



## Molecular Diagnosis of Malaria Parasites Using Polymerase Chain Reaction (PCR) As A Confirmatory Technique

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Received on: February 2017

Revised and Accepted on: November, 2017

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### ABSTRACT

*Confirmation studies of malaria parasites using PCR (polymerase chain reaction) test was carried out at DNA Labs. Fifteen samples were collected from patients attending Fomwan Hospital Badarawa, Kaduna with symptoms of malaria fever as confirmed using microscopy test. Only four samples were positive for Plasmodium falciparum. The other samples were positive for other Plasmodium species. The fever symptoms of some of the patients could be due to other diseases caused by viral or bacterial organisms such as West Nile Fever, the Flue etc. Contamination is also a possible cause for false or negative result, which could happen in three ways: blood sample collection, blood sample preservation, and DNA extraction process. Delay in DNA extraction after blood collection could lead to changes in parasite morphology, which could alter the results. Polymerase Chain Reaction (PCR) for detecting malaria parasite is an expensive but accurate method to detect low levels parasitemia, and has a threshold of detecting 5 parasite/ $\mu$ l, and species specific diagnosis.*

**Key words:** Molecular diagnosis, Malaria parasites, Polymerase chain reaction (PCR), Confirmatory technique.

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### 1.0 INTRODUCTION

Malaria is a mosquito-borne infectious disease of humans as well as other animals caused by the *Plasmodium* parasite. Malaria is likely to have originated from Africa and has coevolved along with its host, the mosquito (Austin, 2008).

First evidence of *Plasmodium* parasite was first recorded in mosquitoes preserved in amber from the Palaeogene period approximately 30 million years old. Malaria was also common in most Europe and North America but now it has been eradicated. For thousands of years, traditional herbs such as; *Artemisia annua*, *Cinchona succirubra* have been used as remedies to treat malaria (Nye, 2002)

There are about 200 species of *Plasmodium* parasites, with about 11 species infecting humans, while other species infect animals including monkeys, rodents and reptiles. This parasite has two hosts in its life cycle: a vector usually a mosquito and a vertebrate host usually humans (Cogswell, 1992). The parasite was first reported by Laveran on November 6, 1885 at a military hospital in Algeria (Carter, 2002).

In the year 2013, 123-283 million malaria infections and deaths in the range of 367, 000-755, 000 were reported globally, while 437, 000 African children were reported dying annually due to malaria infection (WHO, 2014). In 2014, malaria cases were reported in 97 countries and territories. An estimated 3.3 billion people

were at risk of malaria, out of which 1.2 billion are at high risk. In Nigeria, malaria is endemic and 97% of the population is at risk of infection. The World Health Organization estimated 100 million malaria cases with over 300,000 deaths annually and child's mortality rate at 729 per 100, 000 (WHO, 2014).

Between 2010 and 2013, 53 countries of the world reported incidence of mosquito resistance to at least one insecticide especially Pyrethroids which is most frequently used in the control of malaria vector (Misawa, 1985). Similarly, reported cases of parasite resistance to anti-malarial medicines and mosquito resistance to insecticides rendered efforts of controlling malaria ineffective and triggered a rise in global malaria mortality undermining malaria control measures (WHO, 2014).

Rapid and accurate diagnosis is the key to effective management of malaria cases in order to reduce morbidity and mortality caused by delayed or poor management of patients. In many instances, a presumptive diagnosis of malaria is based upon the presence of fever alone which leads to the wrong use of antimalarial drugs. (Pathol, 1994)

Under ideal circumstances, the clinical suspicion of malaria would be confirmed by a laboratory test that is simple to perform, rapid, sensitive, specific, and inexpensive. Hence traditional malaria diagnosis based on the examination of stained blood smears under light microscope remains the gold standard for malaria diagnosis. However, it is labour-intensive, time-consuming, requires technical expertise and the availability of a good quality microscope. Therefore, several alternative methods have been developed for malaria diagnosis including: Polymerase Chain Reaction

method. These methods allow species differentiation and can be used to identify mutations of the parasites genetic makeup, which can be correlated to drug resistance acquired by the parasite. Although microscopy remains the most appropriate method for clinical malaria diagnosis in field settings, molecular diagnostics such as real-time PCR offer a more reliable means to detect malaria parasites, particularly at low levels (Gwerrent 2006).

Malaria disease is among the leading causes of death in Nigeria. Conventional blood test is not enough to make quick diagnosis. Molecular test is done to identify the exact plasmodium species. Malaria parasites have resisted anti-malarial drugs. It reduces the diagnosis time plan and make treatment plan faster. It allows for sensitive and specific detection of Plasmodium species. This study was designed to diagnose the presence of malaria parasite using PCR technique.

## **2.0 MATERIALS AND METHODS**

### **2.1 Study area:**

Blood samples were collected from patients with symptoms of malaria fever as confirmed by microscopy test attending FOMWAN Hospital Badarawa Kaduna.

The experiment was carried out at DNA labs research centre, Katuru Road Kaduna.

### **2.2 Methodology**

The frame of this work is divided into 4 stages that must be done in the same order.

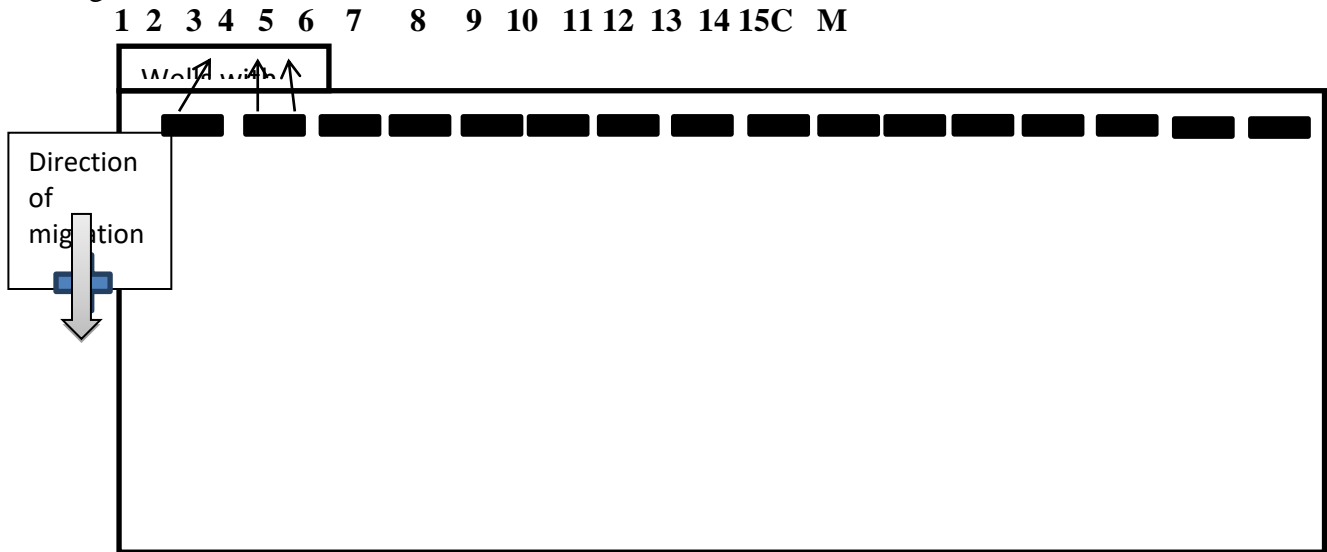
- 1.The blood collection and preparation.
- 2.The polymerase chain reaction technique.
- 3.The gel electrophoresis process.
- 4.Viewing of result under UV trans-illuminator, result reading and interpretation.

### 3.0 RESULTS AND DISCUSSIONS

Naturally, DNA is negatively charged. Therefore it moved towards the positive electrode. And the larger DNA fragments moved slower than the smaller ones. As the DNA fragments migrated towards the positive electrode they formed bands, each band is composed of many identical copies of a particular size piece of DNA. The length of each DNA molecule was measured

by the number of its nucleotide or base pairs.

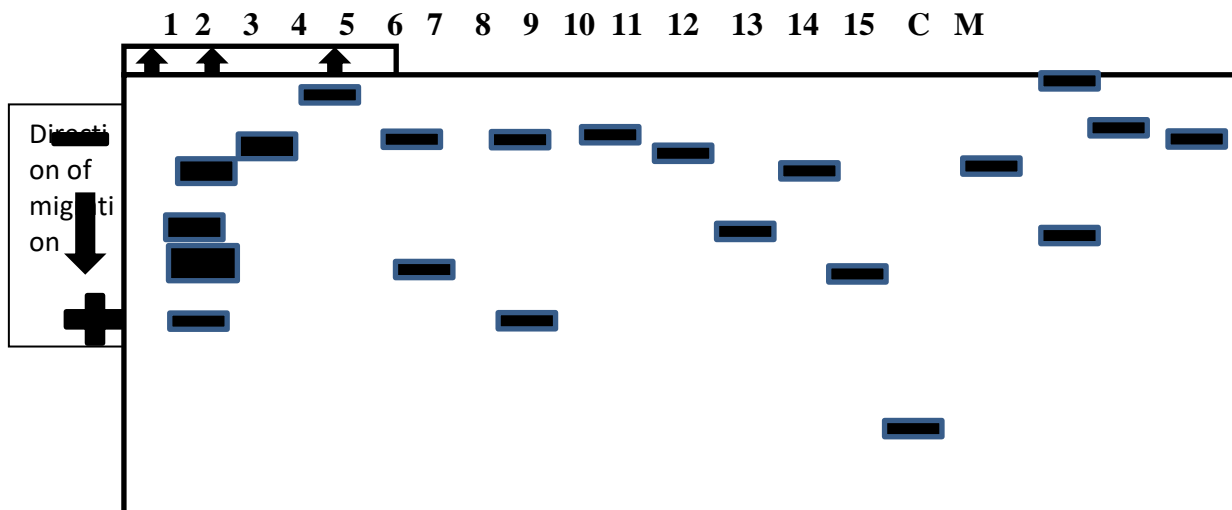
Agarose gel before electrophoresis commenced. The DNA samples were in their respective wells. The C represents control and M representing the molecular ladder of 1-kb plus, which is a mixture of DNA fragments of known sizes usually provided commercially. (Plate i)



**Plate i** Agarose gel before electrophoresis

Agarose gel during gel electrophoresis. The DNA wells migrated from the negative rode

towards the positive rode according to their molecular weight. ( Plate ii)

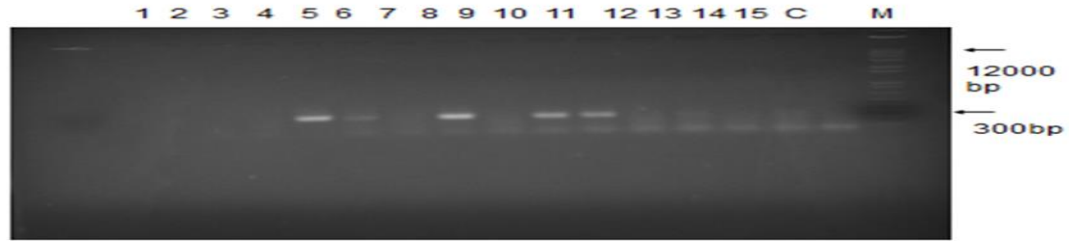


**Fig. 4** The Agarose Gel During Electrophoresis

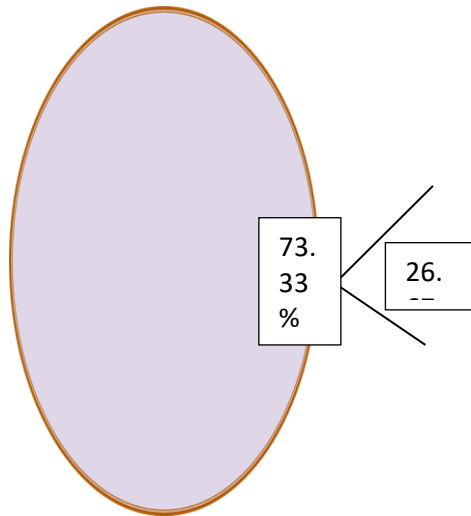
**Plate ii. Agarose gel before electrophoresis**

Agarose gel viewed under UV trans-illuminator with the 15 samples in their respective wells. Although all 15 samples were tested to be positive by microscopy, the PCR test confirmed samples 11,10,8 and

5 to be positive for Plasmodium falciparum (Fig.5). The slight band at the bottom of every sample is a concentration of primer dimer which settles down.



**Plate iii The Agarose Gel at the end of Electrophoresis**



**Fig. 1.** Presence of Plasmodium parasites using the PCR Technique.

**3.1 Discussion**

Molecular tests for malaria diagnosis using PCR have repeatedly been shown to be more accurate and sensitive in detecting malaria parasites compared to microscopy. In this study, out of the 15 samples which were all microscopically confirmed P. falciparum positive only 4 were shown to be positive by

PCR test. Parasite nucleic acids are detected using polymerase chain reaction, although this technique may be slightly more sensitive than smear microscopy, it is of limited utility for the diagnosis of acutely ill patients in the standard healthcare setting. Therefore, PCR is an alternative method of malaria diagnosis that allows for sensitive and specific detection of plasmodium species DNA from peripheral blood. The technique may be more sensitive than conventional microscopy in very low parasitaemia, and is more specific for species identification. It may be particularly useful when subjective microscopy does not permit certain identification of the species present. Malaria PCR can be used in conjunction with a traditional blood film to reach a sound diagnosis and start proper treatment plan (Hanscheid and Grobusch, 2002).

Possible reasons for the negative results with the other samples earlier confirmed could be either of the following:

Blood collection tubes were not probably drawn in specific order to avoid cross-contamination of additives between tubes.

Proper disposal of needles and tubes might also contribute to having accurate blood sample for the microscopy test. Proper labeling of tubes, patients name and identification numbers, knowing exactly what test is required all these help to perform correct blood test. Any error during this stage could affect the overall work that depends on having correct blood sample. This is as ascertained by the Public Health Laboratory Network. (Commonwealth, 2001).

During blood sample preparation for DNA extraction, Centrifugation and separation of serum and plasma from blood cells should be performed within one hour after collection. However, during blood specimen processing certain problems could arise that could alter the specimen and these might affect the result. For example; Hemolysis which is the release of red blood cells into the sample may have occurred. This may be corrected by avoiding freezing the blood before centrifuging, refrigerating the freshly collected blood before clotting. Release of Lactescence which is milky plasma obtained when the blood sample was collected from a patient after having a fatty meal may also be the reason for Negative results.

This can be corrected by avoiding drawing blood from patients who have eaten within 2 hours of collection. (Centre for Diseases Control, 1995).

Another possible reason might be Bacterial Changes which contaminates the specimen from ammonia or urea or reducing the concentration of the chemical components. This could have been avoided by using sterile tools, sterile work tops and wearing protective gloves through the entire process, and promptly processing blood samples. If the specimens are not to be used immediately certain precautions and storage techniques must be made to make sure that the specimen will still be viable when

needed. (National Community For Clinical Laboratory Standards, 1991).

The error leading to the negative results might be during DNA extraction when using water bath, it is important that the temperature does not exceed 90C so as to denature only the protein component of crude extract and not the DNA which is the target isolate.

The DNA extraction method used employs phenolchloroform, and its action is buffered by Ethanol precipitation to prevent oxidized products in the phenol from damaging the nucleic acid. The phenolchloroform must be added in (50%:50%v/v) this will provide good protein denaturation and a tighter interphase between the aqueous and organic phases. (Chomczynski, 1987)

Delay in DNA extraction after blood collection could lead to changes in parasite morphology, which could alter the results.(C.C.A.M.P. 2001)

During Polymerase Chain Reaction, if the DNA extraction was not carried out properly, the PCR technique will yield no results due to the fact that there was no DNA to amplify it in the first place. The primers which are usually commercially provided come with instructions and guidelines on their preparation, failure to adhere to this instruction will give a primer that will provide negative results.(Promega, 2010)

Similarly, the gel used during gel electrophoresis must be left to cool down before Ethidium bromide is added, so as to bind to the DNA molecule and be viewed under UV light. (Loughheed Genetics Laboratory Manual, 2005)

It may also be possible that the symptoms were not due to malaria, because having high fever, joint pain and headache is not an indicator of malaria, other diseases may be responsible. This could explain the negative results obtained.

Another limitation is that this test did not record mixed infections because the primers used are for *Plasmodium falciparum* species only.

As illustrated by results from this study, one of the most notable advantages of molecular method is their higher sensitivity to detect mixed infections and to identify species of malaria parasites accurately. Therefore, while light microscopy is still a convenient technique for the routine malaria diagnosis, molecular tests are suitable complementary tests for the confirmation of species and to detect mixed infections in special studies such as drug efficiency and clinical trials. Microscopic detection of malaria parasites is a standard method for the diagnosis of malaria because of its sensitivity, specificity, and ability to quantify the parasitaemia level under ideal conditions. It is also less expensive than many other malaria diagnostic tools. However, one of the limitations of microscopy is that sometimes it may be difficult to identify the species correctly, especially when slides are not properly prepared or the user has limited training. Microscopy can also fail to detect mixed infections especially when one of the infecting species is present at low levels. Therefore, molecular tests have been used as complimentary tools for the diagnosis of malaria in some reference laboratories so that accurate diagnosis can be made. Recent effort to eliminate malaria has increased the need for introducing molecular tools with high sensitivity capable of detecting sub-clinical levels of parasitaemia in asymptomatic carriers.

#### 4.0 CONCLUSION

Malaria is one of the most important parasitic infections in humans. Consequently, a sensitive diagnostic test for malaria that could be applied at the community level would be useful in controlling the disease.

PCR results are often not available for the diagnosis of malaria infection. The PCR technique is most useful in confirming the species of malarial parasites after the diagnosis has been established by either smear microscopy or Rapid detection kit.

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