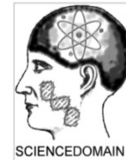
International Journal of TROPICAL DISEASE & Health
21(2): 1-8, 2017; Article no.IJTDH.31517
ISSN: 2278–1005, NLM ID: 101632866

SCIENCEDOMAIN ***international***
www.sciencedomain.org



Molecular Identification of *Plasmodium* Species in Malaria in Zimbabwe by Mitochondrial DNA Sequencing

Willard Mbiri[1] and Nyasha Chin'ombe[1*]

[1]*Molecular Microbiology Laboratory, Department of Medical Microbiology, College of Health Sciences, University of Zimbabwe, P.O.Box A178 Avondale, Harare, Zimbabwe.*

Authors' contributions

This work was carried out in collaboration between both authors. Authors WM and NC designed the whole study and wrote the protocols. Author WM collected and processed blood samples from hospitals. Authors WM and NC did laboratory bench work, analyzed data and wrote the manuscript. Both authors read and approved the final manuscript.

Article Information

DOI: 10.9734/IJTDH/2017/31517
Editor(s):
(1) Nicolas Padilla-Raygoza, Department of Nursing and Obstetrics, Division of Health Sciences and Engineering, Campus Celaya Salvatierra, Mexico.
Reviewers:
(1) Jaffu Othniel Chilongola, Kilimanjaro Christian Medical University College, Tumaini University Makumira, Moshi, Tanzania.
(2) Edward Pfeiler, Centro de Investigacion en Alimentacion y Desarrollo, Unidad Guaymas, Mexico.
Complete Peer review History: http://www.sciencedomain.org/review-history/17680

Short Research Article

Received 10th January 2017
Accepted 27th January 2017
Published 30th January 2017

ABSTRACT

Aim: To identify the *Plasmodium* species circulating in Zimbabwe.
Study Design: Cross-sectional study.
Place of Study: The study was conducted at three malaria referral hospitals in Harare, Zimbabwe from January to May 2016.
Methodology: Blood samples from patients with malaria were collected at three referral hospitals and DNA extracted. *Plasmodium* mitochondrial genomic DNA was amplified by polymerase chain reaction (PCR) and amplicons were sequenced. The sequences were analyzed using bioinformatics tools and *Plasmodium* species identified.
Results: A total of 160 blood samples were collected throughout Zimbabwe through three referral hospitals, of which 130 were malaria-positive and 30 were malaria-negative by microscopy. DNA was extracted from 100 samples (80 malaria-positive and 20 malaria-negative) and amplification of *Plasmodium* mitochondrial DNA was performed. Out of the 100 samples, 78 (78%) showed

*Corresponding author: Email: nyasha.chinombe@gmail.com;

amplification of the expected PCR band of 220 bp. Of the 80 malaria-positive samples, 77 showed mitochondrial genome amplification and of the 20 malaria-negative samples, 1 showed amplification of the expected band. Out of 78 samples that were successfully amplified, 60 were sequenced. Analysis of the sequences showed that all (100%) the selected samples belonged to *Plasmodium falciparum* species.

Conclusion: From the analyzed malaria-positive blood samples, *P. falciparum* was shown to be the only species of *Plasmodium* present. Further studies with larger sample size may need to be done in order to ascertain whether *P. falciparum* is the only species causing malaria in Zimbabwe.

Keywords: Malaria; Plasmodium; species; mitochondrial DNA; sequencing; Zimbabwe.

1. INTRODUCTION

Malaria is a fatal insect-borne tropical disease that continues to pose public health challenges with about 3.3 billion people at risk of being infected by the disease globally [1]. Africa carries the greatest burden of the disease where it is one of the major causes of mortality and morbidity [2]. Malaria is caused by the *Plasmodium* parasite that is transmitted by female *Anopheles* mosquitoes [3]. The main species of *Plasmodium* that are known to cause malaria are *P. falciparum, P. ovale, P. vivax,* and *P. malariae*. *P. knowlesi* was recently identified and is now recognized as an emerging zoonotic species of *Plasmodium* [4]. In Zimbabwe, malaria remains a serious national health problem [5]. With about 50% of the population of Zimbabwe at risk, malaria accounts for around 30% of all outpatient attendances and about 12% of the inpatients [5]. Currently, the routine diagnosis of malaria in Zimbabwe and most African countries is based on clinical symptoms and/or on the use of microscopy and rapid diagnostic tests (RDTs). These approaches lack accuracy and infecting *Plasmodium* species cannot be determined in most cases. The use of molecular methods such as polymerase chain reaction (PCR) and DNA sequencing can increase the ability to diagnose malaria and to identify the different *Plasmodium* species causing the disease. The mitochondrial genome has been targeted for both molecular diagnosis and identification of the different species of the *Plasmodium* parasite [6-8]. The key advantage of using this target is that mature gametocytes have 4-8 mitochondrial organelles per parasite and each organelle can have about 20 copies of the 6 kb mitochondrial genome [9,10]. This potentially makes the amplification of the genome target by PCR very sensitive, even if the parasite level in the blood of patients is very low [7,8]. The aim of this study was to identify the *Plasmodium* species circulating in Zimbabwe by amplifying and sequencing a short hypervariable segment of the *Plasmodium* mitochondrial genome within the cytochrome c oxidase gene.

2. MATERIALS AND METHODS

2.1 Collection and Processing of Malaria-positive Blood Samples

A cross-sectional study was done on residual blood samples collected from malaria-positive and negative patients who attended three referral hospitals (Harare Central Hospital (HCH), Parirenyatwa Group of Hospitals (PGH) and Chitungwiza Central Hospital (CCH)) in Harare, but were from different rural areas of Zimbabwe. The study group included participants with acute malaria symptoms, confirmed as positive and those reported as negative by microscopic examination in hospital laboratories. Patient material used in this study was collected between January 2016 and May 2016. Malaria-positive and negative residual EDTA blood samples were collected from hospital laboratories by researchers of this study. The blood samples were temporarily stored in the hospital laboratories for 7 days before being discarded. To get these samples for our study, we came to collect them within 7 days where they were stored in the cold room at 4°C. To select the samples, laboratory result registers of the hospital laboratories were used in order to identify the samples which had been found to be malaria-positve or malaria-negative by standard microscopy. The selected samples were taken to the Department of Medical Microbiology, University of Zimbabwe and frozen at -20°C pending DNA extraction.

2.2 Total Genomic DNA Extraction from Blood Samples

Genomic DNA was extracted from the blood samples using the Quick-gDNA™ blood MiniPrep Kit (Zymo Research, USA) according to manufacturer's recommendations. Briefly, frozen whole blood specimens stored at -20°C were thawed at room temperature and mixed by vortexing for 4 - 6 seconds. The whole blood

(100 µl) was mixed with 400 µl of genomic lysis buffer and allowed to stand at room temperature for 10 minutes. The mixture was then transferred to a Zymo-Spin IIC TM column with a collection tube and then centrifuged at 10000 xg for 1 minute. The tube with flow through contents was discarded. The Zymo-Spin IIC TM column was washed with 200 µl of Pre-wash Buffer, and by 500 µl of g-DNA Wash Buffer. The DNA was eluted in a total elution buffer volume of 100 µl and stored at -20°C until DNA amplification.

2.3 DNA Amplification of Mitochondrial Genome

Part of the *Plasmodium* mitochondrial genome was amplified using polymerase chain reaction. The PCR in 50 µl volume contained 2x KAPA Taq ReadyMix (25 µl) (Kapa Biosystems, South Africa), forward (1 µl) and reverse (1 µl) genus-specific mitochondrial DNA primers (5′-TCG CTT CTA ACG GTG AAC-3′ and 5′-AAT TGA TAG TAT CAG CTA TCC ATA G-3′) (10 µM) as previously published [11], DNA template (5 µl) and water (18 µl). The KAPA Taq ReadyMix (2X) was a ready-to-use cocktail containing all components for PCR, except primers and template, and contained KAPA Taq DNA Polymerase (1 U per 50 µl reaction), KAPA Taq Buffer, dNTPs (0.2 mM of each dNTP at 1X), MgCl2 (1.5 mM at 1X) and stabilizers. The following cycling program was used for amplification: initial denaturation of 15 minutes at 95°C followed by 40 amplification cycles of 30 seconds at 95°C, 30 seconds at 49°C, and 30 seconds at 72°C, and ending with a final extension step of 10 minutes at 72°C. The quality of PCR products (10 µl) was checked by 2.5% agarose gel electrophoresis.

2.4 DNA Sequencing and Sequence Analysis

PCR-positive samples were selected and sent for DNA sequencing at Inqaba Biotechnical Industries, South Africa. The forward primer was used for the sequencing reaction. The ABI-Prism 3100 Genetic automated Analyzer (Applied Bio systems, Foster City, CA) was used for the sequencing following the manufacturer's instructions. The analysis of DNA sequences to identify the *Plasmodium* species was done using Geneious Basic program (Biomatters, USA) and the Basic Local Alignment Search Tool (BLAST) programs. A phylogenetic tree was drawn using the Geneious Basic program using the Juke-Cantor genetic distance model and UPGMA (Unweighted Pair Group Method with Arithmetic mean) algorithms. BLAST program was used to search *Plasmodium* species in public Genbanks based on our DNA sequences.

3. RESULTS

3.1 General Characteristics of the Study Population

The study population consisted of people of all ages who were at risk of getting malaria in Zimbabwe who visited the three referral hospitals in Harare; Harare Central Hospital (HCH), Parirenyatwa Group of Hospitals (PGH) and Chitungwiza Central Hospital (CCH) with malaria-related symptoms for the period January-May 2016. Harare is a non-malaria zone and so the patients who visited the three hospitals were referred from all over Zimbabwe especially the rural areas. The common symptoms of the patients included moderate to severe shaking, chills, high fever, profuse sweating, nausea, vomiting and diarrhea. Laboratory investigations for the confirmation of malaria diagnosis were ordered and EDTA blood samples were collected from which blood samples were obtained by the researchers. The total number of blood samples collected was 160, of which 130 were positive for malaria and 30 were negative for malaria based on microscopic examination of stained smears. From the samples which were collected at PGH, 60 were positive for malaria and 15 were negative for the disease. HCH had 40 samples were positive and 10 negative for the disease, while CCH contributed the lowest number of samples which were 30 positive and 5 negative for the disease.

3.2 DNA Extraction and Mitochondrial DNA Amplification

Out of 160 blood samples, genomic DNA was extracted from 100 (80 malaria-positive and malaria-negative 20 samples). This represented 62.5% of the total number of collected samples. Amplification of *Plasmodium* mitochondrial DNA was performed on the extracted DNA. Of these (n = 100), 78 (78%) showed amplification of the expected PCR band of 220 bp (Fig. 1). Of the 80 malaria-positive samples, 77 showed amplification and of the 20 malaria-negative samples, only 1 showed amplification of the expected band. Most PCR bands were strong on agarose (Fig. 1).

3.3 DNA Sequence Analysis and *Plasmodium* Species Identification

Out of 78 samples that were successfully amplified for *Plasmodium* mitochondrial DNA, 60 were sequenced. All the 60 sequences were clean (Fig. 2) and were eligible for analysis by Geneious Basic and BLAST programs. There were no ambiguous bases in the sequences, which could affect the identification of the *Plasmodium* species using the bioinformatics tools. The NCBI BLAST tool was used to identify the *Plasmodium* species. Results giving 100% in the sequence identities were assigned as the *Plasmodium* species present in the specimen. For those with results giving identities less than 100%, the species with the highest identity and lowest e- value was selected as the *Plasmodium* species present in the specimen.

Analysis of the sequences showed that all the 60 sequences belonged to *Plasmodium falciparum*. The pairwise identity of 59 out of the 60 sequences was 100% with *Plasmodium falciparum*. Only 1 sequence (M2) was 99.4% similar to *Plasmodium falciparum* sequence. There was a single base difference in M2 at nucleotide position 56 of the sequence where there was base A instead of base T which was present in *Plasmodium falciparum*. A further sequence searches in the Genbanks failed to find sequences which were 100% similar with M2. A phylogenetic tree was drawn to show the genetic relatedness of all the 60 samples (Fig. 3). Representative sequences of all *Plasmodium* species (*P. falciparum, P. malariae, P. ovale, P. vivax and P. knowlesi*) that cause malaria were included in the analysis (Fig. 3). Analysis showed that all the 60 sequences from our malaria samples clusted with *P. falciparum* sequence and were not closely related to *P. malariae, P. ovale, P. vivax* and *P. knowlesi* (Fig. 3).

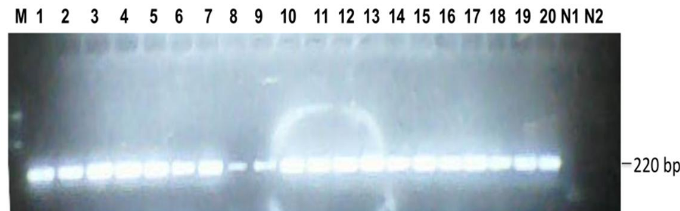


Fig. 1. A representative agarose gel photograph showing amplification of *Plasmodium* mitochondrial DNA from blood

Lane M: DNA marker, Lanes 1-20: amplified Plasmodium mitochondrial DNA from malaria-positive blood, Lanes N1 and N2: negative controls (malaria-negative blood)

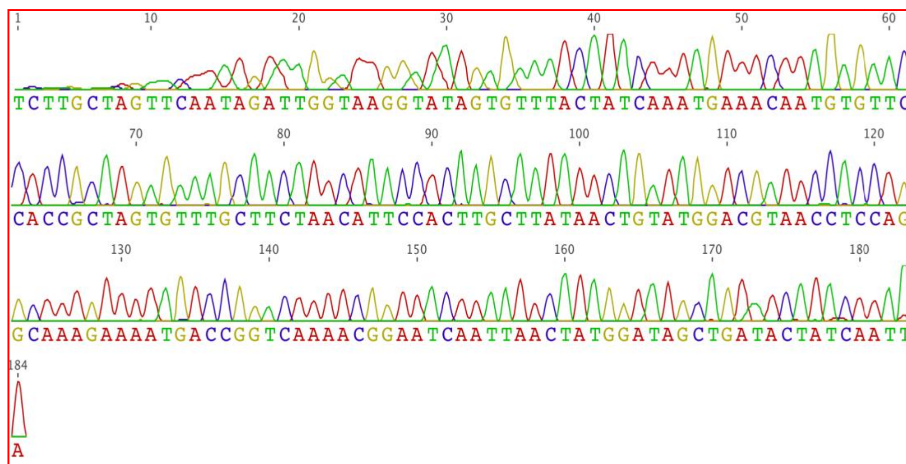


Fig. 2. A representative chromatograph result after sequencing one of the 60 *Plasmodium* mitochondrial DNA amplicons

The peaks in blue, green, grey and red colours represented nucleotides cytosine (C), thymine (T), guanine (G) and adenine (A), respectively

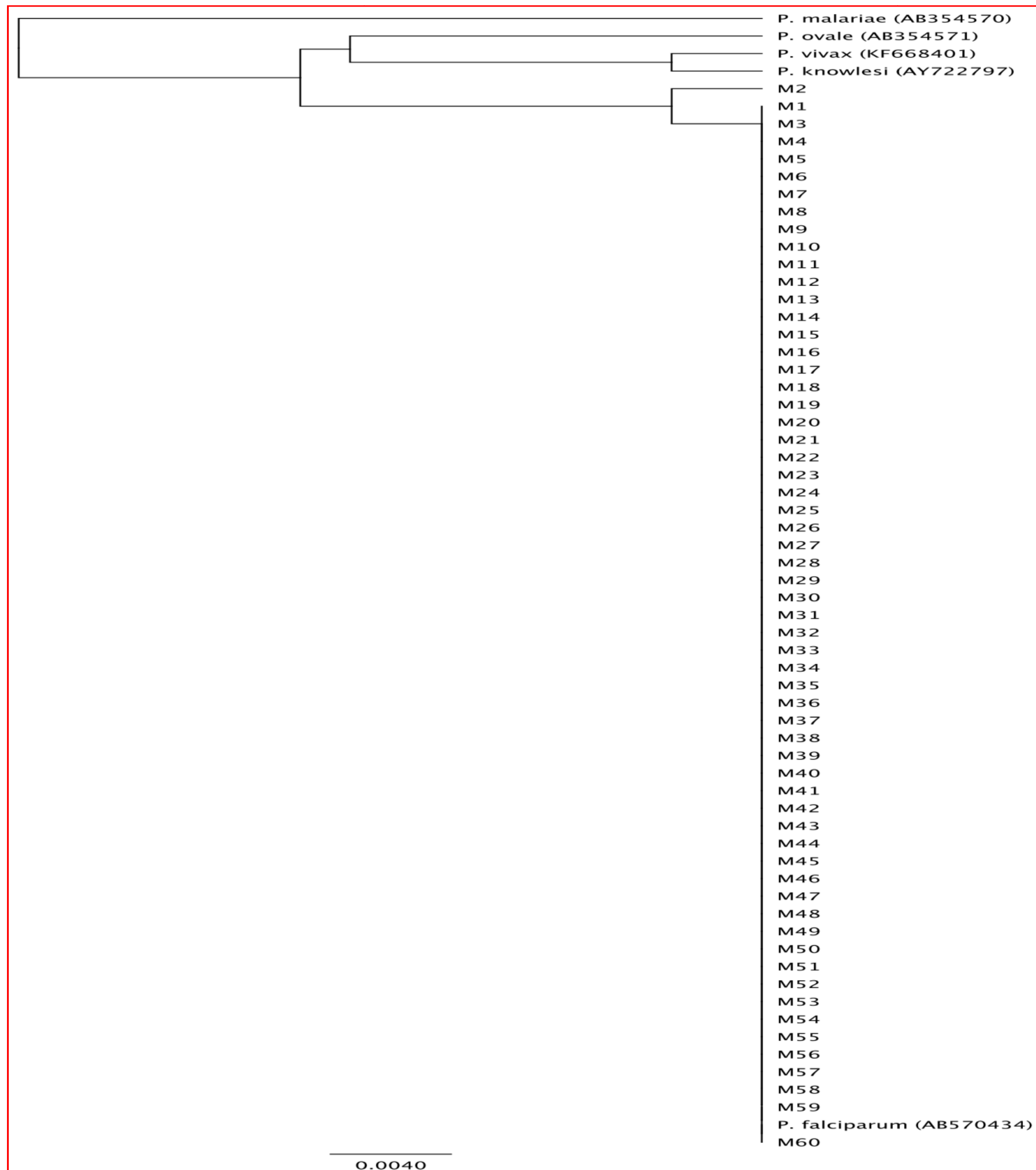


Fig. 3. *Plasmodium* mitochondrial genome sequence-based phylogenetic tree of the 60 malaria-positive blood samples

The DNA sequences were coded M1-M60 for samples 1-60 respectively. The 60 sequences were compared against known sequences of Plasmodium species, P. falciparum (ABS5570434), P. malariae (AB354570), P. ovale (AB354571), P. vivax (KF668401) and P. knowlesi (AY 722797)

4. DISCUSSION

Malaria is a serious disease caused by a parasite belonging to the *Plasmodium* genus and is transmitted by *Anopheles* mosquito vector [12]. In Zimbabwe, the disease is endemic in most rural areas [13]. In this study, blood samples of patients suspected to have malaria were collected at three main referral hospitals. The patients who visited the hospitals came from different parts of Zimbabwe, hence the samples we used in the study were representative of the whole country. Using polymerase chain reaction as a diagnostic tool, we were able to detect

mitochondrial DNA from blood samples that were found to be malaria-positive by microscopy, which is one of the main traditional methods. In 80 samples which were classified as malaria-positive, PCR was positive in 77 of them. It was no clear whether the 3 samples which were negative by PCR were false-positive by microscopy, or PCR method failed to detect the parasite. The PCR method was however able to detect the parasite DNA in one of the samples that had been categorized as malaria-negative by microscopy. This could be a typical case of low parasitemia and polymerase chain reaction was able to detect the presence of parasite DNA. Although traditional methods such as microscopy and RDTs are the mainstay of malaria diagnosis in most endemic areas such as Zimbabwe, they may fail to detect low parasitemia [14]. A study in Ethiopia showed that microscopy and RDT were less sensitive in detecting malaria than the PCR test [15]. Traditional methods are also poor at identifying the different species of *Plasmodium*. DNA amplification and sequencing methods can be used to resolve some of the challenges faced by traditional methods [16]. Such methods have not previously been used in Zimbabwe for detection and speciation of *Plasmodium* species. Targeting mitochondrial DNA for detection of *Plasmodium* has the advantage of increasing sensitivity. That is one of the reasons why most the PCR bands on the agarose gel were strong (Fig. 1). The short mitochondrial DNA fragment of 220 bp was amplified efficiently by PCR and the high copy number of the targeted mitochondrial genome further increased the accumulation of the PCR product. Sequencing product (Fig. 2) also showed that amplification of the target was efficient. Targeting mitochondrial genome of the parasite increases sensitivity because of high copy number [11]. There are up to 150 copies of mitochondrial genome per parasite and PCR should practically be able to detect few parasites in a blood sample [17]. On top of detection, molecular methods can also be used for parasite speciation which cannot be done easily by traditional methods [18].

In this study, all the 60 samples (100%) that were sequenced had *Plasmodium falciparum* species (Fig. 3). None of the samples had *P. ovale, P. vivax, P. malariae or P. knowlesi. P. falciparum* is known to be dominant and widely distributed in many tropical and sub-tropical countries especially those in the Sub-Saharan region [19]. It is also responsible for the deadliest form of malaria [20]. The *P. vivax* species is normally distributed in temperate regions, but is also found in some tropical and sub-tropical areas [19]. *P. ovale* is not very common, but is found in some tropical regions of Africa [19]. *P. malariae* is sporadically distributed and is found in some areas where *P. falciparum* is also found [19]. Previous reports in Zimbabwe indicated that *P. falciparum* was responsible for 97.8% of all malaria cases, followed by *P. malariae* and *P. ovale* with prevalence of 1.8% and 0.3% respectively [21]. It was also previously reported that the main mosquito vector for malaria transmission in Zimbabwe was the female *Anopheles arabiensis* [21]. Other vectors involved in malaria transmission included *Anopheles gambiae*, *An. fenestus, An. quadranulatus* and *An. merus* [21]. It has further been shown that peak transmission of malaria occurred between February and May [21] and this was the time of the year this study was carried out.

Although we found that all blood samples to have only *P. falciparum*, a previous study about three decades ago in Zimbabwe reported that *P. falciparum* had a prevalence of 97% using microscopic examination [22]. Zimbabwe Malaria Programme Performance review of June 2011 also supports the findings of this study reporting *P. falciparum* to be 98% prevalent [23]. Our study is the first to use molecular method in Zimbabwe to speciate the *Plasmodium*. The predominance of *P. falciparum* has also been found in other studies in Africa. A study in Tanzania showed that all patients screened by PCR and sequencing did not have any other species of *Plasmodium* except *P. falciparum* [24]. In a similar study in Mozambique*, P. falciparum* was the most prevalent malaria parasite with greater than 90% of all infections, only 6% were due to *P. malariae* and the remainder made up of mixed infections of *P. falciparum* and *P. malariae* [25]. In another study in Zambia, 88% of all malaria infections were due to *P. falciparum*, 10.6% as mixed infections and 1.4% as non-*falciparum* mono-infections [26]. In Botswana, 98% of all malaria cases were reported to be due to *P. falciparum* and 2% due to *P. malariae* and *P. ovale* [27]. In Angola, *P. falciparum* accounted for 98.8% of all cases [28]. In Burkina Faso, *P. falciparum* has been found to coexist with *P. malariae* and *P. ovale* [29].

5. CONCLUSION

From the few analyzed malaria-positive blood samples collected in Zimbabwe, *P. falciparum* was shown to be the only species of *Plasmodium*

to be present. The other four *Plasmodium* species that cause malaria in humans, *P. ovale, P. vivax*, *P. malariae* and *P. knowlesi* were not detected. A bigger study with a larger sample size may need to be conducted in order to ascertain whether *P. falciparum* is the only predominant *Plasmodium* species circulating in Zimbabwe.

CONSENT AND ETHICAL APPROVAL

This study was approved by Joint Research Ethics Committee (JREC) (Approval no. JREC/28/16) and Medical Research Council of Zimbabwe (MRCZ) (Approval no. MRCZ/B/1080) as per local requirement. No informed consent was signed by patients. The study was exempted from written informed consent because the human-derived samples used constituted non-identifiable residual clinical specimens which were destined for discarding by hospital laboratories. Institutional permission to use the residual samples was sort and granted from the clinical laboratory heads.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. World Health Organization. World Malaria Report. World Health Organization, Geneva, Switzerland; 2014.
2. Breman JG, Alilio MS, Mills A. Conquering the intolerable burden of malaria: What's new, what's needed: A summary. Am J Trop Med Hyg. 2004;71(2 Suppl):1-15.
3. Cohuet A, Harris C, Robert V, Fontenille D. Evolutionary forces on *Anopheles*: What makes a malaria vector? Trends Parasitol. 2010;26(3):130-6.
4. Millar SB, Cox-Singh J. Human infections with *Plasmodium knowlesi*--zoonotic malaria. Clin Microbiol Infect. 2015;21(7): 640-8.
5. Ministry of Health and Child Care. Guidelines for management of malaria in Zimbabwe: Diagnosis, management of uncomplicated and severe malaria. Harare, Zimbabwe; 2009.
6. Polley SD, Mori Y, Watson J, Perkins MD, Gonzalez IJ, Notomi T, Chiodini PL, Sutherland CJ. Mitochondrial DNA targets increase sensitivity of malaria detection using loop-mediated isothermal amplification. J Clin Microbiol. 2010;48:2866-2871.
7. Xu W, Morris U, Aydin-Schmidt B, Msellem MI, Shakely D, Petzold M, Björkman A, Mårtensson A. SYBR Green real-time PCR-RFLP assay targeting the *Plasmodium* cytochrome B gene--a highly sensitive molecular tool for malaria parasite detection and species determination. PLoS One. 2015;10(3): e0120210
8. Isozumi R, Fukui M, Kaneko A, Chan CW, Kawamoto F, Kimura M. Improved detection of malaria cases in island settings of Vanuatu and Kenya by PCR that targets the *Plasmodium* mitochondrial cytochrome c oxidase III (cox3) gene. Parasitol Int. 2015;64:304-8.
9. Preiser PR, Wilson RJ, Moore PW, McCready S, Hajibagheri MA, Blight KJ, Strath M, Williamson DH. Recombination associated with replication of malarial mitochondrial DNA. EMBO J. 1996;15: 684–693.
10. Krungkrai J. The multiple roles of the mitochondrion of the malarial parasite. Parasitol. 2004;129:511–524.
11. Haanshuus CG, Mohn SC, Morch K, Langeland N, Blomberg B, Hanevik K. A novel, single-amplification PCR targeting mitochondrial genome highly sensitive and specific in diagnosing malaria among returned travellers in Bergen, Norway. Malar J. 2013;12:26.
12. Solomon L, Okere HC, Daminabo V. Understanding human malaria: Further review on the literature, pathogenesis and disease control. Rep Opinion. 2014;6(6): 55-63.
13. Ministry of Health and Child Care. National Malaria Control Programme, 2008-2012. Harare, Zimbabwe.
14. Okell LC, Ghani AC, Lyons E, Drakeley CJ. Submicroscopic infection in *Plasmodium falciparum*-endemic populations: A systematic review and meta-analysis. J Infect Dis. 2009;200: 1509–1517.
15. Harris I, Sharrock WW, Bain LM, Gray KA, Bobogare A, Boaz L, Lilley K, Krause D, Vallely A, Johnson ML, Gatton ML, Shanks GD, Cheng Q. A large proportion of asymptomatic *Plasmodium* infections with low and submicroscopic parasite densities in the low transmission setting of Temotu Province, Solomon Islands: Challenges for

malaria diagnostics in an elimination setting. Malar J. 2010;9:254.
16. Osman MM, Nour BY, Sedig MF, De Bes L, Babikir AM, Mohamedani AA, Mens PF. Informed decision-making before changing to RDT: A comparison of microscopy, rapid diagnostic test and molecular techniques for the diagnosis and identification of malaria parasites in Kassala, Eastern Sudan. Trop Med Int Health. 2010;15: 1442–1448.
17. Isozumi R, Fukui M, Kaneko A, Chan CW, Kawamoto F, Kimura M. Improved detection of malaria cases in island settings of Vanuatu and Kenya by PCR that targets the *Plasmodium* mitochondrial cytochrome c oxidase III (cox3) gene. Parasitol Int. 2015;64:304-8.
18. Jeremiah S, Janagond AB, Parija SC. Challenges in diagnosis of *Plasmodium* knowlesi infections. Trop Parasitol. 2014;4:25–30.
19. Gilles HM. Milestones in the history of malaria and its control. In Bruce-Chwatt's Essential Malariology, Eds. Gilles HM and Warrel DA. Arnold, London; 1993.
20. Snow RW, Omumbo JA. Malaria. In: Jamison DT, Feachem RG, Makgoba MW, Bos ER, Baingana FK, Hofman KJ, Rogo KO, editors. Disease and mortality in Sub-Saharan Africa. 2nd Edition. Washington (DC): World Bank. 2006;14.
21. Taylor P, Mutambu SL. A review of the malaria situation in Zimbabwe with special reference to the period 1972-1981. Trans R Soc Trop Med Hyg. 1986;80:12-19.
22. Taylor P. The malaria problem in Zimbabwe. Epidemiology. Cent Afr J Med. 1985;31(9):163-6.
23. Ministry of Health and Child Welfare. National Malaria Control Programme, Zimbabwe Malaria Programme Review Report, Harare, Zimbabwe; 2011.
24. Strøm GE, Moyo S, Fataki M, Langeland N, Blomberg B. PCR targeting *Plasmodium* mitochondrial genome of DNA extracted from dried blood on filter paper compared to whole blood. Malaria J. 2014;13:137.
25. Marques PX, Saúte F, Pinto VV, Cardoso S, Pinto J, Alonso PL, Rosário VE, Arez AP. *Plasmodium* species mixed infections in two areas of Manhiça District, Mozambique. Int J Biol Sci. 2005;1(3):96-102.
26. Sitali L, Chipeta J, Miller JM, Moonga HB, Kumar N, Moss WJ, Michelo C. 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.
27. The Republic of Botswana Ministry of Health. Guidelines for the diagnosis and treatment of malaria in Botswana. Gaborone, Botswana; 2007.
28. Pembele GN, Rivero LR, Fraga J. Detection and species identification of malaria parasites by nested-PCR: Comparison with light microscopy and with SD BIOLINE malaria Ag test in Luanda, Angola. IJTDH. 2015;10(1):1-13.
29. Gnémé A, Guelbéogo WM, Riehle MM, Tiono AB, Diarra A, Kabré GB, Sagnon N, Vernick KD. Plasmodium species occurrence, temporal distribution and interaction in a child-aged population in rural Burkina Faso. Malar J. 2013;12:67.

© 2017 Mbiri and Chin'ombe; This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/4.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Peer-review history:
The peer review history for this paper can be accessed here:
http://sciencedomain.org/review-history/17680