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# SHORT COMMUNICATION

## Comparison of the Anaerocult A and the oil blocking methods for the *in vitro* cultivation of *Entamoeba histolytica*

Nurul Azira MOHD SHAH *MPath*, Wan Nor Amilah WAN ABDUL WAHAB* *MPath*, Siti Farah Alwani MOHD NAWI *MPath*, Zaini MOHD-ZAIN *PhD*, Baha LATIF *PhD* and Rosliza SUHAIMI *MSc*

*Health and Wellbeing Core, Universiti Teknologi MARA (UiTM) 40450 Shah Alam and *School of Health Sciences, Health Campus, Universiti Sains Malaysia*

*Abstract*

*Entamoeba histolytica,* the causative agent for human amoebiasis, is among the most deadly parasites, accounting for the second highest mortality rate among parasitic diseases. Because this parasite dwells in low oxygen tension, for its cultivation, microaerophilic conditions are required to mimick the human gut environment. Several methods developed for optimal growth environment are commercially available and some are conventionally modified in-house which include the Anaerocult A and oil blocking preparation methods. This study was undertaken to compare the reliability of the Anaerocult A and the oil blocking methods in generating anaerobic environment for cultivation of *E. histolytica*. The trophozoites of *E. histolytica* HM1: IMSS strains were axenically cultivated in TYI-S-33 medium in culture incubated anaerobically by using Anaerocult A (Merck) and mineral oil blocking method. The outcomes of both methods were determined by the minimum inhibitory concentration (MIC) of metronidazole against *E. histolytica* by giving a score to the growth pattern of the trophozoites. The reliability of both methods was assessed based on susceptibility testing of *E. histolytica* to metronidazole. The MIC obtained by both anaerobic condition methods was 6.25ug/ml, thus showing that oil-blocking method is comparable to the Anaerocult A method and therefore, considered as a reliable method for generating an anaerobic environment for the cultivation of *E. histolytica.*

*Keywords: Entamoeba histolytica*, cultivation methods

### INTRODUCTION

*Entamoeba histolytica* is the causative agent of amoebiasis. The disease caused by this parasite is the third leading parasitic cause of death worldwide and is more prevalent in tropical and subtropical regions.[1] It infects 10% of the world population and the majority of cases are asymptomatic while 10% of them are symptomatic.[2,3]

Amoebiasis is primarily transmitted via ingestion of water or and food contaminated with mature cysts of *E. histolytica*. Trophozoites are released from the cyst in the intestinal lumen and colonize the large intestine via galactose and N-acetyl-D-galactosamine (Gal/GalNAc)-specific lectin.[4] Upon colonization of intestinal epithelium, encystation of the trophozoites occur followed by the excretion of cysts in the feces to complete the cycle. Trophozoites may also invade the intestinal epithelium and spread to the other organs, particularly the liver.[5]

*E. histolytica* is a known anaerobic organism. However, it is able to tolerate up to 5% oxygen. Therefore, the survival and optimal growth of *E. histolytica in vitro* requires low oxygen tension which mimics the intestinal environment.[6] Under certain conditions, it is also able to utilize the oxygen. Glucose, galactotose and ethanol have been shown to stimulate the respiration of *E. histolytica*.[7]

There are several cultivation methods which are currently commercially available and some in-house preparations to create the anaerobic or microaerophilic environment for cultivation of *E. histolytica in vitro*. The methods include using Anaerocult A and mineral oil-blocking. Anaerocult A has been the frequently used for the cultivation of *E. histolytica.* The mineral oil-

*Address for correspondence:* Dr. Nurul Azira Mohd Shah, Faculty of Medicine, Universiti Teknologi Mara, Sungai Buloh Campus, 47000 Sungai Buloh, Selangor, Malaysia. Email: nazlizainuddin@yahoo.com

blocking method to culture *E. histolytica in vitro* was also reported to be an effective method.[6]

To the best of our knowledge, Anaerocult A has been the frequently used method in cultivation of *E. histolytica*. The mineral oil blocking method is currently being optimized for cultivation of *E. histolytica.* However, the reliability of these methods has not been compared. Therefore our study aimed to compare the reliability of the Anaerocult A and the mineral oil-blocking methods in generating a suitable anaerobic environment for cultivation and simple drug susceptibility testing of *E. histolytica.*

## MATERIALS AND METHODS

The axenically cultured trophozoites of the *E. histolytica* isolate HM-1: IMSS were grown in TYI-S-33 medium under microaerophilic (5–7% $O_2$) or anaerobic conditions. The anaerobic conditions for the growth of *E. histolytica* trophozoites were compared in two incubation methods in the presence of metronidazole; the two incubation methods were the Anaerocult A (Merck) and mineral oil (Nujol®) blocking method.[6]

*Anaerocult A*

Anaerocult A comprises of iron powder that binds to oxygen chemically and citric acid and sodium carbonate to liberate carbondioxide.[8] Stock solutions of metronidazole, 200 µg/ml in TYI-S-33 medium were prepared and stored at -20°C. The stock solution was diluted with the medium to obtain the concentrations of 100, 50, 25, 12.5, 6.25, 3.2, 1.6, and 0.8 µg/mL.

A 100 µl volume of different concentrations of the diluted metronidazole was added to wells of a 96-well flat-bottom, covered tissue culture plate (Greiner Cellstar). Into each of the wells, 160 µl volume of medium containing $5\times10^4$ trophozoites was added. A well containing 100 µl of medium and 160 µl volume of medium containing $5\times10^4$ trophozoites was used as control. The plate was placed in the incubation bag (mini sachet) supplied with the Anaerocult A which generated the anaerobic environment. The incubation bag was sealed with Anaeroclips and placed in a 36°C incubator for 24 hours (modified from Wan Nor Amilah and Alvieno, 2012).[9]

*Mineral oil (Nujol®) blocking method*

Regarding the mineral oil-blocking method, 100 µl of the different concentrations of diluted metronidazole were pipetted into the wells followed by the addition of 100 µl medium containing $5\times10^4$ trophozoites. A well containing 100 µl of the medium and 100 µl of medium containing $5\times10^4$ trophozoites was used as control. To each well, 70 µl volume of sterilized mineral oil was layered on top of the culture medium surface. The plate was placed in a 36°C incubator for 24 hours.

The growth of the trophozoites was observed after 24 hours of incubation by comparing the control and metronidazole-containing wells under an inverted microscope. The number of trophozoites was scored according to Table 1. The trophozoites were closely observed for motility and rounding-up, which is an indication of drug susceptibility.[10] Rounding-up refers to ball-like trophozoites with no pseudopodia and no evidence of movement.[10]

*Minimum inhibitory concentration* (MIC) is defined as the lowest concentration of metronidazole at which a 1+ score was obtained in the majority of triplicate wells. The minimum amoebicidal concentration (MAC) is when there is 99.99% inhibition of the growth of *E. histolytica* growth or death. The MIC and MAC tests were performed in triplicate and repeated three times to obtain accurate results.

## RESULTS

There was no significant difference in the total score observed between Anaerocult A and the mineral oil-blocking method. The MIC and MAC obtained from both methods were 6.25 µg/ml and 12.5 ug/ml respectively (Table 2).

**TABLE 1: Scoring the growth of the trophozoites[10]**

| Score | Description |
|---|---|
| 1+ | Dead or significantly fewer (not > 20% coverage of well surface + >90% rounded up |
| 2+ | 20-50% coverage of the well surface + some motility |
| 3+ | An almost confluent well (>50% coverage of the well ) + much motility |
| 4+ | A confluent well (100% coverage of the well surface) |

**TABLE 2: Comparison of the two anaerobic incubation methods used for observing the MIC and MAC of *E. histolytica***

| | Anaerocult A | | | Mineral Oil | | |
|---|---|---|---|---|---|---|
| **Replicates** | A | B | C | A | B | C |
| MIC (ug/ml) | 6.25 | 6.25 | 12.5 | 6.25 | 6.25 | 6.25 |
| MAC (ug/ml) | 12.5 | 12.5 | 12.5 | 12.5 | 12.5 | 12.5 |
| Control (TYI-S-33 medium + Eh) | 4+ | 4+ | 4+ | 4+ | 4+ | 4+ |
| Blank (TYI-S-33 medium only) | - | - | - | - | - | - |

MIC- minimum inhibition concentration (score 1+); MAC- minimum amoebicidal concentration (99.99% inhibition of growth or dead); Eh- *E. histolytica*

Therefore, there was no significant difference in the reliability of the two methods.

## DISCUSSION

*E. histolytica* is the one of the most common intestinal parasites of humans associated with high fatality worldwide. Amoebiasis causes invasive disease in humans as well as humans become asymptomatic carriers spreading the disease among the general population. The invasive form can also be transmitted through haematogenous spread and invade other organs such as the liver, lungs and brain.[11]

*E. histolytica* requires a special environment for its optimal growth. However, it is able to survive and grow in the presence of up to 5% oxygen and is also able to detoxify the oxygen reduction products in the medium. An anaerobic environment mimicking the lumen of intestine is crucial for optimal growth of *E. histolytica in vitro.* Thus, the most practical method in the cultivation of *E. histolytica* is worthwhile in order to facilitate other related studies, particularly in the areas of molecular studies and diagnostic purposes.[12]

In this study, it was found that both Anaerocult A and mineral oil-blocking methods have shown to produce comparable results in the detection of minimum inhibitory concentration of metronidazole against *E. histolytica.*

In the Anaerocult A, the iron powder present in the system binds to the oxygen. The sodium bicarbonate and citric acid present will initiate the release of carbon dioxide. As a result, the system generates 18% $CO_2$ and 0.1% $O_2$ in an anaerobic jar within 150 minutes.[10] This method was reported to be more reliable in cultivation of *E. histolytica* when compared to Oxoid Anaero Gen and Oxoid Campy Gen methods.[8]

In the mineral oil-blocking cultivation of *E. histolytica*, the mineral oil which overlaid the air-medium interface created a decrease in oxygen tension leading to enhanced parasitic proliferation.[6,13] This method exhibited a greater number of trophozoites and motility which is indicative of higher metabolic rates compared to oil-devoid medium. In addition, the utilisation of mineral oil decreases oxidative stress by down-modulating reactive oxidative species production within the trophozoites. Therefore, the use of mineral oil in the medium makes it completely confluent with the pseudopods which are constantly exhibiting high motility compared to oil-devoid medium.[6]

Nevertheless, Anaerocult A is functional because it is a rapid and easy to perform assay in a laboratory, especially by untrained laboratory personnel. On the other hand, the mineral oil-blocking method costs lower than Anaerocult A system and would be considered as one of alternative inexpensive methods.

In both methods, only small volumes of the media were required. These two methods are useful particularly in determining antiparasitic compound assay as both methods have shown to produce the same scoring. However, Anaerocult A method is considered to be much easier to perform and requires shorter time to generate an anaerobic environment compared to mineral oil-blocking method.

In conclusion, for the cultivation of *E. histolytica* in anaerobic condition, although mineral oil-blocking method is comparable to Anaerocult A, the latter method was found to be more feasible and practical for drug susceptibility testing of *E. histolytica*.

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