A real-time PCR based Malaria-typing assay for the detection and differentiation of all five human pathogenic Plasmodium species

Introduction
Malaria, a vector-borne parasitic disease, which is endemic in most tropical and subtropical countries, is responsible for more than 200 million clinical cases and one million deaths per year. The causative agents of Malaria are eukaryotic apicomplexa of the genus Plasmodium. Within this genus, there are five species known to be pathogenic to humans: Plasmodium falciparum, Plasmodium vivax, Plasmodium knowlesi, Plasmodium malariae and Plasmodium ovale. For optimal clinical management of malaria patients, species-specific diagnosis is required. At present, microscopy of Giemsa-stained thick and thin blood smear represents the golden standard in Malaria diagnostics. Microscopy is known to be sensitive and specific but this technique is highly dependent on the skills of the microscopists. In order to prevent fatalities due to misdiagnosis, fast and reliable assays for Malaria diagnosis down to the species level are required.

Objectives
Here we report on the evaluation of a new molecular diagnostic Malaria-typing assay that allows sensitive detection and differentiation of all five human pathogenic Plasmodium species. The evaluation work included determination of analytical sensitivity (Limit of Detection: LoD) and specificity (cross-reactivity), as well as diagnostic evaluation by analyzing 82 specimen sent in for routine Malaria diagnostic and pre-analyzed by thick blood smear microscopy.

Material & Methods
The new assay consists of two independent reagent mixes which can be used in parallel on most of the standard real-time PCR instruments. The Reagent Mix A is designed to detect and differentiate Plasmodium species that cause non-relapsing malaria: Plasmodium falciparum, Plasmodium knowlesi and Plasmodium malariae. Whereas Reagent Mix B detects and differentiates the Hypnozoites-forming Plasmodium species: Plasmodium vivax and Plasmodium ovale. Both Reagent Mixes contain an Internal Control (IC) that can be used as a nucleic acid extraction control and as a PCR inhibition control. The limit of detection of the assay for the different Plasmodium species was determined by analyzing dilution series of quantified genomic material of the different species and Probit analysis.

Specificity of the assay was determined by analyzing 20 negative blood samples and a collection of DNA and RNA from agents related to Plasmodium species or causing Malaria-related symptoms. For the diagnostic evaluation of the assay the DNA of 82 specimen, pre-analyzed at the Bernhard-Nocht-Institute for Tropical Medicine (BNITM) using Giemsa-stained thick blood smear microscopy and stored at -20°C for different periods of time, was extracted using different extraction methods. Out of the 82 pretested specimens, 62 were Malaria positive (P. falciparum, P. vivax, P. malariae and P. ovale) and 20 Malaria negative.

Results
The limits of detection of the assay for the five human pathogenic Plasmodium species are listed in Table 1.

The detection and differentiation of the different Plasmodium species and the Internal Control in their respective detection channels are shown in Figure 1. All Malaria negative blood samples were analyzed negative with a valid Internal Control signal and the assay showed no cross-reactivity with the samples tested (Table 2).

All specimens pre-analyzed as Malaria positive were tested Plasmodium positive with the new assay and all Malaria negative specimens were tested negative (Figure 2).

Conclusion
Our new real-time PCR based assay for the detection and differentiation of the five human pathogenic Plasmodium species is at least as sensitive and specific as the golden standard method in Malaria diagnostic. Therefore, this assay will be a valuable tool for assisting in the reliable diagnostic of suspected Malaria cases and patient management.

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