-Pf malaria is possible and is comparable with WHO approved nested PCR technique. The sensitivity and specificity of all the lots was found to be 100% and the inter-lot variation between three lots was insignificant. The requirement of 150 µl blood can be met by 2–3 drops of finger prick blood in field conditions. Truelab DUO machine which was provided by Molbio diagnostics, Goa can screen two samples in one hour while the machines with four trays can process four

samples in an hour and thus 32 samples in a day which can be increased as per demand. At present there is no policy for detection of asymptomatic cases or sub-microscopic cases of malaria in India by NVBDCP. This suggests that inability to capture sub-patent cases of malaria in routine surveillance that currently rely on microscopy and/or RDT. Keeping in view that asymptomatic cases are infectious to mosquito vectors² and sub-microscopic malaria cases contribute 20–50% of human to mosquito transmission⁵, the national programme may consider use of this molecular technology for detection of sub-microscopic malaria cases for achieving the goal of elimination. World Health Organization (WHO) (2015) in its strategy for malaria elimination has emphasized the need of research for “assessment of highly sensitive sub-microscopic diagnostic assays for detecting both *P. falciparum* and *P. vivax* parasitaemia”⁹. In view of the results obtained in the present study, the Truenat® assay seems a promising tool for field-based PCR for diagnosis for malaria cases. The advantage of Truenat® assays over conventional PCR is in detection of <5 parasites/µl (Nair *et al* 2016), i.e. sub-microscopic infections which serve as parasite reservoir, are often missed by conventional techniques. As India tar-

gets malaria elimination by 2030, the need of surveillance for low density parasite infections will become even more desirable to achieve the goal.

LIMITATIONS

A limitation of the study is that it did not compare the limits of detection of malaria parasite between the Truenat results and conventional PCR. The goal of the present study was only to compare and contrast the variations in diagnostic results of different lots of Pf and Pv-Pf Truenat kits. Furthermore, the ability of the Truenat kits to accurately detect parasites in mixed Pv-Pf infections was not assessed.

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Conflict of interest: None

REFERENCES

1. NVBDCP. National Framework for Malaria Elimination in India (2016-2030). New Delhi; 2016.
2. Alves FP, Gil LHS, Marrelli MT, Ribolla PEM, Camargo EP, Da Silva LHP. Asymptomatic carriers of Plasmodium spp. as infection source for malaria vector mosquitoes in the Brazilian Amazon. *J Med Entomol* 2005; 42: 777–9.
3. Dal-Bianco MP, Köster KB, Kombila UD, Kun JFJ, Grobusch MP, Ngoma GM, et al. High prevalence of asymptomatic Plasmodium falciparum infection in Gabonese adults. *Am J Trop Med Hyg* 2007; 77: 939–42.
4. Baliraine FN, Afrane YA, Amenya DA, Bonizzoni M, Menge DM, Zhou G, et al. High prevalence of asymptomatic Plasmodium falciparum Infections in a highland area of western Kenya: A cohort study. *J Infect Dis* 2009; 200: 66–74.
5. Okell LC, Bousema T, Griffin JT, Ouedraogo AL, Ghani AC, Drakeley CJ. Factors determining the occurrence of submicroscopic malaria infections and their relevance for control. *Nat Commun* 2012; 3: 1237.
6. Ganguly S, Saha P, Guha SK, Biswas A, Das S, Kundu PK, et al. High prevalence of asymptomatic malaria in a tribal population in eastern India. *J Clin Microbiol* 2013; 51: 1439–44.
7. van Eijk AM, Sutton PL, Ramanathapuram L, Sullivan SA, Kanagaraj D, Priya GSL, et al. The burden of submicroscopic and asymptomatic malaria in India revealed from epidemiology studies at three varied transmission sites in India. *Sci Rep* 2019; 9: 1–11.
8. Shankar H, Phookan S, Singh MP, Suresh Bharti R, Ahmed N, Prakash Yadav C, et al. Prevalence of Asymptomatic and Sub-Microscopic Malaria Infection During Non-Transmission Season: A Community Based Cross-Sectional Study in Districts of North-Eastern Region, India [Preprint]. *SSRN Electron J Elsevier*; 2020.
9. WHO. Global technical strategy for malaria 2016-2030. World Health Organization. 2015.
10. Kaura T, Kaur J, Sharma A, Dhiman A, Pangotra M, Upadhyay AK, et al. Prevalence of submicroscopic malaria in low transmission state of Punjab: A potential threat to malaria elimination. *J Vector Borne Dis* 2019; 56: 78.
11. Nair CB, Manjula J, Subramani PA, Nagendrappa PB, Manoj MN, Malpani S, et al. Differential diagnosis of malaria on Truelab Uno®, a portable, real-time, MicroPCR device for point-of-care applications. *PLoS One Public Library of Science*; 2016; 11.
12. ICMR. National Ethical Guidelines for Biomedical and Health Research Involving Human Participants, 2017. Mathur R, editor. Director-General Indian Council of Medical Research; 2017.
13. Snounou G, Viriyakosol S, Xin Ping Zhu, 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.

Correspondence to: Dr. Ramesh C. Dhiman, ICMR-National Institute of Malaria Research, Sector-8, Dwarka, New Delhi 110077, India, Email: r.c.dhiman@gmail.com

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