Low prevalence of *Plasmodium malariae* and *Plasmodium ovale* mono-infections among children in the Democratic Republic of the Congo: a population-based, cross-sectional study

Stephanie M. Doctor1, Yunhao Liu2, Olivia G. Anderson1, Amy N. Whitesell1, Melchior Kashamuka Mwandagalirwa1,3, Jérémie Muwonga4, Corinna Keeler5, Michael Emch1,5, Joris L. Likwela6, Antoinette Tshefu3 and Steven R. Meshnick1*

**Abstract**

**Background:** In an effort to improve surveillance for epidemiological and clinical outcomes, rapid diagnostic tests (RDTs) have become increasingly widespread as cost-effective and field-ready methods of malaria diagnosis. However, there are concerns that using RDTs specific to *Plasmodium falciparum* may lead to missed detection of other malaria species such as *Plasmodium malariae* and *Plasmodium ovale*.

**Methods:** Four hundred and sixty six samples were selected from children under 5 years old in the Democratic Republic of the Congo (DRC) who took part in a Demographic and Health Survey (DHS) in 2013–14. These samples were first tested for all *Plasmodium* species using an 18S ribosomal RNA-targeted real-time PCR; malaria-positive samples were then tested for *P. falciparum*, *P. malariae* and *P. ovale* using a highly sensitive nested PCR.

**Results:** The prevalence of *P. falciparum*, *P. malariae* and *P. ovale* were 46.6, 12.9 and 8.3 %, respectively. Most *P. malariae* and *P. ovale* infections were co-infected with *P. falciparum*—the prevalence of mono-infections of these species were only 1.0 and 0.6 %, respectively. Six out of these eight mono-infections were negative by RDT. The prevalence of *P. falciparum* by the more sensitive nested PCR was higher than that found previously by real-time PCR.

**Conclusions:** *Plasmodium malariae* and *P. ovale* remain endemic at a low rate in the DRC, but the risk of missing malarial infections of these species due to falciparum-specific RDT use is low. The observed prevalence of *P. falciparum* is higher with a more sensitive PCR method.

**Keywords:** *Plasmodium malariae*, *Plasmodium ovale*, Democratic Republic of the Congo, Rapid diagnostic test

**Background**

Malaria remains a severe global burden, causing an estimated 214 million cases and 438,000 deaths in 2015 [1], a toll that is particularly high in sub-Saharan Africa. Efforts to control malaria depend on diagnostic accuracy and availability, and in recent years the demand for rapid diagnostic tests (RDTs) has been increasingly high. RDTs detect malaria antigens in the blood using immunochromatography. They provide an easy-to-use alternative to microscopy, which requires skilled experts to be optimally effective [2, 3], and a cost-effective and field-ready alternative to PCR, which is more sensitive [4] but requires expensive equipment.

© 2016 The Author(s). This article is distributed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The Creative Commons Public Domain Dedication waiver (http://creativecommons.org/publicdomain/zero/1.0/) applies to the data made available in this article, unless otherwise stated.
Among the most widely used RDTs are those that detect *Plasmodium falciparum* histidine-rich protein 2 (*PfHRP2*). These RDTs are sensitive and specific for parasitaemias above 200 per μl blood [5]. RDTs that use the *Plasmodium* lactate dehydrogenase (pLDH) antigen are designed to detect all species of malaria, but are less sensitive [6–8].

In sub-Saharan Africa, where the vast majority of malaria infections are due to *P. falciparum* [1], RDTs detecting *PfHRP2* are most commonly used. However, mono-infections with non-falciparum malarias (primarily *Plasmodium malariae* and *Plasmodium ovale* in sub-Saharan Africa) may go undetected. In this study, a sub-set of samples from a nationally representative, cross-sectional study of children under age 5 years in the Democratic Republic of the Congo (DRC) were used to determine the prevalence of *P. malariae* and *P. ovale* mixed and mono-infections, and to assess the risk of missed detection due to the use of falciparum-specific RDTs.

### Methods

**Survey methodology and sample collection**

The 2013–14 Demographic and Health Survey (DHS) was a cluster-based household survey in the DRC, which took place between November 2013 and February 2014. As part of the survey, blood samples collected from children under 5 years of age were analysed for malaria infection, without speciation, by light microscopy. The samples were also tested with an RDT targeting *PfHRP2* (SD Bioline Malaria Ag Pf., Standard Diagnostics, Gyeonggi-do, Republic of Korea) and used to make dried blood spots (DBS). From DBS, DNA was extracted and tested for *P. falciparum* infection using a real-time PCR assay targeting the *P. falciparum* lactate dehydrogenase (*pfldh*) gene as previously described [9, 10]. This research was approved by institutional review boards at the Kinshasa School of Public Health and the University of North Carolina at Chapel Hill. Informed consent was obtained from a parent or responsible adult for all subjects.

Samples for this study were randomly chosen from four strata: (1) microscopy-positive, *pfldh* PCR-negative; (2) microscopy-negative, *pfldh* PCR-negative; (3) microscopy-positive, *pfldh* PCR-positive; and, (4) microscopy-negative, *pfldh* PCR-positive (Table 1). To detect a prevalence of 1 % (0, 2, 95 % confidence interval), the minimum sample size was estimated as 362 [11].

**All-Plasmodium real-time PCR assay**

Samples that initially tested negative by *pfldh* PCR underwent a real-time PCR assay that detects all species of *Plasmodium*, targeting the gene encoding the small sub-unit (18S) of the ribosomal RNA gene (heretofore referred to as “All Plasmodium qPCR”). Primer and probe sequences, reaction mixture and cycling conditions for this assay were previously published [12] with the exception that Probe Master qPCR Mix (Roche, Indianapolis, IN, USA) was used (Additional file 1: Table S1). Each sample was tested in duplicate. A sample was considered positive for *Plasmodium* if both of its replicates amplified or if one replicate amplified with a cycle threshold (C_T) value lower than 38.

**Speciation by BLAST**

Samples that tested positive in the All *Plasmodium* qPCR assay were speciated using Sanger sequencing and BLAST. DNA was amplified by nested PCR of *Plasmodium* 18S rDNA as previously described [13], with the outer primers rPLU1 and rPLU5 and the inner, genus-specific primers rPLU3 and rPLU4. Primer sequences and reaction conditions are listed in Additional file 1: Table S1.

Products from this nested PCR were Sanger sequenced (Eton Bioscience, Durham, NC, USA) and queried in the BLAST database (National Center for Biotechnology Information, Bethesda, MD, USA). If a sequence produced a result with at least 50 % query cover and 94 % identity, it was identified as the species with the best match. Otherwise, if a result produced 10 % query cover and 80 % identity, the cleanest part of the sequence, as determined by the authors, was resubmitted to BLAST and if this search fulfilled the initial criteria the species was defined. All sequences that did not produce such a match were labelled ‘indeterminate’ by this method.

**Speciation by species-specific nested PCR**

Samples that were malaria-positive by either the *pfldh* qPCR or the All *Plasmodium* qPCR were tested using species-specific nested PCR as in [13]. The Round 1 reaction used the same primers as used in the genus-specific nested PCR but with Qiagen HotStarTaq Master Mix (Qiagen, Hilden, Germany) (Additional file 1: Table S1). Separate Round 2 reactions were run for *P. falciparum* (primers rFAL1 and rFAL2), *P. malariae* (rMAL1 and rMAL2), and *P. ovale* (rOVA1 and rOVA2) (see Additional file 1: Table S1). The PCR products from each

### Table 1 Sampling strata used in this study

| Strata                  | Total size | Sampled | Fraction (%) |
|-------------------------|------------|---------|--------------|
| Microscopy+/PCR−        | 216        | 15      | 6.9          |
| Microscopy−/PCR−        | 4169       | 301     | 7.2          |
| Microscopy+/PCR+        | 1695       | 89      | 5.3          |
| Microscopy−/PCR+        | 1057       | 61      | 5.8          |
| Total                   | 7137       | 466     | 6.5          |

was defined by the authors, was resubmitted to BLAST and if this search fulfilled the initial criteria the species was defined. All sequences that did not produce such a match were labelled ‘indeterminate’ by this method.

**Speciation by species-specific nested PCR**

Samples that were malaria-positive by either the *pfldh* qPCR or the All *Plasmodium* qPCR were tested using species-specific nested PCR as in [13]. The Round 1 reaction used the same primers as used in the genus-specific nested PCR but with Qiagen HotStarTaq Master Mix (Qiagen, Hilden, Germany) (Additional file 1: Table S1). Separate Round 2 reactions were run for *P. falciparum* (primers rFAL1 and rFAL2), *P. malariae* (rMAL1 and rMAL2), and *P. ovale* (rOVA1 and rOVA2) (see Additional file 1: Table S1). The PCR products from each
speciation reaction were analysed by gel electrophoresis on a 3 % agarose gel to determine positivity for each species in each sample. Samples that did not amplify for any of the three species were labelled ‘indeterminate’ by this method.

The species of each sample was determined based on results from nested PCR. If a sample was indeterminate by nested PCR, the BLAST result was used instead. If the BLAST result was also indeterminate, the sample was removed from the analysis.

Analysis
Data were entered and analysed in Microsoft Excel 2007 (Microsoft, Redmond, WA, USA). Weighted prevalence were calculated as follows: for each stratum the proportion of positive samples in the sub-set was multiplied by the number of samples in the stratum. The sum of these was divided by the total number of samples.

Results
Study population
There were 7137 children under 5 years old with known pfldh PCR, microscopy and RDT results. Of these, a total of 466 samples (6.5 %) were chosen from the four strata listed in Table 1 for use in this study. A sample flow diagram is shown in Additional file 1: Figure S1.

All Plasmodium qPCR
Among the 316 samples that were negative by pfldh PCR, 52 samples were malaria-positive by the All Plasmodium qPCR assay—nine microscopy-positive and 43 microscopy-negative. Including these and 150 samples that were positive by pfldh PCR, a total of 202 malaria-positive samples was analysed for speciation. Out of these, four (2.0 %) were indeterminate (Table 2), giving an analysable population of 198 malaria-positive samples and 462 total samples.

Prevalence of Plasmodium falciparum
Of 198 malaria-positive samples, 190 (96.0 %) were positive for P. falciparum, giving a weighted prevalence in the survey sample set of 46.6 % (95 % CI 44.4–48.9 %) (Table 2). This is slightly higher than the prevalence of 38.6 % by the initial PCR test (pfldh) [10].

Prevalence of Plasmodium malariae and Plasmodium ovale
Of 198 samples, 52 (26.3 %) were positive for P. malariae and 34 (17.2 %) were positive for P. ovale, giving weighted prevalence of 12.9 % (95 % CI 10.0–15.9 %) and 8.3 % (95 % CI 5.7–10.8 %), respectively (Table 2). Geographical distributions of P. malariae and P. ovale infections are shown in Fig. 1. Both P. malariae and P. ovale infections are widely distributed. Eighty-nine percent of individuals with P. malariae or P. ovale infection were also infected with P. falciparum.

Five P. malariae mono-infections and three P. ovale mono-infections were found, giving weighted prevalence of 1.0 % (95 % CI 0.1–1.8 %) and 0.6 % (95 % CI 0–1.3 %), respectively. Of these eight non-P. falciparum mono-infections, six (75.0 %) were negative by RDT.

Discussion
Using a sub-set of 462 samples from the large, cross-sectional DHS, the prevalence of P. malariae and P. ovale among children in the DRC was found to be 12.9 and 8.3 %, respectively, with widespread geographical distributions seen in both species. A recent study of children in Western Kasai, DRC found a similar prevalence of P. malariae (13.8 %) but a lower prevalence of P. ovale (2.4 %) [14], and another study of asymptomatic individuals in six provinces of the DRC found a much lower prevalence of P. malariae (1.0 %) [15]. In 2007 prevalence were 4.9 % for P. malariae and 0.6 % for P. ovale [16]. In general, prevalence found here are higher than those reported in other African countries for P. malariae [17–20] and P. ovale [17, 18, 21]. However, all of these differences could be due to normal geographic and temporal variations as well as differences in the PCR and sampling methods.

Mono-infections of P. malariae and P. ovale appear to be rare in the DRC. In this study, the mono-infection prevalence were only 1.0 and 0.6 %, respectively. In Western Kasai, there were no P. malariae or P. ovale mono-infections reported [14].

Because HRP2-based RDTs detect P. falciparum only, they can result in missed detection of P. malariae and P. ovale mono-infections. Of the eight mono-infections found here, six were negative by RDT. The remaining two were likely recently cleared P. falciparum infections in which the HRP2 antigen was still present, as it can remain in the blood stream for up to 1 month after parasite clearance [22]. Overall, the number of non-falciparum infections missed by the RDT was small as most such cases were co-infections with P. falciparum.

Of 316 samples that were initially negative by pfldh PCR, 52 amplified P. falciparum 18S rDNA by nested species-specific PCR. As a result, the PCR prevalence using both tests was higher (46.6 %) than found using a single test (38.6 %). This is likely because the 18S rDNA assay has a lower threshold of detection. Thus, caution must be used when comparing prevalence rates determined using different PCR and survey methodologies.
Conclusions

*Plasmodium falciparum* remains the most prevalent species of malaria in the DRC, but *P. malariae* and *P. ovale* are endemic at a low rate. While RDTs have limitations, the results presented here suggest that the risk of missing malarial infections because they are mono-infections of *P. malariae* and *P. ovale* is low. However, the development of new RDTs to cover non-falciparum malaria will improve efforts at elimination.
Additional file

Additional file 1. PCR reaction and cycling conditions and sample flow diagram.

Abbreviations

RDT: rapid diagnostic test; PCR: polymerase chain reaction; PfHRP2: Plasmodium falciparum histidine-rich protein II; pLDH: Plasmodium lactate dehydrogenase; DHS: demographic and health survey; PCR: polymerase chain reaction; PfHRP2: Plasmodium falciparum histidine-rich protein II; pLDH: Plasmodium lactate dehydrogenase; DHS: demographic and health survey; DRC: Democratic Republic of the Congo; DBS: dried blood spots.

Authors’ contributions

MK, JM, JLL and AT conducted field and laboratory work to obtain samples. SMD and SRM conceived and designed the experiments. SMD, OGA and ANW performed the PCR assays. SMD, YL and SRM analysed the data. SMD, CK and ME created the Figures and Tables. SMD and SRM wrote the first draft of the manuscript. All authors revised it. All authors read and approved the final manuscript.

Author details

1 Department of Epidemiology, Gillings School of Global Public Health, University of North Carolina, Chapel Hill, NC 27599, USA. 2 Department of Statistics and Operations Research, University of North Carolina, Chapel Hill, NC 27599, USA. 3 Ecole de Santé Publique, Faculté de Medecine, Université de Kinshasa, Kinshasa, Kinshasa, Democratic Republic of the Congo. 4 Programme National de Lutte contre le Paludisme, Kinshasa, Kinshasa, Democratic Republic of the Congo. 5 Department of Geography, University of North Carolina, Chapel Hill, NC 27599-7435, USA. 6 Programme National de Lutte contre le Paludisme, Kinshasa, Democratic Republic of the Congo.

Acknowledgements

We are indebted to the administrators of the 2013–2014 DRC DHS and the participants of this study, without whom this work would not have been possible.

Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

Sharing of individual-level raw data is not permitted under current Institutional Review Board approvals.

Consent for publication

All authors have approved the final draft, contributed to it significantly and commensurate with their status as co-authors, and agree with the decision to seek publication.

Ethics approval and consent to participate

This research was approved by institutional review boards at the Kinshasa School of Public Health and the University of North Carolina at Chapel Hill. Informed consent was obtained from a parent or responsible adult for all subjects.

Funding

National Institutes of Health (grant S01AI107949 to SRM) and National Science Foundation (Grant BSC-1339949 to ME). The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

Received: 6 April 2016 Accepted: 21 June 2016 Published online: 08 July 2016

References

1. WHO. World malaria report 2015. World Health Organization, Geneva, Switzerland. 2015.
2. Mwingira F, Genton B, Kabanywanyi AN, Felger I. Comparison of detection methods to estimate asexual Plasmodium falciparum parasite prevalence and gametocyte carriage in a community survey in Tanzania. Malar J. 2014;13:433.
3. Kilian AH, Metzger WG, Mutschelknauss EJ, Kabagambe G, Langi P, Korte R, et al. Reliability of malaria microscopy in epidemiological studies: results of quality control. Trop Med Int Health. 2000;5:3–8.
4. Kamau E, Tolbert LS, Kortepeter L, Pratt M, Nyakoe N, Muringo L, et al. Development of a highly sensitive genus-specific quantitative reverse transcriptase real-time PCR assay for detection and quantitation of Plasmodium by amplifying RNA and DNA of the 18s rRNA genes. J Clin Microbiol. 2011;49:2946–53.
5. WHO. Malaria rapid diagnostic test performance: results of WHO product testing of malaria RDTs. Round 5 (2013). World Health Organization, Geneva, Switzerland. 2014.
6. Ashley EA, Touabi M, Aher M, Hutagaling R, Htun K, Luchavez J, et al. Evaluation of three parasite lactate dehydrogenase-based rapid diagnostic tests for the diagnosis of falciparum and vivax malaria. Malar J. 2009;8:241.
7. Heutemekers M, Gillet P, Cnops L, Bottieau E, Van Esbroeck M, Maltha J, et al. Evaluation of the malaria rapid diagnostic test SDFK90: detection of both PfHRP2 and Pf-pLDH. Malar J. 2012;11:359.
8. Hendriksen IC, Mtove G, Pedro AJ, Gomes E, Silamut K, Lee SJ, et al. Evaluation of a PfHRP2 and a pLDH-based rapid diagnostic test for the diagnosis of severe malaria in 2 populations of African children. Clin Infect Dis. 2011;52:1100–7.
9. Ministère du Plan et Suivi de la Mise en oeuvre de la Révolution de la Modernité MdISP, ICF International. Enquête Démographique et de Santé en République Démocratique du Congo 2013–2014. Rockville: MPSMRM, MSP et ICF International. 2014.
10. Doctor SM, Liu Y, Whitesell A, Thwai KL, Taylor SM, Janko M, et al. Malaria surveillance in the Democratic Republic of the Congo: comparison of microscopy, PCR, and rapid diagnostic test. Diagn Microbiol Infect Dis. 2016;85:16–8.
11. Dean AG SK, Soe MM. OpenEpi: Open source epidemiologic statistics for public health (2015). www.openepi.com. Accessed 17 June 2016.
12. Taylor SM, Juliano JJ, Trottman PA, Griffin JB, Landis SH, Kitsa P , et al. Molecular malaria epidemiology: mapping and burden estimates of the Democratic Republic of the Congo (2007). PLoS One. 2011;6:e16420.
13. Singh B, Bobogare A, Cox-Singh J, Sounouo G, Abdullah MS, Rahman HA. A genus- and species-specific nested polymerase chain reaction malaria detection assay for epidemiologic studies. Am J Trop Med Hyg. 1999;60:687–92.
14. Gabrielli S, Bellina L, Millardi GL, Katende BK, Totino V, Fulin V, et al. Malaria in children of Tshimbulu (Western Kasai, Democratic Republic of the Congo): epidemiological data and accuracy of diagnostic assays applied in a limited resource setting. Malar J. 2016;15:81.
15. Mvumbi DM, Bobanga TL, Melin P, De Mol P, Kayembe JM, Situmabanza HN, et al. High prevalence of Plasmodium falciparum infection in asymptomatic individuals from the Democratic Republic of the Congo. Malar Res Treat. 2016;2016:5405802.
16. Taylor SM, Messina JP, Hand CC, Juliano JJ, Muwonga J, Tshefu AK, et al. Molecular malaria epidemiology: mapping and burden estimates for the Democratic Republic of the Congo, 2007. PLoS One. 2011;6:e16420.
17. Williams J, Njie F, Cairns M, Bojang K, Coulibaly S, Kayentao K, et al. Non-falciparum malaria infections in pregnant women in West Africa. Malaria J. 2012;11:53.
18. Sititi L, Chipeta J, Miller JM, Moonga HB, Kumar N, Moss WJ, et al. Patterns of mixed Plasmodium species infections among children six years and under in selected malaria hyper-endemic communities of Zambia: population-based survey observations. BMC Infect Dis. 2015;15:204.
19. Geiger C, Agustar HK, Compaore G, Coulibaly B, Sie A, Becher H, et al. Declining malaria parasite prevalence and trends of asymptomatic parasitaemia in a seasonal transmission setting in North-Western Burkina Faso between 2000 and 2009–2012. Malar J. 2013;12:27.
20. Pullan RL, Bukirwa H, Staelke SG, Snow RW, Broker K. Plasmodium infection and its risk factors in eastern Uganda. Malar J. 2010;9:2.
21. Oguike MC, Betson M, Burke M, Nolder D, Stothard JR, Kleinschmidt I, et al. *Plasmodium ovale curtisi* and *Plasmodium ovale wallikeri* circulate simultaneously in African communities. Int J Parasitol. 2011;41:677–83.

22. Abeku TA, Kristan M, Jones C, Beard J, Mueller DH, Okia M, et al. Determinants of the accuracy of rapid diagnostic tests in malaria case management: evidence from low and moderate transmission settings in the East African highlands. Malar J. 2008;7:202.