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Modified Diphasic Liver Infusion Agar: Selective Medium for the Axenic Cultivation of *Entamoeba histolytica*

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

A modified diphasic liver infusion agar medium was prepared manually in the laboratory in order to be used for culturing *Entamoeba histolytica in vitro*. This medium is a good alternative for the commercial one and has the main ingredients for the parasite's growth. *E. histolytica* was successfully cultured in the present study and PCR technique was performed to confirm *Entamoeba* species. The growth of *E. histolytica* trophozoites had started in the third day post-cultivation and reached its highest rate in the seventh. Then the growth started to decrease until the trophozoites completely disappeared after 12-14 days post-cultivation. Cryopreservation using glycerol 10% (V/V) was used in order to preserve trophozoites for later cultivation.

Keywords: E. histolytica; diphasic; liver infusion agar; PCR, trophozoites; cultivation; cryopreservation.

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1. INTRODUCTION

E. histolytica is a parasite causes a parasitic disease known as amoebiasis. It can't be distinguished with the microscope from *E. dispar* which also inhibits the human colon but is considered a nonpathogenic. These two organisms are found worldwide, especially in tropical countries and in those with low sanitation standards. It was estimated that 50 million new cases of amoebiasis are reported annually worldwide with more than 100,000 death cases [1]. Intestinal infection resulting from *E. histolytica* is an important cause of diarrhea worldwide, especially where sanitation conditions are inadequate. This infection mainly affects the colon resulting in a dysenteric syndrome. The optimal growth of *E. histolytica* needs special conditions and environment, that's because of its ability to survive and grow with only 5% oxygen, in addition this parasite can detoxify the products of oxygen reduction. Anaerobic environmental conditions such as those found in the intestines are necessary for the optimal growth of this parasite *in vitro* [2]. Cultivation of *E. histolytica* has a long history. For instance, Williams (1911) documented the cultivation of *E. histolytica* on a medium of crushed brains, liver, kidney and other tissues of rabbits or guinea pigs streaked on nutrient agar [3]. Furthermore, Boeck and Drbohlav developed and used a diphasic egg slant medium for cultivating *E. histolytica* in 1925 [4]. *In vitro* cultivation is very important for diagnostic researches as well as necessary to identify potential therapeutic agents [5]. The aim of this study is the cultivation of *E. histolytica* through an axenic medium.

2. MATERIALS AND METHODS

2.1 Preparation of Modified Diphasic Liver Infusion Agar Medium Used for *E. histolytica* Cultivation

The medium which was used in order to cultivate this parasite was a modified diphasic liver infusion agar medium, with rich nutrients for the parasite's growth. It contains some supplements such as rice flour, that used as a carbohydrate source, bovine serum as a source of lipids, and antibiotics and antifungal to prevent the growth of bacteria and fungi. pH was adjusted to 7.2, which is the optimal pH for the growth of *E. histolytica*.

2.2 Solid Phase

For every 100 ml of distilled water (D.W), the following amounts were added: 1.2 g Liver extract, 1.4 g Agar, 0.3 g Sodium chloride, 0.3 g Disodium hydrogen phosphate, and 0.6 g Peptone special. After dissolving these amounts in 100 ml of distilled water, the mixture was boiled for two minutes in a microwave oven to ensure complete dissolution of the materials. Then 7 ml was put in a 13 ml screw-cap tube, and then sterilized by using the autoclave at 121°C for around 15 –20 minutes and 15 lbs/in. More media was prepared following the same steps several times during the present study. After sterilization, the tubes were tightly closed, and they were left to solidify in slant position then preserved at 4°C till use.

2.3 Liquid Phase

Physiological salt solution was prepared as shown in Table 1.

Materials A, B and C were mixed well together for complete dissolving in DW to generate a 3000 ml volume compound. pH for Physiological salt solution was adjusted at 7.2 then stored at 4°C. Horse serum was used at the beginning of the study but later was replaced with bovine serum because it's easier to obtain. Bovine or horse serum was used as a nutrient supplement, and heat-inactivated at 56°C for 30 minutes in water bath, then sterilized by using 0.45µm filter papers, this sterile serum was put in sterile cups and stored at - 20°C till used. One ml of sterile serum was mixed with 6 ml of the physiological salt solution then this mixture was added to a tube containing 7 ml of slant solid phase. The starch is added to the medium in order to increase the growth rate of phototrophic intestinal protozoon. The grain must be small for ingestion, according to Kibry [6]. A loopful of rice flour was added to each tube of media after rice flour standard autoclave sterilization. Streptomycin 3 mg/ml and penicillin 1500-2000 Iu/ml were added to the media after *E. histolytica's* cultivation to prevent the growth of bacteria that are sensitive to these antibiotics while a drop of nystatin (originally named Fungicidin) was added to each media tube to prevent the growth of fungi.

2.4 Samples Collection

In order to ensure a continuous growth of *E. histolytica*, thirty stool samples were collected from patients who had been diagnosed clinically with amoebic dysentery. These samples were obtained from the United Nations Relief and Work Agency for Palestinian Refugees (UNRWA) clinic in El-shatte' refugee camp and from some private medical laboratories in Gaza. The

samples were delivered to the Islamic University labs within 30 minutes of being collected. First, direct smear examination using saline for each sample was performed. When viable trophozoites were seen, the sample was then cultivated. Some stool samples were divided into four parts. The first part was preserved in 10% formalin to maintain the shape of trophozoites for further examinations. The second part was preserved at -20°C for DNA. The third part was mixed with 10% glycerin for cryopreservation. While the fourth one was kept as a fresh sample and used for immediate cultivation.

2.5 *E. histolytica* Cultivation

When the stool is soft, a small portion at the size of a bean was inoculated well by stabbing into the solid phase, making sure that the sample stretch well in the slant solid phase. But, when the stool is watery or mucoid, 0.350 ml were taken by micropipette then inoculated into the solid phase. Then 7 ml of liquid phase were added to the sample which was inoculated in the slant solid phase. Antibiotic, starch, and antifungal were added as described previously after cultivation. Subsequently, culture tube containing medium, rice flour, antibiotics, antifungal, and stool were incubated vertically at 37Cº for 48 hrs before examination.

After 48 hrs. of *E. histolytica* incubation at 37°C, a drop from the bottom of the cultured tube was taken and examined directly under the microscope. Many subcultures were performed when growth was found; however, if no growth was found, the sample was further incubated for one more week and examined daily. When *E. histolytica's* growth was acceptable, 0.350 ml from the bottom of the sample was taken and added directly to the slant solid phase, then the liquid phase and the supplements were both added whereas when the growth of *E. histolytica* wasn't acceptable (i.e. few trophozoites were seen in the samples under H.P.F), the liquid phase was centrifuged for 4 minutes at 2500 rpm. Then sediment was transferred to a fresh slant solid culture tube then the liquid phase and supplements were both added as described previously. After incubation for an additional 48 hrs., the culture was reexamined.

2.6 Cryo-preservation of *E. histolytica*

In the present study, the method described by Miyata [7] was followed, 10% (v/v), glycerol was used for cryo-preservation of *E. histolytica* then preserved at -70°C. When needed, it was cultured following the same way which was described above for sub-culturing then examined after 24 hrs.

2.7 Preservation of Stool Sample Containing *E. histolytica*

Small amounts of stool containing viable trophozoites were preserved at around (-20°C) in order to obtain a stool sample suitable for DNA extraction that is needed for PCR analysis.

2.8 Polymerase Chain Reaction for *E. histolytica*

The protocol of this study was performed according to (Qiagen Inc., USA). DNA was extracted using the QIAamp DNA Stool Mini Kit according to the manufacturer's instructions.

Table 1. The composition of liquid phase media

Material	Concentration	Amount prepared
A- Disodium hydrogen phosphate (Na_2HPO_4)	0.95 g/100 ml D/W	375 ml
B-Dipotassium hydrogen phosphate (K_2HPO_4)	0.91 g/100 ml D/W	125 ml
C- Sodium chloride (Na Cl)	0.9 g /100 ml D/W	2500 ml

Table 2. The primers used in the present study

Primers	Nucleotide sequences	PCR product size	References
EH1(P1)	P1: ^{5}TCAAAATGGTCGTCTAGGC $^{3'}$	125 bp	[11]
E. histolytica			
EH2 (P2)	P2 :$^{5'}$CAGTTAGAAATTATTGACTTTGTA $^{3'}$	133 bp	
ED1 (P3)	P 3: ^{5}'GATCCTCCAAAAAATAAAGTTT^{3}'		
E. dispar			
ED2 (P4)	P4:$^{5'}$ACAGAACGATATTGGATACCTAGTA $^{3'}$		

2.9 Primers Used in This Study

The primers used in this study are shown in Table 2.

3. RESULTS

3.1 The growth of *E. histolytica*

To monitor the growth of *E. histolytica*, the cultivated stool samples were microscopically examined daily for nearly two weeks after 48 hrs. of incubation. During this period the number of seen motile trophozoites was recorded per (High Power Field) H.P.F as illustrated in Fig. 1. The sample was considered negative if no growth was observed within a week of cultivation. *E. histolytica* growth usually started from the third day, reaching the maximum level of growth on the seventh day, then the number of trophozoites started to decrease and finally disappeared after nearly two weeks of cultivation as shown in (Fig. 2).

3.2 Result of Polymerase Chain Reaction

EH1 (P1) primer was used for testing stool samples in which the targeted PCR product band size was 125 bp. This primer was compared with *E. histolytica's* positive and negative controls. The band size of unknown stool samples was compared with the known band size. The size of unknown samples was 125 bp as shown in (Fig. 3). This was consistent with the positive control size. Based on this, it was confirmed that the used stool samples in the present study were *E. histolytica* not *E. dispar*.

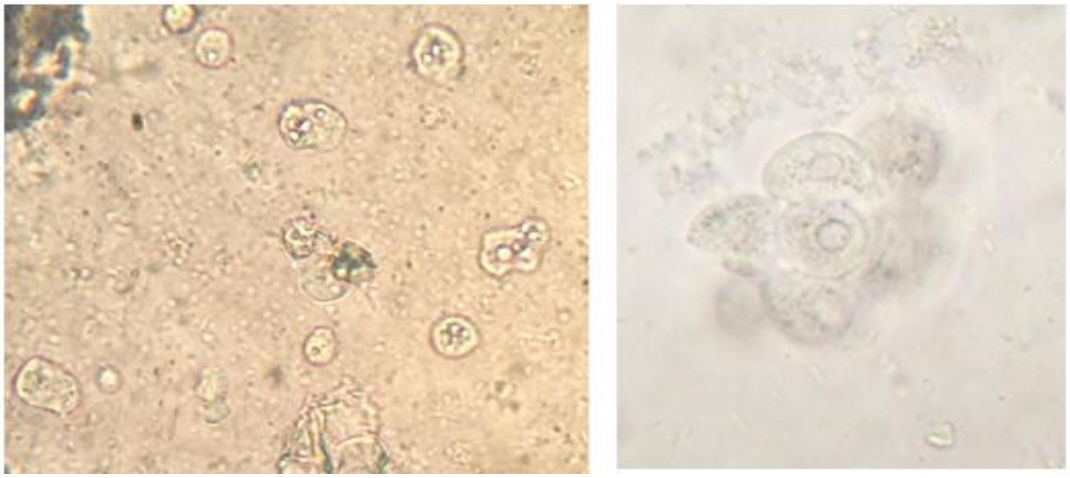

Fig. 1: *E. histolytica* trophozoites grown in modified diphasic liver infusion agar media

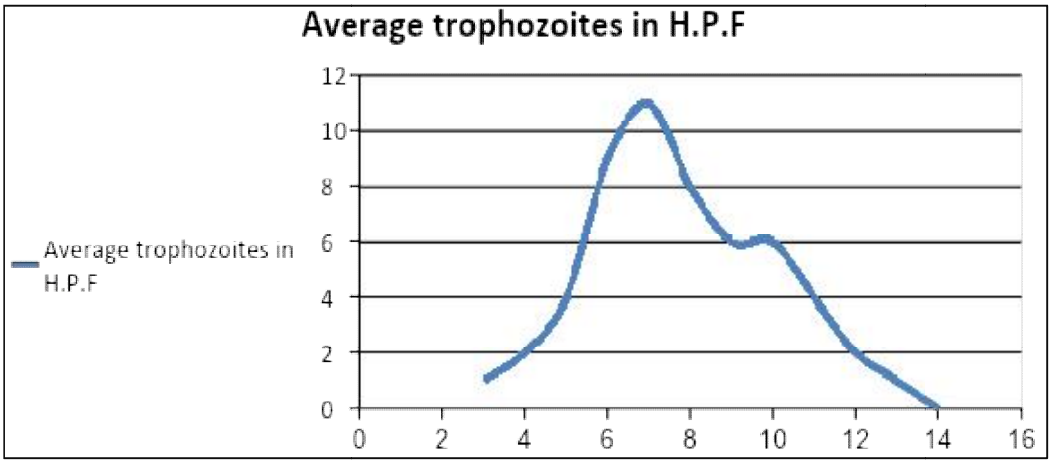

Fig. 2: The average growth of *E. histolytica* trophozoites per H.P.F. per day for 14 days

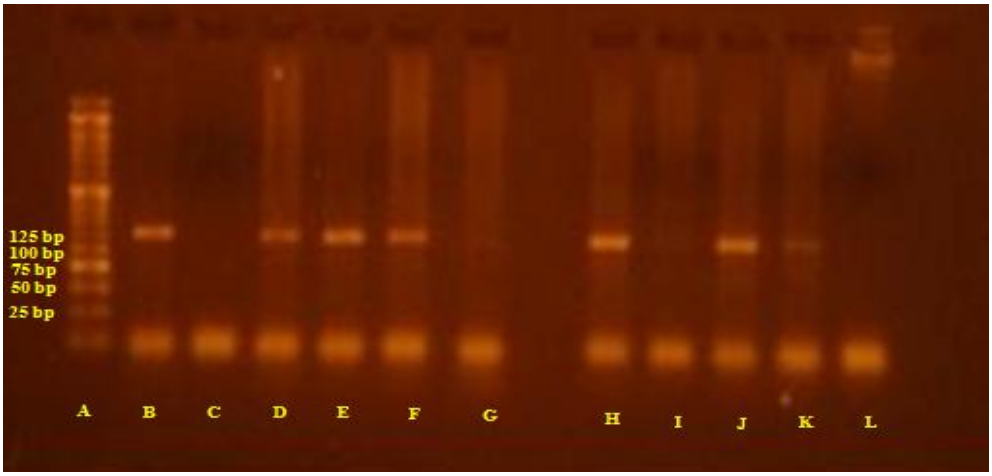

Fig. 3. A representative photograph of ethidium bromide stained 2% agarose gel showing, the PCR products for some stool samples. Lane A; ladder, lane B; Positive control, lane C; Negative control. Lanes D, E, F, H, J, and K were positive for *E. histolytica* while Lanes G, I and L for negative

4. DISCUSSION

In the present study a modified diphasic liver infusion agar media was used for culturing *E. histolytica*. This media was prepared manually with new compounds due to the lack of prepared media for parasite growth in our laboratory such as using liver extract instead of liver infusion and peptone special instead of Protease peptone. Furthermore, bovine serum was used instead of horse serum and rice flour instead of rice starch.

Trophozoites when using this medium lived for long time compared with the other media used for *E. histolytica* cultivation. Xenic, monoxenic and axenic are three kinds of *E. histolytica* cultural systems. Axenic culture, which was performed in the present study, is cultivation of parasite without any other metabolizing cells [8]. Parasite's growth may depends on the number of active *E. histolytica* trophozoites in the cultivated stool sample, the time of sample delivery to the laboratory, the incubation's temperature which should be adjusted to 35-37°C in addition to the pH which necessarily should be adjusted to 6.8-7.2. Furthermore, culture media should contains the main nutrient sources and supplements needed for parasite's growth. This is clearly concluded in this study by repeating the experiments many times using different conditions. Because the pathogenic Amoeba *E. histolytica* and the non-pathogenic *E. dispar* can't be distinguished morphologically. Thus, in order to avoid unnecessary therapy, devising a method to distinguish between them is very important [9]. Polymerase chain reactions (PCR) and many other molecular techniques are used to detect *E. histolytica* DNA in stool samples and fluid withdraws from liver abscess [10]. In the present study, *E. histolytica* was successfully cultured and confirmed using PCR. Al-Hindi et al. [11] reported that 30% of suspected clinical amoebiasis in the study area were negative for *E. histolytica* after analyzing them using (PCR) and they recommended that (PCR) should be used for diagnosis of *E. histolytica* in stool samples. The growth of *E. histolytica* trophozoites started from the third to the fifth day post-cultivation and protozoal growth reached its maximum level between the fifth and the seventh day. However, the growth rate started to decrease until the trophozoites clearly disappeared in 12-14 days. This result is consistent with the study carried out by Cruz et al. [12]. In their study iron-starved trophozoites were cultivated for 13 days in the presence of different iron sources. It is also consistent with a study carried out by Costa et al. [13] they reported that the growth of *E. histolytica* EGG strains surpassed the growth of *E. dispar* strains after 48 hrs. of cultivation while after 72 hrs. the difference reached its significant point. While the *E. dispar* strains suffered growth decrease between 48 and 72 hours, the exponential growth of EGG *E. histolytica* continued until 120 hours of cultivation. So the maximum growth of *E. histolytica* was on the fifth day almost as the maximum growth in this study. The maximum growth period for *E. histolytica* trophozoites which was achieved in this study may be due to

the nutrient-dense media and supplements which increased the nutritional materials in addition to the absence of any bacterial or fungal contaminations.

5. CONCLUSION

This study revealed that the used medium which was prepared manually is a good alternative for the commercial one. As well as, by using this medium the parasite lives longer. In addition, the protocol used in this study to cultivate *E. histolytica* is an ideal protocol for obtaining good growing isolates. Cryopreservation is a good idea in order to preserve the parasite and keep it available for cultivation anytime.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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