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In Vitro Cultivation of *Plasmodium vivax* using McCoy's Medium

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ABSTRACT: Malaria still poses a threat to the health of residents and travellers in tropical countries, which causes high morbidity and mortality. The present study was undertaken for cultivation of the *Plasmodium vivax* in McCoy's 5A medium and observed for their growth. Cultivation of *Plasmodium vivax* was done according to modified method of Jensen and Trager (1980) in immature red blood cells (reticulocytes) at a 5% haematocrit in McCoy's 5A medium. Culture medium was sterilized by filtration through a membrane filter of 0.22 µm porosity after addition of supplements. Complete McCoy's 5A medium was taken in a sterile tissue culture flask and malarial parasites infected blood was added and incubated at 37ºC in 5-10% CO2 incubator. Smears were prepared, stained and examined each day for growth of malarial parasites. Total 22 malaria positive samples were included in this study, out of which 15 were for *Plasmodium vivax* and 7 were mixed Plasmodium species (*Plasmodium vivax* and *Plasmodium falciparum*). Inoculums were incubated at 37°C for 48 hours. After 48 hours of incubation the culture showed 40% growth of *Plasmodium vivax* and no growth of mixed Plasmodium species. McCoy's 5A medium supplemented with L-glutamine, HEPES buffer, NaHCO3, hypoxanthine, 0.5% Albumax II and 50µg/ml Gentamicin was useful media for cultivation of *Plasmodium vivax*.

Keywords: Malaria, *Plasmodium vivax*, Culture, McCoy's 5A medium, Filtration, CO2 incubator.

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INTRODUCTIO

Malaria has been and remains a major public health concern globally. 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 host. 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 (Frederick et al., 2002). There are currently six species of the genus Plasmodium known to infect humans: *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovalecurtisi*, *Plasmodium ovalewallikeri*, *Plasmodium malariae* and *Plasmodium knowlesi*. Of these, *P. falciparum* is the species responsible for most of the mortality and morbidity associated with the disease and it is during the asexual intraerythrocytic stages that most of the symptoms of malaria are manifest. Studies into many aspects of human malaria parasite biology was possible by the development of a method to culture asexual blood stages of *P. falciparum* in vitro in 1976 (van Schalkwyk et al., 2013, Singh et al., 2015).

Plasmodium vivax is the most widespread and, except in equatorial Africa, the most prevalent malaria infection of man. It is widely acknowledged that at least ten times as many cases occur annually. It produces a severe, fulminant illness that can relapse months after the original infection as dormant parasites are released from the liver. Successful therapy requires clearance of both the blood and liver stages (Golenda et al., 1997).

Plasmodium vivax biology lags behind that of other Plasmodium species. Significant differences in details of life cycle, epidemiology, and disease mechanisms of *P. vivax*and other Plasmodium species, such as the early appearance of gametocytes compared with *Plasmodium falciparum*, highlight the importance of detailed species-specific investigations (McClean et al., 2010).

The first in vitro development of malaria parasites was reported nearly 80 years ago by Bass and Johns (1912). They obtained defibrinated blood from patients infected with *P. falciparum* and cultured the sample at 37°C in a glass vial to which a small amount of glucose had been added. Newly developed rings could be observed after one generation time and only occasionally after one to two additional cycles (Bass et al., 1912).

MATERIALS AND METHODS

This prospective study was carried out at Department of Microbiology, MGM Medical College and Hospital, Navi Mumbai, India over a period of one year from January 2013 to December 2013.

Ethical clearance

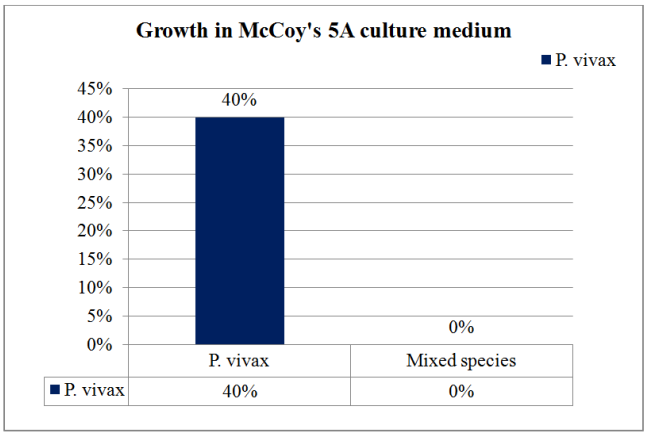
Ethical clearance was obtained from the Institutional Ethical committee of MGM Institute of Health Sciences (Deemed University), Navi Mumbai before starting the project.

Sample Collection

5 ml of blood was withdrawn from antecubital vein by venipuncture in EDTA tube taking all sterile precaution and properly labeled with patient's name, date and time. Both thick and thin smears were examined after staining with Leishman's stain, using standard protocols provided with kit. The blood samples were centrifuged and the plasma was discarded and McCoy's 5A incomplete medium was added in centrifuge tube with RBCs sediment, mixed and again centrifuged. The procedure was done thrice to remove plasma and buffy coat except red blood cells.

Processing of Samples

Total 22 malaria positive samples were subjected to cultivation using McCoy's 5A medium. *Plasmodium vivax* and mixed Plasmodium species were cultured according to modified method of Jensen and Trager (1980) in cord blood containing immature red blood cells (reticulocytes) at a 5% haematocrit in McCoy's 5A medium, supplemented with L-glutamine (4.2mM), HEPES buffer (25 mM), NaHCO3 (25 mM) hypoxanthine (6.8 M), 0.5% Albumax II (Invitrogen) and 50µg/ml Gentamicin (Trager et al., 1976). Culture medium was earlier sterilized by filtration through a membrane filter of 0.22 µm porosity for making stock 500 ml and stored in a sterile screw capped bottle. The working media was again filtered using syringe filter, the filtrate was stored in 125 ml square bottle (GenetixBiotech Asia Pvt. Ltd.), sealed with parafilm (Genetix Biotech Asia Pvt. Ltd.) and stored in refrigerator at 2 - 4°C, which was ready to use. Complete McCoy's 5A medium was taken in a sterile tissue culture flask (Genetix Biotech Asia Pvt. Ltd.) and malarial parasites were cultivated. The inoculum was incubated at 37ºC in 5-10% CO2 incubator (Figure 1). Smears were prepared, stained and examined each day for growth of malarial parasites (Figure 2).



Graph 1. Showing growth of *P. vivax* in McCoy's 5A medium.

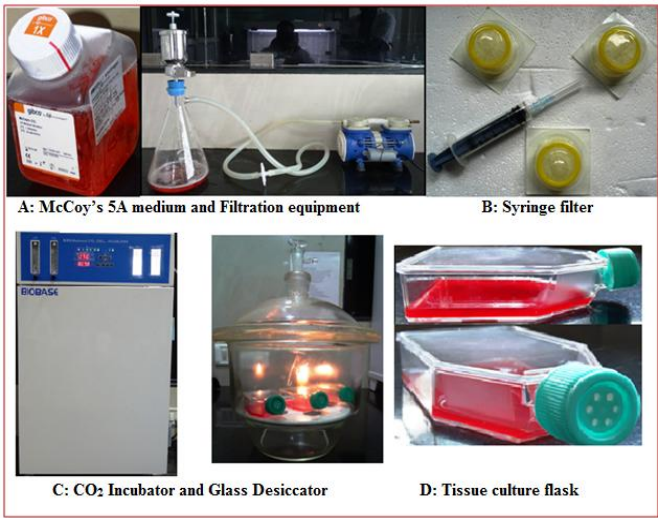


Figure 1. Showing different equipments and McCoy'5A Showing different morphology of *Plasmodiumvivax* after culture on McCoy's 5A medium.

RESULTS

Total 22 malaria positive blood samples were included in this study. As per microscopy report, 15 were positive for *Plasmodium vivax* and 7 were mixed Plasmodium species. These were processed for in vitro cultivation in McCoy's 5A medium supplemented with

Albumax II and cord blood (which is rich in reticulocytes). *Plasmodium vivax* showed 6/15 (40%) growth whereas mixed Plasmodium species showed no growth in culture medium (Graph 1).

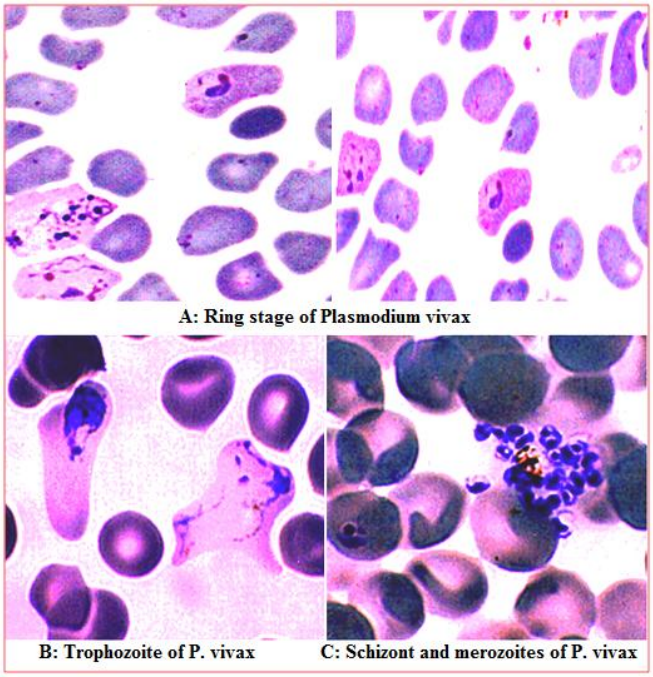


Figure 2. Showing different morphology of *Plasmodium vivax* after culture on McCoy's 5A medium.

Table 1. Comparison of our work with others

Workers	Place	Findings
Present study	Navi Mumbai India	6/15 (40%) *P. vivax*in McCoy's 5A medium Mixed species were culture on McCoy's 5A medium but *P. vivax*did not grown.
Udomsangpetch et al., 2008	Bangkok, Thailand	8 parasites cultured > 30 days. Fluctuation of parasitaemia
Golenda et al., 1997	Washington, DC	Difficulty in culturing *P. vivax*in McCoy's 5A medium. Parasites grow in 48 hours cycle.

DISCUSSION

Only few workers have tried cultivation of malarial parasites. From earlier experiments, it was clear that it is very difficult to grow and maintain culture of malarial parasites and hence it could not be used as routine diagnostic method for detection of malarial parasite. Secondly, *Plasmodium vivax* requires McCoy's 5A medium and in addition, cord blood (rich in reticulocytes cells) as target cells. Immature red cells (reticulocytes cells) are required for further growth of *P. vivax*.

Different workers have used cultivation methods of *P. falciparum* and *P. vivax*for research purpose only i.e. enzyme or virulence factors studies and to compare different nutritional requirements (Table 1).

In our studies, we could grow 6/15 (40%) samples. Udomsangpetch et al. (2008) could grow *P. vivax*parasite after 30 days of incubation and there was fluctuation of parasitaemia. Golenda et al. (1997) also came across many difficulties for cultivation of *Plasmodium vivax* in McCoy's 5A medium but parasites grew in 48 hours of incubation.

There was no growth of malarial parasites in culture in blood samples of mixed species. It appears that these blood samples were of *P. falciparum* which will not grow in McCoy's 5A medium.

CONCLUSION

Plasmodium vivax was difficult to grow in the McCoy's 5A medium. Parasitaemia starts increasing after 2 days of incubation and cord blood containing human immature red blood cells were added for further sub cultivation. This study support the use of McCoy's 5A medium supplemented with L-glutamine, HEPES buffer, NaHCO3, hypoxanthine, 0.5% Albumax II, 50µg/ml Gentamicin and cord blood for cultivation of *Plasmodium vivax* in the presence of 5-10% CO2. This culture method can only be useful for drug sensitivity testing of *P. vivax*.

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