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Original Research Article

Cultivation of malarial parasites: Conceptualising one of the method of diagnosis of malaria

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Abstract

Background: Malaria is one of the diseases where successful diagnosis can lead to successful treatment and would be able to save lives. Cultivation of malarial parasite in a laboratory can aid to diagnosis and research.

Materials and Methods: We are sharing experience of cultivation of malarial parasite in 6 patients who had heavy parasitemia and 6 patients with scanty parasitemia. Successful cultivation could be achieved by continuous culture method using medium RPMI-1640. The cultivation was ended at 48 hours.

Results: The immature schizonts were formed at 24 hours, mature schizonts as 30 hours and rupturing schizonts into new merozoites at 2 days.

Conclusion: In cases of sparse parasitemia, the rings became visible in an enlarged form and thus could be used as one of the methods of diagnosis like we have it for bacterial culture.

Keywords: Cultivation of malarial parasites, Medium RPMI-1640, Malarial schizonts, Scanty malarial parasitemia.

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1. Introduction

WHO malaria report 2023, states that there were an estimated 249 million cases of malaria all over the world. This occurrence is mainly in 85 million malaria endemic areas. Though malaria case incidence has stalled or declined, there are still 89 countries where malaria is endemic.[1] This is one infection where prevention and successful treatment is available but still has substantial health and economic burden, particularly on less developed countries where they are endemic.[2] This disease, with high morbidity and mortality in tropical and subtropical countries, is caused by parasites of the genus *Plasmodium* (and transmitted by a bite of an infected female mosquito of the *Anopheles* species). As travel has become very common now a day, non-immune visitors are at risk to develop malaria. Vivax malaria has more complex agent-host relation, due to hypnozoites, dormant form remaining in liver for long time and causing relapsing malaria.[3] Again, malaria may behave differently in various host factor diversity.[4] Thus, diagnosis of malaria, which can be lifesaving at times; is very important. Unfortunately there is diversity in agent, host and in interaction between them. This makes clinical presentation different; in different individuals. Agent diversity in form of falciparum, vivax, malariae and oval species of human malaria is known, however zoonotic malaria is also threat in recent times.[5]

Since the discovery of Laveran in 1880 of malarial parasites in the blood; examination of blood smear has remained gold standard for diagnosis of malaria even today. Since Automated Hematology system can detect the presence of malaria parasite with high sensitivity, it can be a good option for presumptive diagnosis in endemic areas. Microscopy remains the gold standard to confirm MP in suspected patients. Rapid diagnostic tests have acceptable

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sensitivity and specificity. Thick-thin smear is one of the most rewarding laboratory investigations.[6]

Polymerase chain reaction (PCR)-based assays are used for malaria diagnosis and are considered to be the most sensitive tests; however, the cost may be a factor.[7] The field implementation of advanced techniques for the diagnosis of malaria is a challenge. Again, five to six separate PCR reactions are involved to detect different strains of plasmodia. It may be labor and time consuming. Cross contamination is also a problem. Multiplex PCR systems can be the solution but validation and further research may be needed in laboratory and also in the field for of its applicability.[8]

Cultivation of malaria parasite in laboratory condition could be used for diagnosis and drug sensitivity testing as a macro test. Further research may be needed to involve malaria culture by automated culture system. Idea to present this work is to communicate our idea and experience, such that recent technology inputs can generate a technique of cultivating malaria parasite in an innovative way.

The aim and objective of the study was to cultivate Plasmodium falciparum in artificial media such that it can be used for further diagnostic and research purposes.

2. Materials and Methods

The patient's blood whose peripheral smear showed malarial parasite by conventional microscopy was taken for further cultivation. Cultivation of only *Plasmodium falciparum* parasite was undertaken in a selected group of 12 patients of whom 6 had showed heavy parasitaemia and 6 showed scanty parasitemia on peripheral smear and those who had not taken any anti-malarials.

The selected 12 patients' peripheral blood were examined by staining with Giemsa stain. The thick smear was examined for presence of parasites and thin smear was made for species diagnosis. The following method was adapted for cultivation-

1. 10 ml of venous blood was taken with all aseptic precautions in a sterile test tube. 8 ml of blood was transferred to another test tube to collect autologous serum.
2. 2 ml blood, under sterile condition was transferred to a sterile flask containing glass beads in the inoculation hood. This was defibrinated by rotation of the flask for 5 minutes.
3. Medium RPMI-1640 was reconstituted and sterilized by filtration method, 1 ml of medium was transferred to each sterile screw capped bottles. In these bottles, 9 ml sterile double distilled water was added.
4. Heparin, Gentamicin & Na- bicarbonate was added pH was checked. (7-7.2).
5. Patient's blood was added in the above bottles with 5% autologous serum of the same patient for a good growth of the parasites.
6. This was incubated for 48 hrs. in 10% C02 jar at 37-degree temperature in incubator.
7. Within 24hrs to 48hrs, thick and thin smears were prepared and observed for development of mature schizonts. Before and after using of inoculation hood, it was sterilised by ultra violet light and also cleaned with spirit.
8. The cultivation was done for 48 hrs in each case.
9. Smear was prepared at various time after 24 hrs to find out growth of blood stages of malarial parasite.

3. Results

Observation and analysis of cultivation of P. falciparum in artificial medium- RPMI 1640. [n=12]

1. Cultivation of malarial parasite was successfully done on RPMI 1640, tissue culture medium and continuous serial observation of growth of the malarial parasite could be done.
2. Immature schizonts developed at 24hrs incubation period, fully mature schizonts developed at 30hrs, rupturing of schizonts into new merozoites was seen on 36-48hrs incubation. (**Figure 1**-**Figure 3**)
3. One of the observations which was been made that in patients who have very scanty parasitaemia or very scanty ring which was difficult to interpret on conventional microscopy, the culture method could make them easily detectable. This would be because the rings would become bigger and other stages of falciparum parasite can be visualized which is difficult to detect on peripheral smear because of the process of sequestration. (**Figure 4**-**Figure 5**)

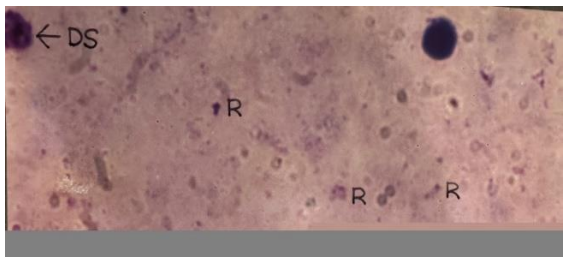

Figure 1: Thick Smear (Giemsa Stain, 1000x) showing developing schizonts (DS) and rings at 24 hrs in RPMI-1640 culture medium

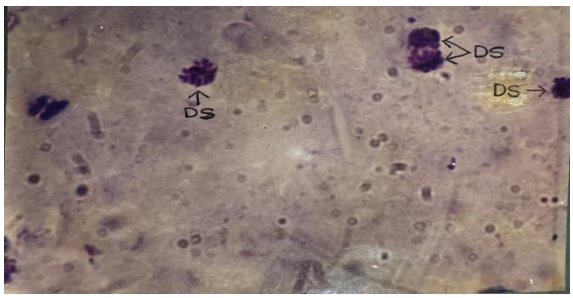

Figure 2: Thick Smear (Giemsa Stain, 1000x) showing developing schizonts at 28 hrs in RPMI-1640 culture medium

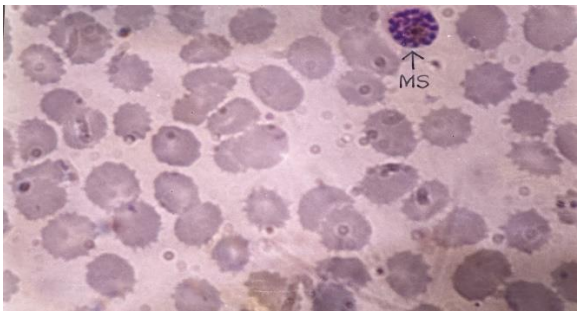

Figure 3: Thick Smear (Giemsa Stain, 1000x) showing (MS) developing mature schizonts at 30 hrs in RPMI-1640 culture medium

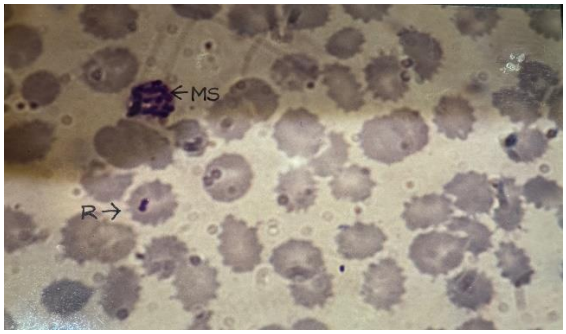

Figure 4: Thick Smear (Giemsa Stain, 1000x) showing (MS) developing mature schizonts and rings mature schizonts at 32 hrs in RPMI-1640 culture medium

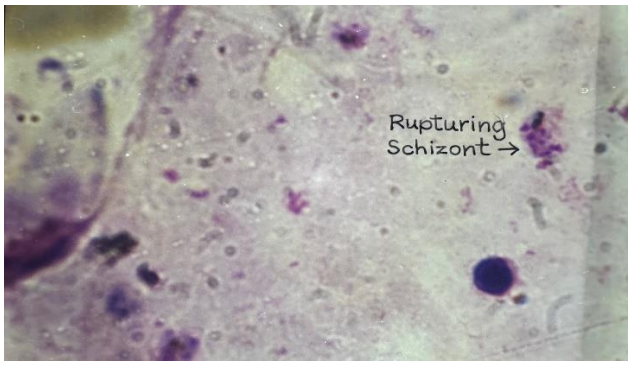

Figure 5: Thick Smear (Giemsa Stain, 1000x) showing rupturing schizonts at 36 hrs in RPMI-1640 culture medium

4. Discussion

As the rapid diagnostic method has come up, the cultivation of malarial parasite has not been studied deeply and the available literatures are sparse regarding this topic.

Continuous culture of P. falciparum is possible which was done in this study.[9] This type of cultivation is one of the pre-requisites for vaccine development, drug sensitivity testing, drug development and various other research on malaria parasite.[10] As a post doctorate thesis and research by first author of this paper, we established the method of cultivation of malaria parasite in 1998.[11]

In vitro cultures of malaria parasites were attempted in 1912 by Bass and Johns; they and their followers obtained a limited multiplication of human Plasmodia.[12] They found that young trophozoites (ring forms) of P. falciparum would develop to schizonts when supported in vitro with 0.5% glucose. Trigg P.I. developed continuous perfusion system for cultivation of malaria parasite inside RBC.[13] Researcher concluded that when pH of the medium was kept constant, the second generation of schizonts can develop. Short term in vitro cultivation was widely and successfully used to study the biochemistry of plasmodia. In 1968 Rieckman and co-workers, established macro- testing method to find out morphological changes due to antimalarials on parasite maturation. Thus it was in vitro testing of drug sensitivity. If the drug is effective than young trophozoite cannot mature to schizont. This is also known as "Schizont maturation test-Macro test, developed by Rieckman".[14] This test was done by cultivation procedure was challenged by increasing concentrations of chloroquine. Based on this macro technique, the sensitive strains could be readily differentiated from parasites exhibiting chloroquine resistance. There are micro test kits available from WHO. In 1976 Trager and Jensen developed the in vitro continuous cultivation of P. falciparum in HEPES-buffered RPMI 1640, a tissue culture media, which was a major advance; the subsequent rapid adoption of this method and its various improvements can be considered as a milestone in the history of malaria research.[14,15]

Non-human plasmodium species are similar to their human counterparts in terms of feeding, and therefore have frequently been used as models for the production of human-infecting species. Trigg, P.I experimented with P. know less strain in cultivation.[13] All four species have been maintained or cultivated in vitro to varying degrees; P. falciparum is the only species for which every step of the life cycle has been established in culture.

In this study, the medium used to initiate the first culture of P. falciparum was RPMI 1640, originally developed for the cultivation of leucocytes. Medium 199 used by Haynes et al also gives good results.[16] Another is Ham's F 12 medium gives equal results. It is more expensive than RPMI1640. So RPMI 1640 medium remains the medium of choice for P. falciparum cultures, as per our experience. A continuous flow method devised by Trager and improved by others permits the maintenance of stock cultures for a long time. The principle of the original 'candle jar' method is to maintain the infected erythrocytes in a relatively simple culture medium, in an atmosphere of 3-4% carbon dioxide and 16% oxygen, such as can be provided in a closed jar in which a candle has been extinguished.[15] The successful cultivation of malaria parasites has become a most valuable tool in malaria research and advanced the understanding of parasite biochemistry, developmental biology, immunology, pharmacology and physiology. This powerful tool will undoubtedly continue to be a boon to research on the organism that continues to be man's greatest scourge.

Drug sensitivity tests in malaria parasites are very important.[17] Cultivation of malaria parasite is required in vitro, for drug sensitivity is a problem of various anti-malarial drugs. We also could cultivate schizont and maturation of malarial parasite was seen in periodical peripheral smear as shown in the slides. The World Health Organisation has standardised both the test procedure and the material.[18] The test is based on the principle that ring forms (young trophozoites) are usually the only asexual stages of P. falciparum found in a patient's peripheral circulation.

5. Limitations

This pilot work is done for the purpose to carry forward the concept of diagnosing falciparum malaria by cultivation of malarial parasites. The hypothesis is put forward that this method can be adopted in problematic cases or as one of the adjunct methods for diagnosing malaria. Further work may be needed for practical application. This work is done in 12 cases of falciparum malaria which a small sample size. Positive results were obtained in 24 to 36b hours and thus it is not equated as a rapid diagnostic test. The clinical applicability may not seem to be superior than another available test. Idea of this work is to conceptualize this method for further research such that this technique can be used for variety of purposes apart from diagnosing malaria. In cases of co-infection with other microorganisms or in cases of PUO, this can become a definitive diagnostic method. This concept for malaria diagnosis is novel however further applicability, validity and research may be needed for its practical use.

6. Conclusions

When cultivating Plasmodium falciparum in 12 instances for this study, we saw that mature schizonts formed within 30 hours, rupturing schizonts into new merozoites after 48 hours, immature schizonts at 24 hours and identifying sparse parasitemia or very scanty rings becoming visible on cultivation. The rings would enlarge and other falciparum parasite stages would become visible, which are challenging to identify on peripheral smears due to the sequestration process. Thus, we conceptualize, like bacterial culture this can be one of the methods which can be adopted in laboratory for scanty parasitemia cases.

7. Source of Funding

None.

8. Conflict of Interest

None.

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