

## Molecular Identification of *Plasmodium* Species in Malaria in Zimbabwe by 18S Ribosequencing

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### Authors' contributions

This work was carried out in collaboration among all authors. Authors NC and WM designed and supervised the study. Author NC and WM performed laboratory experiments. Author DM analysed the sequence data using bioinformatics. All the authors wrote, reviewed and approved the final version of the manuscript.

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### ABSTRACT

**Background:** Malaria remains a major cause of mortality and morbidity in Zimbabwe due to transmission of *Plasmodium* parasite by the *Anopheles* mosquitoes. Globally, several species of *Plasmodium* parasite have been identified, but only *P. falciparum*, *P. ovale*, *P. vivax*, *P. malariae* and *P. knowlesi* are known to cause malaria in humans. No studies have previously been done in Zimbabwe to identify the *Plasmodium* species in malaria using molecular methods. The aim of this study was to identify circulating *Plasmodium* species in malaria in Zimbabwe using 18S ribosequencing.

**Methods:** The study was a cross-sectional survey in Zimbabwe from January to May in 2016. Venous blood was collected from malaria-suspected patients at three referral hospitals and subjected to microscopy and/or serology. Total DNA was isolated and *Plasmodium* species identified by 18S amplification and ribosequencing.

**Results:** A total of 160 patient samples were used in this study. Out of these, 130 were malaria-positive by microscopy and/or serology and 30 were negative. Total genomic DNA was extracted from 100 samples of the patients (80 malaria-positive and 20 malaria-negative samples by microscopy and/or serology). Amplification of the 18S ribosomal RNA gene of *Plasmodium* was performed on 74 malaria-positive and 6 malaria-negative samples. All the 74 samples showed 18S RNA gene amplification and the 6 negative controls did not show amplification. Only 50 amplicons

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were selected for sequencing. Ribosequencing and bioinformatics analyses showed that all (100%) the sequences belonged to *Plasmodium falciparum*.

**Conclusion:** The study was the first to provide molecular evidence of the existence of only *P. falciparum* in Zimbabwe. However, further studies with bigger sample sizes need to be done to ascertain whether *P. falciparum* is the dominant circulating species in malaria in Zimbabwe.

**Keywords:** Malaria; 18S Ribosequencing; *Plasmodium falciparum*; Zimbabwe.

## 1. BACKGROUND

Malaria remains a very serious public health tropical disease worldwide especially in Africa where it is a major cause of mortality and morbidity [1]. Globally, about 40% of the world population live in areas in which malaria is endemic [2]. The disease is caused by parasites that belongs to the genus *Plasmodium* and are transmitted to humans by the *Anopheles* species of mosquitoes [3]. There are five known species of *Plasmodium* that cause human malaria and these are *Plasmodium falciparum*, *P. vivax*, *P. malariae*, *P. ovale* and *P. knowlesi* [4]. Accurate diagnosis and identification of infecting *Plasmodium* species is crucial for provision of realistic management and treatment of malaria. Due to the high morbidity and mortality associated with species such as *P. falciparum* and *P. knowlesi*, their timeous identification is critical so that treatment can be expedited. A considerable number of *P. knowlesi* infections are known to progress to severe malaria in the absence treatment with mortality reaching approximately 2% [5]. In most tropical countries, microscopy and rapid diagnostic tests (RDTs) are always used for diagnosis of malaria, but these methods are poor at identifying the different species of *Plasmodium* [6]. In Zimbabwe, malaria is endemic and microscopy is considered to be the golden standard in diagnosis. Rapid methods are also in use in Zimbabwe for diagnosis of malaria, but to a lesser extent. Molecular methods such as polymerase chain reaction have the potential to overcome the limitations of microscopy and RDTs in identification of *Plasmodium* species in malaria. The aim of the study was to identify *Plasmodium* species circulating in Zimbabwe using malarial parasite 18S ribosomal RNA gene amplification and sequencing.

## 2. MATERIALS AND METHODS

### 2.1 Processing of Malaria-Positive Blood Samples and DNA Extraction

Malaria-positive blood samples were collected and processed as previously described [7].

Briefly, residual venous blood samples which were positive for malaria by microscopy and/or serology were collected from three referral hospitals, that is, Harare Central Hospital (HCH), Parirenyatwa Group of Hospitals (PGH) and Chitungwiza Central Hospital (CCH) in Harare and the patient samples came from different parts of Zimbabwe including rural areas. The blood samples were taken to the Department of Medical Microbiology, University of Zimbabwe and frozen at -20°C until DNA extraction. Total genomic DNA was extracted from the blood samples using the Quick-gDNA™ blood MiniPrep Kit (Zymo Research, USA) according to manufacturer's recommendations. The extracted DNA samples were stored at -20°C until *Plasmodium* DNA amplification.

### 2.2 DNA Amplification of 18S Ribosomal RNA Gene of Plasmodium

The *Plasmodium* 18S ribosomal RNA gene was amplified using consensus primers, 5' TTA AAA TTG CAG TTA AAA CG (forward primer) and 5' CCT GTT GTT GCC TTA AAC TTC (reverse primer) as previously published [8]. The primers targeted all *Plasmodium* species. All polymerase chain reaction reagents and DNA samples were properly thawed and mixed prior to amplification. Each amplification reaction contained 36.8 µl of distilled water, 0.2 µl of DreamTaq polymerase (5U/µl stock) (Inqaba, South Africa), 1 µl of dNTPs (10 mM stock) (Inqaba, South Africa), 1 µl forward primer (10 µM stock), 1 µl reverse primer (10 µM stock) (Inqaba, South Africa), 5 µl of 10x PCR Dream Buffer and 5µl of the template DNA and the total volume of the reaction was 50 µl. The following cycling program was used for amplification: initial denaturation of 5 minutes at 95°C followed by 50 amplification cycles of 30 seconds at 95°C, 30 seconds at 45°C, and 45 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.

### 2.3 DNA Sequencing and Sequence Analysis

After electrophoresis, amplicons with bands of the expected size (277 bp) were selected, packaged and sent to Inqaba Biotech, South Africa for sequencing. Sequencing outcomes were sent back by email to the researchers for bioinformatics analysis. The analysis of DNA sequences to identify the *Plasmodium* species was performed using both Geneious Basic program (Biomatters, USA) and the Basic Local Alignment Search Tool (BLAST) programs. A phylogenetic tree to check the genetic relatedness of the *Plasmodium* species was drawn using the Geneious Basic program. Representative sequences *Plasmodium* species (*P. falciparum*, *P. malariae*, *P. vivax*, *P. knowlesi* and *P. ovale*) downloaded from the Genbank were included in phylogenetic analysis of the sequences.

## 3. RESULTS

### 3.1 Study Subjects

The study subjects were made up of patients of all ages from Zimbabwe who had been confirmed to have malaria by microscopy and/or serology and were recruited from any of the referral hospitals in Zimbabwe, Harare Central Hospital (HCH), Parirenyatwa Group of Hospitals (PGH) and Chitungwiza Central Hospital (CCH) and they had malaria symptoms (fever, shaking, chills, sweating, nausea, vomiting and diarrhea). In this study, we focused more on identifying species of *Plasmodium* in patients who had been confirmed to be malaria-positive by microscopy and/or serology. Hence, the malaria-positive samples were used. The total number of blood samples used in this study was 160 and 130 were positive for malaria by microscopy and/or serology and 30 were negative.

### 3.2 Amplification and Sequencing of 18S Ribosomal RNA Gene of *Plasmodium* Parasite

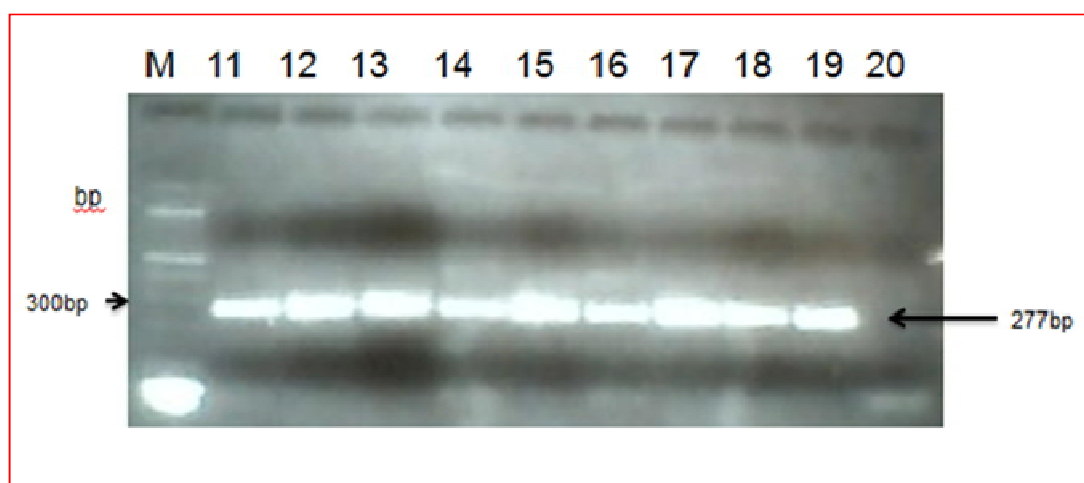
Total genomic DNA was extracted from only 100 samples of the patients (80 malaria-positive and 20 malaria-negative samples by microscopy and/or serology). The 18S ribosomal RNA gene of *Plasmodium* parasite was only amplified from the 74 malaria-positive and 6 malaria-negative samples. Out of the 80, the 74 samples (malaria-positive) showed amplification of the expected

band of 277 basepairs of 18S RNA gene of *Plasmodium*. Fig. 1 is a representative gel showing the amplification of the *Plasmodium* gene. On agarose gels, the amplicon bands were very strongly visible (Fig. 1). No DNA bands were found in the 6 negative control samples (Lane number 20, Fig. 1). Therefore, the PCR targeting 18S rRNA gene reported as positive all the 74 samples initially reported as positive by microscopy and/or serology and this represented 100 % agreement. The same PCR method also reported as negative the 6 patient samples previously reported as negative by microscopy and/or serology and this also represented 100% agreement of microscopy and 18S rRNA gene amplification.

### 3.3 18S Ribosequencing and *Plasmodium* Species Identification

Of the 74 samples that were positive by polymerase chain reaction, 50 were selected for sequencing. Out of these 50 samples sequenced, 47 (94%) were successful and 3 (6%) were not eligible for further sequence analysis. Analysis of the 47 sequences using Geneious Basic and BLAST programs showed that they were all *Plasmodium* DNA sequences. Further analysis showed that all the sequences belonged to the *Plasmodium falciparum* species (Table 1). Analysis of the sequences showed that they were more closely related to *P. falciparum* than to other species and percentage identities with known *Plasmodium* species were however not 100% (Table 1).

All the 47 successfully sequenced 18S rRNA genes were subject to further phylogenetic analysis. The sequences were compared against known *Plasmodium* species *P. malariae* (KJ934252), *P. ovale* (KF018661), *P. vivax* (KC 750244), *P. knowlesi* (KJ917896) and *P. falciparum* (HQ 283219). A phylogenetic tree was drawn and showed the genetic relatedness of our samples and known *Plasmodium* species. All the samples clustered closely with *P. falciparum* (HQ283219). V15 and V25 were more closely related to each other than they were to the rest of the V1 to V49 sequences. There was a distant relationship between the sequences V1 –V50 to the other known *Plasmodium* species, *P. malariae* (KJ934252), *P. ovale* (KF018661), *P. vivax* (KC 750244), *P. knowlesi* (KJ917896). All (100%) the 18S rRNA gene sequences were therefore identified as *P. falciparum* according to phylogenetic analysis.



**Fig. 1. Representative gel electrophoresis showing amplification of 18S RNA gene of *Plasmodium* parasite**

Lane M: DNA marker, Lanes 11-19: amplified *Plasmodium* 18S ribosomal RNA gene, Lane 20: negative control (malaria-negative control), Lane M: 1-kb ladder (Fermentas)

**Table 1. Identification of *Plasmodium* species based on analysis of 47 18S rRNA gene sequences. Three samples failed sequencing. All the other 47 samples that were sequenced were *Plasmodium falciparum* species**

Sequence sample	Closest species identified	% identity	Sequence sample	Closest species identified	% identity
V1	<i>P.falciparum</i>	92%	V26	<i>P.falciparum</i>	93%
V2	<i>P.falciparum</i>	95%	V27	<i>P.falciparum</i>	93%
V3	<i>P.falciparum</i>	93%	V28	<i>P.falciparum</i>	95%
V4	<i>P.falciparum</i>	95%	V29	<i>P.falciparum</i>	93%
V5	<i>P.falciparum</i>	95%	V30	<i>P.falciparum</i>	93%
V6	<i>P.falciparum</i>	94%	V31	<i>P.falciparum</i>	94%
V7	<i>P.falciparum</i>	96%	V32	<i>P.falciparum</i>	95%
V8	<i>P.falciparum</i>	91%	V33	<i>P.falciparum</i>	96%
V9	<i>P.falciparum</i>	95%	V34	<i>P.falciparum</i>	97%
V10	Sequencing failed	N/A	V35	<i>P.falciparum</i>	96%
V11	Sequencing failed	N/A	V36	<i>P.falciparum</i>	97%
V12	<i>P.falciparum</i>	96%	V37	<i>P.falciparum</i>	92%
V13	<i>P.falciparum</i>	95%	V38	<i>P.falciparum</i>	95%
V14	<i>P.falciparum</i>	96%	V39	<i>P.falciparum</i>	93%
V15	<i>P.falciparum</i>	91%	V40	<i>P.falciparum</i>	93%
V16	<i>P.falciparum</i>	95%	V41	<i>P.falciparum</i>	93%
V17	<i>P.falciparum</i>	98%	V42	Sequencing failed	N/A
V18	<i>P.falciparum</i>	98%	V43	<i>P.falciparum</i>	94%
V19	<i>P.falciparum</i>	93%	V44	<i>P.falciparum</i>	94%
V20	<i>P.falciparum</i>	97%	V45	<i>P.falciparum</i>	93%
V21	<i>P.falciparum</i>	95%	V 46	<i>P.falciparum</i>	93%
V22	<i>P.falciparum</i>	95%	V47	<i>P.falciparum</i>	91%
V23	<i>P.falciparum</i>	93%	V48	<i>P.falciparum</i>	91%
V24	<i>P.falciparum</i>	95%	V49	<i>P.falciparum</i>	95%
V25	<i>P.falciparum</i>	92%	V50	<i>P.falciparum</i>	98%

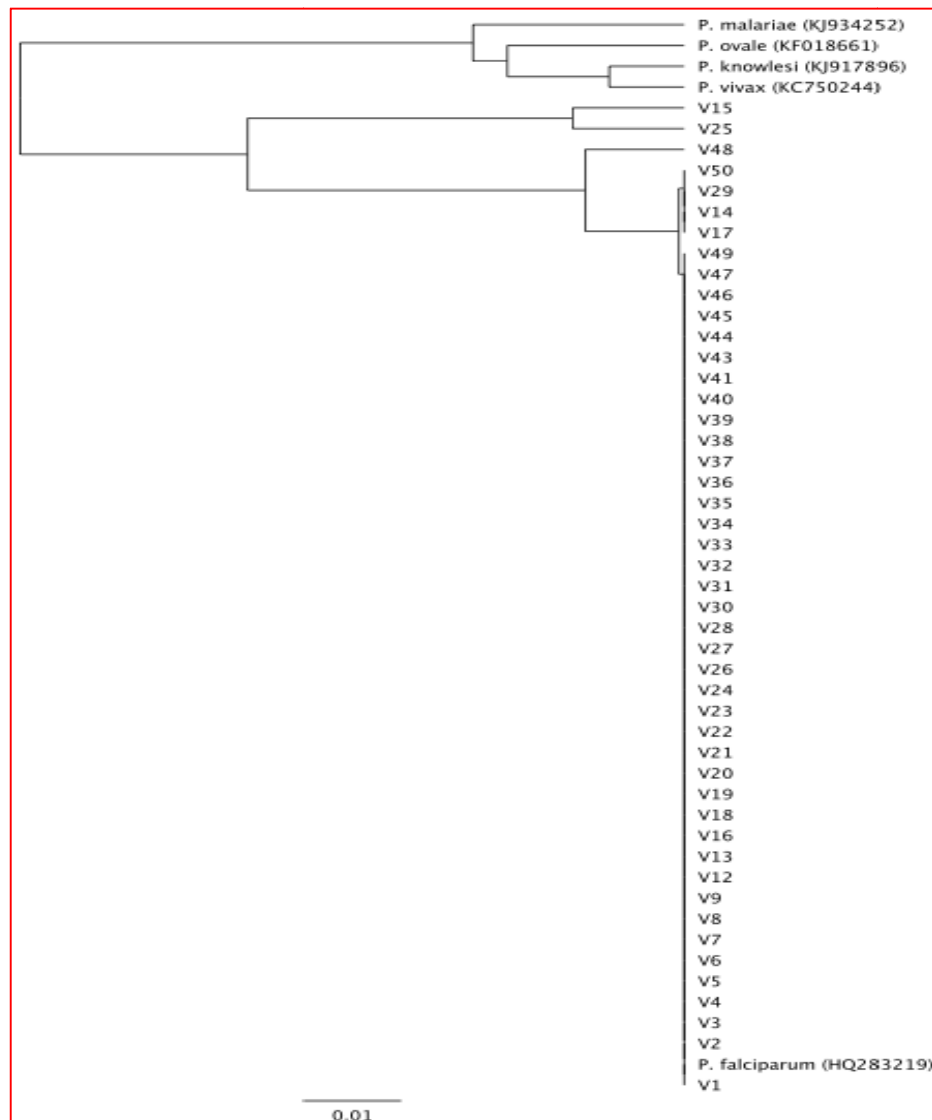


Fig. 2. *Plasmodium* 18S RNA gene-based phylogenetic tree of the 47 malaria-positive blood samples

#### 4. DISCUSSION

Malaria remains a fatal tropical disease especially in developing countries. According to World Health Organization, there were about 435 000 deaths due to malaria globally in 2017 [9]. The disease is caused by the *Plasmodium* parasite that is transmitted by the *Anopheles* mosquito. So far, more than 250 *Plasmodium* species are known to exist, but only five, *P. falciparum*, *P. ovale*, *P. vivax*, *P. malariae* and *P. knowlesi* infect humans [10]. In Zimbabwe, malaria remains one of the key causes of mortality and the cost of intervention (in terms of

its treatment, diagnosis and prevention) is very huge. Accurate and prompt diagnosis and species identification are therefore important for proper management and control of the disease. Traditional approaches such as observation of clinical symptoms and microscopy are the mainstay for malaria diagnosis in most endemic regions including Zimbabwe. Diagnostic methods such as microscopy and rapid tests are however not reliable in identifying the different species of *Plasmodium* parasite in malaria disease. In this study, we were interested in identifying the *Plasmodium* species in malaria samples collected throughout Zimbabwe at referral

hospitals. A molecular method was used where the 18S ribosomal RNA gene of *Plasmodium* genus was targeted for identification of the species. The 18S ribosomal rRNA gene possesses both conserved and variable regions that are not found in the human genome and therefore is a good target for *Plasmodium* speciation [11]. The amplification was successful and DNA bands on agarose were strong. The 18S ribosomal RNA gene of *Plasmodium* parasite is known to exist in several copies (five to eight) and this may generally increase the sensitivity of polymerase chain reaction. The high gene copy number is one of the reasons why the gene has been used in several studies for the detection of *Plasmodium* parasites using molecular methods [8,12,13]. The high copy number of the gene also allows the parasite to be detected even in a single-step polymerase chain reaction. In this study all the samples which were positive by microscopy and/or serology were also positive by 18S ribosomal RNA gene amplification. The 6 negative samples by microscopy were also negative by polymerase chain reaction. This clearly showed 100% agreement between the two methods.

Phylogenetic analysis of the 47 samples showed that all of them were more closely related to *P. falciparum* than to *P. malariae*, *P. ovale*, *P. vivax* and *P. knowlesi*. Various other studies also support the findings obtained in this study. Previously, we used the *Plasmodium* mitochondrial gene for identification of the species and all the samples had *P. falciparum* [7]. Other studies in Africa have also shown the predominance of *P. falciparum* in malaria infections. *P. falciparum* is known to account for more than 85% of the global *Plasmodium* species infecting humans and is responsible for the majority of malaria deaths in Africa [14]. According to WHO (2018), the overall prevalence of the two dominant species, *P. falciparum* and *P. vivax* are 89% and 11% respectively. A study conducted in Zambia showed that 88% of all malaria infections were due to *P. falciparum*, 10.6% were mixed infections and 1.4% as non-falciparum mono infections [15]. In Botswana, 98% of all malaria cases were reported to be due to *P. falciparum* and 2% due to *P. malariae* and *P. ovale* with no reported cases of *P. vivax* [16]. A study in Zimbabwe more than three decades ago reported that *P. falciparum* had a prevalence of 97% using microscopic examination [17]. Zimbabwe Malaria Program Performance review of June 2011 also supports the findings of this study reporting *P. falciparum* prevalence to be

98%. The high percentage of *P. falciparum* reported in this study may be due to the fact that our sample size was small. If more patients had been investigated, maybe other species of *Plasmodium* were going to be identified.

The predominance of *P. falciparum* in malaria in Zimbabwe has some far-reaching implications. The *P. falciparum* is known to be the most virulent species and is responsible for more than 90% of malaria deaths globally [2]. According to World Health Organization, *P. falciparum* accounted for 99.7% of malaria cases in the African region in 2017 [9]. The *P. falciparum* disease also progresses more rapidly than in any of the other *Plasmodium* species infecting humans and is the most serious form of malaria with higher deaths rates. Although molecular methods are not prompt and easily available for routine *Plasmodium* diagnosis, they may help in the management of malaria in critical cases such as in pregnant women where delayed treatment may be fatal. Bearing in mind the potential fatality associated with *P. falciparum* there is greater concern for early and accurate diagnosis and species identification. The molecular method, 18S ribosequencing, was therefore used for the first time in Zimbabwe for the identification of *Plasmodium* species in malaria cases.

## 5. CONCLUSION

The study showed that all malaria-suspected cases in Zimbabwe were caused by *P. falciparum* species. This was the first time molecular evidence indicated that *P. falciparum* was the main cause of malaria in Zimbabwe. A much bigger study is however needed, with more sampling sites throughout the country and over a longer period of time.

## CONSENT AND ETHICAL APPROVAL

The study was approved by the University of Zimbabwe's Joint Research Ethics Committee (Approval no. JREC/28/16) and the Medical Research Council of Zimbabwe (Approval no. MRCZ/B/1080) as required by the university and government. No informed consent forms were signed by patients. The study was exempted from written informed consent by patients because the human-derived samples used in the study constituted nonidentifiable residual clinical specimens which were destined for discarding by hospital laboratories after use. Institutional permission to use the residual samples was however sort and granted from the clinical laboratory heads.

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## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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