

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

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 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: *P. falciparum* was shown to be the only species of *Plasmodium* causing malaria in Zimbabwe. Further studies with increased sample size are needed to ascertain whether *P. falciparum* is only predominant *Plasmodium* species 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 using polymerase chain reaction and DNA sequencing of the *Plasmodium* mitochondrial genome.

34 2. MATERIALS AND METHODS

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36 2.1 Collection and processing of Malaria-positive blood samples

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38 A cross-sectional study was done on residual blood samples collected from malaria-positive and
39 negative patients who attended three referral hospitals (Harare Central Hospital (HCH), Parirenyatwa
40 Group of Hospitals (PGH) and Chitungwiza Central Hospital (CCH)) in Harare, but were from different
41 rural areas of Zimbabwe. The study group included participants with acute malaria symptoms,
42 confirmed as positive and those reported as negative by microscopic examination in hospital
43 laboratories. Patient material used in this study was collected between January 2016 and May 2016.
44 Malaria-positive and negative residual EDTA blood samples were collected from hospital laboratories
45 by researchers of this study. The laboratory result register in the departments of parasitology was
46 used to select positive and negative samples. Laboratory generated identity numbers were then used
47 to link the samples of interest from those which were being temporarily stored pending disposal.
48 Positive samples were identified as those with malaria parasite as proved by microscopy of standard
49 Giemsa or Fields stained thin and thick films. Negative samples were identified as those with no
50 malaria parasite using the same methodology. Collected samples were frozen at -20°C pending DNA
51 extraction.

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53 2.2 Total genomic DNA extraction from blood samples

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55 Genomic DNA was extracted from the blood samples using the Quick-gDNA™ blood MiniPrep Kit
56 (Zymo Research, USA) according to manufacturer's recommendations. Briefly, frozen whole blood
57 specimens stored at -20°C were thawed at room temperature and mixed by vortexing for 4 - 6
58 seconds. The whole blood (100 µl) was mixed with 400 µl of genomic lysis buffer and allowed to stand
59 at room temperature for 10 minutes. The mixture was then transferred to a Zymo-Spin IIC TM column
60 with a collection tube and then centrifuged at 10000 xg for 1 minute. The tube with flow through
61 contents was discarded. The Zymo-Spin IIC TM column was washed with 200 µl of Pre-wash Buffer,
62 and by 500 µl of g-DNA Wash Buffer. The DNA was eluted in a total elution buffer volume of 100 µl
63 and stored at -20 °C until DNA amplification.

64 2.3 DNA amplification of mitochondrial genome

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66 Part of the *Plasmodium* mitochondrial genome was amplified using polymerase chain reaction. The
67 PCR in 50 µl volume contained 2x KAPA Taq ReadyMix (25 µl) (Kapa Biosystems, South Africa),
68 forward (1 µl) and reverse (1 µl) genus-specific mitochondrial DNA primers (3'-TCG CTT CTA ACG
69 GTG ACC-3' and 3'-AAT TGA TAG TAT CAG CTA TCC ATA G-3') (10µM) as previously published
70 [11], DNA template (5 µl) and water (18 µl). The KAPA Taq ReadyMix (2X) was a ready-to-use
71 cocktail containing all components for PCR, except primers and template, and contained KAPA Taq
72 DNA Polymerase (1 U per 50 µl reaction), KAPA Taq Buffer, dNTPs (0.2 mM of each dNTP at 1X),
73 MgCl₂ (1.5 mM at 1X) and stabilizers. The following cycling program was used for amplification of the
74 *Plasmodium* mitochondrial genome: initial denaturation of 15 minutes at 95°C followed by 40
75 amplification cycles of 30 seconds at 95°C, 30 seconds at 49°C, and 30 seconds at 72°C, and ending
76 with a final extension step of 10 minutes at 72°C. The quality of PCR products (10 µl) were checked
77 by 2.5% agarose gel electrophoresis.

78 2.4 DNA sequencing and sequence analysis

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80 PCR-positive samples were selected and sent for DNA sequencing at Inqaba Biotechnical Industries,
81 South Africa. The forward primer was used for the sequencing reaction. The ABI-Prism 3100 Genetic
82 automated Analyzer (Applied Bio systems, Foster City, CA) was used for the sequencing following the
83 manufacturer's instructions. The analysis of DNA sequences to identify the *Plasmodium* species was
84 done using Geneious Basic program (Biomatters, USA) and the Basic Local Alignment Search Tool
85 (BLAST) programs. A phylogenetic tree was drawn using the Geneious Basic program. BLAST
program was used to search *Plasmodium* species in public Genbanks based on our DNA sequences.

86 3. RESULTS

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88 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 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).



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).

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.

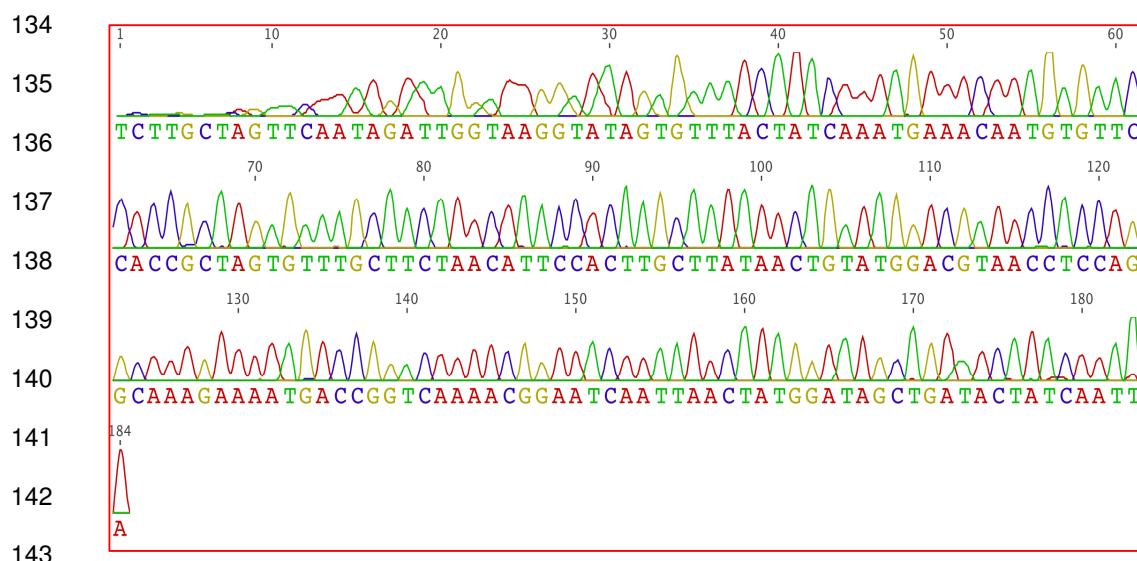


Fig. 2. A representative chromatograph result after sequencing one of the 60 *Plasmodium* mitochondrial DNA amplicons. The peaks in blue, green, grey and red colours represented nucleotides cytosine (C), thymine (T), guanine (G) and adenine (A), respectively.

Analysis of the sequences showed that all the 60 sequences belonged to *Plasmodium falciparum*. The sequence identities with *Plasmodium falciparum* ranged from 96% to 100%. The majorities of sequences from our samples were similar and had 100% similarity with *Plasmodium falciparum* sequences found in the Genbanks. 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 clustered with that of *P. falciparum* (Fig. 3).

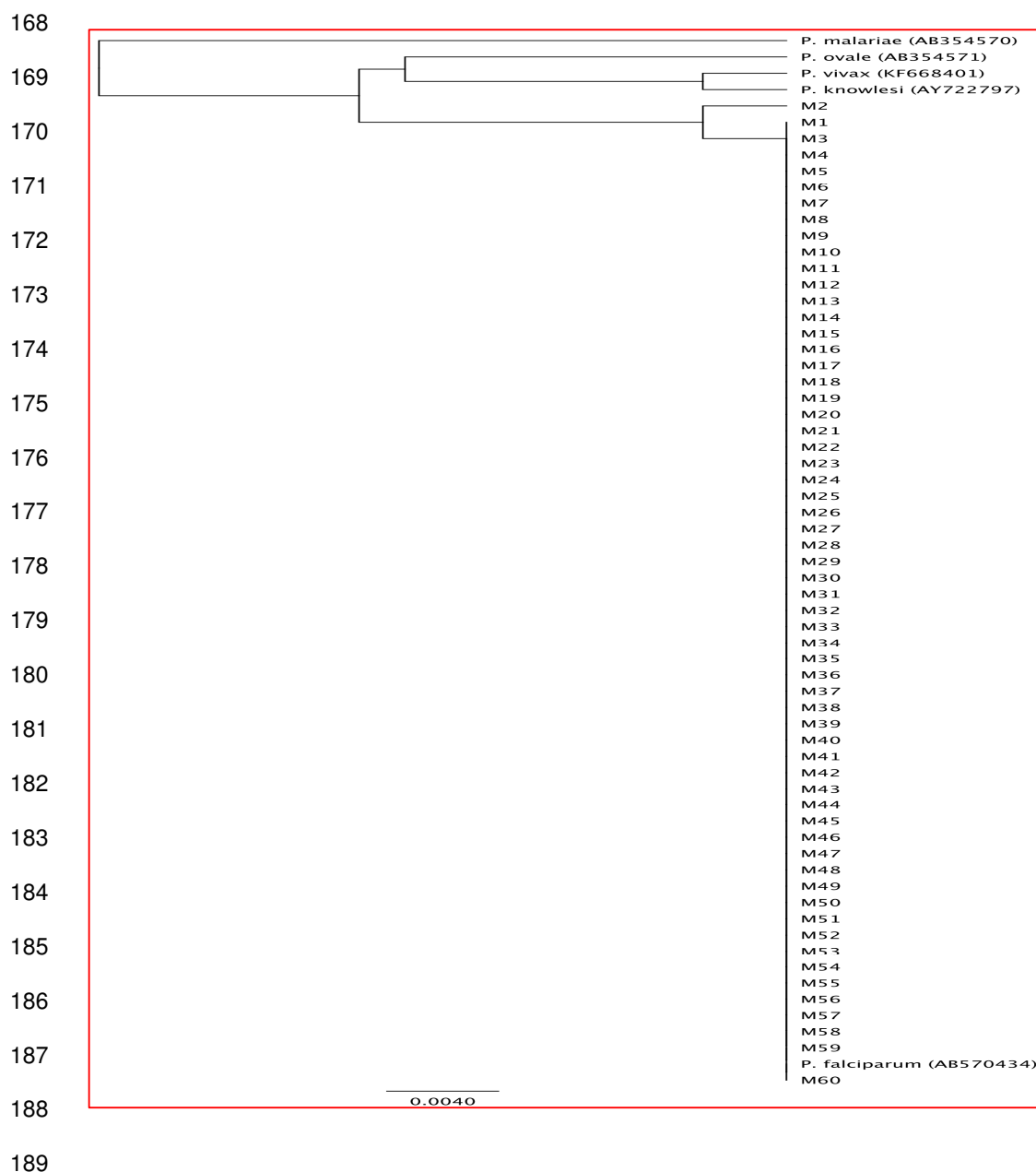


Fig. 3. *Plasmodium* mitochondrial genome sequence-based phylogenetic tree of the 60 malaria-positive blood samples. The DNA sequences were coded M1-M60 for samples 1-60 respectively. The 60 sequences were compared against known sequences of *Plasmodium* species, *P. falciparum* (AB5570434), *P. malariae* (AB354570), *P. ovale* (AB354571), *P. vivax* (KF668401) and *P. knowlesi* (AY 722797).

4. DISCUSSION

Malaria is a serious disease caused by single-celled protozoan organism that belongs to the *Plasmodium* genus. So far, more than 100 species of *Plasmodium* are known and some induce malaria in birds, reptiles and various mammals [12]. Of these species, only five (*P. falciparum*, *P. ovale*, *P. vivax*, *P. malariae* and *P. knowlesi*) are known to infect humans and cause malaria after transmission by *Anopheles* mosquito vector [12]. Accurate diagnosis and identification of the different species of *Plasmodium* are therefore important for epidemiological and surveillance of malaria especially in the tropics where the disease is endemic. In Zimbabwe, malaria is a major public health

issue and is responsible for a substantial number of hospital admissions in the country [13]. Although the country has a well-established malaria national control programme, malaria continues to infect and kill many people every year. The climatic conditions are conducive for transmission of malaria by mosquitoes and about half of the country's population is at risk of getting the disease [13]. The primary objectives of this study were to detect and identify the *Plasmodium* species circulating in Zimbabwe. Blood samples of patients suspected to have malaria were collected at three referral hospitals (Harare Central Hospital, Parirenyatwa Group of Hospitals and Chitungwiza Central Hospital) in Harare, Zimbabwe. The three hospitals are located in Harare province where malaria is not endemic. Hence all malaria cases are referred to these hospitals from district hospitals from rural areas where malaria is endemic. Therefore, the samples we used in the study were representative of the different rural areas of Zimbabwe. We however did not track the origins of each sample we used and this could be a source of bias.

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 not 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

This study confirmed that *P. falciparum* was the main species causing malaria in Zimbabwe. The other Plasmodium species causing malaria in humans, *P. ovale*, *P. vivax*, *P. malariae* and *P. knowlesi* were not detected. Much bigger study may need to be done with sampling sites throughout the country. A larger sample size and much broader study population covering most parts of the country may be required to rule out the presence of other *Plasmodium* species other than *P. falciparum*.

ETHICAL APPROVALS

This study was approved by Joint Research Ethics Committee (JREC) approval number JREC/28/16 (22 April 2016 to 21 April 2017) and Medical Research Council of Zimbabwe (MRCZ) approval number MRCZ/B/1080 (22 June 2016 to 21 June 2017) as per local requirement. The study was exempted from written consent because the human-derived samples constituted non-identifiable residual clinical specimens. Institutional permission to use the residual samples was sort and granted from the clinical and laboratory heads of department.

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