Validation of a new real-time PCR based kit for detection and typing of 5 human pathogenic *Plasmodium* species, including *P. knowlesi* in whole blood specimen

A. Martin-Ramirez[1], E. Escolar-Ramirez[1], K. Rottengatter[2], L.-S. Hecht[2], M. Hess[2,] J. M. Rubio[1];
[1]Centro National de Microbiologia, Madrid/ES, [2]altona Diagnostics, Hamburg/DE

Purpose

Malaria remains an important cause of morbidity and mortality, worldwide. Even in settings in which malaria is not endemic, infections can be seen in individuals, who had travelled to or emigrated from regions with ongoing malaria transmission. Human infection is caused by five *Plasmodium* species, including *P. knowlesi,* which has emerged as a widespread cause of zoonotic human malaria in Southeast Asia, associated with a high risk of severe disease. *P. knowlesi,* is often misdiagnosed as *P. malariae* or *P. vivax,* due to its phenotypic and genetic similarities. There is an obvious need for sensitive and specific detection and typing of the different *Plasmodium* species due to differences in patient management and medication depending on the *Plasmodium* species the patient is infected with.

Here we describe the verification and validation of a new real-time PCR based kit for detection and typing of human pathogenic *Plasmodium* species.

Assay Description

The RealStar® Malaria Screen & Type PCR Kit (altona Diagnostics) is a real-time PCR based kit for the detection and typing of all five human pathogenic *Plasmodium* species, consisting of two reaction mixes (Figure 1). Each reaction mix includes an Internal Control (IC) for monitoring of the efficiency of the nucleic acid extraction process and possible inhibitory effects during PCR.

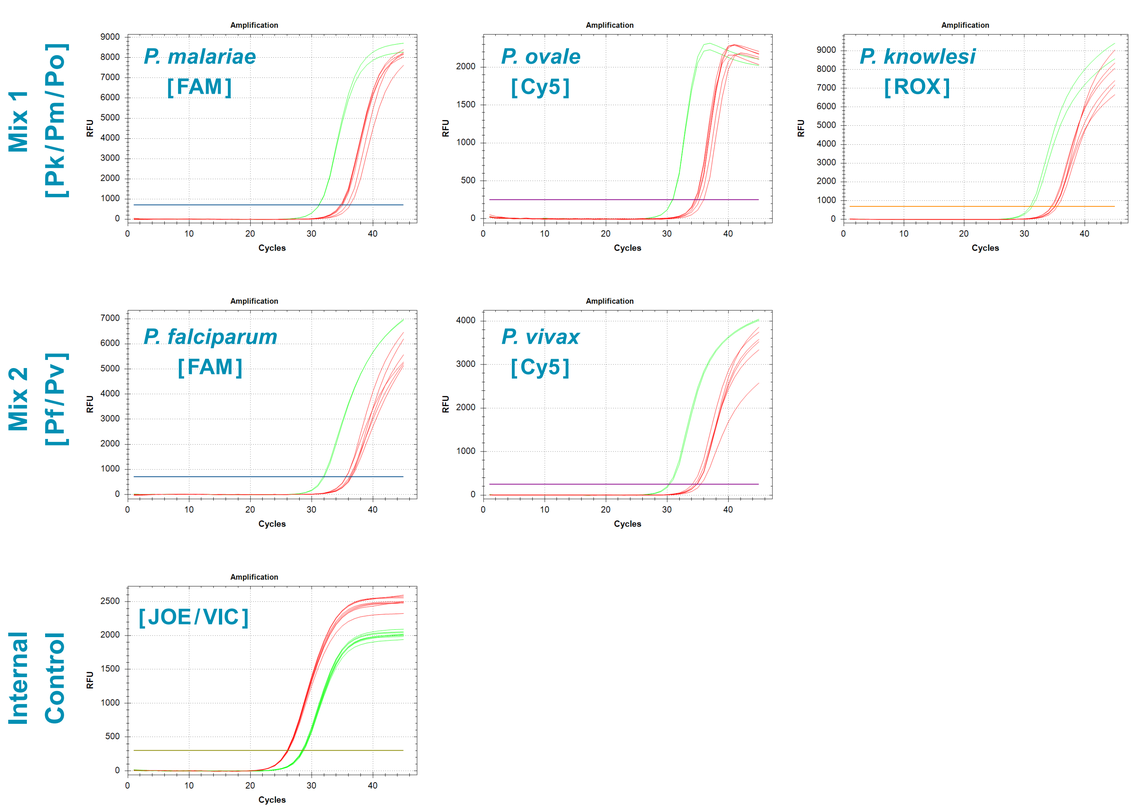

Figure 1: RealStar® Malaria Screen & Type PCR Kit: specific detection and typing of all five human pathogenic *Plasmodium* species and Internal Control.

Analytical Sensitivity

The analytical sensitivity (Limit of Detection; LoD) is defined as the concentration (copies/µl) of *Plasmodium* spp. specific DNA molecules that can be detected with a positivity rate of ≥ 95%.

The LoD of the different *Plasmodium* spp. specific detection systems were determined using half-logarithmic dilution series of *Plasmodium* spp. specific quantified DNA. The analytical sensitivity of the RealStar® Malaria Screen & Type PCR Kit was determined by Probit analysis:

Table 1: Results of the Probit analysis including Limit of detection (LoD) and Confidence Interval (CI)

	LoD [copies/µl]	Confidence Interval (CI)
P. falciparum	0.8065	95% CI 0.4415 to 2.4493 copies/µl
P. vivax	0.7310	95% CI 0.4595 to 1.6243 copies/µl
P. ovale	1.4601	95% CI 0.8929 to 3.2781 copies/µl
P. malariae	0.3619	95% CI 0.2446 to 0.7418 copies/µl
P. knowlesi	2.3477	95% CI 1.3708 to 5.5527 copies/µl

Cross Reactivity

Cross Reactivity of the RealStar® Malaria Screen & Type PCR Kit was analysed by testing a panel of genomic DNA/RNA extracted from parasites related to *Plasmodium* spp. or pathogens and viruses causing similar symptoms as *Plasmodium* spp.. The RealStar® Malaria Screen & Type PCR Kit did not cross-react with any of the DNA/RNA tested.

Table 2: Cross Reactivity of the RealStar® Malaria Screen & Type PCR Kit

Sample ID	Ct FAM	Ct Cy5	Ct ROX	Ct VIC
L. donovani	no Ct	no Ct	no Ct	25.36
L. major	no Ct	no Ct	no Ct	25.40
L. infantum	no Ct	no Ct	no Ct	25.42
T. gondii	no Ct	no Ct	no Ct	25.47
T. cruzi	no Ct	no Ct	no Ct	25.27
T. brucei	no Ct	no Ct	no Ct	25.28
B. microti	no Ct	no Ct	no Ct	25.42
Influenza virus A	no Ct	no Ct	no Ct	25.97
Influenza virus B	no Ct	no Ct	no Ct	25.86
CHIKV	no Ct	no Ct	no Ct	25.27

Validation

A collection of 105 whole blood samples from individual patients was included in the study. All samples were previously analyzed and typed by an established *in-house* workflow at the Centro National de Microbiologia, Madrid, Spain.

The collection comprised 75 specimen with 15 specimen pre-typed as *P. falciparum, P. vivax, P. malariae, P. ovale* and *P. knowlesi,* respectively, 15 specimen previously tested negative for human pathogenic *Plasmodium* species (negative cluster 1) and 15 specimen previously tested positive for other parasites causing diseases with similar symptoms like malaria (negative cluster 2).

The specimen were thawed, nucleic acid was extracted using the QIAamp DNA Blood Mini QIAcube Kit (Qiagen) and eluates were analyzed with the RealStar® Malaria Screen & Type PCR Kit (altona Diagnostics) on an Rotor-Gene® 6000 (Corbett-Research). The Internal Control (IC) of the RealStar® Malaria Screen &Type PCR Kit was added during the nucleic acid extraction process.

Table 3: Result of the validation study

		RealStar® Malaria Screen & Type PCR Kit					
		P. falciparum	***P. ovale***	***P. malariae***	***P. vivax***	***P. knowlesi***	***negative***
Reference Method	***P. falciparum* (n=15)**	15	0	0	0	0	0
	***P. ovale* (n=15)**	0	14	0	0	0	1
	***P. malariae* (n=15)**	0	0	15	0	0	0
	***P. vivax* (n=15)**	0	0	0	15	0	0
	P. knowlesi (n=15)	0	0	0	0	15	0
	negative Cluster 1 (n=15)	0	0	0	0	0	15
	negative Cluster 2 (n=15)	0	0	0	0	0	15

All 30 specimens pre-tested negative for human pathogenic *Plasmodium* species were tested negative with the RealStar® Malaria Screen & Type PCR Kit. The IC showed a valid result in all samples.

74 out of 75 specimen pre-tested positive for a human pathogenic *Plasmodium* species were tested positive and typed identically to the pre-typing results with the RealStar® Malaria Screen & Type PCR Kit. Unfortunately, there was no material left to reanalyze the one discrepant sample.

Conclusion

Our study shows, that the RealStar® Malaria Screen & Type PCR Kit provides a reliable assay with high diagnostic sensitivity and specificity for the detection and typing of human pathogenic *Plasmodium* species. It can be a useful tool in the management of malaria patients.

Contact

altona Diagnostics GmbH, Mörkenstr. 12, 22767 Hamburg, Germany
phone +49 40 548 06 76 – 0 (Germany), info@altona-diagnostics.com
karin.rottengatter@altona-diagnostics.com