parasitologia

MDPI

Article

Sensitivity Assessment of a Multiplex and Real-Time PCR Protocols for the Detection of Malaria in External Quality Control Samples in the Malaria Reference Center in Greece

Nikolaos Tegos [1,*], Christos Goumenopoulos [2], Anastasia Mpimpa [1], Vasilios Papavasilopoulos [1], Stavroula Beleri [1] and Eleni Patsoula [1]

[1] Laboratory for the Surveillance of Infectious Diseases, Department of Public Health Policy, School of Public Health, University of West Attica, 115 21 Athens, Greece; nmpimpa@uniwa.gr (A.M.); vpapavasilopoulos@uniwa.gr (V.P.); smpeleri@uniwa.gr (S.B.); epatsoula@uniwa.gr (E.P.)
[2] MSc Course in Public Health (Specialty: Infectious Diseases-Public Health Laboratories), School of Public Health, University of West Attica, 115 21 Athens, Greece; chrisgoum81@gmail.com
* Correspondence: ntegos@uniwa.gr

Abstract: Background: Accurate malaria diagnosis constitutes a challenging task, necessitating the need for the implementation of targeted and effective diagnostic tools. The purpose of the current study was to evaluate the effectiveness of two different molecular methodologies in terms of sensitivity for the detection of External Quality Assessment (EQA) *Plasmodium* samples. Methods: A total of 104 lyophilized blood samples from 14 different UK-NEQAS (National External Quality Assessment Site) (2016–2021) and eight WHO-NEQAS distributions (2017–2020) were analyzed. An in-house multiplex PCR protocol, followed by single target real-time PCR protocols for all five *Plasmodium* species, was implemented. Results: The multiplex PCR had a success rate of 10/16 and 20/28 for *P. vivax* and *P. falciparum* species, respectively. On the other hand, the respective results for real-time PCR had a success rate of 13/16 (*P. vivax*), 28/28 (*P. falciparum*), 5/8 (*P. malariae*), 8/10 (*P. ovale*), and 10/14 (*P. knowlesi*). *Plasmodium falciparum* samples displayed the highest sensitivity of detection, 0.02 parasites/µL. *Plasmodium vivax* samples displayed a 0.1 parasites/µL cutoff value, greater than the respective value for whole blood samples, while *P. ovale* species displayed a respective cutoff value of 0.05 parasites/µL. Due to the limited number of tested samples, data obtained for *P. malariae* and *P. knowlesi* species samples were inconclusive. Conclusions: Real-time PCR comprises a credible molecular methodology in terms of sensitivity assessment and detection of low parasitemia levels of *Plasmodium* sp. in EQA lyophilized blood samples.

Keywords: malaria; *Plasmodium* species; diagnosis; multiplex PCR; real-time PCR

check for updates

Citation: Tegos, N.; Goumenopoulos, C.; Mpimpa, A.; Papavasilopoulos, V.; Beleri, S.; Patsoula, E. Sensitivity Assessment of a Multiplex and Real-Time PCR Protocols for the Detection of Malaria in External Quality Control Samples in the Malaria Reference Center in Greece. *Parasitologia* **2024**, *4*, 150–161. https://doi.org/10.3390/parasitologia4020013

Academic Editor: Geoff Hide

Received: 2 April 2024
Revised: 10 May 2024
Accepted: 14 May 2024
Published: 17 May 2024

Copyright: © 2024 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

1. Introduction

Malaria represents an ongoing issue of public health concern globally and is associated with increased levels of morbidity and mortality. Only for the year 2022, an estimated 249 million cases, along with 608,000 deaths, were recorded in 85 malaria-endemic countries [1]. These numbers account for an increase of five million cases compared with 2021. The African region continues to carry a disproportionate burden, with an estimated 233 million cases in 2022 (accounting for about 94% of cases globally). Furthermore, children under the age of five years constitute the most vulnerable demographic, with nearly 76% of the deaths taking place in the abovementioned geographical area. Infected female *Anopheles* mosquitoes (i.e., vectors of malaria parasites) transmit the disease to humans. Among the five known *Plasmodium* species, *Plasmodium falciparum* and *Plasmodium vivax* are associated with severity in the clinical outcomes in the majority of the cases, along with causing complications [2,3]. Nevertheless, rare cases of malaria with a severe clinical picture because of *Plasmodium ovale* and *Plasmodium malariae* have also been described in the literature [4,5].

The World Health Organization (WHO) launched a project in 2015 for malaria called GTS (Global Technical Strategy) for the time period 2016–2030 [6]. The primary aim is to reduce mortality rates and global incidence by at least 90% by the year 2030. Therefore, it is imperative to design and implement prompt and reliable diagnostic tools. At present, WHO recommends microscopy and quality-assured RDTs (rapid diagnostic tests) for suspected clinical malaria cases [7]. Nevertheless, these two methodologies are associated with lower sensitivities in asymptomatic patients, resulting in potential underestimation of malaria prevalence [8]. On the other hand, PCR-based assays are characterized by increased sensitivity and specificity, assisting diagnosis of cases of low-density malaria infections, which, in many cases, are subclinical [9]. It is of vital importance to understand that underdiagnosis may lead to delays in the initiation of antimalarial treatment and pose a serious threat to the lives of many patients [10].

The aim of the current study was to evaluate two different molecular methodologies, a conventional multiplex PCR for *P. falciparum* and *P. vivax* detection and single target real-time PCRs for all five *Plasmodium* species, in terms of prompt and sensitive detection of different *Plasmodium* species. A series of external quality control samples from the United Kingdom (UK) National External Quality Assessment Site (NEQAS), along with various WHO-NEQAS distributions, were included in the present study. These samples have been successfully handled in the context of laboratory competence in the external quality control of the Greek Malaria Reference Center. The main goal was to demonstrate the sensitivity levels of multiplex and real-time PCR methodologies in terms of the accurate detection of *Plasmodium* species and preparedness for successful malaria diagnosis in an ISO 15189:2012 certified malaria reference laboratory. Noteworthy, external quality control samples present with a distinct form and constitution compared with the average clinical sample (i.e., blood). External Quality Assessment (EQA) *Plasmodium*-positive samples are prepared either from cultured parasites or from clinical blood samples. On the other hand, EQA *Plasmodium*-negative blood samples are prepared from healthy blood donors and are made available to laboratories participating in specific distribution schemes in the form of lyophilized blood samples in order to facilitate their transport around the world [11]. Therefore, in the current study, we investigated the efficiency and detection sensitivity of two different approaches in molecular malaria diagnosis protocols in a series of EQA samples.

2. Materials and Methods

2.1. EQA Samples

A series of 104 lyophilized blood samples from 14 different UK-NEQAS (time period: 2016–2021) and eight WHO-NEQAS (time period: 2017–2020) distributions of the external quality control program for malaria of UK-NEQAS were used for the purposes of the current study. The distribution of the samples for the UK-NEQAS schemes was 14 negative for malaria samples, along with 42 positive for malaria samples (17 *P. falciparum*, eight *P. vivax*, three *P. malariae*, seven *P. ovale*, seven *P. knowlesi*). The respective distribution for the WHO-NEQAS schemes was 14 negative for malaria samples, along with 34 positive for malaria samples (11 *P. falciparum*, eight *P. vivax*, five *P. malariae*, three *P. ovale*, seven *P. knowlesi*). The identities of all 104 samples, along with the respective parasitaemias, were already available from the respective UK-NEQAS and WHO-NEQAS final reports, as this comparative study was performed retrospectively after the result submission and report of intended outcomes.

All samples were reconstituted with 0.5 mL RNase-free water, according to guidelines of the participating external quality control interlaboratory body [12]. Thereafter, samples were subjected to automated DNA extraction (iPrep, Invitrogen, Van Allen Way Carlsbad, CA, USA), following the manufacturer's instructions. The next step included measurement of the optical density, hence calculating the $A_{260/280}$ ratios, the values of which ranged from 1.08 to 2.22 (Eppendorf biophotometer, Hamburg, Germany).

2.2. β-Globin Internal Control PCR Protocol

An internal control PCR protocol was implemented, amplifying a 260 bp sequence of the human β-globin gene, as previously described [13]. BEI Resource NIAID (Biodefence and Emerging Infections Research Resources Repository, National Institute of Allergy and Infectious Diseases), NIH (National Institutes of Health) strains were used as positive controls in all PCR protocols, namely strains: genomic DNA from *Plasmodium falciparum*, Strain 02000708 Tanzania, MRA-1169G, contributed by Michal Fried [14] and diagnostic plasmid containing the small subunit ribosomal RNA gene (18S) from *Plasmodium vivax*, MRA-178 [15], contributed by Peter A. Zimmerman, respectively.

2.3. Multiplex PCR Protocol

An in-house multiplex PCR protocol was implemented, amplifying simultaneously and in a single reaction, *P. falciparum* and *P. vivax* positive samples, as previously described [16]. This protocol was slightly modified, namely a quantity of 3 μL of genomic DNA corresponding to a total reaction volume of 30 μL. The exact composition of the PCR master mix included 3 μL of PCR Buffer (including $MgCl_2$), 0.6 μL of dNTPs mix, 0.3 μL for each of the three tested primers, 0.1 μL of Taq polymerase, and the remaining quantity with water for molecular use (all reagents supplied by New England Biolabs). Three (3) primers were used: PL3 (5′-ATG GCC GTT TTT AGT TCG TG-3′), PL4 (5′-GGA AAC GGT ACG ATA AGC CA-3′) and PL5 (5′-ACG CGT GCA GCC TAG TTT AT-3′). PL3 amplifies a sequence common to the rRNA genes of *P. falciparum* and *P. vivax*. PL4 is specific for *P. vivax* and PL5 for *P. falciparum*, respectively. An initial 5 min denaturation step at 94 °C was followed by 30 cycles (denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s), and a final elongation step at 72 °C for 10 min (2720 Thermal Cycler, Applied Biosystems). PCR products were then subjected to agarose gel electrophoresis; banding patterns were visualized and photographed.

The amplified PCR products, 346 bps and 266 bps, respectively, were successfully tested by the accredited Greek Malaria Reference Center (ISO 15189:2012), both for whole blood as well as lyophilized samples. BEI Resource NIAID, NIH strains were used as positive controls, namely strains MRA-1169G for *P. falciparum* and MRA-178 for *P. vivax*, respectively, as previously described.

2.4. Real-Time PCR Protocol

The implementation of single target real-time PCR protocols for each one of the five different *Plasmodium* species was applied to the EQA samples to ensure accurate species diagnosis.

The multiplex real-time PCR protocol by Rougemont et al. (2004), as four separate single real-time PCRs, was implemented for the detection of *P. falciparum*, *P. vivax*, *P. ovale*, and *P. malariae* species, respectively [17]. Furthermore, a single real-time PCR protocol by Divis et al. (2010) was implemented for the detection of *P. knowlesi* species [18]. All samples were tested in single real-time PCR protocols, in which targeted forward and a common reverse primer, along with targeted for each species probes, were implemented, as previously described.

A modification, in terms of PCR cycle number, was performed for the five discrete *Plasmodium* species, namely 44 cycles for *P. falciparum* and *P. vivax*, 40 cycles for *P. ovale* and *P. malariae* and 45 cycles for *P. knowlesi*, respectively. Polymerase chain reaction cycling conditions remained the same for all species, namely denaturation at 95 °C for 15 s, followed by a common annealing and extension step at 60 °C for 1 min. BEI Resource NIAID, NIH strains were used as positive controls, namely strains MRA-1169G for *P. falciparum* and MRA-178 for *P. vivax*, respectively, as previously described. Furthermore, a diagnostic plasmid containing the small subunit ribosomal RNA gene (18S) from *P. ovale*, MRA-180, contributed by Peter A. Zimmerman, along with a purified plasmid DNA containing the small subunit ribosomal RNA gene (18S) from *P. malariae*, MRA-179, contributed by Peter

A. Zimmerman, and a genomic DNA from *P. knowlesi*, Strain H, MRA-456G, contributed by Alan W. Thomas, were used [14,15].

All samples were recorded, and the respective Ct values were calculated. The number of 40 cycles was deduced as the cut-off value; namely, a sample was recorded as negative if a curve was found beyond the abovementioned value. Based upon previous publications, a cycle number of <40 was set as the threshold detection level for positive by real-time PCR samples [17]. On the other hand, sample detection in cycle numbers of >40 was set as the threshold detection level for negative by real-time PCR samples.

2.5. Detection of Sensitivity of the Multiplex PCR and the Real-Time PCR Protocols for Malaria Diagnosis in EQA Samples

Following reports of positive results for malaria samples from 14 different UK-NEQAS (time period: 2016–2021) and eight WHO-NEQAS (time period: 2017–2020) distributions (as referred to in Section 2.1), 10-fold serial dilutions for the participating in the current study DNA samples were implemented.

Regarding the multiplex PCR protocol, a 10-fold dilution (1:10) was implemented for the abovementioned *Plasmodium* species samples, taking under consideration the exhibited specificity and sensitivity levels, along with the detection limit for *P. falciparum* and *P. vivax* species in whole blood samples, as previously published [16].

The same rationale was followed for the single target real-time PCR protocols in order to investigate and verify the limit of detection for each one of the five different *Plasmodium* species [17]. A series of 10-fold dilutions were performed (from 1:10 up to 1:10,000), and samples were evaluated, with the 1:10,000 dilution being set as the last dilution value.

3. Results

3.1. β-Globin Internal Control PCR Protocol

A PCR protocol, amplifying a 260 bp PCR product, was implemented successfully for the 104 participating lyophilized blood samples (Figure 1).

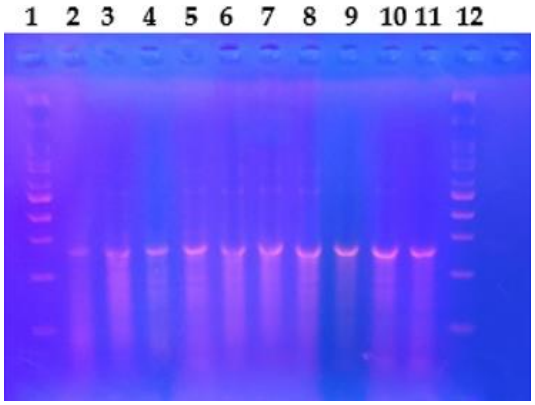

Figure 1. Representative PCR products for the detection of the expected 260 bp fragment corresponding to the β-globin gene (Lanes 1,12: 100 bp DNA ladder, 2–9: lyophilized blood samples, 10: MRA-1169G *P. falciparum* positive strain, 11: MRA-178 *P. vivax* positive strain).

3.2. Multiplex PCR Protocol

The expected 266 bp PCR product, corresponding to the *P. vivax* species, was observed in 10 out of 16 *P. vivax* samples. On the other hand, a 346 bp PCR product, corresponding to the *P. falciparum* species, was deduced for 20 out of 28 *P. falciparum* samples (Table S1).

3.3. Real-Time PCR Protocol

The multiplex real-time PCR protocol by Rougemont et al. (2004), as four separate single real-time PCRs, was implemented for the detection of *P. falciparum*, *P. vivax*, *P. ovale*, and *P. malariae* species, respectively (Table S1) [17].

The *P. falciparum* lyophilized blood samples were successfully detected, with a success rate of 28/28. Thereafter, the *P. vivax* lyophilized blood samples were successfully detected, with a success rate of 13/16. Namely, the target sequence was not detected in three cases, probably due to degradation of the genomic DNA, with the latter samples being excluded from further analysis. Therefore, the number of samples for investigation through serial dilutions was narrowed to 13.

Furthermore, the *P. malariae* lyophilized blood samples were successfully detected, with a success rate of 5/8. The target sequence was not detected in three cases, probably due to degradation of the genomic DNA, with the latter samples being excluded from further analysis. Therefore, the number of samples for investigation through serial dilutions was narrowed to five.

In addition to that, the *P. ovale* lyophilized blood samples were successfully detected, with a success rate of 8/10. The target sequence was not detected in two cases, probably due to degradation of the genomic DNA, with the latter samples being excluded from further analysis. Therefore, the number of samples for investigation through serial dilutions was narrowed to eight.

The fifth and final *Plasmodium* species, namely *P. knowlesi*, was investigated by implementation of a single real-time PCR protocol by Divis et al. (2010) [18]. The *P. knowlesi* lyophilized blood samples were successfully detected, with a success rate of 10/14. The target sequence was not detected in four cases, probably due to degradation of the genomic DNA, with the latter samples being excluded from further analysis. Therefore, the number of samples for investigation through serial dilutions was narrowed to 10.

3.4. Multiplex PCR Results for Serial Dilutions of Plasmodium Samples

A total of 10 samples, positive for *P. vivax*, were subjected to a 1:10 serial dilution. There were four cases that successfully displayed the expected 266 bp PCR product. On the other hand, a total of 20 samples, positive for *P. falciparum*, were also subjected to a 1:10 serial dilution. The results revealed six cases that successfully displayed the expected 346 bp PCR product.

3.5. Real-Time PCR Results for Serial Dilutions of Plasmodium Samples

Positive samples for the five investigated *Plasmodium* species were subjected to a 10-fold dilution range (from 1:10 up to 1:10,000), and samples were evaluated, with the 1:10,000 dilution being set as the last dilution value.

3.5.1. Real-Time PCR Results for Serial Dilutions of *P. falciparum* Samples

All 28 DNA samples were investigated in terms of their serial 10-fold dilutions. Namely, one sample was found negative in all dilutions, six samples were detected as positive up to the first dilution (1:10), five samples were found positive up to the second dilution (1:100), 12 samples were detected positive up to the third dilution (1:1000). In contrast, four samples were positive up to the fourth dilution (1:10,000), respectively. In the experimental design, we decided to include a fifth dilution (1:100,000) for samples that were detected positive in less than 37 cycles in the 1:10,000 dilution. There were two cases where the target sequence was detected in reaction cycle 36. As far as the positive control DNA is concerned, it was detected after the 17th cycle (Figure 2).

Thereafter, the minimum detectable concentration by real-time PCR was estimated. Three out of twenty-eight samples displayed an initial parasite density value of 0.2 parasites/μL up to the first dilution (1:10). Therefore, the concentration value of 0.02 parasites/μL was set as the accepted cutoff value of the tested real-time PCR protocol for the *P. falciparum* EQA lyophilized samples.

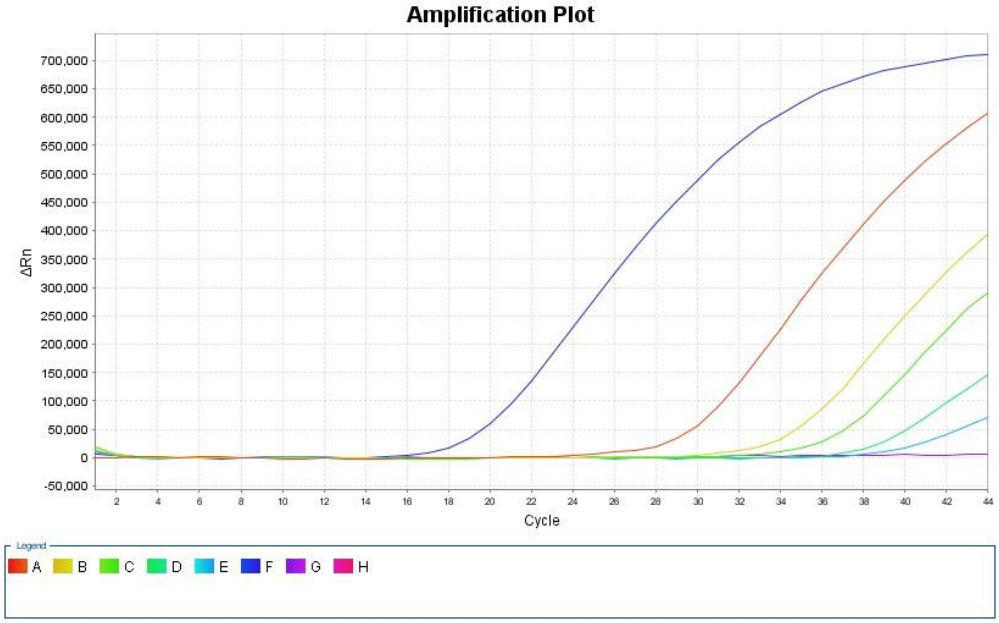

Figure 2. Representative real-time PCR curves for positive *P. falciparum* external quality control lyophilized sample MRA-1169G (From left to right: Positive control, initial concentration of 10^3 parasites/μL in 17th cycle, 1:10 dilution in 27th cycle, 1:100 dilution in 33rd cycle, 1:1000 dilution in 35th cycle, 1:10,000 dilution in 37th cycle, 1:100,000 in 39th cycle).

3.5.2. Real-Time PCR Results for Serial Dilutions of *P. vivax* Samples

The remaining 13 positive samples displayed heterogeneous results in the serial dilution investigation stage. More specifically, two samples did not amplify the expected target sequence after dilution, four samples were detected as positive up to the first dilution (1:10), six samples up to the second dilution (1:100), and only one up to the third dilution (1:1000), respectively. As far as the positive DNA marker is concerned, it was found positive after the 19th cycle.

Thereafter, the minimum detectable by real-time PCR concentration was estimated. Three out of thirteen samples displayed an initial parasite density value of one parasite/μL, two of them up to the first dilution (1:10). Therefore, the concentration value of 0.1 parasites/μL was set as the accepted cutoff value of the tested real-time PCR protocol for the *P. vivax* lyophilized samples.

3.5.3. Real-Time PCR Results for Serial Dilutions of *P. malariae* Samples

The remaining five samples displayed the target sequence only in the undiluted form in one case, whereas four samples displayed the target sequence up to the first dilution (1:10) respectively. The positive marker was detected in the ninth reaction cycle.

Thereafter, the minimum detectable by real-time PCR concentration was estimated. One out of five samples displayed an initial parasite density value of five parasites/μL, up to the first dilution (1:10). Therefore, the concentration value of 0.5 parasites/μL was set as the accepted cutoff value of the tested real-time PCR protocol for the *P. malariae* lyophilized samples.

3.5.4. Real-Time PCR Results for Serial Dilutions of *P. ovale* Samples

The remaining eight samples were further investigated by serial 10-fold dilutions. One sample was found positive only in the undiluted form, four samples were found positive up to the first dilution (1:10), and the remaining three samples were found positive up to the second dilution (1:100), respectively. Finally, the positive DNA marker was detected in the seventh reaction cycle.

Thereafter, the minimum detectable by real-time PCR concentration was estimated. One out of eight samples displayed an initial parasite density value of five parasites/μL until the second dilution (1:100). Therefore, the concentration value of 0.05 parasites/μL was set as the accepted cutoff value of the tested real-time PCR protocol for the *P. ovale* lyophilized samples.

3.5.5. Real-Time PCR Results for Serial Dilutions of *P. knowlesi* Samples

The remaining 10 samples were further investigated by serial 10-fold dilutions. One case of a sample was found positive only in the undiluted form. Two samples were detected as positive up to the first dilution (1:10), four samples were found positive until the second dilution (1:100), two samples were positive up to the third dilution (1:1000) and one sample was positive up to the fourth dilution (1:10,000), respectively. The positive marker was detected in the 27th reaction cycle.

Thereafter, the minimum detectable by real-time PCR concentration was estimated. Two out of 10 samples displayed an initial parasite density value of one parasite/μL, with one of them until the second dilution (1:100). Therefore, the concentration value of 0.01 parasites/μL was set as the accepted cutoff value of the tested real-time PCR protocol for the *P. knowlesi* lyophilized samples.

4. Discussion

The eradication of malaria constitutes a challenging and multifaceted task, relying, among others, upon the use of antimalarial drugs, insecticide-treated bed nets, indoor residual spraying, and the elimination of vector-breeding sites [19]. Malaria manifestations vary greatly among different patient populations and epidemiologic settings, a fact that further complicates definitive diagnosis [20] and imposes an urgent need for prompt and accurate laboratory detection. The generation of false-positive, as well as false-negative results, is regularly recorded in clinical settings. Nevertheless, the lack of consensus guidelines for diagnosis in clinical trials imposes a further obstacle in the current setting.

Microscopy and rapid diagnostic tests are recommended by the World Health Organization (WHO) as basic diagnostic tools for malaria, especially in cases of detecting elevated concentrations of different *Plasmodium* species in blood samples. On the other hand, the detection of low levels of parasitemias and identification at the species level often constitutes a difficult and laborious diagnostic task [21]. This situation is occasionally verified in cases of asymptomatic patients with *P. vivax* and *P. malariae* species [21,22]. Towards that direction, the implementation of molecular methodologies capable of detecting parasitemias under the lower threshold of microscopy (the gold standard for malaria detection and diagnosis) is continuously gaining ground in the foreign literature. Furthermore, molecular research protocols have the added advantage of coping with mixed infections in which more than one distinct *Plasmodium* species are implicated [17].

Molecular techniques of amplification of nucleic acids are currently used in epidemiological studies and research, drug efficacy assessment and tolerance, and diagnostic tools among different laboratory reference centers around the world. Variations in the polymerase chain reaction (PCR), including nested, multiplex, and real-time PCR protocols, amplifying a conserved target sequence in the 18S rRNA gene, increase the possibility of detecting pathogenic organisms in cases of low parasitemia levels [22].

The first part of the study included the implementation of an in-house multiplex PCR protocol, with a detection limit of one *P. falciparum* and three *P. vivax* per μL in whole blood samples [16]. In the case of using dried blood spots (DBS) instead of whole blood samples, the respective minimum detectable concentration corresponds to 10 parasites/μL of the initial sample. This finding has been verified in different molecular protocols, as described in the foreign literature [23]. Furthermore, these deviations between DBS and whole blood samples account for the lack of detection of positive samples, in the order of 10–27%, for the former type of samples. The majority of the non-detectable samples account for the *P. vivax* species, as opposed to the *P. falciparum* species, respectively [24]. Similar deviations

have also been recorded in the evaluation of external quality control DBS and lyophilized blood samples, with the latter case of samples being more credible in terms of detecting the implicated *Plasmodium* species [25]. Based on the above, it can be deduced that the expected limit of detection for lyophilized blood samples was of the order of ≤10 parasites/μL in the case of the implemented multiplex PCR protocol.

After the completion of the sensitivity assessment experiments, the authors could not reach a final conclusion regarding an accepted cutoff value with the implemented conventional molecular protocol for positive lyophilized blood samples, both for *P. falciparum* as well as *P. vivax* species. The above findings are indicative of a much lower than the expected performance of the multiplex PCR protocol; nevertheless, given that the current protocol has already been successfully tested in a large number of clinical blood samples in a time period of more than two decades, as part of the routine check of the Greek Malaria Reference Center, leads to the assumption that the nature of the implemented lyophilized blood samples constitutes the main factor that accounts for the recorded deviations.

The second part of the study included the initial implementation of a verified, in terms of sensitivity, real-time PCR protocol for the detection of four distinct *Plasmodium* species [17]. The discrete efficiency of the current protocol corresponds to 0.2 copies of the respective target sequence for a given *Plasmodium* species/μL reaction. Given that the target sequence of interest is located within seven gene loci in the genome of *P. falciparum* and 5–8 gene loci of the *P. vivax*, *P. malariae*, and *P. ovale*, respectively, we can translate the discrete efficiency of the current protocol in terms of parasitic numbers [17,22]. Namely, the genetic material that is expected to be detected corresponds to 0.03 parasites/μL for *P. falciparum* and 0.04 parasites/μL for the other three *Plasmodium* species, respectively. Furthermore, it has to be mentioned that according to the prerequisites set by WHO, the minimum and acceptable analytical sensitivity of molecular methods for malaria diagnosis is set at two parasites/μL [21]. External quality control program UK NEQAS, which is the one used by the accredited Greek Malaria Reference Center, has also accepted the same concentration threshold with WHO as a proper limit of detection for asymptomatic infections, as well as cases of low parasitemias [25].

A concentration value of 0.02 parasites/μL was set as the accepted cutoff value for the 28 tested *P. falciparum* lyophilized samples. This respective value is very close to the respective value proposed by Rougemont et al. (2004), after implementation in whole blood samples [17].

Investigation of 13 tested *P. vivax* lyophilized samples revealed a concentration value of 0.1 parasites/μL as the accepted cutoff value for that particular *Plasmodium* species. This respective value is slightly larger, as opposed to the respective value proposed by Rougemont et al. (2004), after implementation in whole blood samples [17]. The authors noticed that the discrete efficiency of the respective molecular protocol is slightly smaller in the case of the *P. vivax* species. This comparison has also been recorded in cases of clinical samples of patients with malaria and has been attributed either to the quadruple number of merozoites of the infected with *P. falciparum* erythrocytes or due to the regularly recorded asymptomatic infections of the *P. vivax* species [22]. Similar results, as far as the discrete efficiency is concerned, have also been extracted by other molecular protocols in the detection of *P. falciparum* and *P. vivax* [26].

The next samples to be investigated were five cases of positive for *P. malariae* lyophilized blood samples. A concentration value of 0.5 parasites/μL came up as the accepted cutoff value for that particular *Plasmodium* species. Nevertheless, given the small number of the tested *P. malariae* samples, the authors do not consider these findings as conclusive.

Evaluation of eight lyophilized blood samples for *P. ovale* followed thereafter, with the obtained data revealing one single case that displayed a concentration value of 0.05 parasites/μL. This particular value corresponded to the second serial dilution (1:100) and is very close to the respective value proposed by Rougemont et al. (2004), after implementation in whole blood samples [17]. Studies performed in whole blood samples have shown that the discrete efficiency of real-time PCR protocols displays lower sensitivity of

the *P. ovale* species, compared with *P. falciparum*, and larger compared with *P. vivax*, respectively [26]. This comparison was verified in our study in terms of evaluating lyophilized blood samples of the three abovementioned *Plasmodium* species.

The last stage of this study included the evaluation of 10 lyophilized blood samples for *P. knowlesi*, where a different molecular protocol was implemented by Divis et al. (2010) and tested in whole blood samples [18]. It is important to mention that this protocol was based on the initially used reaction conditions set by Rougemont et al. (2004) [17]. The analytical sensitivity of the implemented protocol, which tested positive for *P. knowlesi* whole blood samples, was set at two copies of the target sequence/μL of the reaction [18]. By following the same way of thinking, i.e., translating the discrete efficiency of the current protocol in terms of parasite numbers, we can estimate the expected detection of genetic material in the order of 0.2–0.4 parasites/μL. The results of the current study revealed, in two cases, a 0.01 parasites/μL concentration value, corresponding to the second serial dilution (1:100). Nevertheless, given the small number of the tested *Plasmodium knowlesi* samples as part of the external quality control samples, the authors consider these findings as primary observations that need to be further verified by processing a larger number of EQA samples.

The results of the current study verify the utility of real-time PCR in terms of prompt detection of different *Plasmodium* species, the origin of which is lyophilized blood. Nevertheless, it has to be mentioned that certain challenges or limitations were encountered during the duration of the experimental workflow. Namely, the nature of the implemented lyophilized blood samples resulted in laboratory deviations for a number of different samples. To be more specific, there were cases of samples being associated with low concentrations after DNA extraction and recorded DNA quantities. In addition to that, a small number of samples had to be excluded from further evaluation due to a lack of obtaining an acceptable DNA quantity. At first, these deviations were attributed to the nature of the lyophilized blood samples. In contrast, it has to be mentioned that there were cases of DNAs that did not amplify with either of the two implemented molecular methodologies (i.e., multiplex and real-time PCR). Another comment that has to be made relates to the cost of implementing that particular molecular technique. Although it outweighs multiplex PCR, it can be characterized as affordable when applied for the detection of a particular *Plasmodium* species, as was the case in the current study. On the other hand, the fact that most malaria-endemic countries lack resource requirements poses an obstacle in terms of the implementation of such a technique [21,25].

To sum up, the preliminary results of the current study highlight the practical applicability of both implemented molecular protocols, namely multiplex and real-time PCR. The former molecular technique is characterized by increased sensitivity and specificity, along with low cost and verified repeatability; therefore, it can be implemented as a screening process in endemic *P. falciparum* settings. Given that this protocol has already been successfully tested by the Greek Malaria Reference Center for whole blood and dried blood spot samples [16], the authors propose the utility of dried blood spots as a proper specimen type for the abovementioned cases. This notion comes in accordance with other literature findings suggesting the implementation of nested PCR as a suitable malaria surveillance molecular tool for dried blood spots collected from people living in low-transmission settings [27]. Hence, these approaches can serve as diagnostic alternatives, enabling asymptomatic submicroscopic infections, being associated with dramatic savings in labor and costs.

As far as the real-time PCR molecular technique is concerned, the results of the current study are promising in the sense that they present similarities to respective findings from testing whole blood samples with the same molecular approach [17]. Therefore, it goes without further notice that targeted real-time PCR protocols can be applicable for testing specific *Plasmodium* species without having to bear the increased cost of multiplex real-time PCR protocols [28,29]. Furthermore, findings from a study in Western Kenya suggest that blood retained from rapid diagnostic tests (RDTs) can be used for molecular quantification

of *P. falciparum* parasite density by using a duplex PCR approach, presenting an alternative approach for malaria-endemic countries [30].

Tremendous progress has been achieved during the past decades in terms of the implementation of innovative molecular protocols for accurate malaria diagnosis. Nevertheless, much has to be accomplished in the future so that these tools can be effective as well and, most importantly, in settings of malaria-endemic countries, accounting worldwide for the vast majority of malaria cases.

5. Conclusions

The results of the current study clearly demonstrated the superiority of the real-time PCR methodology as a credible molecular methodology in the detection of malaria in EQA lyophilized blood samples, even after several serial dilutions. The highest sensitivity was recorded for the *P. falciparum* species, in close proximity to the respective value for whole blood samples. Similar observations were deduced for the *P. ovale* species, whereas a larger cutoff value emerged for the *P. vivax* species, as opposed to the respective value for whole blood samples. On the other hand, multiplex PCR can be applied as a screening tool for endemic *P. falciparum* settings. A final comment, in terms of future research goals, would include the implementation of a larger number of lyophilized blood samples and comparison with whole blood and dried blood spot samples through modern molecular methodologies.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/parasitologia4020013/s1. Table S1: Supplementary File.

Author Contributions: Conceptualization, N.T. and E.P.; methodology, N.T., C.G. and E.P.; validation, N.T., C.G., A.M., V.P., S.B. and E.P.; formal analysis, N.T. and E.P.; investigation, N.T., C.G., A.M., V.P., S.B. and E.P.; writing—original draft preparation, N.T.; writing—review and editing, N.T. and E.P.; visualization, N.T., C.G. and E.P.; supervision, N.T. and E.P.; project administration, E.P. All authors have read and agreed to the published version of the manuscript.

Funding: This publication has received funding by the MSc Program in Public Health, Department of Public Health Policy, University of West Attica.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data are contained within the article and Supplementary Material (Table S1).

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. World Health Organization 2023a. World Malaria Report 2023. 2023. Available online: https://www.who.int/teams/global-malaria-programme/reports/world-malaria-report-2023 (accessed on 26 April 2024).
2. Song, X.; Wei, W.; Cheng, W.; Zhu, H.; Wang, W.; Dong, H.; Li, J. Cerebral malaria induced by *plasmodium falciparum*: Clinical features, pathogenesis, diagnosis, and treatment. *Front. Cell Infect. Microbiol.* **2022**, *12*, 939532–939545. [CrossRef] [PubMed]
3. Mat Salleh, N.H.; Abdul Rahman, M.F.; Samsusah, S.; De Silva, J.R.; Tan, J.H.; Amir, A.; Lau, Y.L. Complications of sub-microscopic *Plasmodium vivax* malaria among Orang Asli in Pos Lenjang, Kuala Lipis. *Trop. Biomed.* **2021**, *38*, 33–35. [CrossRef] [PubMed]
4. Martin Polo, L.; Crespillo-Andújar, C.; Rey Blas, J.R. Acute myocardial infarction secondary to infection by *Plasmodium ovale*. *Med. Clin.* **2020**, *154*, 325–326. [CrossRef] [PubMed]
5. Kotepui, M.; Kotepui, K.U.; Milanez, G.D.; Masangkay, F.R. Global prevalence and mortality of severe *Plasmodium malariae* infection: A systematic review and meta-analysis. *Malar. J.* **2020**, *19*, 274–286. [CrossRef]
6. World Health Organization 2023b. Global Technical Strategy for Malaria 2016–2030. 2021. Available online: https://www.who.int/publications/i/item/9789240031357 (accessed on 1 February 2023).
7. Ballard, E.; Wang, C.Y.T.; Hien, T.T.; Tong, N.T.; Marquart, L.; Pava, Z.; Tarning, J.; O'Rourke, P.; McCarthy, J.S. A validation study of microscopy versus quantitative PCR for measuring *Plasmodium falciparum* parasitemia. *Trop. Med. Health* **2019**, *47*, 49–52. [CrossRef]

8. Ayong, L.; Moukoko, C.E.E.; Mbacham, W.F. Diagnosing malaria: Methods, tools, and field applicability. *Methods Mol. Biol.* **2019**, *2013*, 73–82. [CrossRef]
9. Okell, L.C.; Bousema, T.; Griffin, J.T.; Quédraogo, A.L.; Ghani, A.C.; Drakeley, C.J. Factors determining the occurrence of submicroscopic malaria infections and their relevance for control. *Nat. Commun.* **2012**, *3*, 1237–1245. [CrossRef] [PubMed]
10. Berzosa, P.; Lucio, A.D.; Romay-Barja, M.; Herrador, Z.; González, V.; García, L.; Fernández-Martínez, A.; Santana-Morales, M.; Ncogo, P.; Valladores, B.; et al. Comparison of three diagnostic methods (microscopy, RDT, and PCR) for the detection of malaria parasites in representative samples from Equatorial Guinea. *Malar. J.* **2018**, *17*, 333–344. [CrossRef] [PubMed]
11. WHO. *WHO External Quality Assurance Scheme for Malaria Nucleic Acid Amplification Testing: Operational Manual, Version 1.0, 1 December 2017*; WHO: Geneva, Switzerland, 2017; pp. 1–22. Available online: https://apps.who.int/iris/bitstream/handle/10665/259950/WHO-CDS-GMP-2018.02-eng.pdf?sequence=1&isAllowed=y (accessed on 20 February 2023).
12. UK NEQAS. Distribution Information for UK NEQAS EQA. 2023. Available online: https://ukneqasmicro.org.uk/images/pdf/ReplyForm/DistributionInformationSheet.pdf (accessed on 20 February 2023).
13. Lima, G.F.M.C.; Levi, J.E.; Geraldi, M.P.; Sanchez, M.C.A.; Segurado, A.A.C.; Hristov, A.D.; Inoue, J.; de Jesus Costa-Nascimento, M.; Di Santi, S.M. Malaria diagnosis from pooled blood samples: Comparative analysis of real-time PCR, nested PCR and immunoassay as a platform for the molecular and serological diagnosis of malaria on a large-scale. *Mem. Inst. Oswaldo Cruz.* **2011**, *106*, 691–700. [CrossRef]
14. Park, D.J.; Lukens, A.K.; Neafsey, D.E.; Schaffner, S.F.; Chang, H.-H.; Valim, C.; Ribacke, U.; Van Tyne, D.; Galinsky, K.; Galligan, M.; et al. Sequence-based association and selection scans identify drug resistance loci in the *Plasmodium falciparum* malaria parasite. *Proc. Natl. Acad. Sci. USA* **2012**, *109*, 13052–13057. [CrossRef]
15. Mehlotra, R.K.; Lorry, K.; Kastens, W.; Miller, S.M.; Alpers, M.P.; Bockarie, M.; Kazura, J.W.; Zimmerman, P.A. Random distribution of mixed species malaria infections in Papua New Guinea. *Am. J. Trop. Med. Hyg.* **2000**, *62*, 225–231. [CrossRef] [PubMed]
16. Patsoula, E.; Spanakos, G.; Sofianatou, D.; Parara, M.; Vakalis, N.C. A single-step, PCR-based method for the detection and differentiation of *Plasmodium vivax* and *P. falciparum*. *Ann. Trop. Med. Parasitol.* **2003**, *97*, 15–21. [CrossRef] [PubMed]
17. Rougemont, M.; Van Saanen, M.; Sahli, R.; Hinrikson, H.P.; Bille, J.; Jaton, K. Detection of four *Plasmodium* species in blood from humans by 18S rRNA gene subunit-based and species-specific real-time PCR assays. *J. Clin. Microbiol.* **2004**, *42*, 5636–5643. [CrossRef] [PubMed]
18. Divis, P.; Shokoples, S.; Singh, B.; Yanow, S. A TaqMan real-time PCR assay for the detection and quantitation of *Plasmodium knowlesi*. *Malar. J.* **2010**, *9*, 344–350. [CrossRef] [PubMed]
19. Murphy, S.C.; Shott, J.P.; Parikh, S.; Etter, P.; Prescott, W.R.; Ann Stewart, V. Review article: Malaria diagnosis in clinical trials. *Am. J. Trop. Med. Hyg.* **2013**, *89*, 824–839. [CrossRef] [PubMed]
20. Jagannathan, P.; Kakuru, A. Malaria in 2022: Increasing challenges, cautious optimism. *Nature Commun.* **2022**, *13*, 2678–2680. [CrossRef] [PubMed]
21. WHO. *Evidence Review Group on Malaria Diagnosis in Low Transmission Settings, Geneva, 2013 December 16–18*; WHO: Geneva, Switzerland, 2014. Available online: https://www.who.int/docs/default-source/malaria/mpac-documentation/mpac-mar2014-diagnosis-low-transmission-settings-report.pdf?sfvrsn=5694f918_2 (accessed on 8 February 2023).
22. Gimenez, A.M.; Marques, R.F.; Regiart, M.; Bargieri, D.Y. Diagnostic methods for non-falciparum malaria. *Front. Cell Infect. Microbiol.* **2021**, *11*, 681063–681086. [CrossRef] [PubMed]
23. Aschar, M.; Sanchez, M.C.A.; de Jesus Costa-Nascimento, M.; de Lourdes, R.N.; Farinas, M.; Hristov, A.D.; Lima, G.F.M.C.; Inoue, J.; Levi, J.E.; Di Santi, S.M. Ultrasensitive molecular tests for *Plasmodium* detection: Applicability in control and elimination programs and reference laboratories. *Rev. Panam. Salud Publica* **2022**, *46*, e11–e20. [CrossRef] [PubMed]
24. Mahitticorn, A.; Masangkay, F.R.; Kotepui, M. Comparative performance of PCR using DNA extracted from dried blood spots and whole blood samples for malaria diagnosis: A meta-analysis. *Sci. Rep.* **2021**, *11*, 4845–4853. [CrossRef]
25. Cunningham, J.A.; Thomson, R.M.; Murphy, S.C.; de la Paz Ade, M.; Ding, X.C.; Incardona, S.; Legrand, E.; Lucchi, N.W.; Menard, D.; Nsobya, S.L.; et al. WHO malaria nucleic acid amplification test external quality assessment scheme: Results of distribution programmes one to three. *Malar. J.* **2020**, *19*, 129–140. [CrossRef]
26. Perandin, F.; Manca, N.; Calderaro, A.; Piccolo, G.; Galati, L.; Ricci, L.; Medici, M.C.; Arcangeletti, M.C.; Snounou, G.; Dettori, G.; et al. Development of a real-time PCR assay for detection of *Plasmodium falciparum*, *Plasmodium vivax*, and *Plasmodium ovale* for routine clinical diagnosis. *J. Clin. Microbiol.* **2004**, *42*, 1214–1219. [CrossRef] [PubMed]
27. Hsiang, M.S.; Lin, M.; Dokomajilar, C.; Kemere, J.; Pilcher, C.D.; Dorsey, G.; Greenhouse, B. PCR-based pooling of dried blood spots for detection of malaria parasites: Optimization and application to a cohort of Ugandan children. *J. Clin. Microbiol.* **2010**, *48*, 3539–3543. [CrossRef] [PubMed]
28. Van Bergen, K.; Stuitje, T.; Akkers, R.; Vermeer, E.; Castel, R.; Mank, T. Evaluation of a novel real-time PCR assay for the detection, identification and quantification of *Plasmodium* species causing malaria in humans. *Malar. J.* **2021**, *20*, 314–325. [CrossRef] [PubMed]

29. Lazrek, Y.; Florimond, C.; Volney, B.; Discours, M.; Mosnier, E.; Houzé, S.; Pelleau, S.; Musset, L. Molecular detection of human *Plasmodium* species using a multiplex real time PCR. *Sci. Rep.* **2023**, *13*, 11388–11397. [CrossRef]
30. Robinson, A.; Busula, A.O.; Muwanguzi, J.K.; Powers, S.J.; Masiga, D.K.; Bousema, T.; Takken, W.; de Boer, J.G.; Logan, J.G.; Beshir, K.B.; et al. Molecular identification of *Plasmodium* parasite density from the blood retained in used RDTs. *Sci. Rep.* **2019**, *9*, 5107–5114. [CrossRef]

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.