

Cultivation of *Entamoeba* species in Lock's -egg medium and differentiation of *E.moshkovskii*, *E.dispar* and *E.histolytica* by using single round polymerase chain reaction .

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الخلاصة

يضم جنس الانتاميبيا العديد من الاصناف ، ثلاثة منها هي التوائم (*E. dispar* , *E. mosh kovis* , *E. histolytica*) والتي تستوطن في جوف امعاء الانسان الى القتل الطفيلي للانسان في العالم. اسقطت الدراسات المعاصرة الضوء على اكتشاف *E. moshkovskii* من مرضى يعانون من الالام واعراض في المعدة . عملية زراعة هذه التوائم الثلاثة من الانتاميبيا تمت بنجاح في وسط (Lock's -egg) ، وجد عدد عالي من الادوار النشطة في الوسط الزرع. ان هذه الانواع الثلاثة متشابهة بالشكل تماما ولا يستطيع المجهر من التفريق بينها . لكن مع تطور العلم تم التفريق بينها بواسطة استخدام تقنية تفاعل السلسلة المتبلورة لكشف الDNA لهذه الطفيليات في عينات الغائط باستخدام بادئات وحزم خاصة بكل نوع .

Abstract

The genus *Entamoeba* contains many species, three of which are identical (*Entamoeba histolytica*, *Entamoeba dispar* and *Entamoeba moshkovskii*) reside in the human intestinal lumen. *Entamoeba histolytica* is the causative agent of amebiasis and mortality worldwide in humans. Although recent studies highlight the recovery of *E. dispar* and *E. moshkovskii* from patients with gastrointestinal symptoms. The cultivation of three identical *Entamoeba* species was done successfully in Lock's-egg medium , a high number of trophozoite were found in culture .these three identical *Entamoeba* species did not differentiation by using microscope .but the devolution of scientific they could differentiation by using PCR technique when extraction the DNA in stool samples by using specific bands and primers .

Introduction

Culture techniques for the isolation of *Entamoeba* species have been available for over 80 years. Culture media include xenic (diphasic and monophasic) and axenic systems. Xenic cultivation is defined as the growth of the parasite in the presence of an undefined flora (1). The xenic culture of *E. histolytica* was first introduced by Boeck and Drbohlav in 1925 in a diphasic egg slant medium, and a modification of this medium (Locke-egg) is still used today (1). The epidemiology of *E. histolytica*, *E. dispar*, and *E. moshkovskii* parasitoses remains uncertain, because most of the

existing data were obtained using methods incapable of distinguishing among the three morphologically identical species. *Entamoeba dispar* appears to be about 10 times more common than *E. histolytica*, with most of the 500 million people infected with *E. histolytica*/*E. dispar* carrying *E. dispar* (2). Little is known about the epidemiology and incidence of *E. moshkovskii* infections, as only a few studies have used molecular methods to identify this parasite (2).

Most morbidity and mortality due to amebiasis occur in developing regions

such as Central and South America, Africa, and the Indian subcontinent (3). In Bangladesh, where diarrheal diseases are the leading cause of childhood death, approximately 50% of children have serological evidence of exposure to *E. histolytica* (4). The high association of *E. moshkovskii* with *E. histolytica* and *E. dispar* may have obscured its identification in previous studies. The high prevalence found in this study suggests that humans may be a true host for this amoeba. *Entamoeba moshkovskii*, considered to be primarily a free-living amoeba, is indistinguishable in its cyst and trophozoite forms from *E. histolytica* (the cause of invasive amoebiasis) and *E. dispar* (a common noninvasive parasite), except in cases of invasive disease when *E. histolytica* trophozoites may contain ingested red blood cells. *E. moshkovskii* has so far rarely been shown to infect humans; however, the organism appears to be ubiquitous in anoxic sediments. Although the early isolations of this species were from sewage, *E. moshkovskii* can also be found in environments ranging from clean riverine sediments to brackish coastal pools (5). *Entamoeba* cells are small, with a single nucleus and typically a single lobose pseudopod taking the form of a clear anterior bulge. They have a simple life cycle. The trophozoite (feeding-dividing form) is approximately 10-20 µm in diameter and feeds primarily on bacteria. It divides by simple binary fission to form two smaller daughter cells. Almost all species form cysts, the stage involved in transmission. Depending on the species, these can have one, four or eight nuclei and are variable in size; these characteristics help in identification the species. Uni-nucleated trophozoites convert into cysts in a process called encystation. The number of nuclei in the cyst varies from 1 to 8 among species and is one of the characteristics

used to tell species apart. Since *Entamoeba* does not form cysts *in vitro* in the absence of bacteria (6,7).

Amoebiasis is usually transmitted by the fecal-oral route, but it can also be transmitted indirectly through contact with dirty hands or objects as well as by anal-oral contact. Infection is spread through ingestion of the cyst form of the parasite, a semi-dormant and hardy structure found in feces, since amoebiasis is transmitted through contaminated food and water, it is often endemic in regions of the world with limited modern sanitation systems (8). Although cultivation of intestinal protists has a long history, not all parasites are amenable to growth *in vitro*. few basic studies can be performed beyond morphological or pathological descriptions. Without the ability to cultivate organisms and cultivation is a prerequisite for studies that require large numbers of cells. This is a diverse group of organisms, but many of the principles and even the media used are often common among species. In the clinical diagnostic laboratory setting, cultivation of most of these organisms plays a minor role. Microscopy is still the first choice for identification of these protists in stool samples. However, cultivation is one of the diagnostic methods of choice for *E. histolytica* (9). Establishment of all these organisms in culture is far from a routine procedure and is usually less sensitive than microscopy as a detection mechanism.

Aim of study :

Cultivation of *Entamoeba* species and differentiation the three identical *Entamoeba* (*Entamoeba histolytica*, *Entamoeba dispar* and *E. moshkovskii*).

Materials and Method :

- **Preparation of Xenic Culture Media**

This culture media was prepared according to (11).

- **Rice starch :**
Purified rice starch is important for growth of *Entamoeba* in all parasite media (10).
- **Preparation of Locke's solution .**
- **Preparation of egg slant .**
- **Preparation of Locke's - egg(LE)medium(modification of Boeck and Drbohlav's medium) (11).**

This media composed of Locke's solution and egg slant(diphasic medium)

Cultivation Method:

After complete the prepare Lock's - egg medium added to Each culture tube 0.2 rice starch . Material for inoculation of xenic culture can be prepared by several ways .Most commonly ,stool samples are emulsified in saline and passed through of mesh to removed most of large particles from the material before addition to the culture medium .Culture tubes , containing medium and rice starch are incubated vertically at 35.5 C for 48 h before examination (12).

Isolation of *Entameoba* :

If the culture is have growth ,it is usually helpful to centrifuge the culture . This can be done by chilling

the culture tubes for 5 min. in an ice - water bath, to detach adherent amoebae and transferring the liquid phase to an empty culture tube before centrifugation .drop of sediment can be extracted from the tube for examination on a microscopic slide .

1 Procedure of DNA extraction from stool according to (Bioneer /Korea) .

DNA extraction was performed directly on stool samples by using AccuPrep stool extraction kit (Bioner , Korea) .

The PCR amplification making by used specific primers sequences :

The Amplification of each species-specific DNA fragment started with an initial denaturation at 94°C for 3 min, followed by 30 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min, with a final extension at 72°C for 7 min. Amplified products were visualized with ethidium bromide staining after electrophoresis on 1.5% agarose gels.(14). The primers were designed based on the reported *E. histolytica*, *E. dispar*, and *E. moshkovskii* small-subunit rRNA gene sequences (GenBank accession no. [X64142](#), [Z49256](#), and [AF149906](#), respectively) and was prepared according of Bioneer Oligo synthesis company .The forward primer and the reverse primer as the below table (3-5).

Table (1): The forward and the reverse primers of three identical *Entamoeba* Species .

Primer	DNA sequence		length
<i>E.moshkovskii</i>	F	5'-ATG CAC GAG AGC GAA AGC AT-3'	20
	R	5'-TGA CCG GAG CCA GAG ACG T-3'	19
<i>E.dispar</i>	F	5'-ATG CAC GAG AGC GAA AGC AT3'	20
	R	5'-CAC CAC TTA CTA TCC CTA CC-3'	20

<i>E.histolytica</i>	F	5'-ATG CAC GAG AGC GAA AGC AT-3'	20
	R	5'-GAT CTA GAA ACA ATG CTT CTC T-3'	22

PCR techniques procedure :

From extracted DNA was added 5µl in PCR tube (master mix) with 1.5 µl forward primer and 1.5µl reverse primer , then 12µl from nuclease free water , all volume was completed to 20 µl.

The result:

The table (1) showed the result of *E.moshkovinskii* ,*E.dispar* and *E.histolytica* that grew in culture media and differentiation by single round PCR technique .The growth of *Entamoeba* species was appeared after (4) four days under 36.6°C , 98 samples out of 190 samples were succeed to grow in

culture by using microscopic examination ,the PCR methods to DNA was extracted from positive microscopic *Entamoeba* result in 98 samples , 4 samples to *E.moshkovinskii* , 72 samples to *E.histolytica* and 22 samples to *E.dispar* and the amplification products as in figure (1).

Table (1) : showed the number and percentage of *Entamoeba* species according the PCR methods to DNA was extracted from culture *Entamoeba* .

Number of samples	PCR positive			PCR Negative
	<i>E.hisolytica</i>	<i>E.dispar</i>	<i>E.moshkovinskii</i>	
	72(73.4%)	22(22.5%)	4(4%)	92(48.4%)
Total	98 (51.5%)			

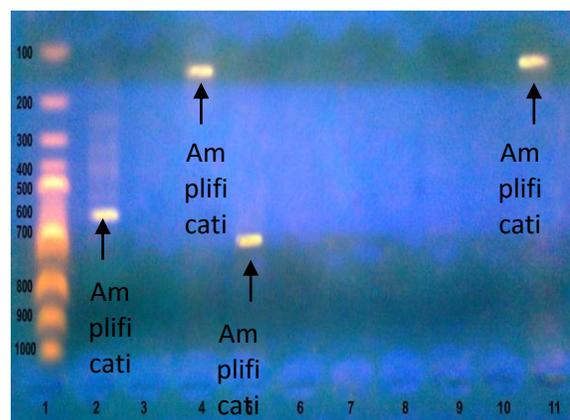


Figure (2) : showed the amplification products of *Entamoeba* species ,Lane
 Figure (1) : showed the amplification products of *Entamoeba* species ,
 (1) DNA ladder, Lane (2) *E. moshkoviskii*, Lane(4 ,11) *E.histolytica* , Lane (5) *E. dispar*.

Discussion

In this study used diphasic media (LE) medium for culturing species of *Entamoeba* under *in vitro* conditions , it is a simple media and have the main components for parasites growth with a rice starch use as a carbohydrate source without any additional of antibiotic was added to media at room temperature for four days ,the number of trophozoite was successfully to grow in culture media . This may be referring that media under study with high nutrients which make trophozoite lived in media (13). A new protocol with results was showed this simple materials and media can *Entamoeba* species cultured with high and new results. This can benefit for future studying on this parasites and also can be used for classify amoeba to strain by using PCR assay. So the present study was revealed that three identical *Entamoeba* species (*E.moshkoviskii* *E.histolytica* and *E.dispar*) were could grow in LE medium . The present study was showed high infection with *Entamoeba histolytica* this indicated of the pathogenicity of parasites. The cause for endemic , hyper endemic, or epidemic amoebiasis in a population is to be found in its epidemiological pattern ,how the agent is maintained and propagated . Therefore , studies must be made to determine whether water , food handlers , person to person contact , or passably reservoir hosts are the responsible factors . Then practical

methods must be set up to control transmission.

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