COMPARATIVE ANALYSIS OF ACON-*Plasmodium falciparum* RAPID MALARIA DIAGNOSTIC TEST WITH ROUTINE MICROSCOPY AMONG SCHOOL CHILDREN AND PREGNANT WOMEN IN A RURAL COMMUNITY IN ENUGU STATE, NIGERIA

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ABSTRACT

There are currently two methods for the direct diagnosis of malaria: the thin blood smear and the thick smear. A third method called the Acon-Plasmodium falciparum (Acon-Pf) (a new cheap malaria rapid diagnostic test) has been developed. This was the first study comparing the three methods in rapid malaria diagnosis among school children and pregnant women in Enugu State, Nigeria, using qualitative and quantitative questionnaires that address their knowledge, attitude, behaviour and practice. In this study, 352 whole blood samples of individuals with suspected falciparum malaria were investigated among pregnant women attending antenatal clinic and school children in rural communities in Enugu State. The following parameters were determined: intrinsic validity, predictive values, species diagnostic power and logistic factors. Acon-Pf had the following characteristics: 91.5 % sensitivity, 87.2 % specificity, 64.3 % positive predictive values, 97.6 % negative predictive values, high malaria infection rate determination of 58.7%, reduced mean packed cell volume (0.25 mmol/L) in malaria patients, correct species diagnosis including both the trophozoite and gametocyte stages of Plasmodium falciparum. The Acon-Pf positive test result ranged from light to thick coloured bands and the time required for the test was 1.3 – 15 ± 5 minutes. The cost per Acon-Pf test cassette at bulk purchase was ₦112.50 (US$0.90) without the service charge as compared with thick smear (₦ 300.00, US$ 2.50) and ₦ 350.00 (US$ 2.90) for thin smear. The study found that Acon-Pf is suitable along side microscopy in the accurate diagnosis of malaria in Enugu State. The use of Acon- Pf and thick smear tests in parallel, first collecting the Acon-Pf results, as it contributes in reading the thin smear result for confirmation of species, diagnosis and assessment of parasitaemia. Thus, Acon-Pf test device is a viable cost effective adjunct to routine microscopy and a reliable option for malaria diagnosis in remote and emergency situations.

Keywords: Acon-Pf, Routine microscopy sensitivity, Specificity, Intrinsic validity, Predictive values, Parasitaemia, Trophozoite, Gametocyte, Diagnostic power, Epidemiology, Logistic factors

INTRODUCTION

Malaria remains a major public health problem in Nigeria, despite decades of a sustained national control programme. One of the major setbacks of this control programme is the lack of a rapid, accurate, affordable and simple means for malaria diagnosis. The two diagnostic approaches currently used i.e. clinical and microscopic diagnosis, however, do not allow for a satisfactory diagnosis of malaria. Clinical diagnosis is the most widely used; however, the symptoms of malaria are very nonspecific and overlap with those of other febrile illnesses (WHO, 1999). A diagnosis of malaria based on clinical grounds alone is therefore unreliable and, when possible, should be confirmed by laboratory tests. Microscopic examination of thick blood film is currently the standard method for malaria diagnosis. This method is relatively simple and has low direct costs, but its reliability is questionable, particularly at low levels of parasitaemia and in the interpretation of mixed infections (Molyneux and Fox, 1993; WHO, 1996). Recently, rapid antigen detection methods have been developed for situations in which reliable microscopy may not be available. These tests are based on the detection of antigen(s) released from parasitized red blood cells (Moody, 2002). In the case of *Plasmodium falciparum*, these new methods are based on detection of *P. falciparum* histidine-rich protein 2. Diagnoses in malaria endemic rural areas are based on clinical symptoms which are non-specific and often difficult to interpret (WHO, 1990; Gilles and Warrell, 1993). This inevitably leads to considerable misdiagnosis and cause less than half of the people with malaria not to receive adequate and effective antimalarial treatment (WHO, 1984; 1990; 2000a).

Research showed that malaria kills as many as 3 million people a year and weaken nearly 300 – 500 million more, mostly in Africa. Children under five years and pregnant women are the most vulnerable to this menace (WHO, 2000b). The burden of malaria in Africa is particularly dangerous, causing 900,000 deaths, and every 30 seconds an African child dies (WHO, 2000b). Everyday, at least 1000 Africans dies of malaria and at least 20 % of all are children under five. The morbidity is high with about 500,000 African children suffering from cerebral malaria and high incidence of mortality during pregnancy and neonatal deaths (within the first 28 days of birth), about 8-14% low birth weight and 3 – 8% of infant deaths in endemic areas of Africa (ASPAD, 2004). Malaria rapid diagnostic tests have the potential to significantly improve the diagnosis of malaria in developing countries (Center for Human Services, 2000). Malaria can be suspected based on a patient's symptoms and physical findings at examination. However, for a definitive diagnosis to be made, laboratory tests must reveal the malaria parasites or their components (WHO, 2005).

Although the peripheral blood smear examination that provides the most comprehensive information on a single test format has been the "gold standard" for the diagnosis of malaria, the immuno-chromatographic tests for the detection of malaria antigens, developed in the past decade, have opened a new and exciting avenue in malaria diagnosis (Chiktara and Ahmed, 2004). However, their role in the management and control of malaria appears to be limited at present.

For monitoring the effectiveness of treatment, the malaria rapid diagnostic tests could therefore be a useful alternative to microscopy, particularly (i) in places where the facilities for microscopy are poor or non-existent and (ii) among hospitalized patients with severe, complicated malaria (in whom parasitaemia and drug response need to be followed very carefully) (WHO, 2000b; Singh *et al.,* 2003). Malaria rapid diagnostic tests can be used by relatively inexperienced persons to diagnose malaria infection in rural areas where facilities for microscopy are not available. In the case of pregnancy, malaria parasites that are sequestered in the placenta may not be detected by smears prepared using peripheral blood samples (Duffy and Fried, 2006). This study investigated evaluated the effectiveness of Acon-Pf rapid malaria diagnostic test and routine microscopy among school children and pregnant women in Enugu State, Nigeria in malaria diagnosis.

MATERIALS AND METHODS

Study Area: Affa is a rural community in Udi Local Government Area of Enugu State, Nigeria. Affa has one of the largest land mass and population of 58,000 people living in scattered farm communities and villages. Semi-subsistence agriculture is the economic mainstay of the Affa people. The inhabitants of Affa lack access to both microscopic diagnosis and malaria rapid diagnostic tests of malaria in the rural health centres. The community is about 70 kilometers away from the district and specialist hospitals located in Udi Local Government Area and Enugu metropolis, respectively. Traveling to these health facilities is difficult and expensive for the poor farmers. The study sites were mapped using the Ach-GIS Global Positioning System (GPS) instrument as follows: Amofia-Agu Dispensary, Affa (Long. 7.25117 E and Lat. 6.56483 N), Affa Health Centre, Affa (Long. 7.32474 E and Lat. 6.60107 N), Cottage Hospital, Affa (Long. 7.32509 E and Lat. 6.60112 N) and Enugu State University of Science and Technology (ESUT) Teaching Hospital (Long. 7.49450 E and Lat. 6.46047 N).

Study Population: A total of 352 subjects were sampled comprising of primary school children aged between (6 and 17) years and pregnant women attending ante-natal clinic. The study was conducted between August and December, 2007.

Ethical Approval: Ethical approval for the study was obtained from the Ministry of Health, Enugu State. At each study site, participation was voluntary and verbal informed consent sought from the subjects and from the parent/guardians of the children to be investigated, before an individual was recruited into the study. The research team consisted of a Medical Practitioner to facilitate the administration of drugs and treatment of infected population. Coartem was administered free of charge, to symptomatic patients who tests positive to the Acon-Pf in the field and fit a standardized diagnostic algorithm that includes fever and headache and/or chills or rigors occurring within the preceding three days, according to World Health Organization standards. Children who were recruited for this study were generally dewormed after being tested, irrespective of the status of the malaria test result with albendazole regimens received from the United Children Education Fund (UNICEF) as a benefit for volunteering towards the survey. There was a strict adherence to all universal precautions and provision of Post Exposure Prophylaxis accordingly as the need arises. Bio-safety issues on handling of needles, disinfections, control of haemorrhages, containment of complications and disposal of equipment and leftovers was strictly adhered to as directed. All sampled data and the research documents were guided with utmost confidentiality.

Study Design: This is an evaluation study in which the results from *falciparum* malaria diagnosis by the (Acon-Pf test cassette) were compared against blood film microscopy and the practicability of the new test in rural and malaria endemic areas assessed. The symptomatic diagnosis of malaria was based on the presence of fever (axillary temperature > 37.5°C) at the time of presentation to the health facilities or within the previous 48 hours, coupled with a history of recent bout of mosquitoes at home and farmlands.

Patients were questioned regarding the nature and duration of recent symptoms. Malaria rapid diagnostic tests and slides were prepared and the malaria rapid diagnostic tests were read. Acon-Pf test cassette, a rapid diagnostic test device manufactured by ACON Laboratories, Incorporated, USA, was used for immunochromatography test. The immuno-chromatographic test (ICT) is based on the detection of histidine rich protein-2 (HRP-2) in the blood. The intensity of malaria rapid diagnostic tests antigen bands were graded 1 (faint) to 3 (equal to or darker than the control band). The slides were re-read by an experienced microscopist, who was not aware of the results of the malaria rapid diagnostic tests, in accordance with WHO criteria (100 thick film fields before negativity declared (WHO, 2001). This was done after storage and transportation

for between two and four weeks at ambient temperature and humidity, as was usual in the area. The ambient temperature of the locality was within the range of 27°C to 29.5°C during the period of the study.

The packed cell volume (PCV) of the pregnant women was determined using Micro-Haematocrit Centrifuge (LAB–TECH, India) at 1000 rpm for 5 minutes to ascertain the effect of the *P. falciparum* malaria on the haemoglobin level of the patients.

The logistic factors that affect the Acon-Pf test cassette which include ambient temperature and humidity, and the effects on transportation and storage on the test cassette. The average times taken for the detection of parasite antigen by the Acon-Pf test cassette, and the type, stage and morphology by the routine microscopy were recorded, respectively.

The comparative cost benefit of using microscopy or Acon-Pf test cassette in diagnosis of malaria was estimated in Naira.

Questionnaires: Questionnaires were administered among the population samples comprising school children and pregnant women to determine their knowledge, attitude, belief and practice (KABP) on malaria prevention and intervention strategies. In an in-depth interview, each schoolchild was asked if he or she had a recent history of fever and, if so, how was the fever treated.

Blood Samples: The venous blood samples were collected from asymptomatic school children and pregnant women to prepare thin and thick smears and diagnose for malaria parasites (WHO, 1991). A tunicate was tied at the upper cubic-forsa of the left arm and using a 5-ml syringe; 2 ml of venous blood was draw and placed in an EDTA bottle. The samples were well packed in the rack and transported in compliance with all ethical regulations. The routine examination included the packed cell volume (PCV) to determine the effect of malaria parasitaemia on the Haemoglobin (Hb) level of patients. Epidemiological and variation rate of parasitaemia was also determined. Careful attention to technique was necessary in the collection of blood and the preparation of blood films. During the study, caution was placed on collection and handling of samples due to a number of viral, bacterial, and parasitological diseases that may be transmitted through the blood (WHO, 1991).

Microscopic Diagnosis: Conventional light microscopy is the established method for laboratory confirmation of malaria (WHO, 1991). The careful examination by an expert microscopist of a well prepared and well stained blood film remains currently the practices for detecting and identifying malaria parasites. The procedure used consists of collecting a finger-prick blood sample, preparing a thick and a thin blood smear, staining of the smear and microscopic examination (preferably with a 100X oil-immersion objective) of the blood smear for the presence of malaria parasites.

The cells in the thin blood smear were chemically fixed to the slide, and the slide is stained with alpha-phenolphthalein stain which better preserves parasite morphology and facilitates detection of parasites in the blood film. The thick blood film increases the sensitivity of the test. The thin blood film was used to quantify and identify parasites to the species level. A high-power microscope (400 times to 1,000 times magnification, with an oil immersion objective) was used to read thick and thin blood films. Between 100 and 200 microscope fields was examined to rule out the presence of parasites in a thick blood film (Cheesbrough and Precott, 1987; Payne, 1988).

Blood film: For routine malaria microscopy, a thin and thick blood smears were made on the same slide after patient information has been recorded in the appropriate register as follows: With the patient's left hand, palm upwards, the third finger was selected. A cotton wool lightly soaked in alcohol was used to clean the finger using firm strokes to remove dirt and grease from the ball of the finger. A clean cotton towel was used to dry the finger, by applying firm strokes to stimulate blood circulation on the finger (WHO, 1991).

With a sterile lancet the ball of the finger was punctured, using a quick rolling action. By applying gentle pressure to the

finger, the first drop of blood expressed was wiped away with dry cotton wool making sure no strands of cotton remain on the finger.

Quickly and by handling clean slides only by the edges, a single and small drop of blood, about this size o was collected by applying gently pressure to the finger, and dropped on to the middle of the slide for the thin film. A thick film was gotten by applying further pressure to express more blood to collect two or three larger drops, about this size o, on to the slide about 1 cm from the initial drop intended for the thin film. The remaining blood was wiped away from the finger with cotton wool (WHO, 1991).

Thick film: Using the corner of the spreader, the two or three more drops of blood were quickly joined and spread to make an even thick film. The blood was not excessively stirred, rather was spread in a circular or rectangular form with 3 – 6 movements.

The smear was allowed to dry in a flat, level position protected from flies, dust, and extreme heat. The dry film was labeled with a pen or marker pencil and not ball point pen was used for writing across the thicker potion of the film, the patients' name or number and date. The dry slides were wrapped in clean dry paper, and dispatch to the laboratory as soon as possible for diagnosis. The slide used for spreading the blood films was routinely disinfected with a spirit-moisted cotton wool to be used on the next patient, another clean slide from the pack being used as a spreader (WHO, 1991).

Thin film: Using another clean slide as a "spreader", and with the slide the blood drops resting on a flat, firm surface, touch the small drop with the spreader and allow the blood to run along its edge. Haven maintained an even contact of blood sample, the spreader is firmly pushed along the slide, away from the largest drops, keeping the spreader at an angle of 45° and not extending to the edges of the slide in order to prevent autoinfection (WHO, 1991).

Staining Blood Films: The blood films have dried overnight and fixed by dipping it in a

container of methanol for a five seconds (WHO, 1991). The thin film with prolonged fixation, it may be difficult to demonstrate Schuffner's dots and Maurer's dots. To permit dehaemoglobinization, the thick film was not fixed; exposure of the film to methanol or methanol vapour was greatly reduced. The slides were placed back to back across an iron bar placed over a staining dish. 3% Giemsa solution was prepared in pH 7.2 buffered distilled. Using a dropper, the stain was gently dropped on the films, until the slides are totally covered. Each stained slide was allowed to stain for 30 minutes. The slides were gently immersed into clean water to float off the iridescent scum on the surface of the stain. The remaining stain was gently poured off, and rinse again in clean water for a few seconds and poured off the water. The slides were removed one by one and placed in a slide rack to drain and dry. The film side was downward, ensuring that film does not touch the slide rack (WHO, 1991).

Rapid Diagnostic Test Procedure (Acon-Pf): The kits were stored at room temperature (27 – 30°C). The test device remained in the sealed pouch until use and was not used beyond the expiry date. The device was not freezed, though could be refrigerated at 2°C as directed by the manufacturer. Schematic representation of the direction for use of the Acon-Pf cassette is shown in Fig. 1. The test procedure recommended by the manufacturers for Acon-Pf test cassettes was followed. The test device, specimen, buffer, and/or controls were allowed to equilibrate to room temperature (15 – 30°C) prior to testing. The test device was removed from the foil pouch and was used as soon as possible to perform the assay within one hour to obtain the best result. The test device was placed on a clean and level surface.

Both finger stick whole blood and venipuncture whole blood were used depending on the situation. The finger stick was mainly used during emergency cases and for children, while the venipuncture was preferable for normal diagnosis and epidemiological purposes (WHO, 2000). Each patient's hand was cleaned with an alcohol swab and allowed to dry. The

hand was massaged without touching the puncture site by rubbing down the hand towards the fingertip of the middle or ring finger. A puncture of the skin was made with a sterile lancet. The first sign of blood was wiped away. Gently the hand was rubbed from wrist to palm to finger to form a rounded drop of blood over the puncture site. An additional 2 to 3 drops of blood were collected by rubbing the finger further over the puncture site. Testing was performed immediately after specimen collection, without allowing the specimen at room temperature for prolonged periods. The whole blood collected by venipunture was stored at (2 - 8°C) in an EDTA bottle if the tests were to be run within 2 days of collection. Such specimens mentioned above, were brought to room temperature prior to testing. The specimens that got frozen were completely thawed repeatedly for more than three times before use. The specimens that were meant to be transported from the remote areas to the diagnostic laboratory were packed in compliance with ethical regulations covering the transportation of aetiological agents. Using a disposable specimen dropper was held vertically; the blood sample was drawn up to the fill line (approximately 10 µL). The blood sample was transferred to the specimen well (S) of the test device and 3 drops of the buffer (approximately 20 µL) was added. The stopwatch timer was then started. A waiting period of 15 minutes was allowed for the coloured line(s) or band(s) of the Acon-Pf test to appear and the result read. The coloured lines appeared with varied intensities and test antigen bands were graded 1 (faint) to 3 (equal to or darker than the control band). No results were interpreted after 20 minutes.

Interpretation of results

Positive: Two distinct coloured lines appear to confirm a positive result. One line was in the control region (C) and another line was in the test region (T). Note that the intensity of the colour in the test line region varied depending on the concentration of the *P. falciparum* present in the specimen. Therefore, any shade of colour in the test region (T) was considered as a positive test result.

Negative: One coloured line appeared in the control region (C). No apparent coloured line appeared in the test region (T).

Invalid: Control line failed to appear. Insufficient specimen volume or incorrect procedural techniques are the most likely reasons for control line failure. In such instance, the procedure was reviewed and the test was repeated with a new test device to rule out all doubts, and otherwise the test would have been discontinued.

Data Analysis

All data were analyzed by Statistical Package for Social Sciences (SPSS). In order to ascertain whether there is any significance differences in the study, data was analyzed using one-way analysis of variance (ANOVA) with a Scheffe and Duncan's New Multiple Range Test post hoc multiple comparison to separate the means that were statistically different. The following key variables were measured:

Disease prevalence: The total number of the individuals infected at the particular period of the study =   Number infected / a + b = Total Number examined (n) / N (Okafor, 2005).

Sensitivity: The ability of the test to detect infected individuals as positive, calculated as TP/(TP+FN) (Mharakurwa *et al.,* 1997).

Specificity: The ability of the test to detect individuals without infection as negative, calculated as TN/(TN+FP) (Mharakurwa *et al.,* 1997).

Positive predictive value: The proportion of the test's positive readings which are truly negative, calculated as TP/(TP+FP) (Mharakurwa *et al.,* 1997).

Negative predictive value: The proportion of the test's negative readings which are truly

negative, calculated as TN/(TN+FN) (Mharakurwa *et al.,* 1997).

False positive rate: The proportion of individuals without infection being missed by the test and falsely ascribed a positive status, calculated as FP/(FP+TN) (Mharakurwa *et al.,* 1997).

False negative rate: The proportion of infected individuals being missed by the test and falsely ascribed a negative status, calculated as FN/(FN+TP(Mharakurwa *et al.,* 1997).

The J-index or overall measure of reliability of a diagnostic test which summarizes both sensitivity and specificity, calculated as (TP×TN-FP×FN)/(TP+FN)(TN+FP). The J-index values lie between 0 and 1, and as the value approaches the ideal; diagnostic ability is poorer the more closely the J-index values approaches 0 (Mharakurwa *et al.,* 1997), where TP = true positives, TN = true negative, FP = false positives and FN = false negatives.

RESULTS

Prevalence: The prevalence data of positive test results of the Acon-Pf and slide microscopy carried out on the same blood sample of children and adult population showed that the prevalence of malaria among the children population below the age of 5 years was highest with parasitaemia levels of 33.8% for Acon-Pf and 28.7% for microscopy. The prevalence rate of the adult population was lower with parasitaemia levels of 21.1% for Acon-Pf and 26.7% for microscopy. A total of 71 and 101 patients tested positive to the Acon-Pf and microscopy, respectively out of the 352 sample size. It also shows that more than half of the sample population of 180 patients tested negative for both the Acon-Pf and routine microscopy (Table 1).

The schematic representation of an Acon-Pf test cassette results conducted showed that the negative test result of patient had only one coloured line which appeared in the control region (C) and there was no apparent coloured line in the test region (T), indicating an actual negative test result. All the positive test results had two distinct coloured lines that appeared in the control region (C), and the other coloured line in the test region (T), indicating an actual positive test result. There was no invalid Acon-Pf test cassette result observed in the study, showing that one coloured line of all the cassettes used appeared in the control region (C) (Figure 1).

P. falciparum **Stages:** The routine microscopy of thin blood film revealed an early stage a *P. falciparum* trophozoite. It is a ring stage with fine chromatin dots and frequently two, but some have one dot. The developing stages can appear two in a red blood cell and in a small, compact and coarse pigmentation present (Figures 2 and 3).
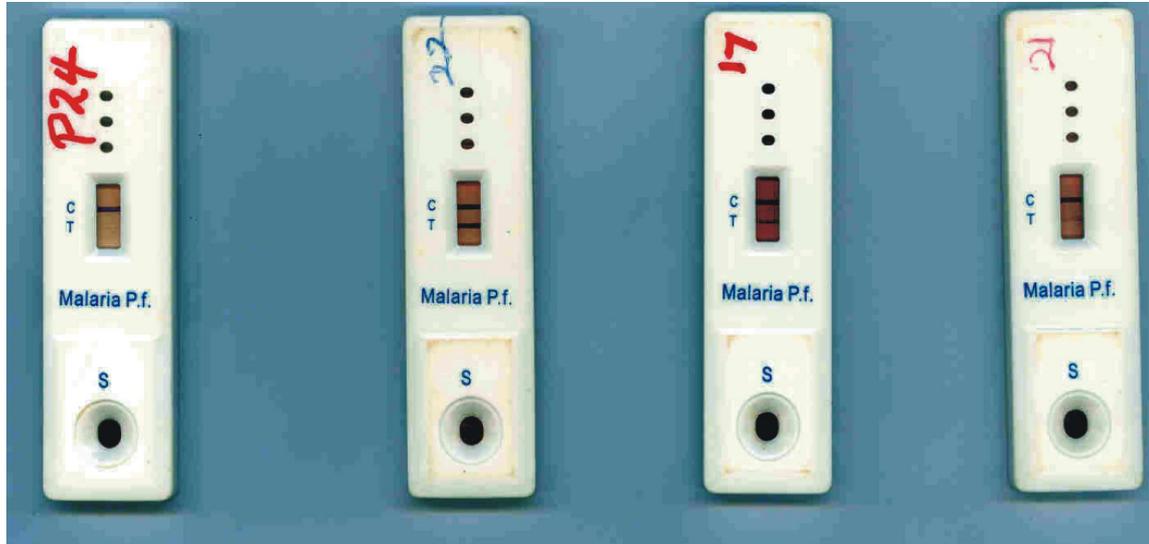
Sensitivity and Specificity of Diagnostic Test Devices: The validity of the both Acon-Pf test cassette and routine microscopic test were considered for same sample size of 352 patients using sensitivity and specificity. The standard deviation validity test was significantly higher in the routine microscopy than the Acon-Pf test cassette (0.71135 versus 0.40184; p <0.05) at (0.1596 − 0.2438) confidence interval, which means Acon-Pf test was more reliable due to minimum variance.

Out of the 352 samples studied, 281(79.8%) tested negative for Acon-Pf test cassettes mean value and standard deviation 0.217 ± 0.591, respectively and 251(71.3%) for routine microscopy with mean value and standard deviation 0.024 ± 0.153, respectively, while 71(20.2%) tested positive for Acon-Pf test cassettes and 55(15.3%) for microscopic test and Acon-Pf test cassettes showed no measurable scanty result, but routine microscopy showed 46(13.1%) scanty results. The mean sensitivity rate was significantly higher in Acon-Pf test cassettes than in microscopic tests (1.21 ± 0.583 versus 0.800 ± 0.403; P<0.05) (Table 1).

Out of the 352 sample studied, the sensitivity rate of the Acon-Pf test cassette was 91.5%, the specificity rate was 87.2%, with positive and negative predictive values at 64.3%

Table 1: The prevalence data of positive test results of the Acon-Pf and slide microscopy

Subjects	Positive		Negative	
	Acon-Pf	Microscopy	Acon-Pf	Microscopy
352	71(20.2%)	101(28.7%)	281(79.8%)	251(71.3%)

Negative Test Result	Thickly Positive Test Result	Light Positive Test Result	Lighter Positive Test Result

Figure 1: Schematic representation of the direction for use of the Acon-Pf cassette (C = control, T = positive test result and S = well).
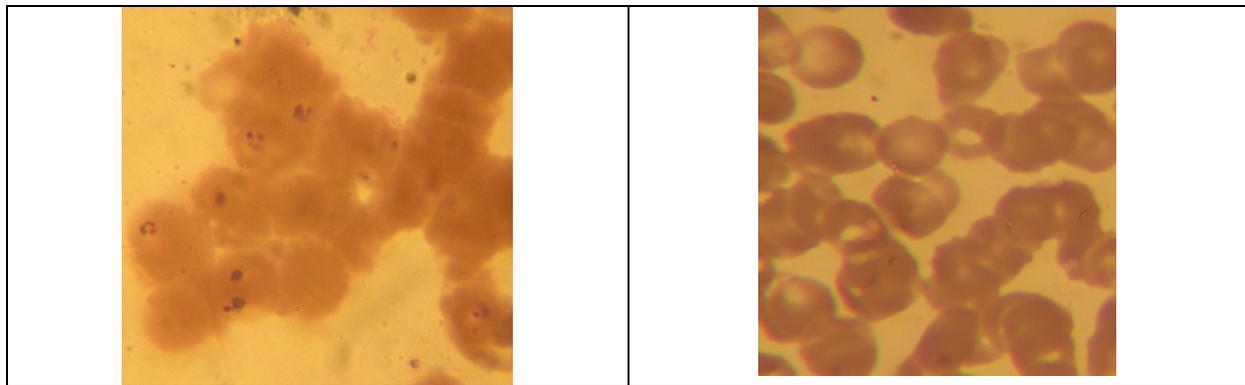

Figure 2: Appearance of *Plasmodium falciparum* Trophozoite stage and species identification in Giemsa's stained thin blood films X1000. (Many parasites are present, mainly small and delicate rings, few rings are with double chromatin dots, the chromatin dot is stained in red colouration, the ring is stained in purple colouration, many rings signifying heavy infection, the size of each trophozoite ring cover about 1/5 of one red blood cell and red blood cell not enlarged. Host red cell are not enlarged, no Schuffner's dots, but cells containing late stage trophozoites show irregular red-mauve staining Maurer's dots (clefts) and more than one parasite in a cell).

Figure 2: Appearance of *Plasmodium falciparum* gametocyte stage and species identification in Giemsa's stained thin blood films X1000. (Crescent (banana) shaped with rounded or pointed ends, but few are oval forms. The chromatin dot is stained in red colouration, chromatin appear as a compact mass near the centre for the female gametocyte (macrogametocyte), chromatin where present, are fine granules scattered throughout the male gametocyte and red blood cell not enlarged.

Table 2: Sensitivity, specificities and positive and negative predictive values recorded for Acon-Pf malaria diagnostic test in the health facilities studied

Type	Subject No	Sensitivity (%)	Specificity (%)	Predictive Value (%) Positive	Predictive Value (%) Negative	FPR (%)	FNR (%)	J-index (%)
Acon-Pf	352	91.5	87.2	64.3	97.6	12.8	8.0	78.7

and 97.6% respectively. The Acon-Pf test cassette used was highly reliable at 78.7% of the sensitivity and specificity tests as indicated through the J-index (Table 2).

Symptomatic diagnosis of the 352 pregnant women and children, showed that 260(79%) did not manifest any of the above symptoms within the past three months as at the time of the study, 20 patients had recent fever alone highest at (5.7%), chill and blisters 0.9% each, headache, dairrheoa and joint pain 0.3% each, weakness and anorexia 0.6% each. Recent fever combined with chill was highest 2.8%, followed by anorexia and weakness 2.0%, fever with headache, and weakness 1.7%, fever with anorexia and/ or vomiting 1.4%, fever with chill and headache and/ or amber urine or joint pain or anorexia or frequent sleeping 1.1%, headache with weakness 1.1%.

Forty six (46) antenatal patients out of the 352 population sampled for symptomatic diagnosis to predict malaria infection in symptomatic patients with recent history of treatment showed low sensitivity (41.6%), but highly specific (80%), low positive value (45.7%), high negative predictive value (77.3%), low false positive rate (20%), moderately high false negative rate (58.4%) and a high J-index (78.7%). The axillary temperature was poorly predictive for negative samples with normal axillary temperatures. The mean axillary temperature among children with Acon-Pf positive at (37.0°C) and Acon-Pf negative at (36.4°C), antenatal patients with Acon-Pf positive at (36.5°C) and antenatal patients with Acon-Pf negative at (35.1°C).

Infection Rate: The analysis of the KABP showed that out of the 46 respondents, 27 antenatal care patients showed the highest frequency of malaria prevalence at an above average percentage of 58.7%, followed by a

combination of malaria and typhoid diseases at 13.0%, other infections have less rate of prevalence compared to malaria and typhoid which are two common and serious tropical diseases (Table 3).

Interviews Results: Eighteen laboratory scientists from the health facilities gave responses to the two alternative diagnostic techniques, knowledge of the Acon-Pf test device and trained on the use of RDTs. All the respondents would use routine microscopy, 88.9% prefer Acon-Pf test device, 38.9% would combine both techniques, 88.9% had knowledge about the rapid malaria diagnostic tests devices and 11.1% have had training on the use of the malaria diagnostic tests devices (Table 4).

Table 3: Diseases associated in the diagnosis and management of malaria among antenatal patients in a semi urban tropical community in Enugu State, Nigeria

Associated Diseases	Frequency	Percentage (%)
Vomiting	2	4.3
Malaria	27	58.7
Diarrhea	2	4.3
Pinworm	2	4.3
Malaria + typhoid	6	13.0
Malaria + Diarrhea	4	8.7
Malaria + Headache	3	6.5
Total	46	100.0

Table 4: Responds on the knowledge of the Acon-Pf test device and training on the use of RDTs

	Frequency	Percentage (%)
First choice Slide Microscopy	18	100
Second choice Acon-Pf	16	88.9
Combined choice	7	38.9
Knowledge of RDTs	16	88.9
Trained on RDT use	2	11.1

The performance of the Acon-Pf test and the test procedures were easy to master and use, with minimum time required. The mean time required for the collection of venous blood samples by a trained phlebotomist were 1.16 minutes and 1.38 minutes from women attending antenatal care and their children, respectively. However, the actual range of time required for a positive test was 0.30 seconds to 15 minutes, and the control band showed at average time of 1.26 minutes depending on the level of parasitaemia and experience of the user, as shown by the intensity of the developing colour.

Logistic Factors: The Acon-Pf test cassettes could detect antigens from the oxalated blood stored for over three days at 30ºC, but exposure to direct sunlight was avoided. Acon-Pf test cassettes were stored in two different environments up to 8 months at a mean ambient temperature of (29.0 ± 3.5 °C) which did not show any adverse effect, thus the procurement, transportation during field work and storage of the Acon-Pf test cassettes did not pose any problem to the quality of the test, and hence does not need a cold chain.

Cost Benefit: The cost per Acon-Pf test cassette at bulk purchase = ₦112.50 ≡ US$0.90, without the service charge (as compared with thick smear = ₦ 300.00 ≡ US$ 2.50; and ₦ 350.00 ≡ US$ 2.90 for thin smear). The cost of a packet of Acon-Pf test device which contains 40 cassettes was ₦ 4, 500.00 ≡ US$ 36.00 as at the time of the study.

DISCUSSION

Despite several *P. falciparum* malaria diagnostic techniques which have been developed currently for the prevention of malaria morbidity and mortality, the incidences of malaria are devastating and decimating the global population, especially among the poor people and the developing countries of the tropical regions. Thus, the rationale for the investigation of the reliability of the Acon-Pf test cassette as an adjunct to the microscopic devices as a simple, inexpensive and sensitive malaria rapid diagnostic test for field and remote areas.

The prevalence of malaria parasitaemia among the sample populations was higher among children less than 5 years, than what was found among the adult population. This may be contributed to low level of malaria induced immunity, late and poor clinical diagnosis especially among those residing in remote and rural communities of study area. The prevalence rates in children were as high as for Acon-Pf (33.8%), slide microscopy (28.7%) and sharply followed by those of the adult population, Acon-Pf (21.1%) and slide microscopy (26.7%), respectively. A mean age of 15.78 years constituted the study population. The malaria parasite species found was *P. falciparum* and two stages were identified as trophozoites and gametocytes. The red blood cells were not enlarged even as the parasites are found in the infected red blood cells. One or more parasites were found in parasitized the red blood cells in slide thin films. The trophozoites showed various shapes which include the ring and comma; while some are rounded. The gametocytes identified showed crescent (banana) shape with the presence of a chromatin on one of the red blood cells.

The Acon-Pf test results showed some negative and positive test results and no invalid test resulted was recorded, which confirm the reliability of the new rapid diagnostic test cassette for prompt malaria diagnosis. This work did not study the quantitative analysis of parasites, however, some of the Acon-Pf

positive test results showed bands which were light to thickly coloured. The intensity of the colour in the test line region (T) may vary depending on the level of the parasitaemia of the *P. falciparum* present in the specimen. More so, in slide microscopy, there were cases with high parasitaemia with asexual stages which turned out to be negative by Acon-Pf test results. The failure of a *P. falciparum* histidine-rich protein-2 that is produced by the asexual stages and gametocytes of *P. falciparum*, which is expressed on the red cell membrane surface, to detect any positive result, may be attributed to low level of parasitaemia and/or error in the interpretation of the colour changes of the rapid diagnostic test result. According to Kakkilaya (2003), other factors that can limit that sensitivity of a rapid diagnostic test result include genetic heterogeneity of *P. falciparum* histidine rich protein-2 expression, deletion of histidine rich protein-2 gene, presence of blocking antibodies for *P. falciparum* histidine rich protein-2 antigen or immune-complex formation, prozone phenomenon at high antigenemia or to unknown causes. In addition, that false negative tests have been observed even in severe malaria with parasitaemia >40000 parasites/μl. Therefore, in cases of suspected severe malaria or complex health emergencies, a positive result may be confirmatory but a negative result may not rule out malaria, especially in cases of asymptomatic malaria. It should be emphasized that *P. falciparum* malaria, a resistant tropical disease, must not be missed because of a false-negative rapid diagnostic test. It has been suggested that in such cases, 1 in 10 dilution of a negative sample with 0.9% sodium chloride solution may help to exclude the prozone phenomenon. Furthermore, any doubtful negative rapid diagnostic test result should always be confirmed by microscopy.

The Acon-Pf was strongly positive and correlated well with the trophozoites stages and degree of parasitaemia. In the blood specimen from patients showing only gametocytes in smear examination, the test was either weakly positive or negative. The weak positivity perhaps was due to persistent low antigenemia. Whether Acon-Pf can detect the early gametocytic stage of parasitaemia is still unclear. This limitation of the test must be appreciated as the patients are in infective stage. The detection of gametocytes by microscopy is relatively easy due to their large size and peculiar morphology. The test could not detect antigen properly from haemolyzed blood or from serum samples.

Rapid diagnostic tests achieved high sensitivity (91.5%), but few cases indicated as positive by rapid diagnostic tests were negative by microscopy. Results of above mentioned diagnostic tests are similar to earlier workers findings of Quitana *et al.* (1998) and Iqbal *et al.* (2001). Sensitivity of a diagnostic test indicates its ability to detect all those infected. The more false negative the test result, the lower the sensitivity of the test. The specificity of a diagnostic test indicates its ability to distinguish one particular infection from the other infections or conditions. The more false positive test result, the lower the specificity of the test. Further analysis of these cases indicated that Rapid Diagnostic Tests were detecting low-level parasitaemia missed by microscopy, and that local slide microscopy in poor health facility without a trained and experienced microscopist, had poor accuracy.

The rapid malaria diagnostic test conducted by experienced personnel demonstrated good sensitivity and negative predictive values in comparison with locally read slides and read in a controlled environment. Specificity and positive predictive values for rapid diagnostic tests are difficult to assess when comparison is made with microscopy as a confirmatory test, which itself has poor sensitivity at low parasite densities. They were probably much higher than this study indicated, for the following reasons. The persistent of the gametocytes of *P. falciparum* following chemotherapy without implying drug resistance and some versions of the test kits targeting Histidine Rich Protein-2 of *P. falciparum* have given false positive results in patients with rheumatoid factor as reported in the work of Whitty *et al.* (2000). Available researches show that Histidine Rich Protein-2 of *P. falciparum* antigen, which is detected by the ICT Malaria Pf/Pv test, may persist for up to 7 to 10 days

after asexual parasite clearance (Shiff *et al.,* 1993; Igbal *et al.,* 2001).

False negative Acon-Pf test may have resulted at parasitaemia < 500/ μl. This can potentially be dangerous, as to miss the diagnosis of malaria in an ambulant, febrile patient may mean that complications develop because appropriate treatment was not instituted in time. False negative rapid malaria diagnostic test results in samples with higher parasitemia have been observed in earlier studies, but the underlying reason was not stated (Anon, 1996; Karbwang *et al.,* 1996; Humar *et al.,* 1997; Palmer *et al.,* 1998; Van den *et al.,* 1998; Hunt-Cooke *et al.,* 1999; Igbal *et al.,* 2002).

In addition, false-negative results by microscopy can occur if patients have undertaken self-medication prior to presentation. It is likely that some of our patients with false-positive results may have performed self-medication with antimalarial drugs during an attack of fever. However, it is unlikely that these factors account for the entire set of false-positive cases. It is more probable that most of the false-positive cases were true positives which were not detected by microscopy, due to sequestration limiting the number of circulating parasites at the time of blood collection or due to the parasitaemia being below the detection limit of approximately 50/μl by microscopy.

The disease infection rate among 46 antenatal patients showed that malaria infection has the highest prevalence rate at an above average percentage of 58.7%, followed by a combination of malaria and typhoid diseases at 13.0%. The public health importance of this high infection rate may increase the level of anaemia, mortality and morbidity rate among children under the age five and pregnant women. This high prevalence rate of malaria demonstrated among the rural subsistence farmers in Enugu State may have exposed a large population of Nigerians to the risk of malaria infection. Therefore, there should be prompt and grass-root intervention programmes to reduce the socio-economic effects on the people through massive education and universal access to malaria control facilities.

The general sensitivity of the Acon-Pf test was found to be high in the sampled sites and communities. The desire for an easy, rapid, cost effective and accurate tests for the detection of resistant malaria *falciparum* infections would never be over emphasized, as Malaria Rapid Diagnostic Test was highly valuable in the communities and among the Health workers and may likely increase the compliance and treatment-seeking behaviour of patients. Rapid diagnostic tests were well accepted and accurately performed by the health workers in the facilities. The high rate of a febrile parasitaemia in this study indicated that large reservoir of barely symptomatic cases existed in the communities.

The symptomatic malaria diagnostic method is not reliable at 21.6% for the sensitivity and specificity test as indicated through the J-index. This is because there could be other infections other than malaria that have similar symptoms. The test will help in early diagnosis of *P. falciparum* malaria, thus enabling the physicians to institute specific treatment of those suffering from *P. falciparum* malaria, and reduce the case fatality rate. Interventions were carried out in the field, thus a presumptive treatment was given to all fever cases and treatment for Pf malaria to those testing positive for *P. falciparum* malaria.

The asymptomatic stage does not automatically indicate that one has no malaria infection. In this same vein, blisters on the lips of the mouth alone can not indicate malaria, all the major symptoms – fever, chill, headache, anorexia and joint pain are indicators of malaria infection. However, blood test using rapid malaria diagnostic test kits and routine microscopy can only indicate infection proper. This is because other infections present similar symptoms like malaria infection.

In the remote and endemic areas of the study, the accuracy of symptom-based diagnosis was poor, as it has been reported by other researchers although specificity in this self-referring sick population should be higher than in the communities as a whole. Local transmission rates and therefore immunity, were low, and this should have increased the symptomatic malaria sensitivity. The

observation of fever alone, and/or fever in combination with chills and/or headache, achieved quite high sensitivities, but both criteria resulted in high rate of over-treatment in symptomatic patients. Any narrower combination of symptoms resulted in sensitivities unacceptable in relation to the detection of a life-threatening illness. The measurement of axillary temperature failed to achieve sufficient sensitivity or specificity to be useful. The data on symptomatic malaria diagnosis shows that children may have higher axillary temperature than adult antenatal patients, reason may be because adults have developed asymptomatic immunity to malaria due to repeated exposure to malaria infection. This study suggested that the symptoms identified can be used as an algorithm for the future identification of symptomatic malaria diagnosis for presumptive treatment in remote and malaria endemic regions.

The attitude of the antenatal patients to diagnosis during the study demonstrated the importance of providing patients with a reliable explanation for their illness. The responses of the rural community members and antenatal women suggested that there is an improved treatment-seeking behaviour and drug compliance. The KABP analysis also showed that there is poor knowledge of the, causes, prevention and control strategies of malaria, such as the use of Long Lasting Insecticide treated bed nets (LLIN). Further findings showed that there was poor attitude to the use of the Long Lasting Insecticide treated bed nets provided to the antenatal patients in the health facilities in the rural community. A rapid blood-based diagnosis at some cost was preferred by rural community members and antenatal patients to both the delayed free slide diagnosis and symptom-based diagnosis, despite the cost to the patient. This cost, approximately the difference between the estimated cost of microscopy to the health service and the bulk wholesale cost of the rapid diagnostic tests, was a substantial sum to families engaged in semi-subsistence agriculture.

The cost-effectiveness of symptom-based diagnosis, rapid diagnostic tests and microscopy, and the proportion of the costs at which the health workers and community members may wish to be buying the Acon-Pf were not investigated, that could be suggested for research.

They can be expected to vary with transmission rates and health service access. In the long term, improved compliance and treatment-seeking behaviour may bring additional economic benefits from rapid diagnostic tests through a reduced burden of illness. The detection of persistent antigen in asymptomatic infection, when fluctuating parasitaemia reduces the sensitivity of microscopy, also offers new possibilities for rapid screening of communities at risk.

In contrast to slide smear that takes up to 60 minutes (30 minutes for filming and staining, and 15 − 30 minutes for microscopy). Microscopy is to great extent subjective and sometimes can lead to misdiagnosis. Investigations on the performance rate of Acon-Pf to microscopy showed high level of awareness of rapid diagnostic test kit to the level of 77.8%, while ignorance to the use of the new rapid diagnostic test kit as much as 77.8%. Those advocating for the new Rapid Diagnostic Test Kit to be made accessible and affordable to Health workers are about 88.9%. The new technique was ethically and culturally acceptable to the volunteers, and may have overwhelming demand at the remote and rural communities in the tropical regions.

This study clearly shows that malaria parasitaemia cannot be easily identified by symptoms alone and that microscopy is a challenge and unreliable in remote areas. The rapid diagnostic test was well accepted by Health workers and community members and was performed accurately by the former and fairly by the latter after little training. It markedly improved diagnostic accuracy and met a desire in the communities for rapid blood-based diagnosis.

The complementary use of immunochromatographic rapid diagnostic tests may be useful, especially during emergency blood transfusion and on-call hours when expert microscopists may not be readily available. The malaria rapid diagnostic tests would reduce the risk of malaria transmission among

asymptomatic and unscreened blood donors, non-immune travelers and in the case of emergency blood transfusion. However, in order to ensure correct interpretation of rapid diagnostic test devices, their inherent limitations have to be well known.

The Acon-Pf could detect antigens from the oxalated blood stored for over 72 hours at 30°C ambient temperature. There was no significant difference in the Acon-Pf test cassette stored in two different environments at relative humidity and temperature as specified in the direction for use by the manufacturer. The Acon-Pf did not demonstrate any adverse effect on the procurement, handling, transportation during field work and storage of the Acon-Pf test cassettes retained the quality of the test, and hence does not need a cold chain. The test result colour lines (T) can remain as long as the control line (C), without fading away.

Depending on the local number of malaria cases seen, laboratory staff should have a low threshold for the decision to perform unsolicited malaria diagnostic tests on suspicious samples, especially if other laboratory tests are abnormal (e.g. thrombocytopenia, presence of atypical lymphocytes, or raised lactate dehydrogenase). The detection of intraleukocytic haemozoin during automated full blood counts is a promising new way to avoid misdiagnosis of clinically unsuspected malaria.

Irrespective of all its advantages, immunochromatographic test device will not be able to replace microscopy. Microscopy is still more flexible and offers the immense advantage of providing species diagnosis and exact parasite densities. The sensitivity of microscopy generally varies with the skill and experience of the slide reader, quality of smear preparation and staining, microscope quality, magnitude of parasitemia, and number of fields read. The potential role of a National Agency for Food and Drug Administration and Control (NAFDAC) – to control and facilitate access to approved rapid diagnostic tests particularly in settings where malaria microscopy is either not available or of poor quality should therefore not be underestimated.

Conclusion: The study shows that Acon-Pf is a reliable test kit for malaria diagnosis in remote areas and for epidemiological studies. The rapid diagnostic test was highly valued in the community and among the Health workers and may increase the compliance and treatment-seeking behaviour of patients. We recommend. A post-donor screening for malaria parasites using the Acon–Pf device is recommended to reduce malaria transmission by transfusion and among travelers. The study advocates for governmental support to researchers for the cultivation of in-vitro rapid diagnostic tests strips here in Nigeria, which may be more sensitive, increase access and may be more tolerant to the prevailing temperature and humidity in the tropical regions. Routine microscopy of thin blood film is still the first choice method for diagnosing malaria because it is specific to all *Plasmodium* species and offers differentiation between parasite growth stages, which are essential for prevention and intervention strategies. If a rapid diagnostic test result is negative and clinical symptoms persists, additional investigation using other clinical methods are recommended. A negative result does not at any time preclude the possibility of malaria infection. Hence, it is recommended that Federal, State, Local authorities and Developmental Partners should assist in promoting and increasing health facility and community access to the current, highly sensitive and specific rapid diagnostic test devices for malaria.

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