# EFFECTS OF ALUMINIUM - MAGNESIUM SILICATE ON THE ANTIPLASMODIAL ACTIVITY OF CHLOROQUINE PHOSPHATE IN MICE.

**BY**

**ELENDU – ELEKE NNENNA PATRICIA**

**REG. NO: PG/M.SC/09/51600**

**DEPARTMENT OF VETERINARY MEDICINE**

**UNIVERSITY OF NIGERIA, NSUKKA.**

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**SUPERVISORS:**

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| **Dr. M.C.O. EZEIBE** | **PROF. C. C. CHUKWU.** |

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# TITLE PAGE

# EFFECTS OF ALUMINIUM MAGNESIUM SILICATE ON THE ANTIPLASMODIAL ACTIVITIES OF CHLOROQUINE PHOSPHATE IN MICE.

# DEDICATION

To my love, Innocent Elendu Eleke, you win my heart each day, and to Nomsy- my special post-graduate studies gift.

## APPROVAL/CERTIFICATION

This is to certify that this work was carried out by Elendu-Eleke, Nnenna Patricia in the Department of Veterinary Medicine, University of Nigeria, Nsukka.

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| _____ | _____ |
| Prof. M.C.O. Ezeibe | Prof. C.C. Chukwu |
| **(Supervisor)** | **(Supervisor)** |
| _____ | _____ |
| Dr. (Mrs.) N.E. Nweze | |
| **(Head of Department)** | **External Examiner.** |

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# ABSTRACT

Effect of stabilizing chloroquine phosphate in a synthetic Aluminium Magnesium silicate (*Nanoparticles*) on antiplasmodial activity of the drug was investigated. Mice infected with *Plasmodium berghei* were treated with chloroquine phosphate at 7 mg/kg, 5 mg/kg and 3 mg/kg dose levels respectively. Two subgroups at each dose level were treated with chloroquine phosphate alone and with a drug formulation of 20% chloroquine phosphate in the synthetic Aluminium Magnesium Silicate (AMS) as an adjuvant. Percentage *Plasmodium berghei* parasitaemia (parasite/ml), Red blood cell (Rbc) count, Heamoglobin concentration (Hb), Rectal temperature (°C) and Body weight of mice in each of the treated groups and of the control were determined and compared. Mean parasitaemia, 4.15± 0.26 of the group treated with 7mg/kg chloroquine phosphate alone was higher ($P<0.05$) than the 3.60 ±0.22 of the control. At 5mg/kg and at 3mg/kg dose levels, the AMS significantly ($p<0.05$) improved ability of chloroquine phosphate to reduce plasmodial parasitaemia from 2.46 ± 0.21 to 1.57± 0.25, and from, 3.82 ± 0.06 to 2.12±0.08 respectively. Mean Hb, 12.95 ±0.25, 12.25 ± 0.27 and 12.68 ±0.18 of the groups treated with 7mg/kg chloroquine phosphate alone, 5mg/kg chloroquine phosphate alone and 5mg/kg chloroquine phosphate in AMS respectively, were significantly ($P<0.05$) higher than 10.43± 2.64 of the only surviving mouse treated with 7mg/kg chloroquine phosphate in AMS and the mean, 10.18 ± 3.00 got in the group treated with 3mg/kg chloroquine phosphate alone.

Mean Red blood cell counts ($\times 10^6$/µl): 9.52 ±2.81, 9.29 ±4.01 and 9.52 ±5.32 of the groups treated with 7mg/kg chloroquine phosphate alone, 5mg/kg chloroquine phosphate in AMS and 3mg/kg chloroquine phosphate in AMS respectively, were also significantly ($P<0.05$) higher than 8.90 ±5.72 of the group treated with 5mg/kg chloroquine phosphate alone and 8.60 of the mouse treated with 7mg/kg chloroquine phosphate in AMS. Least RBC count, 6.34 ± 18.02 was obtained in the group treated with 3mg/kg chloroquine phosphate alone. Mean rectal temperature of 35.56 ±0.82$^0$C and 35.73± 0.38$^0$C of the groups treated with 5mg/kg chloroquine phosphate alone and 5mg/kg chloroquine phosphate in AMS respectively, were significantly ($P<0.05$) lower than 37.92 ± 0.38°C of the mice treated with 7mg/kg chloroquine phosphate in AMS and 36.84± 0.32$^0$C, 36.84 ±0.32$^0$C and 36.16 ± 0.35$^0$C obtained in the groups treated with 7mg/kg chloroquine phosphate alone, 3mg/kg chloroquine phosphate in AMS and in the control respectively. Mean body weight, 32.66± 2.10kg of the group treated with 5mg/kg chloroquine phosphate in AMS was significantly ($P<0.05$) higher than 29.29 ± 0.51kg, 29.17kg and 29.06± 1.95kg obtained in the groups treated with 7mg/kg chloroquine phosphate alone, 7mg/kg chloroquine phosphate in AMS and 5mg/kg chloroquine phosphate alone respectively. It was also significantly ($P<0.05$) higher than 26.65± 0.83kg and 26.35± 0.61kg of the groups treated with 3mg/kg chloroquine phosphate alone and 3mg/kg chloroquine phosphate in AMS respectively. At 7 mg/kg there was mortality with chloroquine phosphate alone (20%) and with the chloroquine phosphate in AMS drug (80%).

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# CHAPTER ONE

## 1.0 INTRODUCTION

## 1.1 MALARIA

Malaria is a mosquito borne infectious disease caused, by a eukaryotic protozoan of the genus *Plasmodium.* Malaria has infected humans for over 50,000 years and *Plasmodium* may have been a human pathogen for the entire history of the species (Joy *et al*, 2003).Close relatives of the human malaria parasites remain common in chimpanzees (Escalante *et al*, 1998).References to the unique periodic fevers of malaria are found throughout recorded history, beginning in 2700BC in China (Cox, 2002).Malaria was so pervasive in Rome that it was known as the ñRoman feverò (Sallares, 2003).The term malaria originated from medieval Italian word: *mala aria* (bad air). The disease was formerly called *ague* or marsh fever due to its association with swamps and marshlands. Other terms used to describe the disease were the shakes, March fever, jungle fever, intermittent fever and chills (Smyth, 1996).

Malaria is widespread in tropical and sub tropical regions. It is presently endemic around the equator, including parts of America, Asia and Africa. However, it is in sub Saharan Africa that 85-90% of malaria fatalities occur (Layne, 2007).Each year, there are approximately between 350 and 500 million cases of malaria, killing between one and three million people in sub Saharan Africa (Snow *et al*, 2005). Majority of the fatalities is among young children under five years of age and pregnant women. Hudson Economic Development Corporation, HEDC (2010) report showed that ninety percent of malaria related deaths occur in sub Saharan Africa and most of the imported infections are acquired from tropical Africa. Malaria cases in Africa account for approximately 90% of the disease in the world (World Health Organization, 1996).Precise

statistics of malaria are unknown, because, many cases occur in rural areas where people do not have access to hospitals or the means of health care. As a consequence, majority of cases are undocumented (Breman, 2001).

Malaria is naturally transmitted by the bite of female *Anopheles* mosquito (Bachou *et al*, 2006). When the mosquito bites an infected person, a small amount of blood is taken, which may contain malaria parasites. These develop within the mosquito and about 6-7 days later when the mosquito takes its next blood meal, the parasites are injected with the mosquitoôs saliva into the person being bitten. The parasites find their way into the hostôs liver. From there, they start to multiply within red blood cells, causing symptoms that include fever and headache. In severe cases, the disease worsens, leading to coma and death.

Malaria is not just a disease commonly associated with poverty but it also causes poverty and is a major hindrance to economic development. The disease has been associated with major negative economic effects on regions where it is widespread (Humphreys, 2001). Poverty is both cause and effect of malaria, since the poor do not have the financial capacities to prevent or treat the disease (Ettling *et al*, 1994). The economic impact of malaria has been estimated to be $2 billion USD every year in Africa. This includes cost of health care, working days lost due to sickness, days lost in education, decreased productivity due to brain damage from cerebral malaria, and loss of investment and tourism (Greenwood *et al*, 2005). In countries with heavy malaria burden, the disease may account for as much as 40% of public health expenditure, 30- 50% of inpatient admissions and up to 50% of outpatient visits (WHO, 2009).

The most economic and reliable diagnosis of malaria is by microscopic examination of blood films. Two sorts of blood films are traditionally used. Thin films are similar to usual blood films and allow species identification, because the parasiteôs appearance is best revealed in this preparation. Thick films allow the

observer to screen a larger volume of blood and are about eleven times more sensitive than the thin film. In effect, picking up low levels of infection is easier on the thick film, but the appearance of the parasite is much more distorted and therefore distinguishing the different species can be very difficult. It is therefore better to use both thin and thick smears while making a definitive diagnosis (Warhurst and Williams, 1996).

For areas where facilities for microscopy are not available or where laboratory staff are not experienced at malaria diagnosis, there are commercial antigen detection tests that require only a drop of blood (Pattanasin *et al*, 2003.) Immunochromatographic tests also called Malaria Rapid Diagnostic tests, Antigen-Capture Assay or ñDipsticksò have also been developed, distributed and field tested for diagnosis of malaria. These tests use finger stick or venous blood, the test taking only about 15 ï 20 minutes. The results are read visually as presence or absence of coloured strips on the dipstick (Pattarasin *et al*, 2003). The threshold of detection by these rapid diagnostic tests is in the range of a 100 parasite/µl of blood compared to about five by thick film microscopy. The disadvantage of this is that dipstick tests are qualitative and not quantitative. They can determine if parasites are present in the blood but do not indicate level of parasitaemia (McCutchan *et al*, 2008).

Molecular methods are also available in clinical laboratories for diagnosis of malaria. Rapid real time assays are also being developed for malaria. An example is the one, based on the polymerase chain reaction (PCR), QT-NASBA (Mens *et al*, 2006). PCR and other molecular methods for diagnosis of malaria are more accurate than microscopy. Their drawbacks are that they are expensive to carry out and require specialized laboratories. Moreover, levels of parasitaemia they determine do not always correlate with progression of the disease, particularly, when the parasite is able to adhere to walls of blood vessels. More sensitive, low ï

tech diagnosis tools are needed to detect low levels of parasitaemia in the field (Mens *et al*, 2006).

Methods used to prevent spread of malaria or to protect individuals in areas where the disease is endemic, include; use of prophylactic drugs, mosquito eradication and prevention of mosquito bites. The continued existence of malaria in an area requires a combination of high human population density, high mosquito population density and high rates of transmission from humans to mosquitoes and from mosquitoes to humans. When any of these is lowered sufficiently, the parasite sooner or later disappears from that area (wikipedia.org, 2010)

Efforts to eradicate malaria by eliminating mosquitoes have been successful in some areas. Draining of wetland which is breeding grounds, for mosquitoes and improved sanitation were adequate for this (www.rollbackmalaria.org, 2009). Use of the pesticide, DDT eliminated malaria from the southern parts of the USA around 1951. Before DDT, malaria was also successfully controlled by removing or poisoning the breeding grounds of mosquitoes or the aquatic habitats of their larva stage. An example was by filling or applying oil to places with standing water. These methods have seen little application in Africa for more than half a century. For this reason, efforts to eradicate malaria in many parts of the developing world have failed .The problem is most prevalent in Africa (Killeen *et al*, 2002).

Introduction of sterile insect technique is emerging as a potential mosquito control method. Progress towards transgenic or genetically modified insects suggests that wild mosquito populations could be made malaria ï resistant. Researchers at the Imperial College, London have been able to create the worldôs first transgenic malaria mosquito (Flamina *et al*, 2000), with the first plasmodium ï resistant species announced by a team at Case Western Reserve University in Ohio in 2002 (Ito *et al*, 2002). Successful replacement of current populations of

mosquitoes with a new genetically modified population relies upon a drive mechanism, such as transposable elements to allow for non ï Mendelian inheritance of the gene of interest. This approach has many difficulties and success is a distant prospect (Knols *et al*, 2002). An even more futuristic method of vector control is the use of lasers to kill flying mosquitoes (Guth, 2009).

Several drugs, most of which are also used for treatment of malaria, can be taken prophylactically. These drugs are taken daily or weekly, at a lower dose than would be used for treatment of a person who had actually contracted the disease. Quinine was used starting in the 17$^{th}$ century as a prophylactic against malaria (Kaufman and Ruveda, 2005). The first effective treatment came from the bark of cinchona tree which contains quinine (Kyle and Shampe, 1974).

Development of more effective alternatives such as quinacrine, chloroquine and primaquine in the 20$^{th}$ century reduced reliance on quinine. Quinine is still used to treat chloroquine resistant *Plasmodium falciparum* as well as severe and cerebral stages of malaria, but is no longer generally used for prophylaxis. Use of prophylactic drugs where malaria ï bearing mosquitoes are present may encourage development of partial immunity (Roestenberg, 2009).

Indoor residual spraying (IRS) is the practice of spraying insecticides on the interior walls of homes in malaria infested areas. After feeding, mosquito species rest on nearby surfaces while digesting the blood meal. So, if the walls of dwellings have been coated with insecticides, the resting mosquitoes will be killed before they can bite another victim to transmit the malaria parasite. The first pesticide used for IRS was DDT (Harrison, 1998). Although it was initially used exclusively to combat malaria, its use quickly spread to agriculture. With time, pest control, rather than disease control dominated use of DDT and its large scale use in agriculture led to evolution of resistant mosquitoes in many regions. The World Health Organization (WHO) currently advises use of 12 different insecticides in

IRS operations. These include DDT and a series of alternative insecticides such as pyrethroids, permethrin and deltamethrin to combat malaria in areas where mosquitoes are DDT resistant and to slow evolution of resistance (World Health Organization, 2006).

Mosquito nets help keep mosquitoes away from people and greatly reduce infection and transmission of malaria. The nets are often treated with an insecticide, designed to kill the mosquito before it has time to search for a way to pass through it. Insecticide ï treated nets (ITN) are estimated to be twice as effective as untreated nets and offer greater than 70% protection compared to no nets (Bachou *et al*, 2006). Although ITN have proven to be very effective against malaria, less than 2% of children in urban areas in sub Saharan Africa are protected by ITNôs. Since the *Anopheles* mosquito feeds at night, the preferred method is to hang a large ñbed netò above the centre of a bed such that it drapes down and covers the bed completely. The distribution of mosquito nets impregnated with insecticides such as permethrin or deltamethrin has shown to be an extremely effective method of malaria prevention and ITNs have been shown to be the most cost effective prevention method against malaria and are part of WHOôs Millenium Development Goals (MDGs). Researchers base their conclusions about the cost ï effectiveness of free distribution on the proven spillover benefits of increased ITN usage (William *and* Hawley, 2003). When large numbers of nets are distributed in a residential area, their chemical additives help reduce the number of mosquitoes in the environment. With fewer mosquitoes in the environment, chances of malaria infection for both recipients and non- recipients are significantly reduced.

Immunity (or more accurately, tolerance) to malaria does occur naturally, but only in response to repeated infection with multiple strains of malaria parasites (Färnert *et al*, 2009). Vaccines for malaria are under development, with no completely effective vaccine yet available. The first promising studies

demonstrating the potential for a malaria vaccine were performed in 1967 by immunizing mice with live, radiation ï attenuated sporozoites. This provided protection to about 60% of the mice upon subsequent injection with normal viable sporozoites (Nussenzweig *et al*, 1967). Since the 1970s, there has been a considerable effort to develop similar vaccination strategies in humans. Although many are under development, the challenge of producing a widely available vaccine that could provide a high level of protection against malaria for a sustained period is still to be met (Kilama and Ntoumi, 2009).

A wide variety of antimalarial drugs are available (Dondorp and Day, 2007). Treatment of malaria involves supportive measures as well as specific antimalarial drugs. Malaria parasite has developed resistance to several antimalarial drugs, most notably chloroquine (Wellems, 2002). Most mammalian malarias can be treated with chloroquine at a dosage of 7mg/kg base for 5 consecutive days (35mg/kg body weight). This can be given as an intramuscular (i.m) injection or per os via nasogastric tubes. The bitter taste of chloroquine precludes putting it in food.

New combinations to use of chloroquine are being investigated since its use in mass drug administrations may have contributed to emergence and spread of resistance, especially, by *Plasmodium falciparum* in humans (Plowe, 2005). Combinations of chloroquine with other drugs have been reported to be more effective than treatment with chloroquine alone (Uhlemann and Krishna, 2005).

Chloroquine needs to be combined with adjuvants for effective treatment of malaria. The three dimensional colloidal structure of Aluminium Magnesium Silicate (AMS) particles known as the ñhouse of cardsò has the ability to stabilize drugs (protects the drugs from destruction, Vanderbilt, 1992). This may prolong bioavailability of the drug and so may improve effect of the drug.

Also AMS is made of platelets that are only 1nm thick. So, it is made of *Nanoparticles*. *Nanoparticles* enhance delivery of drugs across blood brain barrier (Silva, 2008). Aluminium-Magnesium Silicate may in addition to prolonging bioavailability of chloroquine, enhance its delivery to targets including across the blood brain barrier. This may also lead to better treatment of cerebral malaria in humans.

## 1.2 STATEMENT OF THE PROBLEM

1. Malaria affects about 250 million people and causes approximately one million deaths annually (WHO, World Malaria Report, 2008). If the prevalence of malaria stays on its present upwards course, the death rate could be doubled in the next twenty years (Breman, 2001). Tropical and sub-tropical regions of the world- Asia, parts of America and sub-Saharan Africa are greatly at risk.

2. Use of chloroquine in mass drug administrations has led to emergence and spread of resistance, especially by *Plasmodium falciparum* in humans (Plowe, 2005).

## 1.3 AIM OF THE STUDY

To evaluate, the effect of AMS on the antiplasmodial activity of varying doses of chloroquine phosphate.

## 1.4 OBJECTIVES OF THE STUDY

1. To determine the effect of chloroquine and chloroquine in AMS respectively on level of parasitaemia in *Plasmodium berghei* infected mice.

2. To test the effect of AMS on activity of different doses of chloroquine on haematology of mice infected with *P. berghei.*

3. To test the effect of AMS on activity of different doses of chloroquine on clinical signs of malaria in mice infected with *P. berghei.*

## 1.5 HYPOTHESIS

**1.5.1 Hypothesis:** Aluminium-magnesium silicate could improve antiplasmodial activities of Chloroquine phosphate.

**1.5.2 Null hypothesis**: Aluminium Magnesium Silicate has no effect on the antiplasmodial activity of chloroquine phosphate.

## 1.6 SIGNIFICANCE OF THE STUDY

1. The findings of the study could be useful to physicians and veterinarians in management of cases of human and simian malaria.
2. The findings could help solve problem of drug resistance by malaria parasites.
3. The result could be useful in the management of cerebral malaria cases.
4. Dose of chloroquine phosphate needed to treat malaria could be reduced by the findings.
5. Toxicity of chloroquine phosphate could be reduced.

# CHAPTER TWO
# LITERATURE REVIEW

## 2.1 MALARIA

### 2.1.1 CAUSATIVE AGENTS

Causative agents of malaria are members of the genus *Plasmodium* (phylum *Apicomplexa*). In humans, malaria is caused by *P. falciparum, P.malariae, P. ovale, P. vivax,* and *P.knowlesi* (Singh *et al.,* 2004, Mueller *et al.,* 2007). *P. falciparum* is the most common cause of malaria and is responsible for about 80% of malaria cases, and for about 90% of deaths from malaria (Mendis *et al.,* 2001). Parasitic *Plasmodium* species also infect birds, reptiles, monkeys, chimpanzees and rodents (Escalante and Ayala, 1994). There have been documented human infections with simian malaria species, namely *P.knowlesi, P.inui, P.cynomolgi P.simiovale, P.schwetzi, P.brazilianum, P.simium* (Garnham, 1996). However, with exception of *P.knowlesi,* these are mostly of limited public health importance (Collins and Barnwell, 2009). Monkey malaria caused by *P.cyanomolgi* is also transmissible to man but its consequence is not yet well understood (Coatney, 1968).

There are numerous species of avian malaria parasites including *Plasmodium relictum, P.cathemerium, P.gallinaeceum, P.lophurae, P.elongatum, P.juxtanucleare* (McGhee, 1988)

Several cases of so-called amphibian ómalariaô have been reported (e.g. *P.bufonis* in *Bufo americanus*) but the validity of such infections as true malarial parasites still remains to be confirmed (Garnham, 1980).

There are about 25 known species of malaria in reptiles. These have been reported from five continents. They occur commonly in all the major families of lizards and occasionally in snakes, but not in crocodiles, turtles or tuataras. Their life cycles and general biology are poorly understood, the parasites being found in erythrocytes and sometimes in leucocytes (Ayala, 1977).

Rodents also have species of *Plasmodium* that infect them naturally. They fall into three groups. These are *P.berghei* - *P.yoelii* group, the *P.vinkei* group and the *P.chabaudi* group (Cox, 1988).

#### 2.1.1.1 Plasmodium *berghei*:

In 1943, a Belgian antimalarial team began work on the mosquitoes of certain forest districts in Katanga, Congo. The mosquito, *Anopheles dureni* which had a localized distribution (on shady trees by the Kisanga river) was found to be frequently engorged with non- human blood, and to show higher sporozoite index. This engorged blood when tested gave a positive reaction to anti ï rat serum. So blood of rodents in the district was examined for parasites. This led to isolation of a new species of plasmodium, *P.berghei*, in the blood of the thicket rat ï *Grammomys surdaster* (Vincke and Lips, 1948). This was a finding of immense significance for it has been found possible to transmit this species of malaria to a number of laboratory rodents with the result that laboratory research on malaria has been greatly facilitated.

Rodent malarial parasites ï *P.berghei* are models for study of mammalian malaria. They are analogous to malarial parasites of man and of other primates, in structure, physiology and life cycle (Carter and Diggs, 1977).

Rodent malarial parasites are models for in vivo investigation of the developmental biology of human malarial parasites, investigations of parasite ï host interactions, studies on vaccine development and in vivo testing of drugs (Carter and Diggs, 1977).

### 2.1.2 MODE OF TRANSMISSION OF MALARIA:

The malaria parasite is naturally transmitted by bite of female *Anopheles* mosquitoes. When a mosquito bites an infected person, a small amount of blood is taken which could contain malaria parasites. The parasites develop within the

mosquito within one week. When such mosquito bites a person or an animal, the parasites are injected with the mosquitoôs saliva into the host.

The malaria parasite undergoes several developmental changes, multiplying asexually and asymptomatically between two weeks and several months (occasionally years) in the hostôs liver, (wikipedia.org, 2010).

Malaria in humans develops via two phases, exoerythrocytic and erythrocytic phases. The exoerythrocytic phase involves invasion of the hepatic system, whereas the erythrocytic phase involves invasion of the erythrocytes.

In the liver, the parasites differentiate to yield thousands of merozoites, which, following rupture of their host cells, escape into the blood and infect red blood cells, thus beginning the erythrocytic stage of the life cycle (Bledsoe, 2005). The parasites escape from the liver undetected by the body immune system by wrapping itself in the cell membrane of the infected host liver cell (Sturm *et al.,* 2006). The merozoites then invade the red blood cells (Bledsoe, 2005). The asexual stages often seen in blood films are young trophozoites (the ring forms), mature trophozoites and the dividing schizonts that yield another set of merozoites for a new generation (Pasvol and Wilson, 1989, Cogswell, 2000).

It was formerly thought that the merozoites released from a schizont directly ópenetrateô the membrane of red blood cells and then develope.

However, Aikawa and Seed, (1980) have shown that the merozoites enter erythrocytes by endocytosis. This key event in the cycle of *Plasmoduim* has been extensively researched as the inhibition of this process is believed to be crucial in the development of a malaria vaccine.

The process involves recognition and attachment of the merozoites to the erythrocyte membrane and it is suspected that a specific receptor for erythrocyte entry may be involved. If on initial contact with an erythrocyte, the apical complex end of the parasite is not directed towards the blood cell surface, a re-orientation

takes place (Hementin, 1987). After re-orientation, some deformation of the erythrocyte membrane may occur and invagination and endocytosis follows.

A distinct junction is formed between the erythrocyte membrane and the merozoite surface during endocytosis and moves along the confronting membrane. When entry into the host cell is completed, the merozoite is surrounded by a parasitophorus vacuole that has originated from the erythrocyte membrane. This grows with the developing parasite and is retained until formation of the next generation of *Plasmodium* merozotes (Aikawa; 1980).

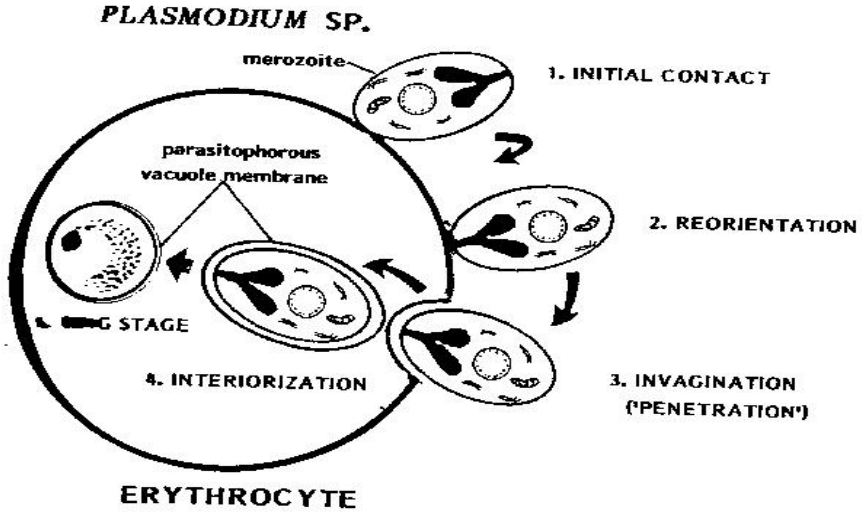



Figure 1: Sequence of events during the invasion of erythrocytes by merozites of *Plasmoduim species* (Hermentin, 1987).

As an environment, the vertebrate red blood cell has certain advantages to offer. It is thin-walled and is in constant motion with the result that absorption of food materials and elimination of waste products of metabolism are relatively easily accomplished. In addition, it contains rich supplies of protein and oxygen. These features which make RBCôs efficient as metabolizing units, also equip them to serve as good habitats for parasites such as the *Plasmodium spp* (Kreier, 1980).

### 2.1.3 CLINICAL SIGNS AND LESIONS OF MALARIA

The most pronounced changes caused by malaria involve the blood and the blood-forming system, the spleen and the liver. Secondary changes can occur in all the other major organs, depending on the type and severity of the infection. The pathological changes are more profound and severe in cases of *P.falciparum* caused malaria. Severe malaria is a complex multisystem disorder with many similarities to sepsis syndrome (Claire *et al*, 2004).

**Clumping of the Red blood cells:** Red blood cells (Rbcs), are the principal sites of infection in malaria. All the clinical manifestations of malaria are due primarily to involvement of the red blood cells. The growing parasite consumes and degrades the intracellular proteins of the Rbcs, mainly haemoglobin. The transport properties of the red cells membrane are altered, cryptic surface antigens are exposed and new parasite derived proteins are inserted. The red cell becomes more spherical and less deformable. In *P.falciparum* infection, membrane protuberances (ñknobsò) appear on the red cell surface in the second 24-hour of the asexual cycle. These knobs extrude a strain specific, adhesive variant protein of high molecular weight that mediates red cell attachment to receptors on venular and capillary endothelia, causing *cytoadherence. P. falciparum* infected red cells also adhere to uninfected red cells to form *rosettes* (Claire et al, 2004).

Although the red blood cell surface adhesive proteins called plasmodium falciparum erythrocyte membrane protein 1($P_f$EMP 1) are exposed to the immune system, they do not serve as good immune targets, because of their extreme diversity. There are at least 60 variations of the protein within a single parasite and effectively limitless versions within the parasite populations (Chen *et al,* 2000) and the parasite switches between a broad repertoire of $P_f$EMP1surface proteins, thus staying one step ahead of the pursuing immune system.

Cytoadherence and rosetting are central to pathogenesis of *P.falciparum* malaria, resulting in the formation of red cell aggregates and intravascular

sequestration of red cells in vital organs including the brain and the heart. This further interferes with the micro circulation and metabolism and allows parasite development, away from the principal host defense, splenic processing and filtration (Chen *et al,* 2000).

This ñstickinessò leading to cytoadherence and rosetting is the main factor which gives rise to haemorrhagic complications of malaria. High endothelial venules (the smallest branches of the circulatory system) can be blocked by attachment of masses of these infected red blood cells. Blockage of these vessels leads to placental and cerebral malaria. In cerebral malaria, the sequestrated red blood cells can breach the blood brain barrier leading to coma (Adams *et al,* 2002).

**Hypovolaemia** is a major feature of severe malaria and when exacerbated by anaemia and microvascular obstruction, is likely to lead to decreased delivery of oxygen to tissues, then anaerobic metabolism and lactic acidosis (Claire *et al*, 2004).

**Anaemia** is a common problem encountered in malaria and it poses special problems in pregnancy and in children. Anaemia in malaria can be due to multiple causes. Repeated hemolysis of infected red blood cells is the most important cause of reduction in haemoglobin levels. Anemia depends on the degree of parasitaemia, duration of the acute illness and the number of febrile paroxysms. It may occur even after 3-5 febrile paroxysms. In humans, *P.vivax* predominantly invades young red cells and the number of cells infected rarely exceeds 2%. *P.malariae* develops mostly in mature red cells and the parasitaemia is rarely greater than 1%. The pathogenesis of malarial anemia is complex and involves multiple processes relating to both destruction of erythrocytes and inhibition of haemopoesis (Claire *et al*, 2004). *P.falciparum* affects red cells of all ages and the parasitaemia can be as high as 20-30% or more. Massive destruction of red cells accounts for rapid development of anemia in *P.falciparum* malaria.

Non parasitized RBCs are also removed from circulation by complement ï mediated lysis and phagocytosis, resulting from immune complex deposition and complement activation (Claire *et al*, 2004).

Increased splenic clearance of parasitized as well as non-parasitized red cells, reduction of red cell survival even after disappearance of parasitaemia, dyserythropoeisis in the bone marrow, drug induced hemolysis etc, also contribute to the anemia (Claire *et al*, 2004). During *P.falciparum* infections, reticulocyte levels are inappropriately low, reflecting suppression of the normal response of erythropoietin (EPO). Some of these mechanisms may perpetuate anemia even after completion of the treatment.

Anaemia of malaria is usually normocytic, hypochromic, with increase in the number of reticulocytes and polychromatophils. Anaemia may be associated with hyperbilirubinemia, due to the haemolytic process. Splenomegally may also be seen (Claire *et al*, 2004). Leucocyte count is usually low or normal in most cases of malaria. Increased leucocyte count indicates secondary bacterial infection. Relative lymphocytosis, monocytosis, eosinopaenia, presence of stab neutrophils are observed with prolonged illness.

**Thrombocytopaenia** is also common in malaria. It has been observed that the platelet count shows moderate decline during the paroxysms of fever. Thrombocytopaenia may be related to sequestration of the platelets in the spleen. Severe thrombocytopaenia, however, indicates severe infection and may herald bleeding syndromes (Claire *et al*, 2004).

**Elevated Erythrocyte sedimentation rate (ESR):** ESR is usually elevated in malaria up to 30-50mm in one hour. Prolonged malaria, severe anaemia is usually associated with higher ESR (Claire et al, 2004).

**Splenomegaly:** The spleen plays an important role in immune response against malarial infection and splenectomy invariably activates a latent infection. Enlargement of the spleen is one of the early and constant signs of malarial

infection. Spleen may become palpable as early as the first paroxysm (Claire *et al*, 2004).

Spleen may be palpable at the early stages of infection, in the right lateral position or even in supine position. Its edge is usually round and hard to palpate and it may be tender. As the disease progresses, the spleen becomes harder, less sensitive and readily palpable. Splenomegaly is common in all types of malaria. Rapid and considerable enlargement of the spleen may sometimes result in splenic rupture, which is a serious complication of malaria (Claire et al, 2004).

**Hepatomegaly:** Enlargement of the liver also occurs early in malaria. The liver is enlarged after the first paroxysm. It is usually firm and may be tender. It is oedematous, coloured brown, grey or even black as a result of deposition of malaria pigment. Malarial hepatitis is characterized by hyperbilirubinaemia with elevation of conjugated bilirubin, increased levels of transaminases and alkaline phosphatase. Being part of the severe falciparum infection, it may be associated with renal failure, anaemia or other complications of falciparum malaria. Malaria is not a proven cause of liver cirrhosis (Claire *et al*, 2004).

**Pneumonia:** Involvement of the lungs occurs in *P.falciparum* malaria. It is secondary to changes in the red blood cells and in the microcirculation. Acute pulmonary oedema is an infrequent but nearly fatal complication of *P.falciparum* malaria, largely due to capillary endothelial lesions and perivascular oedema. Pulmonary capillaries and venules are packed with inflammatory cells and parasitized red cells. The vascular endothelium is oedematous with narrowing of the lumen. Focal or lobar pneumonia and bronchopneumonia can also complicate malaria (Claire *et al*, 2004).

**Alteration of cardiovascular system:** Malaria is commonly associated with cardiovascular function abnormalities. The most frequent changes during a paroxysm, include decrease in blood pressure, tachycardia, muffled heart sounds,

transient systolic murmur at the apex and occasional cardiac dilation. Also, there could be peripheral vasodilation, leading to postural hypotension.

In *P.falciparum malaria*, there could be microcirculatory changes in the coronary vessels. The myocardial capillaries are congested with parasitized red cells, pigment laden macrophages, lymphocytes and plasma cells. Malaria may aggravate a pre existing cardiac dysfunction and may prove fatal to patients already suffering from significant cardiac failure or valvular obstruction (Claire *et al*, 2004).

**Gastro-intestinal tract alteration:** Malaria is often accompanied by nausea and vomiting, mainly central in origin. In acute phase, patient may have anorexia, abdominal distention, and pain in the epigastrum. Sometimes, the abdominal colics may be so severe as to mimic acute abdominal inflammation or appendicitis. Some patients may have watery diarrhoea and the condition may mimic gastro-enteritis or cholera (Claire et al, 2004).

Again, acute colitis may be associated with malaria. Bacillary dysentery, amoebiasis etc may complicate malaria. In falciparum malaria, involvement of splanchnic microcirculation can lead to ischaemia of the gut, mucosal oedema, necrosis and ulceration. This may hamper absorption. These changes in the gut can further lead to absorption of toxins, precipitating septic shock (Claire *et al*, 2004).

**Kidney involvement:** Malaria can cause varied problems in the kidneys. During the acute attack, albuminuria may be seen commonly. Acute diffuse malarial nephritis with hypertension, albuminuria and oedema has also been reported (Claire *et al*, 2004).

**Central nervous system involvement:** Central nervous system manifestations in malaria could be due to pathological involvement of the brain, paroxysms of fever or due to side effects of antimalarial drugs.

The febrile paroxysms are usually accompanied by headaches, vomiting, delirium, anxiety and restlessness. These are as a rule, transient and disappear with normalization of temperature (Claire *et al*, 2004).

Antimalarial drugs, like chloroquine, quinine, mefloquine and halofantrine can also cause various symptoms like dizziness, vertigo, tinnitus, restlessness, hallucinations, confusion, delirium, or even frank psychosis, convulsions etc. Quinine can induce hypoglycemic coma. Artermisinin derivatives are known to cause brain stem dysfunction in animal studies. These factors should be kept in mind while managing cases of malaria (Cann and Verhulst, 1961).

Nervous system gets involved predominantly in *P.falciparum* malaria and only very rarely in other forms of malaria infection. Decreased deformability, increased cytoadherence and rosetting of red cells, occlusion of the microcirculation by the red cell rosettes and their thrombosis, result in cerebral anoxia, development of malaria granulomas and punctate haemorrhages leading to malarial encephalitis and meningoencephalitis (Rowe *et al,* 2009).

### 2.1.4 MALARIA AS A ZOONOSIS

Seven of the species that affect non human primates have been transmitted experimentally to humans: *P.brasilianum, P.cynomolgi, P.eylesi, P.inui, P.knowlesi, P.schwetzi,* and *P.simium* have been reported to be zoonotic. In addition, some species, such as *P.cynomolgi, P.knowlesi, P.simium* and possibly *P.eylesi,* have been found in natural or accidental infections of man (Coatney, 1968, Kreier, 1980, Collins, 1988).

Non human primates can also be infected with natural or adapted strains of the plasmodia that normally affect humans. *Aotus* and *saimiri* monkeys can be infected with *P.falciparum* or *P.vivax,* and chimpanzees can be infected with *P. ovale* (Escalante *et al,* 1998).

Infection of man with plasmodia of non human primates is considered very rare. Literature records only two confirmed human cases acquired under natural conditions from non human primates (de Arruda *et al,* 1989). One was due to *P.knowlesi* infection in Malaysia and another by *P.simium* infection in Brazil. Two other cases have been suspected but not confirmed. One,by *P.knowlesi* and the other, by *P.eyelsi,* both in Malaysia. It has also been discovered that more than 90% of adults in four tribes of northern Brazil had antibodies against P.*brasilanum* or P.*malariae* (these species cannot be differentiated using conventional serology), although the incidence of malaria was very low and parasitaemia was below 0.02% (de Arruda *et al,* 1989). Presence of *P.brasilianum* was confirmed in numerous monkeys and in *Anopheles darlingi* mosquitoes (Deane, 1992) in the area. This finding suggests that *P. brasilianum* infection is occurring in the human population.

*P. knowlesi* was transmitted experimentally to human volunteers by inoculation of blood or by bite of infected mosquitoes. After 170 serial passages in man, the infection became so virulent that the experiment was stopped (Collins and Aikawa, 1977). *P. cynomolgi* inoculated accidentally into humans and then transmitted to man and monkeys by infected mosquitoes, produced low parasitaemia in humans but a moderately serious disease. Infections by *P.brasilianum, P.inui,* or *P.schwetzi* in human volunteers produced low levels of parasitaemia and mild symptoms (Collins and Aikawa, 1977). *P.schwetzi* was also transmitted experimentally to man and it produced a mild disease. Transmission of *P. reichenowi* to man was attempted, but without success (Flynn, 1973).

Deane (1992) reported an accidental human infection with *P.simium* in south- eastern Brazil.

*P.brazilianum* has been found in numerous monkey species of the family cebidae in neotropical regions. The infection rate is about 15% in howler monkeys of the genus *Alouatta,* spider monkeys of the genus *Ateles* and capuchin or white

monkeys of the genus *Cebus*. Prevalence of malaria has been reported to be 10% among simians in the Amazon region and 35% and 18% in the south -Eastern and southern regions of Brazil, respectively. Virtually all the parasites were detected in monkeys of the family *Cebidae* (Deane, 1992).

Among nonhuman primates in Asia and Africa, prevalence of malaria seems to be high in areas with large number of monkeys and appropriate anopheline vectors (Escalante et al, 1998).

Human malaria caused by plasmodia of simian origin resembles a mild and benign infection caused by human plasmodia. In general, the disease is of short duration, parasitaemias are low, and relapses are rare. The periodicity of malarial attacks depends on the parasite species. Malaria in simians is a mild disease that resolves spontaneously in the parasites natural hosts. However, *P.knowlesi* infection is serious in rhesus monkeys and in baboons. In rhesus monkeys (*Macaca mulatta*), *P.Coatneyi*. and *P.fragile* cause neurological signs similar to those that occur in humans infected with *P.falciparum* (Aikawa *et al,* 1992, Fujioka *et al,* 1994). After infection with *P.ovale*, monkeys of the genus *saimiri* develop parasites in the liver but the infections do not progress to erythrocytic stages (Millet *et al,* 1994).

### 2.1.5 MALARIA IN MAN

Apart from the simian malaria parasite which can affect man, there are four species of plasmodium that normally infect man and result in four kinds of malarial fever.

*P.vivax* causes benign, simple or tertian malaria.

*P.falciparum*: leads to aestivo-autumnal, malignant tertian, pernicious quotidian, subtertian or tropical malaria.

*P.malariae*: is the cause of quartan ague, or quartan malaria

*P.ovale*: produces ovale tertian malaria. (Smyth,1996).

Of the above species, *P.vivax* has the widest distribution, being prevalent throughout the tropics and even in the temperate regions. Vivax malaria is characterized by relapses and reappearances of symptoms even after a period of up to 5 years (Snow *et al,* 2005).

Such relapses are due to sudden activation of hypnozoites (Sleeping merozites) in liver cells, (Cogswell, 1992). *P.falciparum* is the most common in tropical and subtropical areas and causes the most dangerous and malignant form of malaria but it does not have relapses. Although the *P.falciparum* malaria and the P.malariae malaria do not show relapses, they are subject to ñrecrudescenceò. That is re - infection after a relatively short period of between 3 months and one year. *P.malariae* is widely distributed but is much less common than *P.vivax* and/or *P.falciparum* (Smyth, 1996).

Table 1: Comparative characteristics of *Plasmodia* of man

| | *P. falciparum* | *P. vivax* | *P. malariae* | *P. ovale* |
|---|---|---|---|---|
| Incidence in world malarial infections | 50 per cent | 43 per cent | 7 per cent | Rare |
| Erythrocytes attacked | Any indiscriminately | Reticulocytes only | Older erythrocytes only | ? |
| Exo-erythrocytic forms | Do not persist | Persist as hypnozoites | Do not persist | Persist as hypnozoites |
| Schizogony cycle (h) | 48 | 48 | 72 | 48 |
| Rings | ⅕–⅙ diam. r.b.c. Multiple infections common | ⅓ diam. r.b.c. Often 2 rings or more per r.b.c. | ⅓ diam. r.b.c. Double infections rare | As *P. malariae* |
| Late trophozoite | Medium, compact; band forms frequent; vacuole inconspicuous; rare in peripheral blood | Large, very amoeboid; prominent vacuole | Small, not amoeboid; often band-shaped; vacuole inconspicuous | Small, not amoeboid; vacuole inconspicuous |
| Mature schizont | Smaller than r.b.c. Rare in peripheral blood | Larger than r.b.c. | Smaller than r.b.c. | Larger than in *P. malariae* |
| Number of merozoites | 12–24, usually 8–12 | 8–16, usually 12–15 | 6–12, usually 8 | 6–12, usually 8 |
| Microgametocytes* | Crescents usually sausage-shaped | Spherical; compact | Similar to *P. vivax* but smaller and less numerous | As *P. malariae* |
| Macrogametocytes | Crescents often longer and more slender | Spherical; compact | Similar to *P. vivax* but small and less numerous | As *P. malariae* |
| Alterations in r.b.c. | Normal size; appear 'brassy'. Maurer's dots or 'clefts' may be visible; rare in peripheral blood | Enlarged and pale; Schüffner's dots present | May appear smaller; fine dots (Ziemann's dots) occasionally seen | r.b.c. oval; Schüffner's dots prominent and appear early |

*Usually smaller and less numerous than macrogametocytes.

(Smyth,1996).

***P.falciparum*** is the most important malaria parasite, producing a disease that runs an acute course and often terminates fatally. It is a significant cause of abortion or stillbirth and even death in non-immune pregnant women (Trampuz *et al.,* 2003). It is responsible for about 50% of all the malarial cases throughout the world. Its distribution is restricted to warm and tropical countries.

Special features of its life cycle include:

- It attacks erythrocytes of all ages, indiscriminately so that high density of parasites is rapidly reached. In extreme cases, up to 48% of the red cells of the victim are parasitized.
- It leads to multiple infections (polyparasitism) thus resulting in several ring forms of the parasite in a cell.
- Later stages of its asexual cycle, that is, its growth to schizonts, do not occur in the peripheral blood as in other forms of malaria, except in severe cases. So, only rings and crescents are found in blood films. After about 24 hours, its ring form and its older trophozoites have a tendency to clump together and adhere to the visceral capillary walls and may become caught up in vessels of the heart, intestines, brain or bone marrow in which the later asexual stages are completed. This behaviour, together with the fact that the subtertian malaria is more toxic, are the principal reasons why this type of malaria is very dangerous (Warrell *et al.,* 1990).
- Its sporulation is not well synchronized as in other species.So, its fever paroxysms last longer.
- Its exoerythrocytic forms do not persist in tissues and hence relapses do not occur. (Smyth, 1996).

***P.vivax*** causes the benign tertian form of malaria and is responsible for about 43% of all cases in the world. It also has the widest geographical

distribution. Although generally, it is not life threatening, it can cause severe acute illness. Characteristics of ***P.vivax*** infection include the following:

- The degree of infection is low, for only the young immature cells (reticulocytes) are attacked. Only about 2% erythrocytes of the victim are parasitized.
- Periodicity of the asexual cycle is closely synchronized.
- Hypnozoites develop in liver. So relapses may occur (Tait and Sacks, 1988).

***P.malariae*** is a relatively rare parasite, producing quartan malaria. It is responsible for about 7% of malaria in the world. Characteristics of its infection include:

- Infected erythrocytes are not larger in size than uninfected ones. At times, they are even smaller.
- The parasite attacks mature erythrocytes more often and rarely attacks reticulocytes. So, the density of parasites is very low. Only 0.2% of the patientôs erythrocytes are parasitized.
- It is often difficult to distinguish between a large trophozoite and an immature gametocyte in patients of *P.vivax* (Smyth, 1996).

***P.ovale*** is a species rarely encountered. It is confined essentially to the tropics and subtropics although it has been reported from many continents (White, 1989). The type of fever it produces (ovale tertian) is milder than the benign tertian fever of *P.vivax*.

- It resembles *P.malariae* morphologically in most of its stages.
- Changes it produces in infected erythrocytes are similar to those produced by *P.vivax.*
- Its hypnozoites develop in the liver. So relapses may occur (Tait and Sacks, 1988).

### 2.1.5.1. CLINICAL SIGNS OF MALARIA IN MAN:

The signs and symptoms of malaria include fever, shivering, arthralgia (joint pain), vomiting, anaemia (caused by hemolysis), hemoglobinuria, retinal damage and convulsions (Beare *et al*; 2006).The classic symptom of malaria is cyclical occurrence of sudden coldness, followed by rigor and then fever and sweating.These last four to six hours and reoccur every two days in *P. vivax* and *P.ovale* infections, or every three days in *P.malariae* (Malaria facts, 2006). *P.falciparum* can have recurrent fever, every 36 ï 48 hours or a less pronounced but continuous fever. For reasons that are poorly understood, but that may be related to high intracranial pressure, children with malaria frequently exhibit abnormal posturing, a sign which suggests severe brain damage (Idro *et al,* 1990).

Malaria has also been reported to cause cognitive impairments, especially in children. It causes widespread anaemia and if this happens during period of brain development it can lead to brain damage. This neurologic damage results from cerebral malaria to which children are more vulnerable to (Holding and Snow, 2001, Boivin, 2002). Cerebral malaria is associated with retinal whitening (Maude *et al*, 2009). This is a useful clinical sign in distinguishing malaria from other causes of fever (Beare *et al*, 2006).

Consequences of severe malaria include coma and death if untreated. Children and pregnant women are especially vulnerable. Splenomegally, severe headache, cerebral ischaemia, hepatomegally, hypoglycemia, and haemoglobinuria with renal failure may occur. Renal failure causes ñblackwater feverò, when haemoglobin from lysed red blood cells leaks into the urine (Trampuz *et al*, 2003).

Pregnant women are especially attractive to mosquitoes (Lindsay *et al*, 2000) and malaria in pregnant women is an important cause of stillbirths, infant mortality and low birth weight (van Geertruyden *et al*, 2004). This is particularly so in *P.falciparum* infection, but it can also occur in infections by other species, such as *P. vivax* (Rodriguez ï Morales *et al*, 2006).

### 2.1.6 MALARIA IN ANIMALS:

**2.1.6.1 SIMIAN MALARIA**: Species of *Plasmodium* reported to infect primates in old world (Malaysia, South East Asia, India, Sri Lanka) are *P.schwetzi, P.knowlesi, P.cynomolgi, P.simiovale, P.gondale, P.jefferyi, P.hylobati, P.silvaticum, P. inui, and P.youngi* .In the new world (Central and south America, Brazil) only *P.brasilianum* and *P.simium* have been reported to infect monkeys (Cogswell,2000).

Table 2: Common *Plasmodium* species that infect primates.

| Species | Host | Geographic range | Periodicity | Relapses |
|---|---|---|---|---|
| *P. brasilianum* | Monkey | Central and South America | Quartan | Uncertain |
| *P. coatneyi* | Monkey | Malaysia | Tertian | No |
| *P. cynomolgi* | Monkey | Southeast Asia | Tertian | Yes |
| *P. eylesi* | Gibbons | Malaysia | Tertian | Uncertain |
| *P. falciparum* | Humans | Tropics | Tertian | No |
| *P. fieldi* | Monkey | Malaysia | Tertian | Yes |
| *P. gonderi* | Monkey | Central Africa | Tertian | No |
| *P. hylobati* | Gibbons | Malaysia | Tertian | No |
| *P. inui* | Monkey | India and Southeast Asia | Quartan | No |
| *P. knowlesi* | Monkey | Malaysia | Quotidian | No |
| *P. reichenowi* | Monkey | Central Africa | Tertian | No |
| *P. schwetzi* | Monkey chimpanzees | Tropical Africa | Tertian | Yes |
| *P. simiovale* | Monkey | Sri Lanka | Tertian | Yes |
| *P. simium* | Monkey | Brazil | Tertian | Uncertain |

Periodicity = interval between fever attacks:

Quartan = three days

Tertian = two days

Quotidan or malignant tertian = few hours to two days.

(Pan American Health Organization, 2003.).

### 2.1.6.1 CLINICAL SIGNS OF SIMIAN MALARIA

Monkeys infected with malaria parasites usually have subclinical disease, making identification of carriers difficult. It is only when monkeys are immunosuppressed or splenectomized that they develop clinical signs of malaria including jaundice, anorexia, listlessness, fever and anaemia. Splenomegaly may occur in immune suppressed animals when their spleens are intact.

Clinical signs of chills and fever seen in malaria are responses to toxins expressed during release of plasmodium merozoites from red cells. Pregnant animals may experience more severe anaemia, which often has a measurable impact on health of their fetuses (Cogswell,2000).

### 2.1.6.2: AVIAN MALARIA:

There are many species of plasmodium that infect avian spp. Of these, only few have been used for laboratory studies. These are *Plasmodium relictum* and *P.cathemerium* . These two occur more commonly in nature than other species (Smyth, 1996). Other species of avian malaria parasite include *P.gallinaecium, P.lophurae, P.elongatum, P.circumflexum* and *P.juxtanucleare.*

Avian *Plasmodium spp,* are often not host specific and infect a wide variety of domestic and wild birds in most parts of the world, leading to high losses. Asymptomatic infections in birds in endemic areas can be spread through mosquitoes and cause fatal disease in both resident birds and those newly introduced. Invertebrate hosts are ornithophilic mosquitoes usually *Culex, Culiseta* or *Aedes* spp (McGhee, 1988).

### 2.1.6.2.1: CLINICAL SIGNS IN AVIAN MALARIA:

Infection with Plasmodium species in poultry may be asymptomatic or cause illness characterized by weakness, lassitude, dyspnea, anaemia, abdominal distention, occular haemorrhage, and death. Death results from blockage of capillaries in the brain or in other vital organs by exoerythrocytic schizonts in endothelial cells.

**Gross postmortem lesion:**

Liver and spleen are markedly enlarged and often discoloured (dark brown to black). In birds that die of acute malaria, organs may be slightly haemorrhagic and many schizonts are found in capillaries of brain, lungs, liver and spleen at microscopy (The Merck Vet. manual, 1998).

### 2.1.6.3: MURINE MALARIA:

Four *Plasmodium* species infect rodents in parts of Africa. These species are *P.vinckei, P.chaubadi, P.yoelli* and *P.berghei.* These species fall into three groups, P. berghei ï P.yoelli group, P.vinckei group and the P.chaubadi group (Cox, 1988). Of all the species, *P. berghei* is the most widely used laboratory plasmodium model.

The four rodents` malaria parasites are principally parasites of thicket rats. Among many thousands of rodents examined in Africa, only few have been found infected with plasmodia. *P.berghei* has been isolated from three different species of thicket rats *Grammomys surdaster, Praomys jacksoni*, and *Leggada bella*, while other plasmodium species have been isolated from *Thamnomys rutinals* (Killck ï Kendrick, 1978). *P.berghei* infects laboratory hamsters, rats, and mice. A suitable mosquito vector for *P.berghei,* which is widely used in the laboratory, is *Anopheles stephansi* (Sinden, 1996).

#### 2.1.6.3.1: CLINICAL SIGNS OF MURINE MALARIA:

Rodent malaria parasites do not cause apparent clinical disease unless the animals are splenectomized or they are severely stressed.However, in some strains of laboratory mice and rats, infection with rodent malaria parasites could cause death within 1- 3 weeks and many animals may show cerebral complications. But in its natural hosts, *P.berghei* causes chronic infections, with low parasite densities. In some mouse strains, there could be cerebral complications but between rodent strains, significant differences exist in susceptibility to plasmodial infections (Killick-kendrick, 1978). Some laboratory rats show no clinical signs to plasmodial infection and are able to clear the parasites from the blood and recover (Carter and Diggs, 1977).

Mice may show signs such as ataxia, abnormal posturing, depression and anorexia (Carter and Diggs, 1977)

### 2.1.7 RELATEDNESS OF RODENTS` MALARIA PARASITES TO OTHER MAMMALIAN MALARIA PARASITES

1. The entire life cycle, as well as the morphology of the different developmental stages of all mammalian malaria parasites are similar (Carter and Diggs, 1977). The life cycles and the different developmental stages of all mammalian malaria parasites are also highly comparable (Aikawa and Seed, 1980). Mammalian malaria parasites share the following characteristics:
    - They infect Anopheline mosquitoes and have haploid sporozoites which invade and develop only in liver cells.
    - After multiplication in the liver, the parasites (merozoites) invade and multiply in red blood cells.

- In the blood, a relatively small percentage of parasites develop into gametocytes, the precursor cells of the haploid gametes.
- Fertilization and development of the diploid zygote into ookinetes occur in the mid-gut of the mosquito.
- Mature ookinetes penetrate the cells of the mid-gut wall of the mosquitoes and develop into oocysts on the outside of the midgut. (Lin *et al,* 2000).

2. The genome organization of rodent and human malaria parasites is similar. (Thomspon*,* 2001). The genome of both *P. falciparum* and the four rodent parasites are organized into 14 linear chromosomes. They posses identical telomeric repeats [CCCT (A/G) AA], organized into tandem arrays (Carlton*,* 1998). The chromosomes are compartmentalized into relatively conserved internal regions (core regions) flanked by highly polymorphic subtelomeric regions (Thompson*,* 2001).
3. No differences in metabolic pathways between mammalian malaria parasites have been reported (Barnwell and Weitheimer, 1989). Similarly, resistance of mammalian malaria parasites, (including rodent parasites) to some anti-malarial drugs and to other specific inhibitors have been reported. (Homewood and Neame, 1980).
4. ósmallô differences that exist between the mammalian malaria parasites are only related to interaction of the parasites with their hosts (Cowman, 2000). These differences have significant impact only on host-parasite interactions and they influence features, such as pathology, virulence and immune escape (Pinder*,* 2000).

## 2.2. CHLOROQUINE

Chloroquine is a 4-aminoquinoline drug used in the treatment or prevention of malaria.

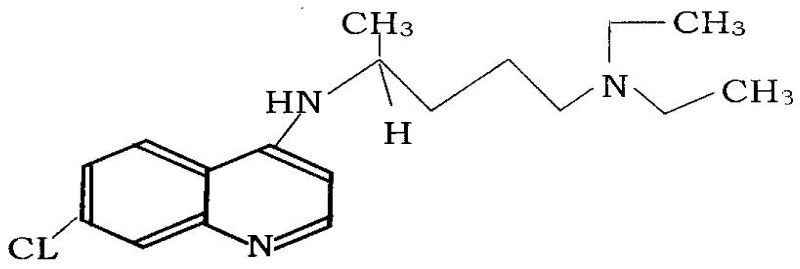



Figure 2: Chloroquine molecule. (Wikipedia.org.2011.)

Systematic (IUPAC) name Nôï (7 chloroquinolin 4 ï yl) ï N, N-diethyl pentane -1, 4, diamine.

**Chemical data:**

Formula = $C_{18}H_{26}CIN_3$

Molecular mass = 319.872g/mol

Pharmacokinetic data: Metabolism - In liver, Half life - 1-2 months

Chloroquine was discovered in 1934 by Hans Andersag and his co-workers at Bayers laboratories and was then named ñResochinò (Krafts *et al*, 2012). During world war II, United States government-sponsored clinical trials for anti-malarial drugs development and it was shown that chloroquine had significant therapeutic

value as an antimalarial drug and was introduced into clinical practice in 1947 for prophylactic management of malaria.

Chloroquine can be used for preventing malaria from *Plasmodium vivax, P ovale* and *P.malariae*. Popular drugs based on chloroquine phosphate (also called nivaquine) are chloroquine FNA, Resochin and Dawaquin. Combination of chloroquine with other drugs has been reported to be more effective than treatment with chloroquine alone. Research for substances to combine with chloroquine for better treatment of malaria is still going on (Uhlemann and Krishna, 2005).

Most mammalian malarias can be treated with chloroquine at a dosage of 7mg/kg body weight given for 5days (total = 35mg/kg). This can be given as an intramuscular injection or per os via nasogastric tubes. The bitter taste of 4-aminoquinolines precludes putting it in food.

Chloroquine is effective against the circulating trophozoites (feeding) stages of the parasites but does not affect hepatic stages nor circulating gametocytes (Hempelmann, 2007).

### 2:2:1 MECHANISM OF ACTION OF CHLOROQUINE

Inside red blood cells, the malarial parasites degrade hemoglobin to acquire essential amino acids, which the parasite requires to construct its own protein and for energy metabolism. Digestion is carried out in the vacuole of the parasite cell.

During this process the parasite produces the toxic and soluble molecule *heme*. The heme moiety consists of a porphyrin ring molecule called Fe (II)-protoporphyrin IX (FP). To avoid destruction by this molecule,the parasite biocrystallizes heme to form hemozin, a non toxic molecule. Hemozion collects in the digestive vacuole as insoluble crystals (Hempelmann, 2007).

Chloroquine enters the red blood cells inhabited by the parasites and the digestive vacuole by simple diffusion. It becomes protonated to $CQ^{2+}$ as the digestive vacuole is known to be acidic (pH = 4.7). Because chloroquine cannot leave by diffusion,it caps hemozoin molecules and prevents further biocrystallization of heme thus leading to heme build up. Chloroquine binds to heme or FP to form what is known as the FP-chloroquine complex. This complex is highly toxic to cells and disrupts membrane functions. Action of the toxic FP and FP-chloroquine complex results in cell lysis and ultimately the parasite drowns in its own metabolic products (Martin *et al,* 2009).

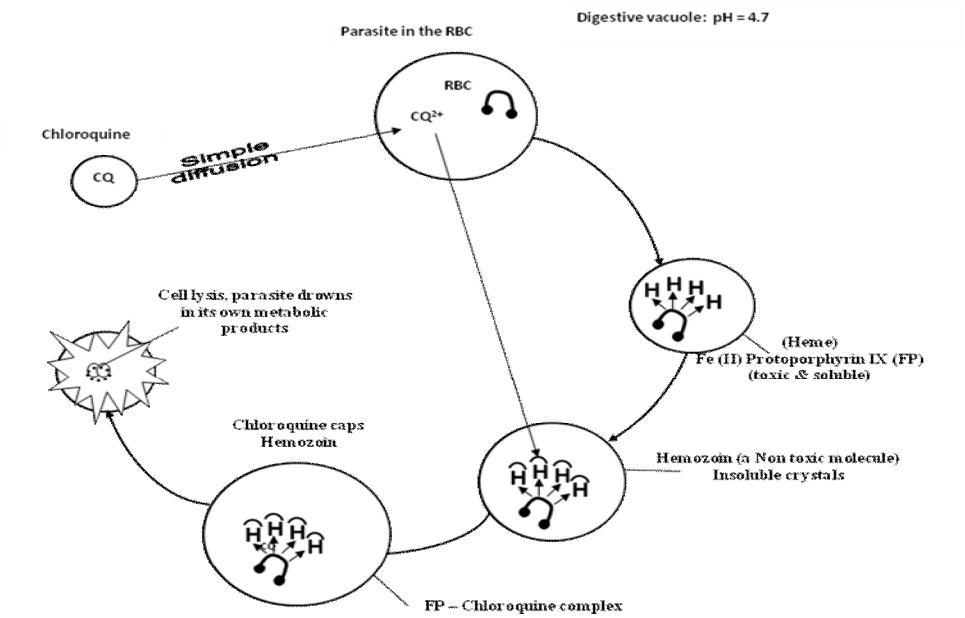



Figure 3: Mechanism of action of chloroquine (Martins *et al*, 2009).

## 2.2.2 CLINICAL USES OF CHLOROQUINE

- Chloroquine has been used for a long time in the treatment or prevention of malaria. New drugs to combine with chloroquine are being investigated for since its use in mass drug administrations may have contributed to emergence and spread of resistance especially by *Plasmodium falciparum* in humans (Plowe, 2005).

- Choloroquine is also used in autoimmune disorders, such as rheumatoid arthritis and lupus erythematosus. This is because it suppresses the immune system (Uhlemann and Krishna, 2005).
- Chloroquine is currently being investigated as anti retroviral drug and as a potential antiviral agent against chikungunya fever (Savarino *et al.,* 2003).
- The radiosensitizing and chemosensitizing properties of chloroquine are beginning to be exploited in anticancer strategies in humans (Savarino *et al.,* 2006, Sotelo *et al,* 2006).

### 2:2.3 SIDE EFFECTS OF CHLOROQUINE TREATMENT

At the doses used for prevention of malaria, side effects of chloroquine include stomach ache, itch, headache, nightmares and blurred vision.

Chronic use of chloroquine is also reported to cause retinopathy (eye toxicity) (Yam and Kwok, 2006).

The daily safe doses of chloroquine to avoid eye toxicity can be computed from oneôs height and weight (Yam and Kwok, 2006, numerical example.com, 2008).

Chloroquine induced itching is very common among Black Africans (70%), but much less common in other races. It increases with age, and is at times so severe as to lead to stoppage of drug therapy. It also increases during malaria fever and its severity correlates to plasmodial parasitaemia (Ajayi, 2000).

When doses are extended over a number of months, it is important to watch out for a slow onset of ñchanges in moodsò (i.e. depression, anxiety). These may be pronounced with higher doses used for treatment (Ajayi, 2000). Chloroquine tablets also have unpleasant metallic taste. So, therapeutic index for chloroquine is small (Cann and Verhulst, 1961).

## 2:2.4. ANAEMIA IN CHLOROQUINE TREATMENT

Haemoglobin is a protein that carries oxygen in the blood from the lungs to other parts of the body of the subject. (MedicineNet.com 2000)

Malaria parasites degrade haemoglobin to acquire essential amino- acids, which they require to construct its own protein and for energy metabolism. Hempelmann (2007) reported that the parasites produce heme, a toxic and soluble molecule consisting of a porphyrin ring molecule, called Fe (II) ï protoporphyrin IX( FP).With introduction of Chloroquine, a complex known as the FP- Chloroquine complex forms. This complex is highly toxic to cells and disrupts membrane functions. Action of the toxic FP- Chloroquine complex results in cell lysis and ultimately the parasite drowns in its own metabolic products (Martins *et al*, 2009).

Chloroquine acts only on parasites in the blood not on those in organs e.g. liver. Action of Chloroquine destroys infected red blood cells with the parasite (Hempelmann, 2007).

Chou and Fitch (1980) reported that the lytic effect of FP (Iron (II) protoporphyrin IX) on red blood cells is enhanced by chloroquine. Also, Hempelmann and Zhang (1987) reported that high doses of chloroquine without immune stimulant medication and other supportive treatments is toxic and causes anaemia. It causes erythropoietic suppression and dyserythropoiesis (Wildig *et al*, 2006).

Chloroquine has also been reported to inhibit uptake of iron, leading to iron deficiency anaemia. Iron inhibition by chloroquine is dose dependant (Emerson *et al*, 2001).

## 2:2.5 CHLOROQUINE TOXICITY

Chloroquine and hydroxychloroquine belong to the quinoline family. They are related drugs with different therapeutic and toxic doses. They have similar clinical indications and uses and their toxicity result in retinal damage (Yam and Kwok,2006).

Initially, chloroquine was indicated for the prevention and treatment of malaria only, but recently, it is being used in treating rheumatoid arthritis, systemic/ discoid lupus erythematosus, and other connective tissue disorders. Dermatologists also use chloroquine for treatment of cutaneous lupus (Manolette and Hampton, 2011)

Expanded use of Chloroquine for other diseases has resulted in prolonged therapy and higher daily dosages leading to cumulative doses greater than those used in antimalarial therapy. The first reports of retinal toxicity attributed to chloroquine appeared during the late 1950ôs. Some patients with retinopathy may be asymptomatic. When they are symptomatic, visual symptoms include the dimness, flickering or flashing lights of yellow, cyclopegia, amblyopia, blindness, photophobia (Manolette and Hampton,2011).

Other signs of chloroquine toxicity include, nausea, abdominal pain,vomiting, skin rashes, pruritus, and sensitivity to ultraviolet light (Manolette and Hampton,2011).

Rarely, neurological symptoms such as vertigo, tinnitus, irritability, cranial nerve palsies and myasthenia-like muscle weakness may manifest in chloroquine toxicity ( Manolette and Hampton, 2011)

## 2:2.6 PLASMODIUM RESISTANCE TO CHLOROQUINE

Malaria death rates have been rising in recent years due to resistance to drugs by the parasite.

Chloroquine has a very high volume of distribution.It diffuses into the bodyôs adipose tissues. It is known to be a lysosomotropic agent, meaning that it accumulates preferentially in the lysosome of cells in the body (Hempelmann, 2007).

The lysosomotropic character of chloroquine is believed to account for much of its anti-malarial activity. The drug concentrates in the acidic food vacuole of the parasite and interferes with essential processes (Chen *et al*, 2011).

High concentrations of chloroquine kill malaria parasites living in the cells. Researchers in Liverpool school of Tropical Medicine have reported that a protein called $P_f$CRT inside malaria parasites enables them to become resistant to anti-malarial drugs, by creating a ñback doorò which leaks the drugs out of the parasite. The $P_f$CRT is believed to be the master gene that controls the parasiteôs resistance to a variety of anti-malarial drugs including artemisine- based drugs (Johnson, 2004).

Ward *et al* (2004) observed that most malaria drugs are more expensive than chloroquine, putting them off the reach of healthcare workers in developing countries.

Research effort is now aimed at developing new drugs based on chloroquine structure, which cannot be gotten rid off through the ñback doorò. Whatever modifications made to improve efficacy of chloroquine must ensure that it remains cheap and yet effective (Ward *et al*, 2004).

## 2:3 ALUMINIUM MAGNESIUM SILICATE

Aluminium magnesium silicate (AMS) is a natural ore which occurs as mineral deposits in India and the United States of America. It occurs as an off-white or creamy white, odourless, tasteless, soft slippery small flakes or fine powder. Its physical properties include:

- Density- 2.41g/cm
- Moisture content ï 6.00 - 9.98%
- Insoluble in water, alcohol and organic solvents.
- Natural smectite clays (bentonite) (Vanderbilt,1992)

**Chemical formula** ï $2Al_2Mg_3(SiO_4)_3$

The complex compound is a polymeric complex of aluminium, magnesium, silicon, oxygen and water. It is composed of three lattice layers of octahedral alumina and two tetrahedral silicate sheets. The Aluminium is often substituted to varying degrees, by Magnesium, Potassium or Sodium to balance the electrical charges. Iron, lithium, calcium and carbon could be present in smaller quantities. Natural Aluminium-Magnesium Silicate contains a lot of impurities which could be injurious to animals or humans when used by them. It could be treated to reduce the impurities and separate out the colloidal fractions.
The refined colloidal dispersion is then drum-dried to form small flakes which are then micro-atomized to form the powder grade. The essence of the water-washing of this smectite clay is to optimize purity and performance (Vanderbilt, 1992).

AMS is marketed as Veegum® or pyropes ®. Other synonyms include, Aluminosilic acid, Carisob, Magnabite, Magnesium- Aluminium silicate (Vanderbilt, 1992).

AMS is generally regarded as non toxic and none irritating and thus has been used in drug formulation for many years. Aluminium Magnesium Silicate (Veegum®) is safe even when consumed in high doses by animals and man (Wai *et al*, 1996). It has been used in pharmaceutical formulations of tablets, ointments and creams. It is also used as adsorbent, stabilizing agent, tablet and capsule disintegrant, tablet binder and viscosity increasing agent ( Wai *et al*, 1996).

**2:3:1. THE SYNTHETIC AMS**

Two AMS related minerals found in Nigeria-

- Aluminium silicate ($Al_4(SiO_4)_3$) and
- Magnesium silicate ($Mg_2SiO_4$)

have chemical structures similar to Aluminium Magnesium Silicate and are safe to both humans and animals (Smith,1984). They are already approved medicines in use, both in veterinary and human practice.

To synthesize a pure form of Aluminium Magnesium silicate, devoid of impurities, Aluminium silicate and Magnesium silicate were reacted as in the chemical equation:

$$Al_4(SiO_4)_3 + 3Mg_2SiO_4 \rightleftharpoons 2Al_2Mg_3(SiO_4)_3$$ (Ezeibe; 2009)

AMS stabilizes suspensions- i.e. makes a compound to stay in same chemical and atomic state and not easily disintegrated (Vanderbilt, 1992). The way in which AMS functions as a stabilizer is as a result of its smectite clay structure which enables it to hydrate in water to form the desired colloidal structure. It is also due to possession of both negative and positive electrical charges by its molecules.

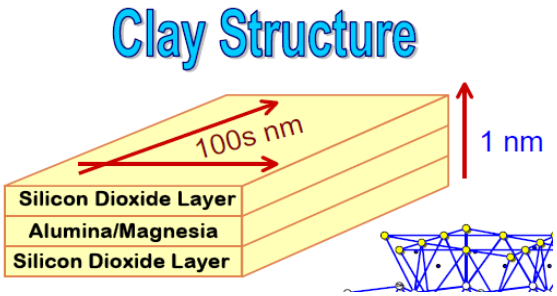
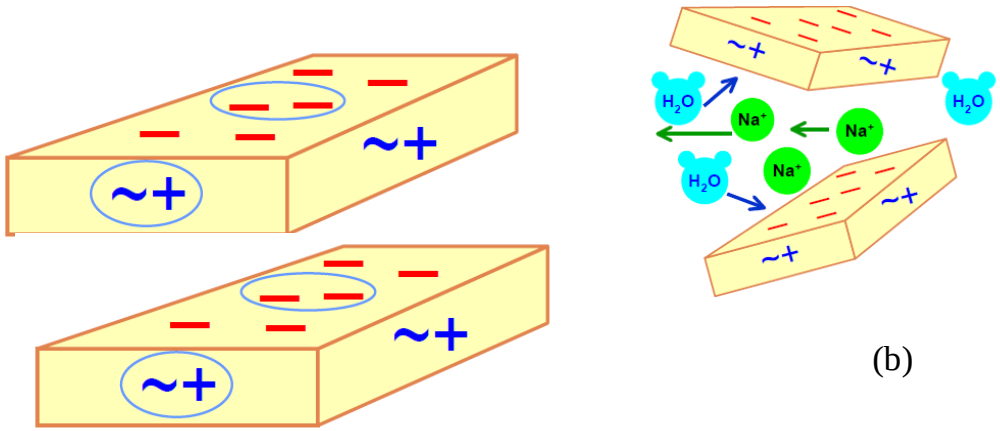



Fig 4: Smectite clay platelet of AMS (Vanderbilt,1992).

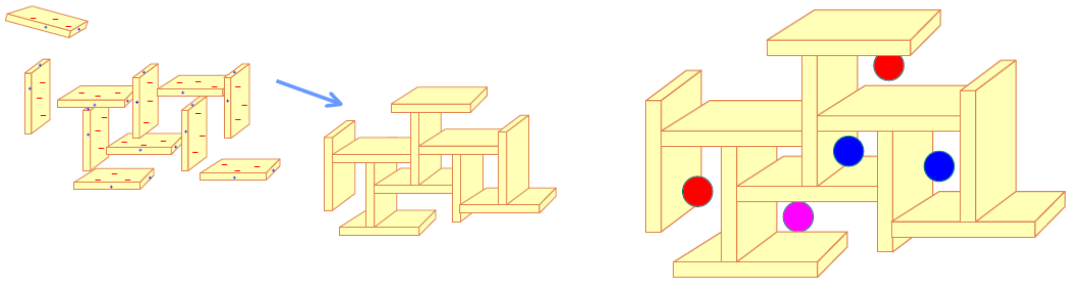
Faces of the platelets are negatively charged. The edges have slight positive charges, due to lattice discontinuities. The net platelet charge is negative. This property makes AMS- a good binding agent. When hydration occurs, water penetrates between the platelets, forcing them further apart, then cations diffuse away from the platelet faces. The diffusion of cations from between platelets and the osmotic movement of water into the spaces promote delamination until platelets are completely separated (Fig.5a and b).




(a) (b)

Fig 5: Platelet charges and hydration

Once the platelets are separated, the weakly positive edges are attracted to the negatively charged platelet faces. A three dimensional colloidal structure commonly called ñhouse of cardsò forms. This has ability to trap and segregate solids (suspensions), oils (emulsions) and gases as foam or mousse.



(House of cards entrap drugs)

Fig 6: Alignment of platelets charges (Positive and negative edges) get attracted leading to formation of ñhouse of cardsò (Vanderbilt, 1992).

By trapping active ingredients of drugs in the ñhouse of cardsò, AMS stabilize drugs (prevents them from destruction by metabolic processes). If drugs are prevented from destruction, their half-lives would be prolonged and bioavailability of ingested drugs could be higher, thereby possibly improving their therapeutic effects.

**2:3:1:1 THE SYNTHETIC AMS - A *NANOPARTICLE***

In Nanotechnology, a particle is defined as a small object that behaves as a whole unit with respect to its transport and properties.

Particles are further classified according to diameter of at least one of their sides (Granqvist *et al,* 1976). Coarse particles are those with a side in the range of 10,000 and 2,500 nanometers. Fine particles have at least a side of 2,500 and 100 nanometers. Ultrafine or ñ*Nanoparticles*ò are those with at least one of their sides between 1 and 100 nanometers (Hayashi Ch *et al*, 1997).

Although the size of most molecules would fit into the above classification, individual molecules are usually not referred to as *Nanoparticles*. The properties of many conventional materials change when formed from *Nanoparticles.* This is

typically because *Nanoparticles* have greater surface area per weight than larger particles which causes them to be more reactive to some other molecules.

Molecules of Aluminium- Magnesium Silicate are made of platelets that are only 1nm thick (Vanderbilt, 1992). So the synthetic AMS is made of *Nanoparticles* (Ezeibe *et al,* $2012_b$). *Nanoparticles* prolong bioavailability of drugs and enhance delivery of drugs across the blood brain barrier (Silva, 2008).

Also, AMS is reported to be a zeolite and that zeolites absorb water and drugs without change in their crystal structures (Mineral Gallaries, 2007). These lead to prolongation of bioavailability of drugs and when bioavailability of drugs is prolonged, their effects improve (Brent *et al,* 2001).

### 2:3:2 THE SYNTHETIC AMS AS ANTIVIRAL AGENT

Molecules of Aluminium- Magnesium Silicate (AMS) have two electrically charged ends. One end has positive electrical charges while the other has negative electrical charges. Viral genomes are made of a number of positively charged ions, such as Na+, Mg2+, K+ while their phosphate component is negatively charged. Some viruses have net negative charges while others have net positive charges. It does not matter which charge a virus has, it can still find a point to adsorb to, on AMS (Ezeibe, 2009).

Anti viral properties of the synthetic Aluminium- Magnesium Silicate (AMS) have so far been investigated with seven viruses including, *Peste des Petits Ruminants virus*(Ezeibe *et al*,$2009_a$), *Newcastle Disease virus* (Ezeibe *et al*,2011), *Infection Bursal Disease virus* (Ezeibe *et al*,$2009_b$ ), *Egg Drop Syndrome 76 virus*(Ezeibe *et al*,$2010_a$) *Canine Parvovirus* (Ezeibe *et al*, $2010_b$) and *Avian influenza virus* (Ezeibe *et al*, $2012_a$).

In all of the above experiments, significant reductions in viral titres were reported (Ezeibe *et al*, 2011). Other observations such as increase in Mean Death

Time of chick embryos innoculated with the viruses, reduction both in morbidity and mortality rates of experimental animals infected with the viruses and treated with the synthetic AMS, reduction in seroconversion abilities of the viruses after in vitro treatment with the AMS, suggest that the AMS inhibited activities of the viruses (Ezeibe *et al*, $2011_a$).

### 2:3:3 THE SYNTHETIC AMS AS A STABILIZING AND POTENTIATING AGENT FOR OTHER DRUGS.

Aluminium- Magnesium Silicate is reported to be a drug stabilizer (Vanderbilt, 1992). By stabilizing drugs, it protects them against destruction by metabolic processes. It achieves this, by trapping active ingredients of drugs in the ñhouse of cardsò. The resulting effects would be that high concentrations of drugs remain for longer periods in blood of treated animals.

Aluminium- Magnesium Silicate has already been reported to potentiate sulphadimidine against coccidiosis ï a protozoan disease (Ezeibe *et al*, $2011_b$). By incorporating sulphadimidine with the synthetic AMS, its therapeutic dose was reduced from 1.0g/litre to 0.4g/litre of drinking water (Ezeibe *et al*, $2011_b$). Also, by formulating 20% Ampicillin with the AMS and 20% Piperazine citrate with the AMS, 7.5mg of Ampicillin trihydrate per kg and 82.5mg of Piperazine citrate per kg were reported to produce better effect than 10mg per kg of Ampicillin and 110mg per kg of Piperazine citrate respectively (Ezeibe et al,$2012_c$, Ezeibe et al,$2012_d$).

# CHAPTER THREE

# MATERIALS AND METHODS

## 3.1 MATERIALS:

**3:1:1 Animals** - Thirty ï five male albino mice, aged 8-10 weeks and weighing 20-30 grams each, were used for the study. They were kept in clean cages in the animal house of the Department of Veterinary Medicine, University of Nigeria, Nsukka. The animals were humanely cared for and acclimatized for one week before the study. Feed and clean water were provided ad libitum.

### 3:1:2 *PLASMODIUM BERGHEI* PARASITES

*Plasmodium berghei* obtained from National Institute for Pharmaceutical Research and Development (NIPRD), Abuja, Nigeria were maintained in donor mice and used for experimental infection of the mice.

### 3:1:3 CHLOROQUINE PHOSPHATE

Chloroquine phosphate powder obtained from IECA laboratories limited, Mumbai, India was used for the study.

**3:1:4 ALUMINIUM MAGNESIUM SILICATE**. The AMS was synthesized by the reaction of Aluminium silicate and Magnesium silicate

[$Al_4(SiO_4)_3 + 3Mg_2SiO_4 \rightarrow 2Al_2Mg_3(SiO_4)_3$- Ezeibe, 2009].

## 3:2 METHODS:

### 3:2:1 Experimental Design

The study was conducted at three (3) dose levels to test effect of AMS on chloroquine at 7mg/kg body weight, 5mg/kg body weight and at 3mg/kg body weight.

### 3:2:2 Grouping

The animals were randomly assigned into seven (7) groups of five (5) mice each with two (2) groups for a phase of the experiment.

Six infected groups were treated either with Chloroquine Phosphate alone or with Chloroquine Phosphate in AMS while a group was infected but was not treated.

For each of the treated groups treatment was initiated 10 days post infection (PI).

Group 1(a) was treated with 7mg/kg of chloroquine phosphate alone,

while 1(b) was treated with 7mg/kg of chloroquine phosphate in AMS.

Group 2(a) was treated with 5mg/kg of chloroquine phosphate alone,

while 2(b) was treated with 5mg/kg of chloroquine phosphate in AMS.

Group 3(a) was treated with 3mg/kg of chloroquine phosphate alone,

while 3(b) was treated with 3mg/kg of chloroquine phosphate in AMS.

Group 4 was infected but was not treated (Control).

### 3:2:3 EXPERIMENTAL INFECTION OF MICE WITH *PLASMODIUM BERGHEI:*

The mice were infected by intraperitoneal (IP) inoculation of 1ml of blood of the donor mouse containing $2 \times 10^8$ parasitized RBC per ml, diluted in normal saline.

### 3:2:4 METHODS OF MONITORING PARAMETERS

#### 3:2:4:1 CLINICAL SIGNS

**Body temperature** ï Rectal temperature of the mice was measured with clinical thermometer in degree Celsius (°C). The mice were restrained by an assistant and the stubby bulb end of the thermometer lubricated with light Vaseline (petroleum jelly) was then gently inserted into the rectum with a twisting motion. The thermometer was directed against the upper wall of the rectum and held in place for one minute. It was then gently removed and wiped with cotton wool and the temperature of each mouse was then read and recorded. This was done daily.

**Body weight** ï weight of each mouse was measured in grams (g). The mouse was placed on an electronic weighing balance. Weight of the mouse appeared automatically and was read and recorded. Body weight was measured weekly.

#### 3:2:4:2 PARASITAEMIA

Both thin and thick films/smears of blood were used for assessment of parasitaemia. These were stained by Giemsa staining technique.

Number of parasitized cells in relation to 10,000 RBCs was counted daily for mice in each group, to determine parasitaemia.

Parasitaemia (P) was then expressed as the parasite/ml of blood using the formula:-

$$P = \frac{\#\text{Infected RBCs} \times \text{Total RBC}}{1000} = \text{parasite/ml.}$$

(World Health Organization, 1991)

**Preparation of Thin Blood Smears:**

A drop of blood from the infected mice mixed with EDTA was deposited at one end of a microscope slide. A spreader slide was placed in front of the drop of blood and angled at about 25-30° with the surface of the first slide.

The spreader slide was then drawn back into the drop of blood, causing the blood to run along the interface between the two slides. Then, the spreader slide was pushed steadily with an even rapid motion toward the far end of the other slide, to obtain a thin, even spread blood film. This was then air dried.

**Preparation of Thick Blood Smears:**

A drop of blood from the infected mice mixed with EDTA was deposited on the middle of a microscope slide with an applicator stick. It was spread in circular motion toward the periphery. This made a thick but even blood film. It was then air dried.

**Giemsa staining of thin and thick blood films:**

The air dried blood films were:

- Fixed in methanol for 1 minute
- Washed in tap water and flooded with Giemsa stain freshly diluted 1 in 10 with buffered distilled water of pH=7.2 for 25- 30 minutes.
- Tap water was then run on the slides to float off the stain and to prevent deposition of precipitates on the films.
- The slides were kept vertically to allow water to drain-off.
- The slides (blood film) were examined using a x100 objective (oil immersion) of the microscope.

### 3:2:4:3 HAEMATOLOGY

The orbital bleeding technique was used to collect blood weekly from the mice for haematology. About 0.5- 0.6ml of blood was collected from the medial canthus of each mouse into EDTA sample bottles for determining the following parameters.

**Packed cell volume** (PCV) this was determined using the microhaematocrit method (Coles, 1986).

Each blood sample was drawn into heparinized capillary tube with one end of the tube sealed with plastercin. The tube was then inserted into a groove of a Hawksley haematocrit centrifuge and spun at 10,000 revolutions per minute (rpm) for five minutes.

The PCV was read with a reader supplied with the centrifuge (Coles, 1986).

**Haemoglobin (Hb) concentration:** was measured using the cyanmethaemoglobin method (Van Kampen and Zijlstra, 1961). 5ml of the standard solution of cyanmethaemoglobin reagent (Drabkinôs solution) was added to 0.02 ml of the blood. The figures obtained were compared with that from the spectrophometer (standardized with a haemoglobin standard to obtain optical density at 540 mµ).

**Total RBC count**ï Total RBC was counted using the improved Neubauer Counting Chamber technique (Schalm et al; 1975).

**RBC count**: Blood mixed with EDTA was drawn into an RBC pipette to the 0.5 mark. Any excess blood was wiped from the surface of the pipette before drawing the diluting fluid into it to the 100 mark. The blood was thus diluted 1:200 in the solution. The RBC pipette was then gently rocked for 2 minutes to mix well. After several drops are discarded from the pipette, its tip was wiped clean and the Neubauer chamber was filled by allowing fluid to run from the tip of the pipette under the cover-slip, being careful not to overfill the chamber.

Cells in the 5 small squares were counted under high, dry objective lens. The RBCs counted in the 5 squares were multiplied by 10,000 to obtain total number of red cells per cubic mm.

### 3.2.5 DATA ANALYSIS:

The data obtained for the different treatment groups were tested for difference by one way analysis of variance (ANOVA) and post hoc test was carried out by the least significant difference (LSD) method. Hypothesis was accepted at significance level of $P < 0.05$.

# CHAPTER FOUR
# RESULTS

**Survivability:** At 7 mg/kg chloroquine alone, 80% of the treated mice survived compared with 20% that survived in the group treated with 7mg/kg chloroquine in AMS. At 5mg/kg and 3mg/kg dose levels all the mice treated with chloroquine alone and those treated with chloroquine in AMS survived. Survivability was also 100% in the control group (Table 6).

**Parasitaemia (%):** Mean parasitaemia of 4.15± 0.26 for the group treated with 7mg/kg chloroquine phosphate alone was higher ($P<0.05$) than 3.60 ±0.22 of the control (Table 3). At 5 mg/kg and 3 mg/kg dose levels, the AMS significantly($p<0.05$) improved ability of chloroquine to reduce plasmodial parasitaemia from 2.46 ± 0.21 to 1.57± 0.25, and from 3.82 ± 0.06 to 2.12±0.08 respectively. Mean parasitaemia levels of the control group and the group treated with 3mg/kg chloroquine phosphate alone had no significant difference (Table 3).

**Haemoglobin (Hb) concentration**: Mean Hb values of 12.95 ±0.25, 12.25 ± 0.27, 12.68 ±0.18, 12.98± 0.47 of the groups treated with 7mg/kg chloroquine alone, 5mg/kg chloroquine alone and 5mg/kg chloroquine in AMS, 3mg/kg chloroquine in AMS respectively, were significantly ($P<0.05$) higher than 10.43 of the only surviving mouse treated with 7mg/kg chloroquine in AMS and the mean, 10.18 ± 3.00 got in the group treated with 3mg/kg chloroquine alone (Table 4).

**Red blood cell counts:** Mean Red blood cell counts, 95.19 ±2.81, 92.91 ±4.01 and 95.23 ±5.32 of the groups treated with 7mg/kg chloroquine phosphate alone, 5mg/kg chloroquine phosphate in AMS and 3mg/kg chloroquine phosphate in AMS respectively, were also significantly ($P<0.05$) higher than 88.99 ±5.72 of the

group treated with 5mg/kg chloroquine alone and 85.55 ± 7.83 of the mouse treated with 7mg/kg chloroquine in AMS and 88.74± 2.99 of the control group. Least RBC count of 63.39 ± 18.02 was obtained from the group treated with 3mg/kg chloroquine alone (Table 4).

**Packed cell volumes (PCV)**: PCV 42.35±1.57 of the group treated with 7mg/kg Chloroquine phosphate alone did not vary from 38.93±1.94, 38.10±1.12, 38.15±0.56, 40.60±1.21 and 40.35±1.31 of the mouse treated with 7mg/kg chloroquine phosphate in AMS and those of the groups treated with 5mg/kg chloroquine in AMS, 3mg/kg chloroquine alone, 3mg/kg chloroquine in AMS and the control respectively. The group treated with 5mg/kg chloroquine phosphate alone had a significantly lower mean PCV of 35.45±1.04 which did not vary from those of the mouse treated at 7mg/kg chloroquine in AMS and the groups treated with 5mg/kg chloroquine in AMS and 3mg/kg chloroquine alone (Table 4).

**Body temperature** :Mean rectal temperature of 35.56 ± 0.82$^0$C and 35.73± 0.38$^0$C of the groups treated with 5mg/kg chloroquine alone and 5mg/kg chloroquine in AMS respectively, were significantly (P<0.05) lower than 37.29 ± 0.38°C of the group treated with 7mg/kg chloroquine in AMS and 36.84± 0.32$^0$C, 36.84 ±0.32$^0$, 36.84± 0.32°C, 37.25± 0.32°C and 36.16 ± 0.35$^0$C obtained in the groups treated with 7mg/kg chloroquine alone, 3mg/kg chloroquine in AMS, 3mg/kg chloroquine alone and in the control respectively (Table 5).

**Body weight:** Mean body weight of 32.66± 2.10kg for the group treated with 5mg/kg chloroquine in AMS was significantly (P<0.05) higher than the 29.29 ± 0.51kg, 29.17kg and 29.06± 1.95kg obtained for the groups treated with 7mg/kg chloroquine alone, 7mg/kg chloroquine in AMS and 5mg/kg chloroquine alone respectively. It was also significantly (P<0.05) higher than 26.65± 0.83kg and 26.35± 0.61kg obtained for the groups treated with 3mg/kg chloroquine alone and

3mg/kg chloroquine in AMS respectively. Highest mean body weight value of 35.91± 0.64kg was obtained for the control group.

**Table 3:** *Plasmodium berghei* (parasitaemia) in mice treated with chloroquine phosphate stabilized with a synthetic Aluminium-Magnesium Silicate.

| | **PARASITAEMIA (%)** | | | | | | |
|---|---|---|---|---|---|---|---|
| | **Infected treated Groups** | | | | | | |
| Weeks post treatment | **1 a**7mg/kg CQ | **1b**7mg/kg CQ-AMS | **2a** 5mg/kg CQ | **2b** 5mg/kg CQ-AMS | **3a** 3mg/kg CQ | **3b** 3mg/kg CQ AMS | Control (infected untreated group) |
| Week 0 | 4.50±0.40 | 4.24±0.25 | 2.62±0.49 | 1.12±0.23 | 3.88±0.23 | 2.24±0.25 | 3.64±0.18 |
| Week 1 | 4.40±0.39 | 4.60±0.40 | 2.20±0.39 | 1.16±0.27 | 3.96±0.18 | 2.04±0.29 | 2.96±0.18 |
| Week 2 | 4.30±0.53 | 4.00±0.00 | 2.97±0.20 | 2.12±0.27 | 3.68±0.29 | 1.92±0.26 | 3.85±0.10 |
| Week 3 | 3.38±0.14 | 2.80±0.00 | 2.06±0.29 | 1.86±0.28 | 3.76±0.35 | 2.28±0.18 | 3.93±0.35 |
| Mean $\overline{x}$ | 4.15 ±0.26$^{c}$ | 3.91 ±0.39$^{abc}$ | 2.46±0.21$^{abc}$ | 1.57±0.25$^{a}$ | 3.82±0.06$^{bc}$ | 2.12 ±0.08$^{ab}$ | 3.60±0.2$^{bc}$ |

Different superscripts $^{abc}$ in a row indicate significant differences between the means at the level of probability: $P$ Ò0.05

**Table 4:** Haematologic values of *P. berghei* infected mice treated with chloroquine phosphate stabilized with a synthetic Aluminium-Magnesium Silicate .

| Haematologic Parameters | **Chloroquine Phosphate Treatment** | | | | | | |
|---|---|---|---|---|---|---|---|
| | 7mg/kg CQ | 7mg/kg CQ-AMS | 5mg/kg CQ | 5mg/kg CQ-AMS | 3mg/kg CQ | 3mg/kg CQ AMS | Control |
| Mean RBC ($\times 10^6$) | 95.19± 2.81$^{b}$ | 88.55±7.83$^{ab}$ | 88.99±5.72$^{ab}$ | 92.91±4.01$^{b}$ | 63.39±18.02$^{a}$ | 95.23±5.32$^{b}$ | 88.74±2.99$^{ab}$ |
| Mean Hb (g/100ml) | 12.95±0.25$^{b}$ | 10.43±2.64$^{a}$ | 12.25±0.27$^{b}$ | 12.68±0.18$^{b}$ | 10.18±3.00$^{a}$ | 12.98±0.47$^{b}$ | 13.3±0.50$^{b}$ |
| MeanPCV (%) | 42.35±1.57$^{(b)}$ | 38.93±1.94$^{(ab)}$ | 35.45±1.04$^{(a)}$ | 38.10±1.12$^{(ab)}$ | 38.15±0.56$^{(ab)}$ | 40.60±1.21$^{(b)}$ | 40.35±1.31$^{(b)}$ |

Different superscripts $^{abc}$ in a row indicate significant differences between the means at the level of probability: $P$ Ò0.05

**Table 5:** Rectal Temperature and Body weight of *P. berghei* infected mice treated with chloroquine phosphate stabilized with a synthetic Aluminium-Magnesium Silicate.

| Parameter | **Chloroquine Treatment** | | | | | | |
|---|---|---|---|---|---|---|---|
| | 7mg/kg CQ | 7mg/kg CQ-AMS | 5mg/kg CQ | 5mg/kg CQ-AMS | 3mg/kg CQ | 3mg/kg CQ AMS | Control |
| Mean Body Weight (g) | 29.29±0.51$^{ab}$ | 29.17±3.38$^{ab}$ | 29.06±1.95$^{ab}$ | 32.66±2.10$^{bc}$ | 26.65±0.83$^{a}$ | 26.35±0.61$^{b}$ | 35.91±0.64$^{c}$ |
| Mean Rectal Temp. ($^{0}$C) | 36.84±0.32$^{ab}$ | 37.29±0.38$^{b}$ | 35.56±0.82$^{a}$ | 35.73±0.38$^{a}$ | 37.25±0.32$^{b}$ | 36.84±0.23$^{ab}$ | 36.16±0.35$^{ab}$ |

Different superscripts $^{abc}$ in a row indicate significant differences between the means at the level of probability: *P* Ò0.05

**Table 6:** Survivability of *Plasmodium berghei*- infected mice treated with chloroquine phosphate stabilized with synthetic Aluminium -Magnesium Silicate .

| Dose of Chloroquine. | Survivability (%) |
|---|---|
| 3mg/kg(CQ) | 100% |
| 3mg/kg-AMS(CQ-AMS) | 100% |
| 5mg/kg(CQ) | 100% |
| 5 mg/kg-AMS(CQ-AMS) | 100% |
| 7 mg/kg(CQ) | 80% |
| 7 mg/kg-AMS(CQ-AMS) | 20% |
| Control | 100 % |

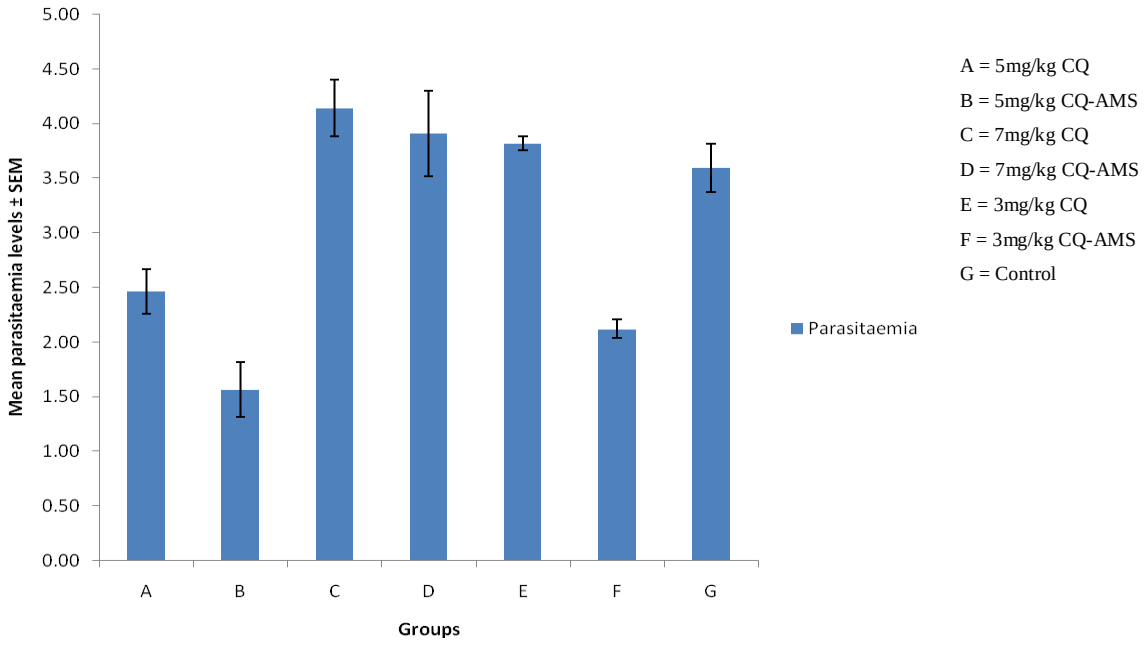



Figure 4: *P.berghei* parasitaemia of mice treated with chloroquine phosphate stabilized with a synthetic Aluminium-Magnesium silicate.

# CHAPTER FIVE

# DISCUSSION

The results showed that Aluminium ï Magnesium Silicate improved ability of chloroquine phosphate to reduce plasmodial parasitaemia at the doses of 5mg/kg and 3mg/kg. This could lead to clearance of the parasite and so reduce chances of development of resistance against the drug (Wellems, 2002).

Vanderbilt (1992) reported that AMS is a stabilizing agent. To stabilize means to protect from destruction. The results of this work suggest that AMS may have protected chloroquine phosphate from being rapidly degraded by metabolic processes. So bioavailability of chloroquine may have been prolonged in the treated mice hence the increased mortality at 7mg/kg and improved clearance of the parasites at 5mg/kg and 3mg/kg.

AMS is also a zeolite (Mineral Gallaries, 2007) and zeolites are reported to deliver drugs to desired targets. So both prolongation of bioavailabilty of chloroquine and delivery of the drug to target may be responsible for the improved effects of the drugs noticed.

Chloroquine has been reported to have no effect on parasites in the liver and other organs and tissues (Hempelman, 2007). It acts only on parasites in the blood. Its toxic effects also lead to both immune suppression and iron deficiency anemia (Emerson *et al*, 2001). Once level of any antimalarial drug, used in treatment, reduces in blood of treated animals, malaria parasites in liver, spleen and lungs return to blood circulation (Emerson *et al*, 2001). It is a possibility that rapid multiplication of parasites that returned to blood circulation, as a result of immune suppression from chloroquine toxicity and reduction in blood volume due to anaemia, were responsible for the high parasiteamia recorded in the two groups treated with chloroquine at the dose of 7mg/kg.

In medical practice, chloroquine is usually given with other drugs, such as iron and vitamins, to help reduce its toxicity and immune suppression. In this work, the supportive treatments were not given. So, immune suppression due to chloroquine toxicity may be responsible for higher parasitaemia seen in the groups treated at 7mg/kg than in the control. Also, there was no mortality in the group of untreated mice infected with *P.berghei* . So, the deaths recorded in those groups treated at chloroquine dose of 7 mg/kg may have also been due to chloroquine toxicity.

The significantly higher parasitaemia of the only survivor in the group treated at 7 mg/kg with chloroquine stabilized in the AMS and the higher mortality rate recorded in that group than that of the group treated with chloroquine alone, suggest that the AMS may also have worsened chloroquine toxicity at 7mg/kg.

RBC counts and Hb values of the groups of mice treated with 7 mg/kg chloroquine alone, 5 mg/kg chloroquine in AMS and 3 mg/kg chloroquine in AMS were high, indicating that those mice had no anemia but the group treated with 5 mg/kg chloroquine alone and the control had slight anemia. Anemia was more pronounced in the group treated with 3 mg/kg chloroquine alone and in the only survivor of the mice treated with 7 mg/kg chloroquine in AMS. Both chloroquine toxicity and plasmodia infection cause anemia (Emerson *et al*, 2001; Wildig *et al*, 2006).

Anemia of the groups treated with 5 mg/kg and 3 mg/ kg chloroquine alone may be due to ineffective treatment of the plasmodial infections while the anemia of the mouse treated with 7 mg/kg chloroquine in AMS may be due to chloroquine toxicity.

The high mean rectal temperature and low mean body weight recorded in the group treated with 3 mg/kg chloroquine alone and in the mouse treated with 7

mg/kg chloroquine in AMS may also be due to ineffective treatment of plasmodia infection and chloroquine toxicity respectively.

The control group had the highest mean body weight, had only slight reduction in RBC count and in Hb. Also mean rectal temperature was slightly high in that group. These findings agree with the report of Carter and Diggs (1977) that plasmodium infection in rats and mice do not produce serious clinical disease.

**Summary and Conclusion:**

Aluminium-magnesium Silicate (AMS) improved ability of chloroquine phosphate to reduce *P. berghei* parsitaemia at lower doses but worsened chloroquine toxicity at 7 mg/kg.

At 5mg/kg chloroquine phosphate, stabilizing the drug with AMS produced best effects against Plasmodium and against the clinical signs of the infection.

**Recommendation:**

With AMS, dose of chloroquine phosphate should be reduced from 7mg/kg to 5mg/kg. Also supporting chloroquine phosphate treatment with administration of vitamins and iron to the sick animal or human being could reduce its toxicity and improve its antiplasmodial effects.

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