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Susceptibility testing of some antimalarial drugs on *Plasmodium spp*.

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ABSTRACT

The susceptibility of Plasmodium falciparum to Artesunate, Amalar and Chloroquine was evaluated by monitoring for schizont formation. Blood samples were collected from five asymptomatic patients within the age bracket of 18 and 26, who are students of Anambra State University Uli, in the month of July. The number of parasites observed in each sample using microscopic technique and Giemsa stain are 120,132,116,25, and 47 for samples A,B,C,D, and E respectively. All the parasites were still in their trophozoite stage with their characteristic double chromatin dots. The parasite density was estimated to be 6000, 6500, 6000, 1500, and 2500 for samples A, B, C, D, and E respectively. Antimalaria susceptibility testing by schizont maturation inhibition technique revealed the number of schizonts found in the two different dilutions of the three drugs applied. In the first dilution which was 50mg of drug in 0.5ml distilled water, the values were 5, 3, 3, 0, and 1 for samples A, B, C, D, and E respectively for Chloroquine. For Amalar, the values were2, 2, 3, 0, and 0 for samples A, B, C, D, and E respectively. For Artesunate the values were 1, 0, 1, 0, and 0 for samples A, B, C, D, and E respectively. For the control, the values were 10, 8, 10, 2, and 3 for samples A, B, C, D, and E respectively. the second dilution which was 25,mg of drug in 0.5ml of distilled water, a number of 7,6, 4, 1 and 2 schizonts were observed in samples A, B, C, G and E respectively for chloroquine. For Amalar, the numbers of schizonts observed were 5, 5, 4, none and 1 foe samples A, B, C, D and E respectively, and for Aztesunate, the numbers were 1,3, 2, non and none for samples A, B, C, D and E respectively. From the results obtained, it inferred that Plasmodium faciparum is susceptible to Artesunate. Amalar and Chloroquine and that Artesunate is best in the treatment of 1 falciparum, followed by Amalar while Chloroquine is offered just good.

Keywords: *Susceptibility Testing, Plasmodium falciparum, Artesunate, Amalar, Chloroquine.*

INTRODUCTION

Plasmodium parasite, the causative agent of malaria [1] has posed a major problem in the entire world. It has been one of the greatest burdens of mankind especially in the tropics from South America to the Indian peninsula, with a mortality rate that is unequalled by other emerging diseases [2]. There are four species of *Plasmodium*, they are *falciparum*, *ovale*, *malariae*, and *vivax*. All of these cause malaria but *P. falciparum* is more dangerous than others[2]. The control or eradication of malaria has been very difficult to achieve mainly because they have ability for resistance to chemoprophylatic and chemotherapeutic agents [3]. Over the past decades, reports have documented the emergence of P.vivax that is resistant to chloroquine. The first case of P.vivax resistance to chloroquine was reported in Papua New Guinea in 1989. Further cases were reported in India, Indonesia, Thailand as well as South America [4]. It is therefore important to conduct a susceptibililty testing of a selected *Plasmodium* species to some antimalaria drugs for the effectiveness of treatment of the infection. *P. falciparum* is the species of choice because it is more prevalent in Nigeria than the other species; secondly, it can be effectively cultivated in vitro [5]. The aim of this work therefore, is to determine the susceptibility of *P. falciparum* to Artesunate, Amalar, and Chloroquine using two different concentrations of these drugs.

MATERIALS AND METHODS

Collection of samples

Three milliliters (3mls) of blood was collected from five asymptomatic patients and was placed in bottles containing EDTA (Ethylene Diamine Tetra Acetic acid) anticoagulant reagents. The patients were the students of Anambra State University, Uli with age range between 18 and 26 years. They had no history of antimalarial drug intake 2 weeks before the collection of the venous blood samples as this might affect the results. The study was carried out in the month of July when there is increase in the number of mosquito bites leading to increased parasiremia.

Identification of parasite species

The parasite species were identified by preparing thick and thin blood films on microscope slides using the Giemsa staining techniques as was described by [6].

For thick films, two drops of blood were dropped at the center of a clean grease-free microscope slide to cover an area of 15mm in diameter. The smears were allowed to air-dry after which they were flooded with 120% Giemsa stain. They were allowed to stay for 10 minutes before the stains were washed off with water. The thick films were used to detect the presence of malaria parasites in the blood samples. Parasite species, morphology and parasitemia were also assessed by the microscopic examination of the thin films which were also done with the 10% Giemsa stain. The microscopic examination of the thin films was done using the oil immersion objective lens (100x objective).

The following are the microscopic features of *P.falciparum* stages or forms which are used for its identification as was described by [7] and [6]

(1) **Trophozoites:** These consist of small rings and occasionally larger rings in heavy infections. They often have double chromatin dots and may lie on red blood cell membranes (i.e. accole forms).
(2) **Schizonts:** These are only seen occasionally in severe infections and they consist of 2 or 4 merozoites. They may be pigmented.
(3) **Gametocytes:** are banana shaped and rounded forms may be seen if films dry slowly.

For the preparation of thin films, a drop of blood placed on the glass slides and was spread along the edge of the cover slip which served as a spreader. The films were allowed to air-dry and stained with 10% Giemsa stain, was allowed to stay for 10 minutes before it was washed off with water. The thin films were used for more detailed morphological analysis, species differentiation and the determination of parasite density.

Counting parasite numbers

The number of parasites present in each of the samples were estimated from the thin films prepared using the Giemsa staining technique. The slides were mounted on the microscope stage and each of the samples was viewed through ten high power fields. The total number of parasites in the ten high power fields (HPF) were counted and divided by the average number of parasites in that sample of blood.

$$\text{Average number of parasites in sample} = \frac{\text{Total number of parasites in the ten HPF}}{\text{Number of fields viewed (ten)}}$$

Estimation of Parasite Density

The parasite density in ach of the sample was also determined by multiplying the average number of parasites in the sample by a factor of 500. The factor of 500 was proposed by [8] and [9].

Antimalarial Drug Susceptibility Assay

The following drugs were assessed for their in vitro antimicrobial actions on the *Plasmodium* species isolated. They are artesunate (dihydroartemisinin 12-α-succinate), amalar (sulfadoxine and pyrimethamine) and chloroquine. These drugs were purchased from the shelf (pharmacy store). The names of the manufacturing companies were withheld. Stock solutions of the antimalarial drugs were prepared. The drugs were made to be of equal concentrations of 100mg/ml by dissolving in the appropriate amount of distilled water. 50mg (1 tablet) of artesunate was dissolved in 0.5ml of the distilled water, 250mg of chloroquine was dissolved n 2.5ml of the distilled water while 525mg of amalar as dissolved in 5.25ml of distilled water. They were allowed to dissolve and the suspensions were shaken thoroughly to get homogenous solutions.

Two-fold serial dilutions of each of the drug suspension were prepared by adding 1ml of the suspension into 1ml of distilled water in the first test tube, from the first tube, 1ml of the solution was taken and added into the second tube already containing 1ml of distilled water. This is done to the fifth tube for all the drugs.

The method used for the in vitro susceptibility test was the schizont maturation inhibition technique. *Plasmodium falciparum* infected blood samples were centrifuged at 2000 revolutions per minute (2000 rpm) for five minutes. After centrifugation, the plasma and buffy coats (leukocyte interface) were discarded and 0.2ml of the packed red blood cells was dispensed into thirteen test tubes for each of the samples. The tubes contained different concentrations of the drugs in duplicates. A control (tube without drugs) was set up for each of the samples. 1ml of the physiological saline prepared was dispensed into each of the tubes. This was done because the physiological saline contains the same concentration of solutes as the body fluid. After addition, lids were placed over the tubes and the tubes were shaken gently to dissolve the drugs. The samples were incubated at 37^0C for 42 hours. At the end of incubation, thick and thin blood films were prepared from the samples in each well (tube) including those of the controls. The number of schizonts or gametocytes in the control tubes was compared with that in the other tubes containing different concentrations of the antimalarial drugs.

RESULTS AND DISCUSSION

Table 1: The number of parasites observed in the samples

Sample	Total number of parasites	Number of fields viewed	Average number of parasites in the sample
A	120	10	12
B	132	10	13
C	116	10	12
D	25	10	3
E	47	10	5

This is the result of parasite number estimation

The number of parasites observed in each of the samples are shown in the table below.

Table 2: The parasite density of each sample

Sample	Average number of parasites per HPF	Multiplication factor	Parasite density
A	12	500	6000
B	13	500	6500
C	12	500	6000
D	3		1500
E	5	500	2500

This is the result of parasite density estimation.

All the parasites seen sere still at their trophozoite or ring stages with their characteristic double chromatin dots.

Table 3: The number of schizonts found in the control samples

Control Sample	Number of schizonts counted
A	12
B	13
C	12
D	3
E	5

This is the number of Schizonts in the control samples

The tables below show the number of schizonts found in the different concentrations of the anitmalarial drugs after the in- vitro susceptibility testing.

Table 4: The **number of schizonts found in the first dilution of the antimalarial drugs**

Sample	Chloroquine	Amalar	Artesunate
A	5	2	1
B	3	2	None
C	3	3	1
D	None	None	None
E	1	None	None

This is the result of Schizonts number found in the 50mg/ml of the Drugs

Table 5: The number of schizonts found in the second dilutions (25mg/ml)

Sample	Chloroquine	Amalar	Artesunate
A	7	5	1
B	6	5	3
C	4	4	2
D	1	None	None
E	2	1	None

This is the result of Schizont number in 25mg/ml of the drugs

For the matured forms of the parasites, both the schizonts and the gametocytes were counted since the schizont stage is not easily seen in peripheral blood except in heavy parasitemia levels.

The thick and thin blood films prepared and viewed through the microscope revealed that *P. falciparum* has high prevalence in this part. The number of *P. falciparuim* observed in each blood sample are shown in Table 1. Shows that the parasite has a high prevalence in this part of the Country more than the other species of *Plasmodium* that infect humans. This agree with the findings of [10] who studied the prevalence of malaria parasitemia among blood donors in Owerri, Imo State Nigeria. *P. falciparum* is indeed the prevalent parasite in the area because the method by which it was isolated, [6] is not specific for it alone but also reveals all types of the parasites that may be inn the blood sample. The sampling and the examination for plasmodium spp was done in the month of July, when these is increase in the number of mosquito bites leading to increased parasitemia. At the time of this sampling too, the students were asymptomatic yet the results indicated presence of the parasites in the magnitude observed. This indicate that *P. falciparum* in endemic in this part of the Country. The parasite densities in each of the blood samples are presented on Table 2. This shows the seriousness of malaria activity in the people of this country and requires a very drastic approach towards eradicating the parasite. The parasites in the control tubes. Table 3 which were suspended in the physiological saline all naturalized from the ring or trophozoite forms to the schizonts or gametocyte forms. This indicates that the physiological salime is a good medium for the growth in vitro of the parasites. Expectedly too, careless exposure of aquom materials in homes would constitute favourable habitat for mosquitoes leading to infection subsequently.

The susceptibility testing using Artesunate, Amalar and Chloroquine are presented on Table 4 and 5. They show that the three drugs had antimalaric or inhibitory effect on the parasites. However, Artesunate is shown to the most effective followed by Amalar and lastly chloroquine. The result in Table 4 is a presentation of the effect of 50mg each of the drugs per ml of distilled water. Artesunate completely inhibited the parasite in sample B, D, and E. their average number in these samples were 13, 3, and 5 for samples B, D, and E respectively. This shows that Artesunate is very effective. The result on Table 5 shows that the Artesunate at 25mg per ml of distilled water inhibited all the parasites in samples D and E while it reduced the parasites in sample B to 3. This shows that increased concentration of the drug is necessary for desired positive effectiveness in the treatment of the infection. It implies that the drugs inhibited the maturation of the trophotozoite to the schizont stage. Since in the control Table 3, almost all the initial trophozoites grew to the schizonts and gametocytes. The least inhibitory effect of chloroquine on the parasites could be associated with the development of resistance by the parasite. This is in agreement with Katzuna, 2007 who pointed out that the parasite have developed some mechanisms on their membranes which help them to extrude the chloroquine molecules out of their cell surface.

CONCLUSION

In conclusion, all drugs studied, Artesunate, Amalar and Chloroquine have inhibitory effect on *P. falciparum.* Effectiveness of the drugs improved with higher concentrations of the drugs. Artesunate, showed best inhibition followed by Amarlar and the least was Chloroquine.

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