Screening of antimalarial drugs: An overview

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ABSTRACT

Efforts to discover and develop new antimalarial drugs have increased dramatically in recent years mainly because of resistance to existing antimalarial drugs. Selection of candidate drugs for clinical trials in man and the design of clinical protocols are based upon consideration of data from a battery of preclinical test systems. All compounds are assessed initially in one or more primary screening models. A compound which is considered 'active' by well established criteria in primary screening test is considered for further evaluation in successively more clinical tests. At the end of each stage of testing, a decision is taken to advance the compound to the next stage or discontinue it. Primary screening tests should have optimal sensitivity, a high degree of reproducibility, high throughput, requiring a minimum quantity of test compound and should bear low cost. As there is growing need for newer and more efficacious antimalarial drugs especially in tropical countries, more sensitive and economical screening models are needed. This review is an update of various conventional and latest *in vitro* and *in vivo* screening methods being used for evaluation of antimalarial compounds

KEY WORDS: Hypoxanthine uptake, rodent malaria model, *Plasmodium berghei*, primate model.

Introduction

Malaria is one of the oldest recorded disease in the world. In the 18th century the Italians associated malaria with 'bad air' – *mala ria* from where the name malaria is derived. It is a protozoal disease caused by parasites of the genus Plasmodium and transmitted to man by certain species of infected female Anopheline mosquito. Malaria remains one of the most important disease of developing world, killing 1-3 million people and causing disease in 300-500 million people annually worldwide.[1] In India, the National Malaria Eradication Programme started in 1958, achieved near complete disappearance of the disease in 1960s. However, due to development of insecticide resistance among mosquitoes and other factors, it staged a comeback in the 70s and continues to prevail in endemic or sub-endemic proportions in different regions. Clinically, malaria manifests as fever, chills, prostration and anemia. Severe form of the disease may lead on to delirium, metabolic acidosis, cerebral malaria, multi organ system failure, coma and death.

Sporozoites inoculated with the bite of mosquito leads to development of blood stage infection (trophozoites) and gametocyte generation which are infectious for mosquito. Gametocytes in human blood are taken up by the mosquito leading to fertilization and zygote formation in mosquito midgut. This is followed by production of haploid sporozoite that invades the salivary glands of the mosquito and is subsequently transmitted back to humans due to the bite.

Since last two decades, malaria control and treatment has been complicated by the emergence of resistance to widely used antimalarial drugs such as chloroquine. Drug resistance has been defined as the ability of parasite strain to multiply or survive in the presence of concentration of a drug that normally inhibit their multiplication or kill the parasite. To combat the problem of resistance, newer drugs are needed. Indeed, an unprecedented number of malaria discovery and development projects are now underway, involving many new drug targets for antimalarial therapy (Table 1). The goal is to develop safe and affordable drugs to counter the spread of malaria parasite resistant to existing drugs. This article is an endeavor in providing hands on information to post graduate and research students about various *in vitro* and *in vivo* screening methods being followed or recommended for antimalarial drug development.

In vitro methods for screening antimalarial compounds

In vitro screens for activity, constitute a key component for antimalarial drug screening. It is based on the ability to culture *Plasmodium falciparum* in human erythrocytes *in vitro*. The development of techniques for continuous cultivation of *Plasmodium falciparum* is a reliable source, for continuous stock culture of parasite, apart from drug screening and long term assessment.

Plasmodium falciparum can now be maintained in continuous culture in human erythrocytes incubated at 38^0C in RMPI 1640 medium with human serum or albumax (a lipid rich bovine serum albumin). Albumax appears to reduce both the rate at which erythrocytes deteriorate *in vitro* as well as

Table 1

Targets for antimalarial chemotherapy

Target location	*Pathway / mechanism*	*Target molecule*	*Existing therapies*	*New compounds*	*References*
Cytosol	Folate metabolism	Dihydrofolate reductase	Pyrimethamine, proguanil	Chlorproguanil	23,24
		Dihydropteroate synthase	Sulphadoxine, dapsone		
	Glycolysis	Thymidylate synthase		5-fluroorotate	25
		Lactate dehydrogenase		Gossypol derivatives	26
		Peptide deformylase		Actinonin	27
	Protein synthesis	Heat shock protein 90		Geldanamycin	28
	Glutathione metabolism	Glutathione reductase		Enzyme inhibitors	29
	Signal transduction	Protein kinases		Oxindole derivatives	30
	unknown	Ca^{2+}-ATPase	Artemisinins		31
Parasite membrane	Phospholipid synthesis	Choline transporter		G25	32
	Membrane transport	Unique channels	Quinolines	Dinucleoside dimers	33
		Hexose transporter		Hexose derivatives	34
Food vacuoles	Haem Polymerization	Haemozoin	Chloroquine	New quinolines	35,36
	Hemoglobin hydrolysis	Plasmepsins		Protease inhibitors	37,38
		Falcipains		Protease inhibitors	39,40
	Free radical generation	Unknown	Artemisinins	New peroxides	41,42
Mitochondrion	Electron transport	Cytochrome c oxidoreductase	Atovaquone		43
Apicoplast	Protein synthesis	Apicoplast Ribosome	Tetracyclines, clindamycin		44
	DNA synthesis	DNA Gyrase	Quinolones		
	Transcription	RNA Polymerase	Rifampin		
	Type II fatty acid biosynthesis	FabH		Thiolactomycin	45
		FabI/PfENR		Triclosan	46,47,48
	Isoprenoid synthesis	DOXP reductoisomerase		Fosmidomysin	49
	Protein farnesylation	Farnesyl transferase		Peptidomimetics	50,51
Extracellular	Erythrocyte invasion	Subtilisin serine proteases		Protease inhibitors	52,53

DOXP, 1-deoxy-D-zylulose 5-phosphate; PfENR, *Plasmodium falciparum* enoyl-ACP reductase.

pH drift when cultures are exposed to ambient air. Continuous culture was made possible by the observation that parasites develop better in a settled layer of red cells with a continuous slow flow of medium over it. In this method, a suspension of human AB group erythrocytes is inoculated with a small amount of *falciparum* infected *Aotus* monkey blood. Type AB blood is used because it can be mixed with *Aotus* blood without danger of agglutination of *Aotus* cells. This suspension is placed in flow vials, which provide flow of medium at 2 ml/hr over the settled cells and an atmosphere of 7% CO_2 and 5% O_2 The cultures are diluted with fresh human red cells on the fourth day and then every third or fourth day as growth continues.[2] Depending on the cell line selected, parasites propagate 3-8 fold every 48 h, thus care must be taken to avoid parasite cultures attaining too high a parasitemia (i.e. percentage of parasitized erythrocytes). Most lines grow optimally at 0.5-4% parasitemia. Parasites are most suitable for drug assays when there is 2-5% parasitemia with mostly ring stages and few or no gametocytes.[3] All stages of the erythrocytic cycle of parasite are present in the culture. Infectivity can be demonstrated by the inoculation of culture material intravenously into splenectomized Aotus monkey.

Culture of *Plasmodium falciparum* is now being used to study the mode of entry of parasite into erythrocytes, screening of new drugs, to isolate and characterize strains and clones, to identify immunogenic antigens and genome of parasite. Several well characterized strains can be made available, either from academic laboratories[4] or through website www.malaria.mr4.org

Materials and Methods

^{3}H Hypoxanthine uptake

^{3}H Hypoxanthine uptake is a standardized model (wherein ^{3}H Hypoxanthine is used (which is taken up by parasite for purine salvage and DNA synthesis) to determine the level of *Plasmodium falciparum* growth inhibition. Radiolabelled hypoxanthine uptake by parasite is an indicator of its growth and multiplication. Parasites are cultured in the presence of different concentration of test compounds in media containing reduced concentration of hypoxanthine, after which ^{3}H Hypoxanthine is added for an additional incubation period before cell harvesting and measurement of radioactivity by a 1205 Betaplate reader. Mean counts per minute (cpm) are generally in the range of 20,000-60,000, with the acceptable minimum of 10,000.

$$\%\ \text{reduction in } {}^3\text{H Hypoxanthine uptake} = 100 \times \frac{(\text{geometric mean cpm of no drug sample}) - (\text{mean cpm of test samples})}{(\text{geometric mean cpm of no drug sample})}$$

Percent reductions are used to plot percentage inhibition of growth as a function of drug concentration. IC_{50} are determined by linear regression analyses on the linear segments of the dose response curve.[4] It is the most commonly used method for assessing antimalarial efficacy of a compound *in vitro.* Its shortcomings are, the method is expensive, complicated and involves usage of radioactive substance.

Giemsa stained slide method (MIC method)

Giemsa stained slide method is a low cost alternative for testing small number of compounds. Parasites are incubated with test compound and then parasitemia of control and treated groups are compared by counting Giemsa stained parasites by light microscopy.

In this model, parasites are incubated in a 5% suspension of erythrocytes with an initial parasite density of 1-2% at 37^{0}C. A sealed incubation chamber, continuously gassed with a mixture of 2% O_2, 8% CO_2, 90% N_2 is used. Increase in the proportion of infected RBCs is assessed at the end of 72 hr incubation period in control samples and at various concentrations of each drug. This method relies on a morphological criterion of response and reports a single concentration as the end point i.e. concentration of a drug in the first sample showing complete inhibition of growth. This measurement is classically known as the Minimum Inhibitory Concentration (MIC), method which is suitable for distinguishing susceptible and resistant isolates.[5]

Other in vitro methods

There are various other *in vitro* methods for assessing antimalarial efficacy of test compounds like Flow cytometry and measurement of LDH activity of *Plasmodium falciparum*. Flow cytometry takes advantage of the fact that human erythrocytes lack DNA. In this technology, parasites are fixed after appropriate period of incubation with test compounds, then either the parasitized cells are stained with hydroethidine (which is metabolized to ethidium53) or the parasite nuclei are stained with DAPI (42, 6-diamidino-2- phenylindole). Counts of treated and control cultures are then obtained by flow cytometry. Appropriate gating can also allow one to distinguish different parasite stages in erythrocyte. This relatively simple assay provides high throughput and has replaced older methods at some centres, but requires expensive equipment. Measuring LDH activity of *Plasmodium falciparum* as an assessment of parasitemia by colorimeter is a less standardized method.[6] This assay is based on the observation that the lactate dehydrogenase enzyme of *Plasmodium falciparum* has the ability to rapidly use 3-acetyl pyridine NAD (APAD) as a coenzyme in the reaction leading to the formation of pyruvate from lactate. Presence of *P. falciparum* at parasitemia levels of 0.02% from *in vitro* cultures can be detected by measuring the development of APADH.

Isobologram analysis

Combination chemotherapy is now being increasingly advocated for the management of malaria. Appropriately chosen combinations can be additive or synergistic and can reduce the selection of drug resistance. To assess the effects of combinations, Isobologram analysis can be performed.[7,8] This is conducted using standard dose response assays over a range of individual drug concentration, using either checkerboard technique[9] or fixed ratio method.[8] This *in vitro* analysis has been useful in identifying clinical combinations like atovaquone and proguanil as well as determining the potential of low activity compounds such as azithromycin.

Micro-test (Mark III)

With the rapid spread of antimalarial drug resistance over the last few decades, the need for monitoring has increased. The most commonly used method for the antimalarial *in vitro* testing for resistance is Micro-test (Mark III).[10] It provides information on the quantitative drug response of *P. falciparum* irrespective of the patient's immune system. The *in vitro* test can be carried out with several drugs, in a Micro test kit with 12 X 8 wells, predosed* with

Chloroquine	:	1 – 64
Mefloquine	:	2 – 128
Quinine	:	4 – 256
Amodiaquine	:	0.25 - 16.0
Artemisinin	:	0.15 - 150.0
Sulfadoxine (SDX)/ pyrimethamine (PYR)	:	10 - 10 000
Pyrimethamine (PYR)	:	0.125 - 125.0

*all drug concentrations are expressed as pmol per well

Patient's blood sample is inoculated in the wells and incubated with suitable medium. The number of schizonts with 3 or more nuclei out of a total of 200 asexual parasites is counted and compared with control well.

For monitoring the level and spread of resistance, molecular diagnostic methods for detecting resistant parasite have been proposed.[11] These methods are suitable for use on a large number of samples in malaria endemic areas and have major advantage over *in vitro* tests that require parasite cultivation which take days to perform.[12] These molecular tools are based on the detection by PCR of point mutation in the parasite genome responsible for *in vitro* resistance.

Advantages of in vitro methods

1. Precise and efficient
2. Rapid
3. Large number of compounds can be evaluated at the same time
4. Synergism or antagonism with drug combinations can be studied
5. Better assessment of intrinsic activity of a drug.

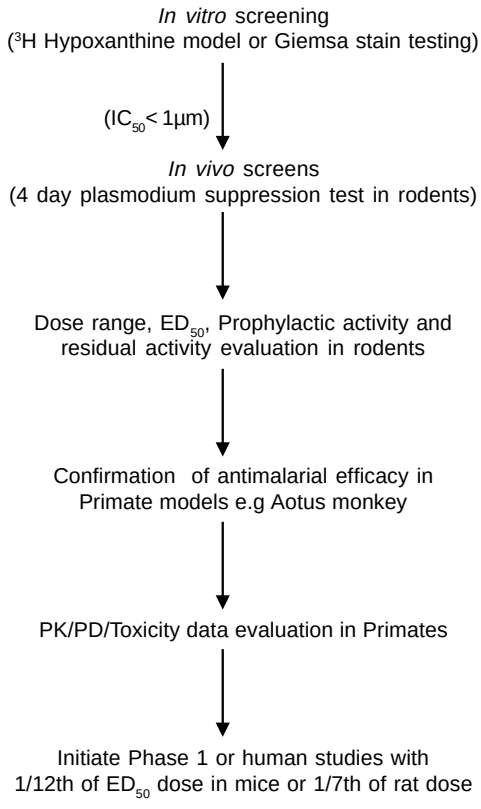
Limitations of in vitro methods

1. Drugs acting through active metabolite cannot be studied.
2. Non reproducibility of pharmacokinetic effects.
3. Toxic compounds also get selected.
4. Expertise and infrastructure needed
5. Lack of clinical correlation.

In vivo methods for screening antimalarial compounds

Compounds effective in *in vitro* screening tests (i.e. those with $IC_{50} < 1\ \mu M$) are taken up for *in vivo* evaluation (Flowchart 1). Plasmodium species that cause human disease are essentially unable to infect non primate animal models. So, *in vivo* evaluation of antimalarial compounds begins with the use of rodent malaria parasite (Table 2). *Plasmodium berghei, P. yoelii, P. chabaudi, P. vinckei* have been used extensively in drug discovery and early development.[4],[13] Choice of rodent malaria species and mouse strains need to be considered during experimental design and interpretation. *P.chabaudi* and

Flowchart 1. Screening process of antimalarial compound.



P.vinckei generate a high parasitemia and produce synchronous infections (propogation of specific stage), enabling studies on parasite stage specificity. *P.chabaudi* and *P.vinckei* are more sensitive than *P.berghei* to iron chelators and lipid biosynthesis inhibitors.[14],[15]

Rodent models:

a) Plasmodium berghei 4 day suppression test:

This is the most widely used preliminary test, in which the efficacy of a compound is assessed by comparison of blood parasitemia and mouse survival time in treated and untreated mice.[2] NMRI (Naval Medical Research Institute) mice free from *Eperythrozoon coccoides* and *Haemobartonella muris* are maintained at 22^{0}C at 50-70% humidity, fed with diet containing *p*-aminobenzoic acid 45 mg/kg and water *ad libitum*. Mice contaminated with *Eperythrozoon coccoides* survive infection with *P. berghei* longer than clean mice whereas the presence of *Haemobartonella muris* tends to accelerate the malaria infection. On day 0, mice are injected with 0.2 ml of aliquot (2X10^7 parasitized erythrocytes, *Plasmodium berghei* ANKA strain) intravenously or intraperitoneally into experimental group (five each). Vehicle treated mice (control group) is compared with the test drug treated group. In the third (positive control) group chloroquine (reference drug) is administered. The drugs are prepared at required concentration, as a solution or suspension containing 7% Tween80/3% ethanol and administered 2-4 hr post infection by appropriate routes.

On day 1 to 3, the experimental groups are treated again (with the same dose and same route) as on day 0. On day 4, 24 h after the last dose (i.e. 96 h post-infection), blood smears from all animals are prepared with Giemsa stain. Parasitemia is determined microscopically by counting 4 fields of approximately 100 erythrocytes per field. For low parasitemias (<1%), up to 4000 erythrocytes have to be counted. The difference between the mean value of the control group (taken as 100%) and those of the experimental groups is calculated and expressed as percent reduction or activity using the following equation:

$$\text{Activity} = 100 - \frac{\text{mean parasitemia treated}}{\text{mean parasitemia control}} \times 100$$

For slow acting drugs, additional smears should be taken on days 5 and 6, to determine parasitemia from which the activity is calculated accordingly. Untreated control mice typically die approximately one week after infection. For treated mice the survival-time (in days) is recorded and the mean survival time is calculated in comparison with the untreated and standard drug treated groups. Mice without parasitemia on day 30 of post-infection are considered cured. Compounds identified as active in this test are progressed through several of the following secondary tests. In the '*Dose ranging full four day test*', compounds are tested at four doses, by different routes of administration, to determine ED_{50} value by plotting the log dose against probit activity. This test also leads to information about relative potency and bioavailability. In the '*onset/recrudescence test*', mice are administered with single dose of the test compound on day 3 of postinfection, subcutaneously. Control mice receive the suspension vehicle alone. Blood smears are prepared at intervals of 12 h, 24h and then daily till day 33, Giemsa stained and assessed for parasitemia. Results are expressed in terms of rapidity of onset of activity, time to onset of recrudescence, increase of parasitemia and duration of survival (in days). Compounds are also tested for prophylactic activity by administrating them prior to infection, followed by daily examination of smears.

b) Hill's test for causal prophylaxis and residual activity:[13]

In this model, mice (Charles River strain) are inoculated with *P. yoelii* (N67 strain) sporozoites from *A. stephensi* (Edinburgh strain). Sporozoites are harvested 8-11 days after the infected blood meal. Whole mosquitoes are grounded in a Tenbroech grinder with Tyrode's Ringer solution. Each mouse receives approximately 10^4-10^5 sporozoites intravenously in a total volume of 0.2ml. For a compound to be considered truly causal prophylactic it must pass through four different phases, the last of which meticulously tests for residual effects upon blood stage parasites.

Phase 1. This basic procedure involves detection of causal prophylactic activity of the test compound in mice. Test compound is administered 3hr after a sporozoite inoculation. During the ensuing 14 day period, blood films are taken until 2% parasitemia is achieved. If parasitemia is not detected for 14 days the compound is considered to be fully protective.

Phase 2. Compound is then tested for residual activity directed against blood stage parasites by administrating a single dose of the test compound 48hr before 10^4 trophozoites are injected intravenously. If the time interval to reach 2% parasitemia is similar to that of the control group,

Table 2

Characteristics of rodent malaria infections

Plasmodium species	*P. berghei*	*P. yoelii*	*P. chabaudi*
First isolated	1948 (Zaire)	1965 (CAR)	1965 (CAR)
Cycle	asynchronous	asynchronous	synchronous
Periodicity	22-25 h	22-25 h	24 h
Host cells	reticulocytes	reticulocytes	Mature RBC
Merozoites per schizont	12 to 18	12 to 18	6 to 8
Primary use	Drug screening	Liver stage biology and vaccine studies	Mechanisms of drug resistance & antigenic variation

then it is considered that no residual activity has occurred.

Phase 3. Compounds suspected of prolonged residual activity are tested by administering sporozoites followed by the drug 3 h later. After an additional 48h have elapsed, 0.2 ml blood is removed from each mouse and injected intraperitoneally into a clean mouse. Blood films from recipient mice are examined for a 14 day period or until patency develops. Residual activity is noted if less than 50% of the recipients develop parasitemia. A compound has no residual activity if 75% or more recipient mice develop patent infection.

Phase 4. An additional procedure is also done to clarify whether or not a compound has residual effect on erythrocytic stages during the 48hr period of drug exposure *in vivo*. If no residual effect is seen then these parasites remain infective. To determine if the erythrocytic parasites are still viable, mice are injected intravenously with 10^4 trophozoites 48 h after the compound administration. After an additional 3-4 h, 0.2ml of blood is removed and injected to clean recipient mice. Blood films are taken and a comparison of the time interval to reach 2% parasitemia is made with control mice, if the time interval is similar, it reflects that no permanent damage has been done to the parasites and thus no residual activity is present.

Sporonoicidal activity testing

Eperythrozoon free albino mice are administered with i.p inoculum of *Plasmodium yoelii nigeriensis* N 67 parasite on day 0. Female *A. stephensi* mosquitoes are placed in containers (about 25 in each container) and fed with 4% sucrose for the first five days after hatching. They are kept at a constant temperature of 24^0C and 75% humidity. On third day following infection by parasitized blood(10^7 infected red cells), mosquitoes which were starved for 24 h are allowed to feed on mice. Before mosquitoes are allowed to feed, presence of mature gamets is confirmed on blood film of mice. The mice are then anaesthetized with a single dose of 60 mg/kg of sodium pentobarbitone i.p. and laid on top of mosquito containers, so that the insects can feed on them through the gauze or membrane covering container for about 30 min.[13]

On the seventh day after the blood feed, a sample of each batch is removed and dissected. Midguts are examined with the aid of semi-dark ground illumination and oocyst counts are made. The mean count for each batch is calculated. To determine the inhibitory effects of a drug on oocyst development, it is dissolved in 4% sucrose and fed *ad libitum* to the insects following the blood meal. Usually a batch of 10 surviving *A. stephensi* for each drug concentration and for each control is sufficient to provide data from which the level of drug activity can be calculated from a comparison of mean oocyst counts in treated and control batches.

A new *in vivo* mice model for antimalarial efficacy is recently designed which comprises of use of immunocompromised mice.[16] It can support *Plasmodium falciparum* infection as it lacks T and LAK **(**Lymphokine activated killer cell**)** cells. Immunodeficient mice have been widely used as xenogenic transplantation models allowing *in vivo* investigation of human cells and organs. In this model, *P. falciparum* parasitized human red blood cells (*P. falciparum*-huRBC) can be grafted into immunodeficient BXN laboratory mice *(P. falciparum*-huRBC-BXN). For immunomodulation dichloromethylene diphosphate (Cl_2MDP) is administered and is responsible for reducing tissue macrophages. The increase in polymorphonuclear neutrophils which occurs with parasitic infection is controlled by using an NIMP-R 14 monoclonal antibody. This is the first rodent model in which *Plasmodium falciparum* infection can be maintained. Test drugs are administered when parasitemia becomes stable for 6 days and smears show predominance of ring forms.

Avian models

At least 447 species of birds have been found infected with malarial parasite. The avian parasite infects nucleated erythrocytes whilst the mammalian species infect non-nucleated blood cells. The invertebrate vectors of avian species are usually mosquitoes of the genera *Aedes* or *Culex* and the mammalian species are confined to the *Anopheles* host. Most striking are the pre erythrocytic stages of the avian parasites, which are found in the mesodermal tissues and require two generations in the host, whereas the mammalian species are found in the liver parenchyma cells with only one generation. The effect of quinine on avian malaria was reported by Wasielewski in 1904, parasites being killed *in vitro* by a concentration as low as 1:10,000 at room temperature.[17] Most of the early experiments on bird malaria was carried out in sparrows, chaffinches, pigeons, linnets, larks and canaries. However, the utility of *P. gallinaceum* and *P. lopharae* as experimental models has led to young chickens and ducks becoming hosts of choice. Young chickens 5-7 day old are the most suitable, being susceptible, easy to handle and only weighing about 50 gm, thus proving economical.

Over time immemorial there have been many attempts to

treat malarial fever with the wearing of charms and bracelets. It is interesting to note that Baranger and Filer (1953) using rings, bands or spirals of various metals placed around chicks infected with *P. gallinaceum* found some protective effects.[17],[18] Not only was there a prolonged survival time when compared with the untreated birds, but the asexual blood parasites were also retarded. Even more intriguing was that the removal of the band was followed by an increase in parasitemia on the following day! Copper, iron or gold were the most effective.

Method: Direct inoculation of infected blood is usually employed for routine maintenance or to inoculate a number of animals for chemotherapy experiments. When only one or two birds need to be infected, a sufficient quantity of parasitized blood can be obtained from the leg vein or wing vein of an infected bird. When large number of chicks is to be infected from a single donor, blood is aspirated directly from the neck vein or from the heart. The total number of parasitized erythrocytes used to infect experimental animals is dependent on virulence of the strain and the route of inoculation. For production of slow infections which takes around 7 days to become patent, parasitized erythrocytes are injected into peritoneal cavity directly below the sternum, intramuscularly into the breast muscle, or subcutaneously into the neck region. For production of fast, easy and uniform infection, parasitized erythrocytes are injected intravenously into the neck vein; this fast infection leads to death in 5 to 7 days. Drugs may be administered to chicks orally, intravenously or intramuscularly, however, the oral route is the method used more frequently. Experimental birds are infected intravenously and receive the first dose of drug on day 1 followed by twice daily for the next 3 days. Blood films are made on day 4. Parasitemia achieved on day 4 in treated and untreated control group is then compared.

Avian models have become unpopular primarily due to the introduction of the rodent models, as mammalian models are more relevant to human infections.

Primate models

Owl monkey (*Aotus trivirgatus*) and the squirrel monkey (*Saimiri sciureus*) have served as experimental models for antimalarial drug research.

Utility of primate models

1. Confirmation of rodent efficacy results.
2. Provide clear prediction of drug effect in humans.
3. Checks vaccine efficacy

These primate hosts are primarily used for screening of antimalarial drugs. They also serve as faithful models to investigate various complications associated with malaria. *Aotus* is one of the WHO recommended model for studies in malaria, and these are the only models which can sustain malarial infection caused by *Plasmodium falciparum* and *P. vivax.*

Plasmodium cynomolgi rhesus model

This model runs a close parallel to *P. vivax* infection in man. It can be used to evaluate causal prophylactic, blood schizonticidal and hypnozoitocidal activity of a test compound in one model only.[19] Young, tuberculin negative rhesus monkeys weighing 3.5 to 6 kg are used. Infected anophelines are grounded in a 1:1 mixture of saline and normal monkey serum and gross debris is removed by light centrifugation. Each animal receives i.v. inoculum of approximately 500,000 sporozoites in 2 ml of fluid. Drugs suspended in 50ml of water are administered through a stomach tube once daily, commencing the day before infection and continuing up to day 8. Blood films are made daily till 4-6 weeks. At the end of 6 weeks if the animals are still negetive, they are rechallenged with a similar inoculum to prove their susceptibility to infection. All animals becoming infected during 4-6 weeks observation period and also infected animals from rechallenged group are further treated as soon as the parasitemia level reaches 0.1 to 0.5%.

The animals receive 7 daily oral doses of the drug and blood films are examined daily during and after therapy up to a total of 30 days. If still negative, the animals are then examined twice weekly until they relapse. If the parasites are not cleared by the drug during the primary attack, the animals are given 7 daily doses of 5mg/kg of chloroquine. If no relapse follows chloroquine administration, it indicates that the test drug has destroyed the hypnozoites. When no relapse occurs within 8-12 weeks, the animals are splenectomized. Failure to develop further parasitemia within 4 weeks of this procedure indicates that the animals are radically cured since, in 99% of those that are not splenectomized, the infection relapses within 3 weeks of splenectomy.

Since last 10 years, considerable progress has occured in the development of malarial vaccine. More than 40 distinct antigens in the various cycle stages of the parasite have been proposed as potential vaccine candidates. Owl and squirrel monkeys are excellent models for testing vaccines. Various studies have shown that there are three readily apparent advantages of monkey trials over human trials with proposed malarial vaccine: they provide efficacy data before clinical grade production; they might prevent the need for existing field trials; they might provide the data to validate *in vitro* assays.[20]

The Clinical Trials Center at the Walter Reed Army Institute of Research, Silver Spring is involved with ongoing Challenge study. The Challenge study is being conducted to determine safety and immune response of candidate malarial vaccine.[21] After vaccination, it is important to see whether the vaccines are able to protect people against malaria. In order to do this, volunteers are infected with a fully drug-sensitive strain of malaria. In addition to vaccinated volunteers, a number of unvaccinated controls take part in the Challenge study to make sure that the infection system works and to act as a comparison. Volunteers are exposed to the bites of five malaria-infected mosquitoes. These mosquitoes are placed in paper cups onto which the volunteers rest their arm for five minutes. Untreated malaria could be very serious and so the volunteers are followed-up very carefully (up to twice per day). At each of these visits a blood sample is taken and examined for malaria. At the first sign of malaria, the volunteer is immediately treated with the antimalarial drug chloroquine. Volunteers getting full protection from the malaria vaccine would not develop the disease.

The aim of the malaria Challenge study is to establish whether vaccination regimes offer protection against malaria infection. Protection may be 'partial' or 'complete': Complete

protection is where vaccinated volunteers do not develop malaria in the Challenge study. Naturally, all the unvaccinated control volunteers develop malaria so that it becomes very clear that the infection system has worked. Partial protection is when there is a delay in the onset of malaria in the vaccinated volunteers compared to the unvaccinated controls. This means that the body's immune system is controlling the infection to start with but is ultimately overwhelmed.

Malaria vaccine development is a very difficult and challenging task due to various reasons. First, the size and genetic complexity of the parasite presents thousands of antigens to the human immune system. Due to antigenic diversity, identification of a useful target for vaccine development has been complicated, second, the parasite changes through several life stages even while in the human host, presenting a different subset of molecules for the immune system to combat with each stage. Third, the parasite has evolved a series of strategies that allow it to confuse, hide, and misdirect the human immune system. Finally, it is possible to have multiple malaria infections of not only different species but also of different strains at the same time.[22]

Conclusion

Malaria models have been established in a variety of laboratory animals, short and long term plasmodium culture systems have been extensively used for chemotherapeutic studies. A variety of avian, rodent, simian and human plasmodium models are used for screening and evaluation of candidate antimalarial compounds. The choice of malaria model depends upon sensitivity, reproducibility and breadth of response to known antimalarial drug and also on practical consideration such as required rate of testing, technical complexity, quantity of test compound needed and cost per test. Host factors such as natural resistance and immune competence also influence the efficiency of test compounds in different models. Each malaria model system has its individual characteristics, and no single model exists which can be said to be entirely predictive for humans.

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