MOLECULAR DETECTION OF THE *PLASMODIUM FALCIPARUM* OBTAINED FROM OUT-PATIENTS FROM SELECTED HOSPITALS IN KADUNA STATE

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ABSTRACT

Molecular detection of *Plasmodium falciparum* based on PCR amplification is generally very specific and sensitive test for determining the species of Plasmodium present in the blood of an individual than the microscopy-based diagnosis from blood smears. Thirty-two (32) microscopic malaria positive blood samples were collected from some patients between November 2013 to March 2014 from three major hospitals.Plasmodium DNA was extracted from the 32 blood samples collected from the malaria-positive patients confirmed by microscopy and the DNA amplification was done by using Polymerase Chain Reaction (PCR). The amplified COX3 gene of each malaria isolates were further characterized using Agarose gel electrophoresis while sequence identification was performed by using GenBank's BLAST algorithm. After the completion of the agarose gel electrophoresis the bands indicating amplified Cox3 gene by PCR was observed in four (4) plasmodium positive blood samples out of the 32 samples analyzed. Amplified band for COX3 gene was located at 300bp position on the DNA ladder on the agarose gel plate for sample 1, 9 and 24 all from Kagarko general hospital while sample 28 was from GwannaAwan general hospital. The BLAST results showed that the P. falciparum DNA sequences aligned at 98-99% similarity with those deposited in the GenBank confirming the parasite isolated from the patients were P. falciparum. The use of PCR diagnosis to compliment microscopy examination of stained blood smears in our medical centres is strongly recommended so that an accurate detection of malaria parasites in blood will help to institute proper drug therapy.

Keywords: Malaria-positive patients, *Plasmodium falciparum*, COX3 gene, GenBank, PCR, Kaduna State

INTRODUCTION

Malaria is a life-threatening disease caused by parasites that are transmitted to people through the bites of infected female Anopheles mosquito (WHO, 2016). It is a mosquito-borne disease caused by parasitic protozoans belonging to the Plasmodium type (Caraballo, 2014). The World Health Organization (WHO) estimated that there were 214 million documented cases of malaria in 2015, resulting in the death of about 438,000 people. About 90% of these deaths occurred in WHO Africaregion, with children less than five years of age accounting for about 70% of total mortality (WHO, 2015).Global efforts to control and eliminate malaria have saved an estimated 3.3 million lives since 2000, reducing malaria mortality rates by 42% globally and 49% in Africa (WHO, 2014).

Five species of malarial parasitein humansnamely: *Plasmodium ovale, Plasmodium vivax, P. malariae,P. falciparum and P. knowlesi* cause infections in humans. The P. knowlesi specie is implicated in the pathology of macaque's malaria and it is also zoonosis. *Plasmodium falciparum* is the most common identified specie causing severe disease in Nigeria (Kalu*et al.,* 2012).

Molecular detection of *P. falciparum* based on PCR amplification is generally a very specific and sensitive test for determining the species of *Plasmodium* present in the blood of an individual than the microscopy-based diagnosis from blood smears (Buppan*et al.,* 2011). The advent of molecular diagnostic tools using amplification of multi-copy target genes such as 18S rRNA, mitochondrial genes in all stages of Plasmodium species has substantially improved malaria diagnosis. Detection of parasite genetic material through PCR techniques has become a more frequently used tool in the diagnosis of malaria as well as the diagnosis and surveillance of drug resistance in malaria (Ricke*et al.,* 2000). Specific primers have been developed for each of the five species of human malaria (Purfield*et al.,* 2004).Furthermore,polymerase chain reaction inhibitors usually affect PCR through interaction with DNA or interference with the DNA polymerase. Inhibitors can escape removal during the DNA purification procedure by binding directly to single or double-stranded DNA (Nwuba*et al.,* 2002). Alternatively, they can reduce the availability of cofactors (such as Mg^{2+}) or interfere with their interaction with the DNA polymerase (Alaeddini, 2012).

In Nigeria, malaria is an endemic disease with seasonal variation in different geographical zones of the country. More than 90% of the total population is at risk of malaria and at least 50% of the population suffers from at least one episode of malaria per year, beyond the impact on pregnant women and children (NMCP, 2014). Studies on malaria and especially detection of *Plasmodium falciparum* in several hospitals and communities in Nigeria has revealed great success in the reduction of malaria burden. Accurate diagnosis however still remains a challenge (Ikpa*et al.,* 2017and Kalu*et al.,* 2012).

In Kaduna State, there has been a number of studies on malaria (Abubakar*et al.,* 2019; Obafemi&Gidado, 2014; Maina*et al.,* 2017), but gaps still exist concerning accurate identification of the *Plasmodium falciparum*parasite. A comprehensive study is expected to provide baseline information which will be useful in the effective formulation of adequate control measures, which could help to quickly diagnose malarial disease in the localities and towards achieving the Millennium Development Goals, (FMoH, 2008). Thiswork is therefore aimed at assessing the effectiveness of malaria parasite identification by PCR method among selected hospitals located in three local government areas in Kaduna-Nigeria.

MATERIALS AND METHODS
Study Area
Kaduna State is located between Latitude 10°30.59" N of the equator and Longitude 7°29.47" E of the Greenwich Meridian, (Max Lock Group, 2003).   Kaduna State is in the sub-humid area of the guinea savanna; it occupies part of strategic central position of the northern part of Nigeria and shares common borders with Zamfara state, Katsina state, Niger state, Kano state, Bauchi state and Plateau State, (Fig 1).

The State occupies an area of approximately 48,473.2 square kilometers and has a population of more than 6 million in 2006, (NPC, 2006). It has 23 Local Government Areas which are divided each into three senatorial zones namely: northern, central and southern senatorial zones.
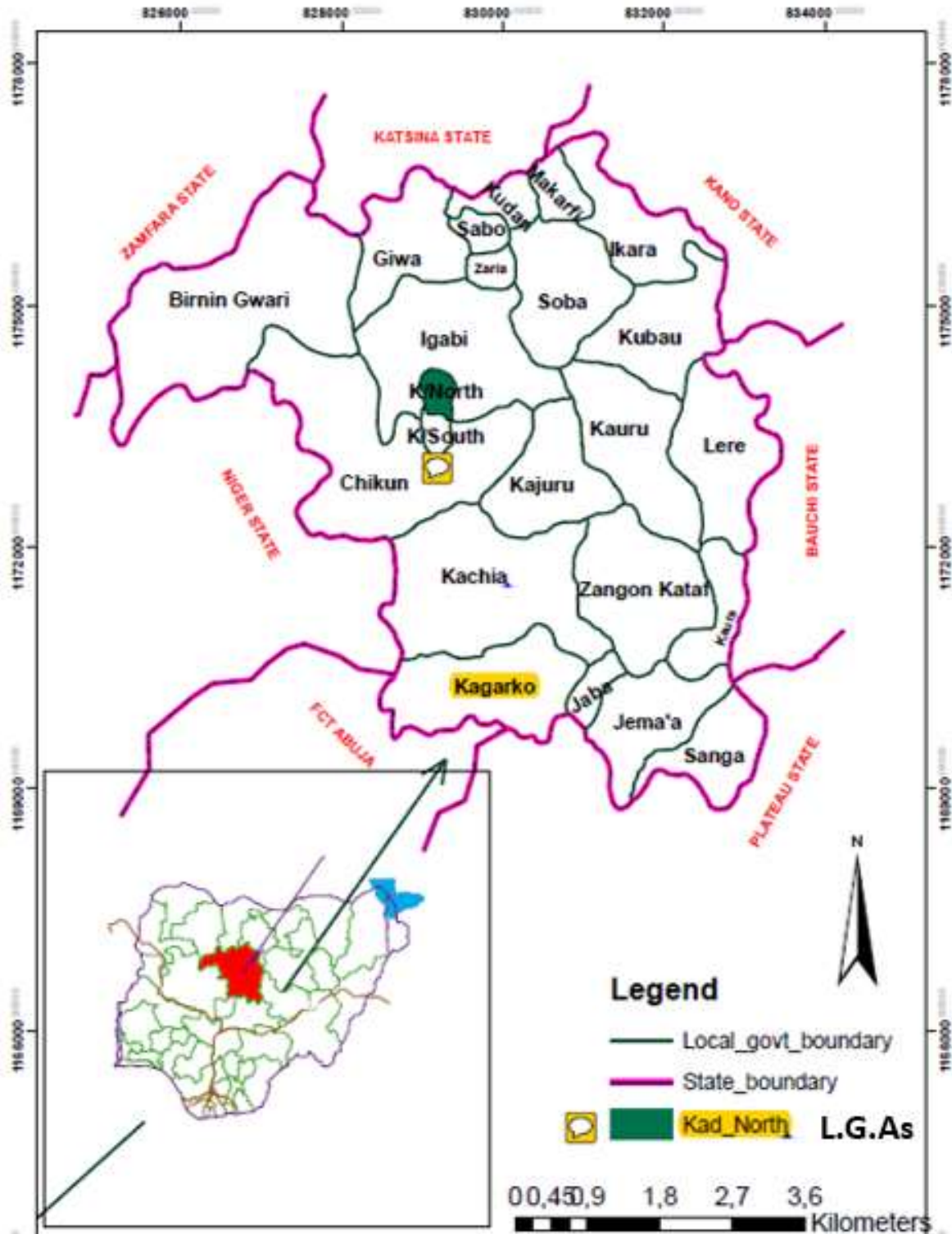

Figure 1: Map showing locations of selected hospitals in Kaduna state. (Source: Geography Department, KASU, 2014).

Sample Collection and DNA Extraction

Thirty-two (32) microscopic malaria positive blood samples were collected according to the method of Kaluet al. (2012 from some groups of out- patients between November 2013 to March 2014 from three major hospitals namely: GwamnaAwan general hospital (Kaduna South LGA), Kagarko general hospital (Kagarko LGA) and Jowako Specialist hospital (Kaduna North LGA) of Kaduna State. Plasmodium DNA was extracted from 32 blood samples collected from malaria-positive patients confirmed by microscopy. These collections were divided into four (4) groups (A, B, C&D) (which indicates the different periods the samples were collected between November 2013 to March 2014) each group containing eight malaria positive patient's blood samples by microscopy.

Plasmodium species genomic DNA was extracted from 1ml of parasitaemic venous blood as described by SNOUNOU *et al.* (1993). Red blood cells were lysed with saponin and, after centrifugation, the parasite pellet was incubated in lysis buffer (10-mM Tris-HCL, pH 8.0, 20-mM EDTA, pH 8.0, 0.5% sodium dodecyl-sulphate, and 0.5mg/mL Proteinase E) at 37°C for 18hours. DNA was obtained by phenol extraction and ethanol precipitation, and resuspended in TAE buffer (10-mM Tris-HCL, pH 8.0, 0.1-mM EDTA, pH 8.0). Finally, 2uL of the suspension was used for PCR amplification.

Polymerase Chain Reaction amplification of isolated plasmodium parasite.

Polymerase chain reaction analysis was carried out for 32 samples of suspected *Plasmodium falciparum* positive brought from the hospitals. These samples were divided into 4 groups of 8 samples in each group.

The oligonucleotides primers used for this study was designed by AmpliTaq Gold, Applied Biosystems, in Foster City, CA, USA. These primers (pf1 forward and pf2 reverse) were employed to amplify the mitochondrial COX3 gene for the characterization of *P. falciparum* using the thermal cycler machine (Perkin Elmer 9700/2400, UK). Half a microlitre (0.5ul) each of the forward(pf1) (5'-TTA AAC TGG TTT GGG AAA ACC AAA TAT ATT-3') and the reverse(pf2) (5'-ACA CAA TGA ACT CAA TCA TGA CTA CCC GTC-3') primers was pipetted and added to 2.5mM MgCl2, 640μM deoxynucleotide triphosphate (dNTPs), buffer 10x, 1U of Taq polymerase (AmpliTaq Gold; Applied Biosystems, Foster City, CA, USA) and 2μL of DNA samples to make a final volume 20ul.The PCR cycling protocol began with an initial 5-minutes DNA denaturation step at 94°C; 35 cycles of 94°C for 30sec, annealing at 50°C for 30secs,extention at 72°C for 1 minute; and a final extension at 72°C for 5mins. All reaction mixtures were preserved at 4°C until it was time for analysis as previously reported by Kloos*et al.* (2006). The amplified COX3 gene of each malaria isolate was further characterized using Agarose gel electrophoresis.

Agarose gel electrophoresis.

Using a digital weighing balance, 1.5g of 2% agarose powder was weighed out and dissolved by heating in 100mls 1X Tris acetic acid and ethylenediaminetetraacetic acid (TAE buffer) (pH 7.9) in a microwave oven set for 2 minutes. The gel was taken out and 8ul ethidium bromide stock was added and allowed to cool sufficiently to 20°C. The 8ul ethidium bromide stock was constituted from 100mg ethidium bromide tablet dissolved in 10mls distilled water and stored in foil-covered bottle to protect from light. This was added and mixed with the agarose gel as described by Jyothi*et al.* (2012). Care was taken while handling because it is a mutagen. The gel was poured into cassette tray with a comb inserted. Gels were allowed 30minutes to solidify at room temperature. A 100mls in 1.0X Tris Acetate Buffer (TAE) was added to the tank and the combs removed by pulling it out straight up. The tank was filled with 1.0X TAE until the gel was covered. Then the first gel well was carefully loaded with 1kilobase (1Kb) plus DNA ladder, the subsequent wells were loaded with the PCR products and run at 100-120 volts for 30mins to 1hour as described by Jyothi*et al.* (2012). Then the PCR products were visualized under UV trans-illumination at 302nm on gel documentation system (Syngenta, USA). A sample was considered positive at 300 base-pair (bp) for COX3 gene of *P. falciparum*, as was detected by the 1Kilobase (1Kb) plus DNA Ladder.

Sequence Determination of COX3 gene. The amplified COX3 gene of each *Plasmodium falciparum* isolate was processed for sequencing. The sequence of the Cox3 gene was determined with a Dye terminator sequencing kit (Applied Biosystems), and the product was analyzed with an ABI Prism DNA sequencer (ABI). The COX3 gene sequences of each isolate obtained in this study were compared with known Cox3 gene sequences in the GeneBank (NCBI) database as described by Jyothi*et al. (2012).*

RESULTS

After the completion of the agarose gel electrophoresis the bands indicating amplified Cox3 gene by PCR was observed in four (4) plasmodium positive blood samples out of the 32samples analyzed. Polymerase Chain Reaction analysis of the first 8 *P. falciparum* positive blood samples for Plate I showed an amplified band for COX3 gene located at 300bp position on the DNA ladder on the agarose gel plate for sample 1 from Kagarko general hospital. However, the result of the same molecular analysis for Plate II of the eight (8) blood samples that were microscopically positive for *P. falciparum* showed a PCR amplified band at 300bp for sample 9 from Kagarko general hospital. The result of the same molecular analysis for Plate III of the eight (8) blood samples that were microscopically positive for *P. falciparum* showed a PCR amplified band at 300bp for sample 24 from also Kagarko general hospital. Finally, PCR analysis of the last 8 *P. falciparum* positive blood samples Plate IV, an amplified band for COX3 gene was located at 300bp position on the DNA ladder on the agarose gel plate for sample 28 from GwannaAwan general hospital.

The *P. falciparum*COX3 sequences result obtained from this study and also subjected to BLAST search using the National Centre for Biotechnology Information (NCBI) Genebank for confirmation. The sequences alignment, gave 98-99% result similarity with *P. falciparum* sequences alignment the Genbank data base of NCBI.

GROUP A
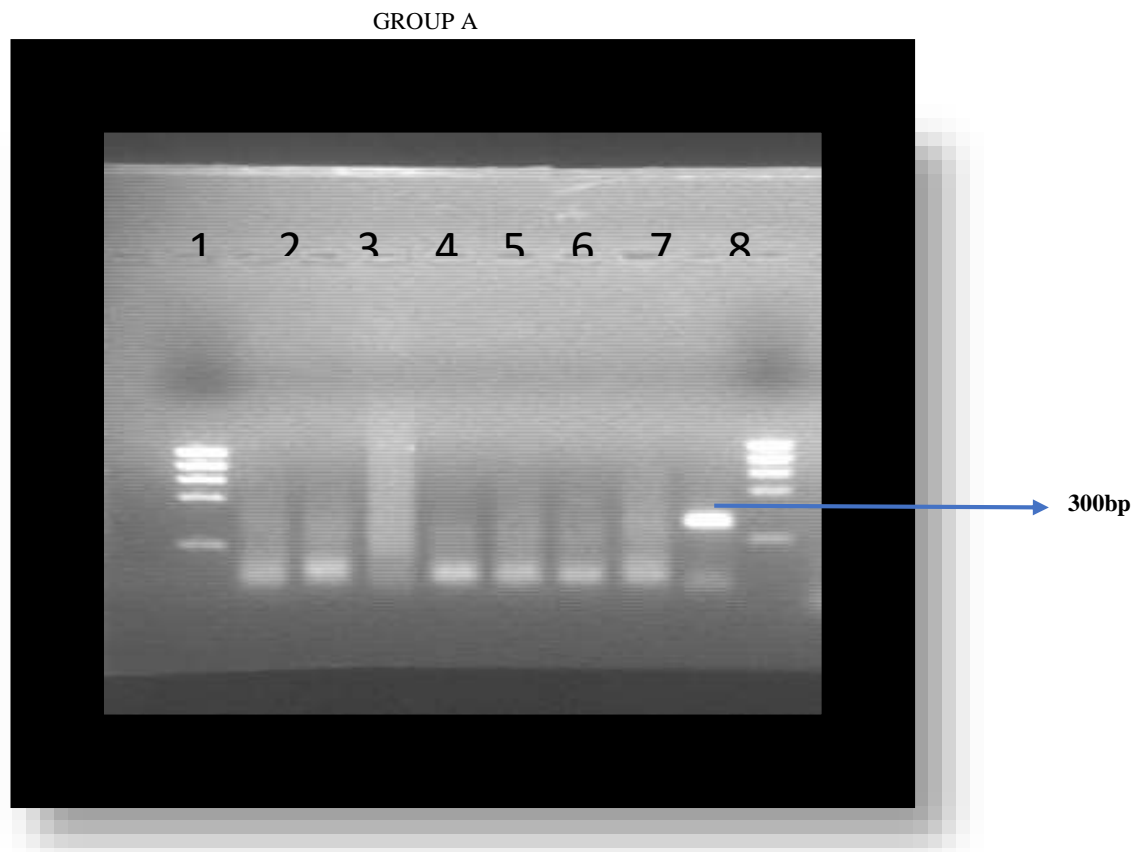
PLATE I: Electrophoregraph of PCR products amplified from genomic
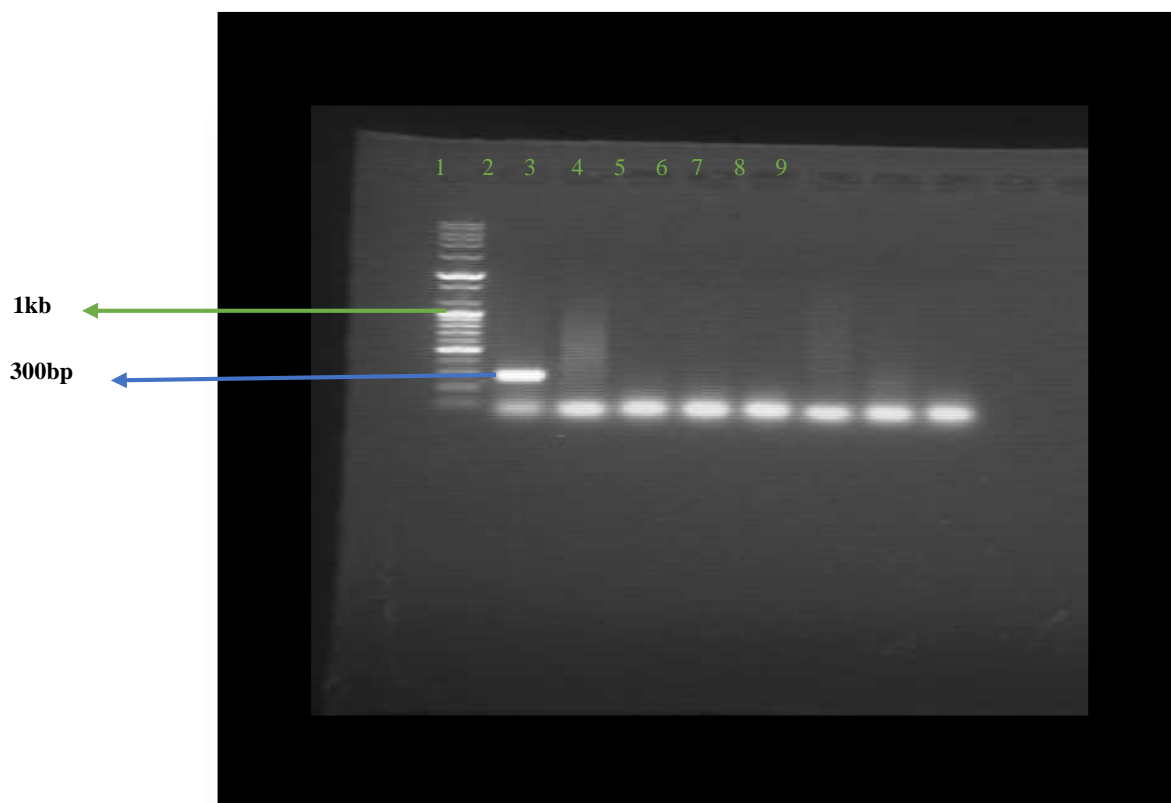DNA extracted from 8 *Plasmodium falciparum* positive blood samples.
    Lane 1 and 10 DNA ladder (1Kb plus)
    Lane 2 and 3, GwannaAwan Hospital (Sample 1 and 2)
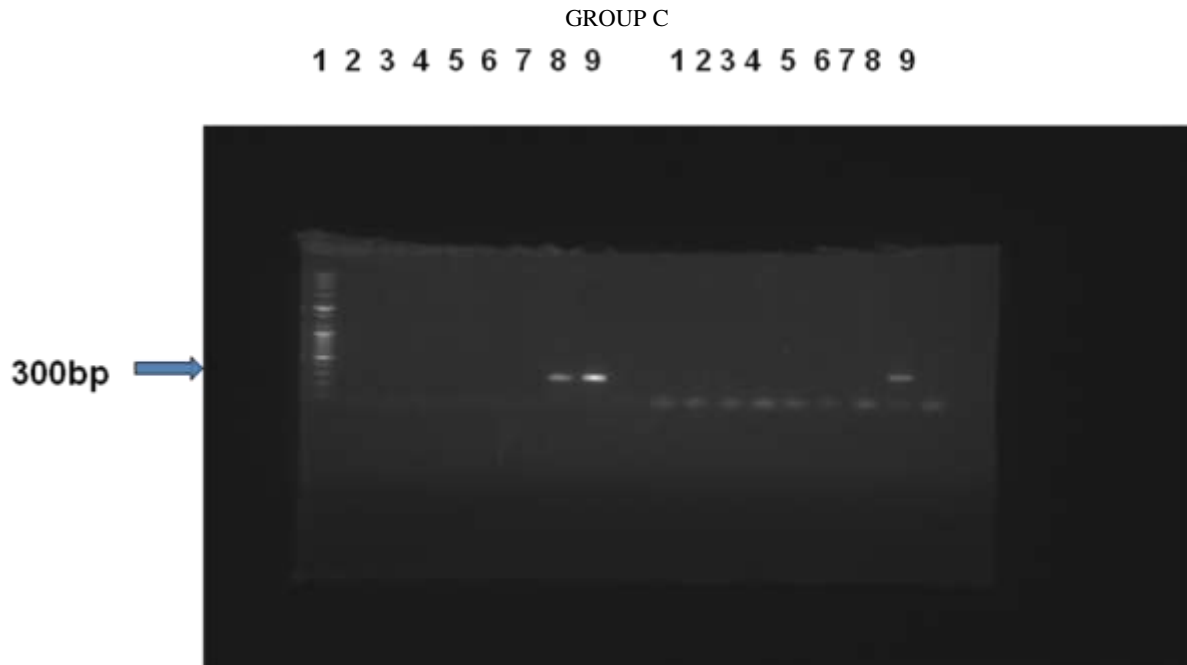   Lane 4 -6,Jowako Hospital (Sample 3, 4 and 5)
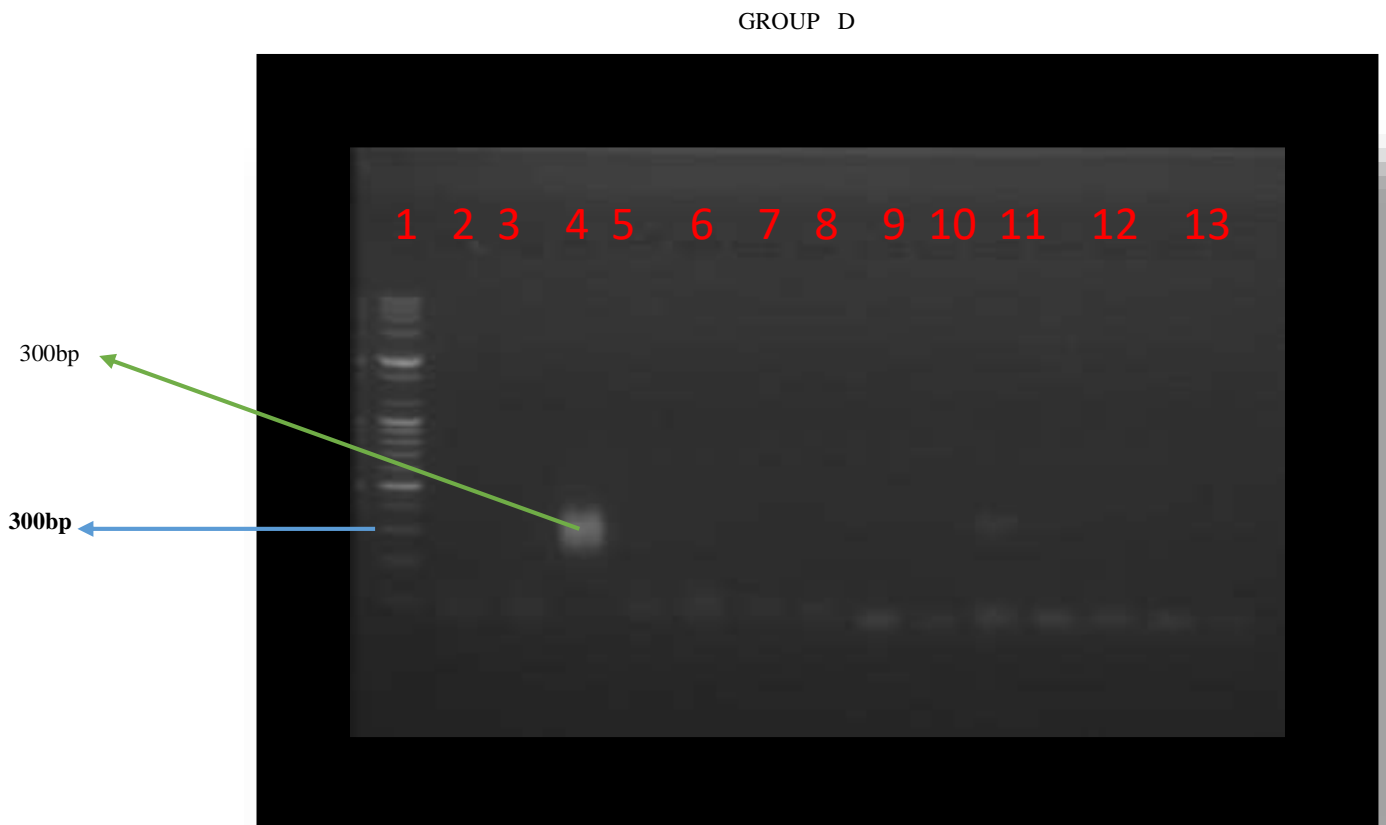   Lane 7-9, Kagarko general Hospital (Sample 6, 7 and 8)

GROUP B

Plate II: Electrophoregraph of PCR products amplified from genomic DNA extracted from 8 *Plasmodium falciparum* positive blood sample.
          Lane 1 DNA ladder (1Kb plus)
          Lane 2 – 4-Kagarko general Hospital (Sample 9, 10 and 11)
          Lane 5-7- Jowako Hospital (Sample12, 13 and 14)
          Lane 8-9- GwamnaAwan Hospital (Sample 15 and 16)

GROUP C

Plate III: Electrophoregraph of PCR products amplified from genomic DNA extracted from 8 *Plasmodium falciparum* positive blood samples.
          Lane 1 DNA ladder (1Kb plus)
          Lane 2 and 3- GwamnaAwan Hospital (Sample 17 and18)
          Lane 4 -6- Jowako Hospital (Sample 19, 20 and 21)
          Lane 7-9- Kagarko general Hospital (Sample 22, 23 and 24)

GROUP D

Plate IV: Electrophoregraph of PCR products amplified from genomic DNA extracted from 8 *Plasmodium falciparum* positive blood samples.

Lane 1 DNA ladder (1Kb plus)
Lane 2 – 4-Jowako Specialist Hospital (Sample 25, 26 and 27)
Lane 5-6- GwannaAwan general Hospital (Sample 28 and 29)
Lane 7-9- Kagarko general Hospital (Sample 30, 31 and 32)

DISCUSSION

This study reports that the PCR identification technique performed on the *Plasmodium falciparum* positive microscopic diagnostic test confirmedonly fourcases (12.5%) of *Plasmodium falciparum* out of the 32 blood samples.Molecular detection of *P. falciparum* based on PCR amplification is generally very specific and sensitive test for determining the species of *Plasmodium* present in the blood of an individual than the microscopy based diagnosis from blood smears (Buppan*et al.,* 2011).It may be that the 28 blood samples observed to be microscopically positive for *Plasmodium falciparum* were all false positive artifacts while only the four (12.5%) blood samples were truly positive for *Plasmodium falciparum* as observed using *PCR* . Also, it may be that the 28 blood samples observed to be microscopically positive for *Plasmodium falciparum* were not *Plasmodium falciparum* as observed using PCR but other plasmodium species like Plasmodium malariae. According to Okwa *et al*. (2009) even though *Plasmodium falciparum* accounts for 95% of all the infections in the country, *P. malariae* also accounts for the remaining 5%. Which may be the case in the result observed in this study.

Furthermore, PCR may not have detected DNA in the blood this may be due to a number of enzyme inhibitors in the patient's blood that may have interfered with the target amplified Cox3 gene hindering it from been amplified (Alaeddini, 2012).

This BLAST result in the study confirms the plasmodium parasite that were analyzed were truly *P. falciparum* species.

CONCLUSION

Although microscopy remains the most appropriate method for clinical malaria diagnosis in field settings, molecular diagnostics such as PCR assay based offer a more reliable means to detect malaria parasites and mixed infections.

The BLAST results showed that the *P. falciparum* DNA sequences aligned at 98-99% similarity with those deposited in the GenBank confirming the parasite isolated from the patients were *P. falciparum.*

RECOMMENDATIONS

1. The use of PCR diagnosis to compliment microscopy examination of stained blood smears in our medical centres is strongly recommended so that an accurate detection of malaria parasites in blood will help to institute proper drug therapy.
2. Laboratory scientists in health centres should be trained in the use of molecular tools in diagnosis of infectious diseases especially malaria.

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