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Research Article

Comparison of Two Antimalarial Brands on Plasmodium Culture

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Abstract

Malaria is a major threat to the public health and economic development of many nations. While *P. falciparum* causes most malaria-induced mortality worldwide, *P. vivax* is the major cause of malaria morbidity. In this study we have compared two different brands of Arteether (EMAL and MATCH) for treating *P. vivax* initially. Arteether™ is a useful drug against chloroquine-resistant *P. vivax* malaria, without an increased incidence of toxicity. It has a longer half-life and has more lipophilic properties than artemether™, which aids its accumulation in brain tissues for the treatment of cerebral malaria. The study revealed that there was a significant difference in the effectiveness of these two formulations even though they were labeled to have similar indications. These indicate the need for prior examination of these kinds of products for their intended activity. The results of this study have provided evidence to support the better effectiveness of EMAL- brand over MATCH-brand. As EMAL has less viscosity, very good redispersibility, larger zone of inhibition. By observing all of these properties we can conclude that EMAL is a better formulation than MATCH, and can show better antimalarial property in patient suffering from Plasmodium malaria.

Keywords: Antimalarial, Plasmodium species, Zone of inhibition, Indications, Mortality, Half-life

INTRODUCTION

On a global scale, malaria has been and remains a major public health concern¹. The disease is caused by parasitic protozoa of the genus *Plasmodium*. The life cycle of this organism is complex, with the parasite alternating between sexual reproduction in an invertebrate (mosquito) host and asexual reproduction in a vertebrate host². In addition to mammals as vertebrate hosts, birds and reptiles also serve as hosts for malarial parasites. The portion of the life cycle in the mosquito is the sporogonic phase, leading to formation of sporozoites which are injected by the vector into the vertebrate host at time of feeding. Sporozoites give rise to the schizogonic phase, with proliferation of the parasites in erythrocytic and exoerythrocytic sites. The parasite is extracellular during its sporogonic phase, shifting to an intracellular location during the schizogonic stages of development. In vitro cultivation of the parasite requires simulating conditions in the mosquito vector for the sporogonic phase of the life cycle and, for the schizogonic phase, conditions promoting growth in exoerythrocytic and erythrocytic locations of the vertebrate hosts. In this review, we attempt to bridge some of the earlier literature to recent developments relating to in vitro growth of *Plasmodium* spp. Both human and non-human malarial parasites are dealt with in this section, even though the latter are not of clinical significance. Non-human *Plasmodium* spp. share nutritional characteristics with their human counterparts, and have often served as models for cultivation of species infecting humans. Since this review does not deal in detail with the extensive literature on cultivation of *Plasmodium* spp., the interested reader is referred to the

more comprehensive treatments of cultivation of the various stages in the parasite life cycle (see, for example, references^{3,4} and for reviews of the exoerythrocytic, erythrocytic, and sporogonic stages, respectively)⁵. When introduced into the bloodstream of a vertebrate host by a mosquito, sporozoites enter into the exoerythrocytic phase of development. In mammals, this occurs in hepatic cells, while in avian hosts, the parasites invade cells of the reticuloendothelial system². In mammalian malarial, the invasive stage for erythrocytes, the merozoite, passes through a developmental sequence beginning with a characteristic ring stage and leading to formation of a multinucleate schizont. Further development of the schizont leads to formation of multiple merozoites, which upon rupture of the infected host cell, invade other erythrocytes. In avian malarial, after exiting from cells of the reticuloendothelial system, the merozoites can either reinvest reticuloendothelial cells or enter erythrocytes. Parasites can be recovered from the peripheral bloodstream of the vertebrate host, where they go through a cycle of repeated invasion of erythrocytes with characteristic synchrony to increase the level of parasitemia in the host bloodstream. Humans are infected by four species of malaria parasites: *P. falciparum*, *P. vivax*, *P. ovale*, and *P. malariae*. To a greater or lesser extent, all four species have been cultured or maintained in vitro; *P. falciparum*, however, is the only species for which all life cycle stages have been established in culture⁶. That's why artemisinins (a new class of antimalarial) are now available for use because it has shown little or no resistance yet⁷. It is more costly than other drugs, but they provide an effective and potential treatment to roll back malaria.

However, if we use this class intensively as a monotherapy, resistance may evolve quickly (as like with other drugs).

EXPERIMENTAL WORK

Preparation of malaria culture

Malaria culture is the method to grow malaria parasites outside the body *i.e.* in an *ex vivo* environment. *Plasmodium falciparum* is currently the only human malaria parasite that

has been successfully cultured continuously *ex vivo*. Although attempts for propagation of the parasites outside of humans or animal models reach as far back as 1912⁸, the success of the initial attempts was limited to one or just a few cycles. The first successful continuous culture was established in 1976⁹. Initial hopes that the *ex vivo* culture would lead quickly to the discovery of a vaccine were premature. However, the development of new drugs was greatly facilitated¹⁰.



Figure 1: Malaria culture

Preparations

Prepare malaria culture medium (MCM) and Tris-buffered Hanks' (TH) for washing cells.

Albumax complete medium:

10.43 g RPMI 1640 powder (Gibco)

25 ml 1 M HEPES solution or 6 g HEPES (Gibco)

2 g NaHCO₃

0.5 ml gentamicin (from 50 mg/ml stock)

5 g Albumax II

Add distilled water to 1 liter. Filter-sterilize. Use within 10 days, store at -20 °C.

Comment: For growing parasites from patient blood, use 10 g of Albumax for 1 liter of complete MCM. The vast majority of cultures will survive at least 2 weeks. It is also important to avoid serum in the culture for preparation of crude parasite antigen, all strains can be adopted to Albumax II medium^{11,12}.

Alternative MCM:

10.43 g RPMI 1640 powder (Gibco)

25 ml 1M HEPES or 6 g HEPES

2 g NaHCO₃

0.5 ml gentamicin (from 50 mg/ml stock)

Add distilled water to 1 liter. Filter-sterilize and store at -20 °C in 45-mL aliquots.

For complete MCM (cMCM), add 5 ml of human blood type AB+ serum (inactivated at 56°C for 60 min; then stored at -20°C) to 45 ml of medium. Complete MCM can be used for up to one week if stored at 4°C.

MCM can also be made from commercial liquid RPMI with sodium bicarbonate and HEPES buffer (Gibco). Just add 5 ml of 100× L-glutamine (Gibco) and 0.25 ml gentamicin (Gibco) to a 500 ml bottle of the RPMI.

TH (0.15 M Tris-buffered Hanks') (pH 7.2):

2.11 g Tris-HCl

0.2 g Tris-base

7.88 g NaCl

Dissolve in distilled water and bring volume to 1 liter. Mix 1 volume of Tris buffer with 1 volume of Hanks.

In vitro cultures in tissue-culture flasks

- Wash the erythrocytes 3 times in TH or RPMI 1640 to remove CPD, serum, and leukocytes if present. Dilute to 5% hematocrit with cMCM in small flasks of 25 cm² (0.2 ml of packed cells to 4 ml of cMCM) or in 75-cm² flasks (1.0 ml to 20 ml).

- Add parasites to an appropriate parasitemia.

- Put the flask in a candle jar and loosen the screw cap. Produce low oxygen by burnt out candle and place the jar at 37 °C.

- Replace the MCM every day (not necessary the day after subcultivation).

- Subculture the cultures 2 times/week.

Subcultivation

- Stain a drop of the culture with acridine orange (10 µg /ml) on a glass slide and put on a coverslip or by Giemsa staining of a thin smear.

- Count the parasitemia (*i.e.*, the percentage of infected cells).

- Prepare freshly washed O+ blood in cMCM (5% hematocrit) and add it to the culture to obtain a parasitemia of not more than 1%, preferably 0.1 to 0.5% if two cycles until next subculturing, 0.5 to 1% if one cycle. Parasitemia should never exceed 15%^{13,14}.

Method

Candle jar

Infected human red blood cells are incubated in a culture dish or flask at 37°C together with a nutrient medium and plasma, serum or serum substitutes¹⁵. A special feature of the incubation is the special gas mixture of mostly nitrogen (93% nitrogen, 4% carbon dioxide, 3% oxygen) allowing the parasites to grow at 37°C in a cell incubator¹⁶. An alternative

to gasing the cultures with the exact gas mixture is the use of a candle jar. The candle jar is an airtight container in which the cultures and a lit candle are placed. The burning candle consumes some of the oxygen and produces carbon dioxide (CO₂), which acts as a fire extinguisher. Carbon dioxide content in fresh air varies between 0.036% and 0.039%, at an app. 5% CO₂ concentration the candle stops burning. The number of parasites increased by a factor 5 approximately every 48 hours (= one cycle). The parasitemia can be determined via blood film, to keep it within the wanted limits, the culture can be thinned out with healthy red blood cells¹⁷. The original method for the successful *ex vivo* propagation of *P. vivax* described culture parasite under static conditions (Trager-Jensen method)⁹. James B. Jensen joined Trager's laboratory as a post-doctoral fellow in 1976. He decided to employ a candle jar instead of the CO₂ incubator. In the summer of 1976 Milton Friedman, a graduate student in the Trager lab who was working in the MRC laboratories in The Gambia arranged for a sample of human blood infected with *P. vivax* of sent New. This was diluted with RPMI 1640 (which turned out to be the best of the commercial media) in Petri dishes, placed in a candle jar and incubated. The line grew very well and became FCR-3/Gambia, one of the most widely used strains. Later, other lines would be established using similar methods and the impact of continuous cultivation of *P. vivax* phenomenal especially for the testing of putative antimalarials and for deciphering its genes¹¹. A number of subsequent reports (from as far back as the early 1980s), showed that cell suspension (using a shaking-incubator) significantly increased culture growth. Continuous agitation has also been shown to improve other parameters of culture growth relevant to researchers, such as the prolongation of culture synchrony after synchronization procedures, and a reduction of the rate of multiple infections. Despite this, the practice of culturing the parasite under static conditions remains widespread. The greatest value of the candle jar method is that it can be used in laboratories almost anywhere in the world where there is an incubator, a candle and a desiccator¹⁹. Around 60% parasitized cells can be obtained using optimized culturing conditions⁷. Recent studies of *P. falciparum* isolated directly from infected patients indicate that alternative parasite biological states occur in the natural host that are not observed with *ex vivo* cultivated parasites²⁰.

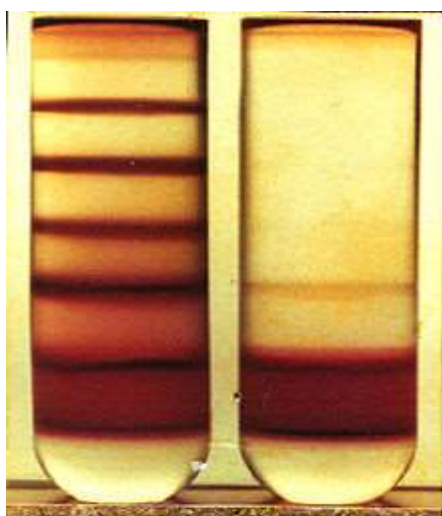


Figure 2: Concentration of *P. vivax* infected erythrocyte discontinuous density gradient centrifugation in Percoll¹⁸

Concentration of infected cells

To achieve synchronization and/or concentration of the parasites in culture several methods have been developed. A discontinuous Percoll gradient procedure can be used to

isolate infected red blood cells because red cells containing plasmodia are less dense than normal ones. Young trophozoites coincided with erythrocytes in a broadband corresponding to densities from 1.075 to 1.100 g/ml, whereas schizonts were concentrated at a density approximating 1.062 g/ml²¹. There are studies, however, that suggests that some strains of *P. vivax* affected in their capacity of invasion after being exposed to this chemical. The difference between diamagnetic low-spin oxyhemoglobin in uninfected red blood cells and paramagnetic hemozoin in infected red blood cells can also be used for isolation. Magnetic columns have shown to be less harmful for the parasite and are simple and adjustable to the needs of the researcher^{22, 23}. The column is mounted in a potent magnet holder and the culture flowed through it. The column traps the erythrocytes infected with the latest stages of the parasites, which can then be eluted when the column is removed from the magnet. It is a simple method that does not need expensive equipment and it does not seem to affect the parasites as to their invasion capabilities afterwards²⁴.

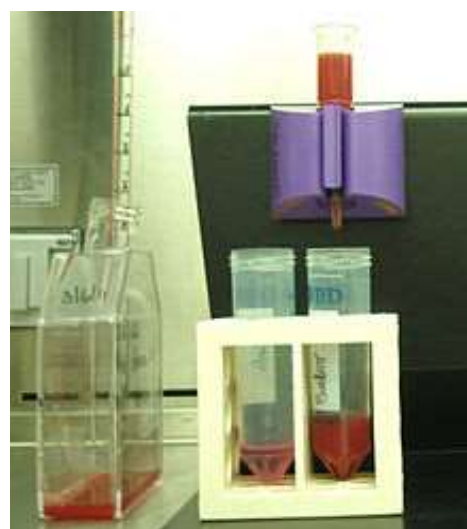


Figure 3: Magnetic collection of *P. vivax* blood^{24, 25}

RESULTS AND DISCUSSIONS

Preliminary examination

The result of above study are summarized in table preliminary evaluation of the products showed that sample B was having less viscosity along with a very good redispersibility as compared to sample A.

Sr. No.	Parameters	Sample A (MATCH)	Sample B (EMAL)
1	pH	5	4
2	Weight per ml (mg/ml)	70	70
3	Viscosity (cp)	61	52
4	Redispersibility	61	50

No. of inversions through 180 degree required uniformly redisperse the sediment layer

Antimalarial activity

Sterile cup and bouror method were used to compare both brands. Hole were made in organism impregnated nutrient blood agar plates were incubated at 37 degree for 24hr and different concentration of sample A and B were placed in two different agar plates, and the zone of inhibition was measured after inoculation, the plates were incubated for 48hr and zone of inhibition was measured. The study revealed that there was

a significant difference in the effectiveness of these two formulations even though they were labeled to have similar indications. These indicate the need for prior examination of these kinds of products for their intended activity.

Antimalarial activity of alpha beta arteether brands

Zone of inhibition (mm)

Sr. No.	Concentration	Sample A (MATCH)	Sample B (EMAL)
1	10mg	5	7
2	15mg	7	8
3	20mg	11	13
4	25mg	14	16

CONCLUSION

The results of this study have provided evidence to support the better effectiveness of EMAL- brand over MATCH- brand. These can be proving on the basis of following points:- EMAL having less viscosity as compared to MATCH which ensures less painful administration. EMAL having very good redispersibility as compared to MATCH which offers uniform dispersion of drug. EMAL shows larger zone of inhibition as compared to MATCH which shows better effectiveness. By observing all of these properties we can conclude that EMAL is a better formulation than MATCH, and can show better Antimalarial property in patient suffering from Plasmodium malaria.

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