

## Original Research Article

## Evaluation of the Efficacy of Artemether/Lumefantrine/Doxycycline Combination against *Plasmodium berghei* in Mice

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**Abstract: Background:** Malaria is common in regions like sub-Saharan Africa and South East Asia, where it results in millions of mortalities per year. Resistance to currently available antimalarials further compounds the problem. Drug combination has been demonstrated to be effective in overcoming resistance to antimalarials. This study aimed to assess the efficacy of the combination of Artemether/Lumefantrine (AL) and Doxycycline (DX) against *Plasmodium berghei* infection in mice. **Methods:** Thirty Adult Swiss albino mice were inoculated with the chloroquine (CQ)-sensitive *P. berghei* NK65 strain. The curative test involved oral administration of any of DX, AL, AL/DX, or Chloroquine (CQ) to mice with established infections, while the suppressive test was performed by treating mice shortly after parasite inoculation. Parasitemia levels were monitored using thin smears stained with Giemsa solution. Mean Survival Time (MST) was determined for each group. **Results:** In the curative test, DX, AL, and AL/DX exhibited parasitemia inhibitions of 81.4%, 94.5%, and 93.2%, respectively, while CQ achieved 93.5% inhibition. MST was significantly extended by DX, AL, and AL/DX treatments compared to negative control (NC). In the suppressive test, parasitemia inhibitions were 79.9%, 96.1%, 95.5%, and 94.9% for DX, AL, AL/DX, and CQ, respectively. The administration of DX, AL, and AL/DX also significantly prolonged MST compared to NC. **Conclusion:** The study demonstrates that the drug combination, particularly AL/DX, exhibits significant antimalarial effects in both curative and suppressive tests. The combination treatments led to substantial reductions in parasitemia levels and extended mean survival time, indicating their potential as effective antimalarial strategies. These findings highlight the promising role of drug combinations in addressing malaria infections and suggest avenues for further investigation and development of combination therapies.

**Keywords:** Artemether, lumefantrine, doxycycline, *Plasmodium berghei*, Malaria, Parasitemia.

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## INTRODUCTION

Malaria remains a major public health concern, causing substantial morbidity and mortality worldwide, particularly in low and middle-income countries, especially in sub-Saharan Africa, where vulnerable communities bear the brunt of its devastating impact [1, 2]. Despite significant efforts and progress in malaria control and elimination, the emergence and spread of drug-resistant malaria parasites have become a formidable challenge [1-4]. In response to this threat, the World Health Organization (WHO) has recommended the use of artemisinin-based combination

therapies (ACTs) as the first-line treatment for uncomplicated malaria [1, 2, 5, 6]. WHO currently recommends six different ACTs for uncomplicated *Plasmodium falciparum* malaria [2, 5]. Although there have been reports of emerging parasite resistance to artemisinin, ACTs remain the best available treatment for confirmed malaria cases [2, 4]. Vigorous measures are needed to sustain the efficacy of ACTs and combat antimalarial drug resistance [2, 4, 6]. Artemether/Lumefantrine (AL) has emerged as one of the most effective and widely used ACTs for the treatment of uncomplicated malaria. Artemether, a derivative of artemisinin, rapidly reduces the parasite

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biomass, while lumefantrine, a longer-acting partner drug, clears the remaining parasites and prevents recrudescence [7]. The combination therapy has demonstrated high efficacy and an acceptable safety profile in clinical trials [8]. However, the development and spread of resistance to artemether and lumefantrine in some regions underscore the need for continuous surveillance and the exploration of novel treatment strategies. Doxycycline, a tetracycline antibiotic, has shown promise as an adjunct therapy in combination with ACTs for the treatment of malaria [9]. Beyond its antibacterial properties, doxycycline has been demonstrated to have antimalarial activity against the asexual blood stages of *Plasmodium* parasites [10]. The drug interferes with protein synthesis within the parasite, leading to its death.

Notably, doxycycline exhibits an additional advantage of inhibiting the development of *Plasmodium* gametocytes, the sexual stages of the parasite that are responsible for malaria transmission back to mosquitoes [11]. Studies investigating the potential benefits of combining doxycycline with ACTs have reported promising results. Co-administration of doxycycline alongside AL has shown improved parasite clearance and reduced gametocyte carriage compared to AL alone [12]. Additionally, this combination has been suggested to delay the emergence of drug resistance, making it a potentially valuable tool in malaria control efforts [13]. To evaluate the efficacy and safety of the Artemether/Lumefantrine/Doxycycline combination, preclinical studies using animal models are essential. Murine models, particularly *Plasmodium berghei*-infected mice, have proven to be valuable tools in assessing the potential of new antimalarial therapies before advancing to human clinical trials [14]. *P. berghei* shares important genetic and biological similarities with human malaria parasites, allowing researchers to study the pathogenesis of malaria and the potential effects of novel therapeutic interventions [15]. Given the promising preclinical evidence and the need to address the ongoing challenge of drug-resistant malaria, investigating the Artemether/Lumefantrine/Doxycycline combination in a murine model infected with *P. berghei* is of utmost importance. This study aims to evaluate the efficacy of this combination regimen. Should the combination demonstrate efficacy and safety in this preclinical setting, it holds the

potential to be a valuable addition to the armamentarium of antimalarial treatments, aiding in the global efforts to combat malaria and reduce its burden on vulnerable populations.

## MATERIALS AND METHODS

### Drugs and Dose Selection

Artemether/Lumefantrine (AL) (manufactured by IPAC Laboratory, India), Chloroquine (CQ) (manufactured by Evans Medical Nigeria Plc), and Doxycycline (DX) (manufactured by Ranbaxy Laboratories Ltd, India) were administered in the following doses: AL at 2.3/13.7 mg/kg based on Georgewill *et al.* [16] study, CQ at 10mg/kg based on Somsak *et al.* [17] study, and DX at 2.2 mg/kg based on Gaillard *et al.* [18] study.

### Animals

The experimental animals consisted of 30 adult Swiss albino mice weighing between 20-30 g. These mice were sourced from the animal unit of the Department of Pharmacology, Faculty of Basic Clinical Sciences, College of Health Sciences, University of Port Harcourt, Rivers State. Throughout the study, the mice were provided with proper housing and adequate nutrition in accordance with recommended standards. A period of two weeks was allowed for the mice to acclimatize to the laboratory environment before the commencement of the study. All handling and care of the mice adhered strictly to the guidelines outlined in the international animal care and welfare guidelines [19].

### Parasites Inoculation

For the induction of malaria in the experimental mice, the CQ-sensitive *Plasmodium berghei* (*P. berghei*) NK65 strain was utilized. The *P. berghei* parasites were obtained from the Malaria Research Laboratory at the Centre for Malaria Research and Phytomedicine, University of Port Harcourt, Rivers State, Nigeria. To maintain the parasite population, previously infected mice served as donor mice, and the parasites were sustained through continuous intraperitoneal passage of blood from the donor mice to uninfected mice every 4 days, as described by Adzu and Haruna [20]. The percentage parasitemia was determined using the following formula:

$$\% \text{ Parasitemia} = \frac{\text{Number of parasitized red blood cells (RBCs)}}{\text{Total number of RBCs count}} \times 100$$

### Protocol for Antiplasmodial Test

#### Protocol for curative test

The standard method of Ryley and Peter [21] was followed to evaluate curative activity. Standard inoculum of  $1 \times 10^7$  *P. berghei* parasitized red blood cells was injected intraperitoneally into mice on the first

day. After 72 hours (3 days), mice were divided into five groups of six mice in each group (30 mice in total) and treated by oral administration as follows; Group A1 (Normal control) was treated with 0.2ml of normal saline daily for 5 days. Group A2 (Positive control) was treated with 10mg/Kg of Chloroquine (CQ) daily for 5

days, Groups A3-A5 were treated with 2.2mg/kg of Doxycycline (DX), Artemether/Lumefantrine (AL) (2.3/13.7 mg/kg), and AL/DX (2.3/13.7mg/kg / 2.2mg/kg) daily for 5 days respectively. Thin smears were prepared with blood from the tail of animals and stained with 10% Giemsa solution of each mouse for 5

days, to monitor the parasitemia level in each group. The stained slides were examined microscopically with an oil immersion objective of 100× magnification power. The percentage parasitemia and inhibitions were calculated using the formula shown below;

$$\% \text{ Inhibition} = \frac{\% \text{ Parasitemia of negative control} - \% \text{ Parasitemia of treated group}}{\% \text{ Parasitemia of negative control}} \times 100$$

Mean survival time for each group was determined arithmetically by finding the average survival time (days) of mice (post-inoculation) over a period of 30 days (D0–D29).

**Protocol for suppressive test**

The suppressive test was performed as described by Knight and Peters [22]. Thirty adult Swiss albino mice were inoculated with blood containing  $1 \times 10^7$  *P. berghei* and randomized into 5 groups (B1-B5) of 6 mice each. The mice were treated after 3 hours of inoculation as follows: Group B1 (Negative control) and group B2 (Positive control) were orally treated daily with normal saline (0.2mL) and CQ (10mg/kg), for 4 days, respectively. Groups B3-B5 were orally

treated with DX (2.2mg/kg), AL (2.3/13.7 mg/kg), and AL/DX (2.3/13.7mg/kg / 2.2mg/kg) daily for 4 days respectively. On day 5, tail blood samples were collected from the mice and thin smears were prepared on slides and stained with 10% Giemsa stain. The stained slides were examined microscopically with an oil immersion objective of 100x magnification power. The percentage parasitemia and inhibitions were calculated as explained above.

**Determination of Mean Survival Time (MST)**

From the time of inoculation with *P. berghei* until death, mortality of each mouse was monitored and recorded. Mean survival time (MST) was determined using the formula below.

$$MST = \frac{\text{Sum of survival time of all mice in a group Days}}{\text{Total number of mice in that group}}$$

**Statistical Analysis**

Data was analyzed using Statistical Package for Social Science (IBM SPSS, version 24). Values were expressed as mean ± SEM (Standard error of mean) of n=6. Values were analyzed using one-way ANOVA, followed by Tukey’s post hoc test. P values less than 0.05, 0.01 and 0.001 were considered significant.

**RESULTS**

**Curative Antiplasmodial Test**

Treatment with DX, AL, or AL/DX led to significant reductions in parasitemia levels compared to the negative control (NC) with varying levels of statistical significance (p<0.05, p<0.01, and p<0.001), as indicated in Table 1. Specifically, DX, AL, and AL/DX demonstrated parasitemia inhibitions of 81.4%, 94.5%, and 93.2%, respectively, whereas the standard drug CQ achieved a 93.5% inhibition. Additionally, MST was significantly extended in mice treated with DX, AL, and AL/DX, as compared to NC, with corresponding levels of significance (p<0.05, p<0.01, and p<0.001), as outlined in Table 1.

**Tables 1: Curative antiplasmodial effect of artemether-lumefantrine-doxycycline on *Plasmodium berghei* infected mice**

Group	Parasitemia (%)	Inhibition (%)	MST (Day)
NC	32.72±1.69 <sup>c</sup>	0.0%	8.67±1.33 <sup>a</sup>
CQ	2.12±0.24 <sup>a</sup>	93.5%	23.45±0.98 <sup>d</sup>
DX	5.81±0.46 <sup>b</sup>	81.4%	16.78±2.32 <sup>b</sup>
AL	1.80±0.40 <sup>a</sup>	94.5%	28.16±1.09 <sup>e</sup>
AL/DX	2.23±0.14 <sup>a</sup>	93.2%	26.00±1.45 <sup>c</sup>

NC: Negative Control, CQ: Chloroquine, DX: Doxycycline, AL/DX: Artemether-Lumefantrine/Doxycycline. Values are expressed as Mean ± SEM, n: 5, a p<0.05 when compared to NC, b p<0.01 when compared to NC, c p<0.001 when compared to NC, d p<0.001 when compared to CQ, e p<0.05 when compared to CQ.

### Suppressive Antiplasmodial Test

The treatment with DX, AL, and AL/DX resulted in significant reductions in percentage parasitemia levels, with respective levels of statistical significance ( $p < 0.5$ ,  $p < 0.01$ , and  $p < 0.001$ ), when compared to the negative control (NC), as presented in Table 2. These treatments yielded parasitemia

inhibitions of 79.9%, 96.1%, 95.5%, and 94.9% for DX, AL, AL/DX, and CQ, respectively, as outlined in the same table. Furthermore, the administration of DX, AL, and AL/DX substantially extended mean survival time (MST) with levels of significance ( $p < 0.5$ ,  $p < 0.01$ , and  $p < 0.001$ ) relative to NC, also depicted in Table 2.

**Tables 2: Suppressive antiplasmodial effect of Artemether-lumefantrine-doxycycline on *Plasmodium berghei* infected mice**

Group	Parasitemia (%)	Inhibition (%)	MST (Day)
NC	19.19±1.60 <sup>c</sup>	0.0%	22.03±1.89 <sup>a</sup>
CQ	0.98±0.15 <sup>a</sup>	94.9%	34.28±2.03 <sup>c</sup>
DX	3.85±0.40 <sup>b</sup>	79.9%	27.71±2.43 <sup>b</sup>
AL	0.75±0.31 <sup>a</sup>	96.1%	38.66±2.00 <sup>c</sup>
AL/DX	0.86±0.11 <sup>a</sup>	95.5%	29.53±3.45 <sup>b</sup>

Values are expressed as Mean ± SEM, n: 5, NC: Negative Control, CQ: Chloroquine, DX: Doxycycline, AL/DX: Artemether-Lumefantrine/Doxycycline, a  $p < 0.05$  when compared to NC, b  $p < 0.01$  when compared to NC, c  $p < 0.001$  when compared to NC, d  $p < 0.001$  when compared to CQ, e  $p < 0.05$  when compared to CQ.

## DISCUSSION

Malaria remains a formidable health challenge in developing regions spanning sub-Saharan Africa and South East Asia. The escalating prevalence of *Plasmodium* species' resistance to numerous antimalarial drugs, coupled with the mounting resistance of mosquitoes to insecticides, and the persistent absence of viable vaccines, collectively amplify the intricate nature of the ongoing fight against malaria [23, 24]. Consequently, an imperative arises to urgently uncover alternative medications showcasing novel mechanisms or to amalgamate existing antimalarial drugs. This pursuit aims to surmount the prevailing challenges in the field. The current investigation aimed to explore the potential enhancement of antimalarial efficacy through the combination of AL/DX, by evaluating its impact in *P. berghei*-infected mice. The study adopted an in vivo model to account for potential prodrug dynamics and the intricate role of the immune system in combatting malaria infection. The choice of *P. berghei* was fitting, as it has been employed in antiplasmodial research to anticipate experimental treatment responses, rendering it a suitable candidate for this study [25]. The study employed both suppressive and curative tests to comprehensively evaluate the efficacy of the drug candidate. The suppressive test gauges the impact of the drug on early-stage infections, while the curative test assesses its effectiveness against established infections. This dual approach offers a comprehensive understanding of the drug's potential activity across different infection stages [26, 27].

In the present study, in the curative test, treatment with DX, AL, and AL/DX resulted in significant reductions in parasitemia levels compared to

the negative control (NC), with varying degrees of statistical significance ( $p < 0.05$ ,  $p < 0.01$ , and  $p < 0.001$ ). Specifically, DX, AL, and AL/DX exhibited substantial parasitemia inhibitions of 81.4%, 94.5%, and 93.2%, respectively, while CQ achieved a 93.5% inhibition. Moreover, administration of DX, AL, and AL/DX significantly extended mean survival time (MST) compared to NC, with corresponding levels of significance ( $p < 0.05$ ,  $p < 0.01$ , and  $p < 0.001$ ). In the suppressive test, treatment with DX, AL, and AL/DX led to significant reductions in percentage parasitemia levels relative to the negative control (NC), with respective levels of statistical significance ( $p < 0.5$ ,  $p < 0.01$ , and  $p < 0.001$ ). Parasitemia inhibitions of 79.9%, 96.1%, 95.5%, and 94.9% were achieved by DX, AL, AL/DX, and CQ, respectively. Furthermore, the administration of DX, AL, and AL/DX significantly extended mean survival time (MST) compared to NC, with varying levels of significance ( $p < 0.5$ ,  $p < 0.01$ , and  $p < 0.001$ ). The prolongation of MST by AL/DX was best when compared to individual doses of DX, AL, and CQ. The observed antimalarial effects of the drug combination, particularly the AL/DX treatment, could potentially be attributed to multiple mechanisms of action. While the specific interactions between these drugs and the *Plasmodium* parasite would require further detailed investigation, several plausible mechanisms can be considered [28, 29]. AL/DX combination might harness synergistic effects between AL and DX, resulting in a more potent and effective antimalarial response compared to either drug used alone [30, 31]. This synergy could potentially enhance their individual antimalarial properties, offering a combined attack on different stages of the *Plasmodium* life cycle [32, 33]. While AL primarily targets the erythrocytic stages, DX's broader range of action could



involve intracellular forms in the liver and asexual stages in red blood cells [34, 35].

DX, as a tetracycline derivative, is recognized for inhibiting protein synthesis in bacteria. Although the antiplasmodial mode of action of DX is not clear, but studies have suggested the inhibition of mitochondrial protein, nucleotides and deoxynucleotides syntheses in *Plasmodium* [36, 37]. Hence, its exact mode of action might differ in *Plasmodium* and the possibility of DX disrupting essential protein synthesis processes in the parasite remains. Moreover, DX's immunomodulatory effects could enhance the host's immune response, contributing to the antimalarial effect [38, 39].

The combination might impact the bioavailability of AL when used alongside DX, potentially leading to higher concentrations of active components in the bloodstream [40, 41]. This increased bioavailability could enhance the antimalarial response. Additionally, the combination could perturb crucial metabolic pathways within the parasite, influencing processes such as heme detoxification or the electron transport chain [42, 43]. Additionally, DX could play a role in improving the uptake of AL into infected cells or the parasite itself, amplifying drug exposure and effectiveness [44, 45]. Moreover, the combined effects of AL and DX targeting distinct aspects of the parasite's survival mechanisms could deter the development of resistance. This is due to the increased challenge the parasite would face in simultaneously countering two different drug actions [46,47]. The observed antimalarial effects of the AL/DX combination likely stem from a combination of factors, including potential synergistic actions, multistage targeting, disruptions in key metabolic pathways, altered immune responses, and enhanced drug uptake [48,49]. It is important to emphasize that the precise interactions between AL and DX within the *Plasmodium* infection context require further investigation. This discussion underscores the complexity of the mechanisms involved and highlights the need for more in-depth molecular and biochemical studies to unravel the intricate interactions driving this promising antimalarial effect.

## CONCLUSION

The findings from this study indicate that the combination treatments, particularly AL/DX, show promising potential as effective antimalarial strategies. Both the curative and suppressive antimalarial tests demonstrated significant reductions in parasitemia levels when treated with DX, AL, and AL/DX compared to the negative control. This suggests that these treatments have the capacity to inhibit parasite growth and multiplication, essential factors in combating malaria infections. Additionally, the observed extension of mean survival time (MST)

further underscores the potential therapeutic impact of these treatments. The substantial parasitemia inhibition rates achieved by DX, AL, and AL/DX, comparable to the standard drug CQ, suggest that these treatments could be valuable alternatives for managing malaria infections. The findings offer insight into the potential of drug combinations to enhance antimalarial efficacy and potentially overcome issues of drug resistance that have emerged with monotherapy. Overall, these results hold promise for further exploration and development of combination therapies for more effective malaria management.

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