A Biochemical Assay Provides Better Diagnosis for Active Malaria Infection

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Abstract

Malaria is a disease caused by *Plasmodium* parasite and it is the most deadly parasitic disease affecting humanity. Detection of this microscopic organism using currently available techniques in the face of reducing quality of testing reagents is increasingly becoming quite challenging especially in low prevalent settings and low density infections. The absence of a particular detection method devoid of shortcomings and pitfalls of significant order prompted the development of a simple biochemical in vitro diagnostic technique for malaria detection known as AfinUrimal. In this study we examined the efficacy of AfinUrimal in detecting malaria using stained blood microscopy method as the reference standards for detecting active infection on 397 patients with febrile illness in Jos, Nigeria in the year 2022. Sensitivity/specificity of AfinUrimal method against stained blood microscopy method was 99.42/92.0% respectively. The analysis showed that biochemical detection of malaria infection using AfinUrimal provides greater sensitivity and specificity useful for diagnostic, epidemiological and research purposes.

Introduction

Jos is the capital city of Plateau state Nigeria. Plateau state is located in the middle belt region of Nigeria in West Africa and considered a malaria endemic area with year round transmission of the parasite which peaks around the mid-months (May-August) of the year. The need for prompt diagnosis to bring about prompt treatment needed to prevent morbidity/mortality arising from malaria infections prompted the development of an easy to use, non invasive, painless, bloodless, affordable and convenient test (AfinUrimal) for malaria. AfinUrimal is a novel biochemical urine malaria test to detect malaria infection using urine instead of blood. Flexibility enhanced in AfinUrimal enables rapid malaria testing, not only at the points of care and conventional laboratories, but also encourage testing anywhere and at anytime yet obtain valid result that will direct informed therapeutic care, (Armstrong-Shellenberg et al., 1987).

Presently and predominantly in highly endemic areas, presumptive diagnosis based on clinical observation has been the basis for therapeutic management of patients suspected of having malaria infection, (Moody(a), 2002), and this has been proven to be unreliable and contributes to malaria over-diagnosis resulting in wastage of anti malaria drugs on patients who do not need them, (Kahama-Maro et al., 2011) and extension of morbidity period, creating chances for further transmission from disease carriers.

Stained blood microscopy method is considered the gold standard but requires well-trained, competent malaria microscopists and rigorous maintenance of functional infrastructures plus effective quality control and quality assurance (Wongsrichanalai et al., 2007). The RDTs that detect malaria antigens such as *Plasmodium falciparum* histidine-rich protein 2 (*PfHRP2*), *Plasmodium* aldolase (*PALD*), *Plasmodium* lactate dehydrogenase (*PLDH*), *Plasmodium falciparum* hypoxanthine-guanine phosphoribosyl transferase (*PfHGPRT*) and *Plasmodium* glutamate dehydrogenase (*PGDH*) are widely used at the point of care due to portability, (Abdalla et al., 2019) however there are some limitations to the RDTs that have to do with relatively long half-life of the antigens after clearance of the infection (Dalrymple et al., 2018) and use of blood which not every patient is comfortable with. Polymerase chain reaction method is
mostly used for research purposes to detect infections especially at low parasitemia missed by stained blood microscopy and RDT methods, (Lucchi et al., 2016). In a study involving another urine malaria test that detects \textit{PfHRP2}, it was indicated that the test had sensitivity of 83.75% and specificity of 83.48% with turnaround time of 25 minutes though different malaria parasite densities showed different sensitivities as follows: \( \leq 100 \text{ parasites/µl of blood} \) 0%, \( \leq 200 \text{ parasites/µl of blood} \) 50% and \( \geq 201 \text{ parasites/µl of blood} \) 89.71% (Oguonu et al., 2014).

Simple, accurate and timely detection of active malaria infection by utilizing malaria pigment, a metabolic product of the parasite is the basic operation of AfinUrical. This metabolite is effectively cleared from peripheral blood (macrophages) shortly (72 hours) after clearing the parasite (Kemsner et al., 2009) and much shorter time from plasma. As the quest to have a simple and reliable malaria test continues, AfinUrical was developed with the objectives of providing ultra sensitive, specific, robust, scalable, goodness of use and interpretable result with short turnaround time, all these objectives were met by detecting malaria pigment from urine of exposed and infected persons. A couple of wet chemistry techniques have been used to study malaria pigment in the field of drug screening assay for antimalaria (Ncokazi et al., 2005) and quantitative determination of phagocytosis of parasitized erythrocytes and of malaria pigment (Schwarzer, Turrini & Arese, 1994) but not much has been seen in the area of malaria diagnosis until the development of AfinUrical. However, AfinUrical is not the first test to utilize malaria pigment but it is the first wet chemistry test designed to be deployed for routine laboratory use for patient diagnosis. In their work, Obisesan et al studied on a method that makes use of electrochemical nanosensor to detect the synthetic form of the malaria pigment as malaria biomarker. (Obisesan et al., 2019). In another study, de Melo et al (de Melo et al., 2021) analyzed the performance of a sensitive hemozoin-based malaria diagnostic test (Gazelle™) device that is based on the magneto-optical concept for the detection of paramagnetic hemozoin by depolarizing light without any reagent. Detection can be done by optical methods using dark-field microscopy (Jamjoom, 1983), polarization microscopy (Lawrence & Olson 1996), or flow cytometry (Hanscheid, Valadas & Grobusch, 2000).

As against these methods, AfinUrical is a biochemical test to detect malaria pigment from urine and has proven to be highly efficient even at a very low parasite density encompassing qualitative, quantitative and semi-quantitative applications. Results can be quantitatively measured on colorimeter. Semi quantitatively results can be matched to a colour chart provided with the test kit. Parasite density has been found to be directly proportional to the optical density. Malaria pigment is produced in increasing amounts by the parasite during the blood stage of its cycle and therefore marks severity and prognostic factor for malaria infection (Hänscheid et al., 2008). Unlike the conventional malaria RDTs which make use of malaria proteins such as \textit{Plasmodium falciparum} histidine-rich protein 2 (\textit{PfHRP2}), \textit{Plasmodium} aldolase (\textit{PALD}), \textit{Plasmodium} lactate dehydrogenase (\textit{PLDH}), \textit{Plasmodium falciparum} hypoxanthine-guanine phosphoribosyl transferase (\textit{PfHGPRT}) and \textit{Plasmodium} glutamate dehydrogenase (\textit{PGDH}) which detect malaria from blood, (Ragavan et al., 2018, Krampa et al., 2020 and Jain et al., 2014), AfinUrical detects malaria pigment from urine. Malaria pigment is an insoluble crystallite that does not provide species-specific diagnosis. It provides universal diagnostic information essential for malaria

# Materials And Methods

## Study Design

Blood and urine samples from a total of 397 hospital patients of all ages attending Bingham University Teaching Hospital, Jos, Plateau State Nigeria were enrolled during malaria transmission season from May to July, 2022. Those suspected of having malaria were referred for malaria test where blood was used for stained blood microscopy test and the urine samples were treated to AfinUrimal test.

## Ethics Statement and Informed Consent

Ethical approval for this study was obtained from Research and Ethics Committee of Bingham University Teaching Hospital (BUTH) Jos, Plateau State Nigeria. All study participants and their parents / guardians were enrolled after giving verbal informed consent in English and Hausa languages for blood specimen and urine sample collection.

## Data and Sample Collection

All patients were recruited regardless of body temperature reading. Details taken from the patients included age, sex and hospital registration number. About 2 ml of blood sample was collected into ethylenediamine tetraacetic acid (EDTA) bottle. Thick blood smears were prepared on microscope slide for stained blood microscopy while urine samples were collected into universal container for every patient.

## Laboratory Assays

### AfinUrimal Test

A measured volume of urine was added to a clean transparent glass test tube. To this was added the AfinUrimal reagent running down through the inner wall to the bottom of the tube. Without mixing a characteristic color immediately develops at the bottom of the tube signifying a reaction between the reagent and malaria pigment. This could be easily seen when the tube is viewed against a white paper placed against a bright environment or white light source. This color is seen within the first five seconds of the test. A solution with a homogenous colour is formed when this is mixed properly and is used to determine the optical density (OD) of the reaction after 15-20 minutes (figure 1).

A modification of this test consists of a treated graduated Pasteur pipette which is used to take up a predetermined volume of urine samples from a container and made to mix properly in the pipette then discharged onto the sample space of AfinUrimal test strip and allowed to migrate by capillary action to the result window where malaria pigment announces its presence instantly if the patient has malaria,
otherwise, patient is negative. Results were read within ten minutes by comparing with the colour chart provided with the test kit (figure 2). Just like in the tube method, a good observer can read off the result right from the pipette.

A proposed modification of this test involves using a microfluidic system for reading and quantification of the reaction from any of the methods is ongoing.

A colour chart provided with the test kit helps to estimate the severity of infection which is proportional to parasite density (PD) and optical density (OD). Optical and parasite densities provide handy quantification of the level of parasitemia (table 1). For the purpose of this study, spectrophotometric reading of the results is matched with microscopy observation as follows: where OD reads $0.09 \leq 0.18$ nm PD is equivalent to $1 \leq 9$ parasites /µl in 100 oil immersion fields of thick blood smear and can be considered to be in ‘trace’ amount. $0.18 \leq 0.36$ is equivalent to $10 \leq 99$ and can be scored +, $0.36 \leq 0.54$ is equivalent to $100 \leq 999$ for ++, OD of $0.54 \leq 0.72$ is equivalent to $1000 \leq 9999$ for ++++, OD $0.72 \leq 0.90$ is equivalent to $10000 \leq 99999$ for ++++ and OD of 0.90 is equivalent to $\geq 100000$ parasites /µl of blood and can be scored $>++++$. Parasite density is estimated as:

$$PD = \frac{Number\ of\ parasites\ counted\ in\ a\ given\ section\ of\ the\ slide}{WBC\ simultaneously\ counted} \times Total\ WBC\ count\ of\ participant\ /\mu l.$$  

Equation 1.

The + scoring methods as used in this study were purposely to estimate parasite densities based on individual's WBC count /µl. Instances where slides have no parasites in 100 oil immersion fields are scored “Negative” while between 1 and 9 parasites in 100 oil immersion fields of thick blood smear are scored “Trace” (Table 1). However, a WHO publication titled “Basic Malaria Microscopy” Part 1 Learner’s guide provides that + is equivalent to 1 to 10 and ++ is equivalent to 11 to 100 parasites per 100 oil immersion thick film fields. Also that +++ is equivalent 1 to 10 and ++++ is equivalent to more than 10 parasites per single oil immersion field of thick film.

Parasite densities for all participants were estimated using their precise white blood cell (WBC) count. Optical density and parasite density of each sample were used to establish the level of infectivity shown on the color chart (table 1) used in observing the strip. The value of the blank used to zero the instrument was the mean of those that were malaria negative (data not shown), with a range of 0.03-0.13 OD.

Table 1: Color Chart for AfinUrimal Semi Quantitative Analysis.
Stained Blood Microscopy

A thick blood smear was made on a clean grease-free microscope slide for the stained blood microscopy method. Giemsa stain was diluted to 10% and the diluted portion was used to stain the thick blood smears for 15 minutes, rinsed in distilled water and dried. The slides were viewed under light microscope at X100 objective and X10 eye piece for presence of malaria parasites and the estimate of parasite density in each slide.

White Blood Cell (WBC) Estimation

For the purpose of estimating the parasite density, total white blood cell was estimated from blood samples for all participants using the improved Neubauer counting chamber. A portion of the thick film where white cells are evenly distributed and parasites well stained is selected for examination and at least 200 WBCs were counted concurrently with all the asexual forms of the parasite in the fields covered, equation 1.

Validation of AfinUrimal

As part of validation activities to assess clinical usefulness and value addition of AfinUrimal test to care of patients. Qualitative and quantitative assessments were carried out to determine the diagnostic validity of the test results from this method. This was ensured so that the result from the test will enable quick and effective decision regarding treatment and provision of preventive measures patients need to take. Interested parties are encouraged to further this study at various scientific dimensions just as obtains with other malaria RDTs.

Data Analysis

The individual’s test result using AfinUrimal were classified as true positive (TP), true negative (TN), false positive (FP), false negative (FN) using stained blood microscopy as the reference standard.
In order to determine the sensitivity i.e., the probability that a truly malaria infected individual tests positive by AfinUrimal is given by the equation:

$$\text{Sensitivity} = \frac{\text{True Positive}}{\text{True Positive} + \text{False Negative}} \quad \text{(Equation 2)}.$$ 

Specificity i.e., the probability that a truly uninfected individual will test negative using AfinUrimal is given by the equation:

$$\text{Specificity} = \frac{\text{True Negative}}{\text{True Negative} + \text{False Positive}} \quad \text{(Equation 3)}.$$ 

The positive predictive value (PPV) of AfinUrimal was also determined. This tells the probability that those individuals testing positive by AfinUrimal were truly infected by malaria parasite and is given by the equation:

$$\text{PPV} = \frac{\text{True Positive}}{\text{True Positive} + \text{False Positive}} \quad \text{(Equation 4)}.$$ 

The negative predictive value (NPV) of AfinUrimal which implies that those individuals testing negative by AfinUrimal were actually not infected by malaria parasite and is given by the equation:

$$\text{NPV} = \frac{\text{True Negative}}{\text{True Negative} + \text{False Negative}} \quad \text{(Equation 5)}.$$ 

Accuracy: the percentage of tests that were correctly detected by AfinUrimal is given by the formula: Items classified correctly/All items classified, or

$$\text{Accuracy} = \frac{\text{True Positive} + \text{True Negative}}{\text{Total Number of participants}} \quad \text{(Equation 6)}.$$ 

Precision: number of correct positive results/Number of all positive result (i.e., \(TP/TP+FP\)), or, \(TP/Actual\ Result\).

$$\text{Precision} = \frac{\text{True Positive}}{\text{True Positive} + \text{False Positive}} \quad \text{(Equation 7)}.$$ 

Error rate: the frequency of errors occurred, defined as "the ratio of total number of data units in error to the total number of data units transmitted". As the error rate increases, the data transmission reliability decreases. It is given by the formula:

$$\text{Error rate} = 1 - \text{Accuracy}, \quad \text{or}$$

$$\text{Error rate} = \frac{\text{False Positive} + \text{False Negative}}{\text{Total number of participants}} \quad \text{(Equation 8)}.$$
In each case, the 95% confidence interval (95% CI) of the respective probabilities was also calculated. (Banoo et al., 2010, Liu et al., 2016).

**Results**

A total of 397 participants were enrolled into the study. 51.2% male and 48.8% female. Participants below 15 years of age were 13.60%, 15-19 years 23.43%, 20-29 years 30.23%, 30-44 years 16.62% and 45 years and above were 16.12% (table 2). Every study participant had their AfinUrimal alongside stained blood smear microscopy test results. Those testing positive to microscopy and AfinUrimal methods were 346 (87.15%) and 349 (87.91%) respectively (table 3).

Table 2: Age Distribution of Study Enrollees.

<table>
<thead>
<tr>
<th>Age (Years)</th>
<th>Total</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 15</td>
<td>54</td>
<td>13.60</td>
</tr>
<tr>
<td>15-19</td>
<td>93</td>
<td>23.43</td>
</tr>
<tr>
<td>20-29</td>
<td>120</td>
<td>30.23</td>
</tr>
<tr>
<td>30-44</td>
<td>66</td>
<td>16.62</td>
</tr>
<tr>
<td>45 &gt;</td>
<td>64</td>
<td>16.12</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>397</td>
<td>100</td>
</tr>
</tbody>
</table>

Table 3: Summary of Results.

<table>
<thead>
<tr>
<th></th>
<th>Stained Blood Microscopy</th>
<th>AfinUrimal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>(%)</td>
</tr>
<tr>
<td>Positive</td>
<td>346</td>
<td>(87.15)</td>
</tr>
<tr>
<td>Negative</td>
<td>051</td>
<td>(12.85)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>397</td>
<td></td>
</tr>
</tbody>
</table>

To recognize the performance characteristics of AfinUrimal, the number of true positive (TP) i.e, the number of participants who tested positive when microscopy is positive were 345 (86.90%). False positive (FP) i.e, the number of participants who tested positive when microscopy is testing negative were 4 (1.01%). True negative (TN) is 46 (11.59%) i.e, the number of participants who tested negative when
microscopy is testing negative, while false negative (FN) i.e, the number of participants who tested negative when microscopy is testing positive is 2 (0.50%) (table 4).

Table 4: Measure of Diagnostic Parameters.

<table>
<thead>
<tr>
<th>Justification</th>
<th>AfinUrimal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
</tr>
<tr>
<td>True positive (TP)</td>
<td>345</td>
</tr>
<tr>
<td>False positive (FP)</td>
<td>04</td>
</tr>
<tr>
<td>True negative (TN)</td>
<td>46</td>
</tr>
<tr>
<td>False negative (FN)</td>
<td>02</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>397</strong></td>
</tr>
</tbody>
</table>

Findings from the study showed the performance characteristics of AfinUrimal as: sensitivity 99.42% and specificity 92.0%, Positive predictive value (PPV) 98.85% while its negative predictive value (NPV) is 95.83%. The diagnostic accuracy of AfinUrimal was found to be 98.49%, precision 98.85% and error rate 1.51% (table 5, Equations 2-8).

Table 5: Summary of Diagnostic Performance of AfinUrimal.

<table>
<thead>
<tr>
<th>Sensitivity</th>
<th>Specificity</th>
<th>PPV</th>
<th>NPV</th>
<th>Accuracy</th>
<th>Error rate</th>
<th>Precision</th>
</tr>
</thead>
<tbody>
<tr>
<td>99.42%</td>
<td>92.0%</td>
<td>98.85%</td>
<td>95.83%</td>
<td>98.49%</td>
<td>1.51%.</td>
<td>98.85%</td>
</tr>
</tbody>
</table>

Discussion

This clinic based study was a deliberate effort to obtain the real-time performance expected of the new diagnostic test for malaria infection with the aim of deploying it for routine use for diagnosis and management of individuals exposed or infected with malaria disease. The high positive rate exhibited by the biochemical detection technique for malaria infection by AfinUrimal showed it has higher sensitivity rate than microscopy method especially at low parasite density. Stained blood microscopy method is more specific than other methods including PCR (Plucinski et al., 2018), especially when carried out by expert malaria microscopist. Observation made from this clinical study on AfinUrimal positions it as a
A veritable tool for detecting active malaria infection. In a comparative study analysis in DR Congo, sensitivities of available methods were as follows: stained blood film microscopy 76.7%, RDT 86.9%, and PCR 94.6% and specificities of 97.2%, 88.1% and 94.6% in that order (Doctor et al., 2016) where prevalence rate by PCR method was 38.6%. Parasite prevalent among children under 5 years in this area has been put at 0.2% (Agency National de la Statistique at de la Demographine, 2015).

Some limitations are associated with this study: in as much as the sampling population was appropriate for evaluating diagnostic methods, the sampling population were mostly those people residing in the study area which is mostly considered endemic region, and therefore findings cannot be generalized to the overall global population and may differ from those seen in cross-sectional survey among asymptomatic persons and those in low prevalent areas. Two of the samples showed false negative result with AfinUrimal in the presence of high malaria parasite densities (41,150 and 102,724 parasites / μl) with blood stained microscopy method. One of these samples was from a 2 year old baby who could not produce her urine in the facility while the other sample was run when the reagent was suspected to have faded after a prolonged exposure. A repeat test of the second sample with a fresh reagent returned a positive result. It is important to note that hydration state of patient plays significant role in the concentration of the pigment as poorly hydrated patient with highly concentrated urine tend to yield higher ODs and vice versa.

Application of AfinUrimal as one of the test options available for malaria detection provides user the advantage of conducting a bloodless, painless, non-invasive and rapid test for malaria while achieving high sensitivity, specificity, accuracy and quantitative result with low error rate of 1.51%.

A user can make a choice between the available methods depending on their interest. For instance, high sensitivity of AfinUrimal favours it over stained blood microscopy method for parasite detection with LoD of about <5 parasite /μl of blood based on observation from hemozoin colorimetric assays while the high specificity and differential ability of stained blood microscopy method make it most suitable for research purposes. Stained blood microscopy has sensitivity of 50-500 parasite /μl (Moody(b), 2002). A PCR method has been reported to have LoD of approximately 0.0001 parasites /μl (Romay-Barja et al., 2015) and another reported 2-5 parasites /μl (Pedro et al., 2018).

The use of AfinUrimal with positive predictive value of 98.85% provides that those requiring treatment can be easily identified and treated, key to eliminating malaria since there won't be transmission when there are no parasites.

**Abbreviations**

LoD: level of detection

OD: optical density

PD: parasite density
Declarations

Acknowledgement

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Competing interests

The authors have no competing interests to declare.

Authors’ contributions

Ifeanyichukwu Okeke conceptualized and coordinated the study. Ifeanyichukwu Okeke and Okeke Cosmas drafted the manuscript, Peter Chinonso and Victoria Chinelo assisted with the laboratory analyses and figures preparation.

Funding

Not applicable.

References


Figures

Figure 1

Display of AfinUrimal Test Results by tube method: (a) before mixing, (b) After mixing.
Figure 2

AfinUrimal Testing Procedure.