



ISSN: 2789-1089 EISSN: 2789-1097

NTU Journal of Pure Sciences

Available online at: <https://journals.ntu.edu.iq/index.php/NTU-JPS/index>



In vitro cultivation and preservation of *Entamoeba histolytica* on TYI-S-33 medium

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Article Information

Received: 30-08-2023,
Accepted: 28-09-2024,
Published online: 12-10-2025

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Key Words:

Entamoeba histolytica,
TYI-S-33 media,
Cultivation,
Parasite counting,

ABSTRACT

An enormous protozoan parasite found worldwide, particularly in unsanitary areas, is called *Entamoeba histolytica*, and considered the main causes for intestinal infection. Amoebiasis is worldwide in distribution and is the third most common cause of death due to parasitic infection after malaria and schistosomiasis. In the current study, stool samples were taken from a total of 270 patients who were attending to Azadi Teaching Hospital of Kirkuk City. The participants aged from (3 month to 79 years old) that suffering diarrhea, abdominal pain, vomiting, dehydration and, flatulence. The study was done between October 2022 and April 2023. By general stool examination identified macroscopically colors that are classified to yellowish, brownish, greenish and bloody. and consistency for liquid, soft and solid as well as microscopic examination for presence of blood and pus cell and identified cyst or trophozoite or both of *E. histolytica* and then isolate and cultivated and maintaining in TYS-S-33 media selective for *E. histolytica* and add healthy human inactivated serum instate of bovine serum to the media.



Introduction

E. histolytica is a parasitic protozoan that causes amoebiasis, a disease that affects the large intestine and can lead to dysentery, liver abscesses, and other serious complications. Its life cycle is relatively simple as it is divided into two basic phases: the active motile phase, which is the feeding phase (Trophozoite), and the dormant resistant phase, which is the infective saccular phase (cyst). The parasite causes intestinal amoebiasis, amoebic colitis, amoebic dysentery, or extraintestinal amoebiasis [1]. The infection spreads all over the world, and the incidence is high in tropical and subtropical regions, as well as in regions with a low level of health and culture [2]. Since they spread from person to person, intestinal parasites are a significant cause of illness and death. This is especially true in developing nations where poor sanitation conditions and a lack of knowledge lead to tainted water supplies and food [3].

The most frequent way that parasites infect the GI tract is through fecal-oral transmission, whether it be through food, water, or hands when feces contaminate them and then go to the mouth [4]. Approximately 90% of people with amoebiasis range from the disease to form Intestinal (luminal form), while dysentery and an invasion of the pathogen occur in less than 10% of infection cases, which can be fatal [5].

Infection with *E. histolytica* occurs when feeding stages adhere to the epithelial layer of the large intestine, as these stages possess virulence factors such as the adhesion factor Lectin, which helps the parasite attach to host cells, as well as its possession of the enzyme lysing foreign proteins, cysteine proteinase, which dissolves cells, as well as its containment of amoebic holes that it creates holes in cell surfaces and the enzymes phospholipase and hemoglobinase are important virulence factors of this parasite [6]. Infection with the parasite stimulates the host's natural immune response and pyrolytic enzymes secreted by the mucous membranes lining the gastrointestinal tract, bowel movement and the acidic environment of the stomach, it is one of the natural defenses shown by the body against the parasite, especially in this strain phenotypically identical to *E. histolytica* [7]. Upon microscopic examination, but genetically different from it and also endemic in the large intestine [8].

In addition to the existence of several cases in which the *E. histolytica* parasite settles in the intestinal cavity in a commensal manner without attacking the tissues, causing what is known as asymptomatic infection, and people, in this case, are considered carriers of the disease, and the infection can be transmitted to another person through food contamination. Water is excreted in the feces of carriers of the disease or is transmitted as a venereal disease, especially in homosexuals [9]. The purpose of this study is to:

1. Identified and isolated of *E. histolytica* from diarrheal patients' stool from different age groups.
2. Cultivate parasite (*Entamoeba histolytica*) in vitro in special media (TYI-S-33).

Materials and methods

270 stool samples from patients who were attending to Azadi Teaching Hospital of Kirkuk City. Those aged from 3 months to 79 years old that suffer diarrhea, abdominal pain, vomiting, dehydration and flatulence. The study was done between October 2022 and April 2023 to isolation *Entamoeba histolytica* and cultivated in selective media for *E. histolytica* TYI-S-33.

Components of TYI-S-33

TYI-S-33 media is a culture medium that is commonly used to grow and maintain *Entamoeba histolytica* in the laboratory. The medium is composed of a variety of nutrients and growth factors that support the growth and multiplication of the parasite. The TYI-S-33 medium is composed of yeast extract, trypticase peptone, iron, glucose, bacteriological agar [10]. To prepare the complete medium add 10 to 15 ml of heat-inactivated adult bovine serum to TYI broth. Note that fetal bovine serum is not acceptable, as fetuin is toxic to the parasite. Use the complete medium within 7 days [11].

Procedure

The preparation of media as ingredients and recommended dosage of serum for TYI-S-33 is typically 13 ml per tube because established cultures inoculated for axenic *E. histolytica* are typically modest. Each isolate uses a different amount of serum because fetuin is poisonous to the parasite, fetal bovine serum is unacceptable [12]. To prepare the medium, the ingredients are mixed together in the appropriate proportions and sterilized by autoclaving (autoclave for 15 min at 121°C under a pressure of 15 lb./in²). Sterilization of the test tube and every culture condition was performed under laminar flow sterilization as part of the culture method (13). Allowed to cool add tween, antibiotic, and vitamins to the medium after sterilization by Millipore filter. contains only those vitamins known to be required by the parasite. It is also available commercially [14]. Measure the pH

of the media with a pH meter (must be 6.8), add HCL if pH is high(alkaline)or NaOH if pH is low(acidic), but not both to gather [15]. Add 10 to 15 ml of heat-inactivated healthy human serum to TYI broth instate of bovine serum because is difficult to obtain it. Use the complete medium within 7 days, dispense into screw-cap borosilicate glass culture tubes (16 by 125 mm) [13].

In most cases 13 ml of TYI-S-33 per tube is the correct amount, as inoculate for axenic *E. histolytica* are generally small in established cultures. The percentage of serum used varies among isolates but is usually either 10 or 15% [12]. The *E. histolytica* culture can then be inoculated onto the surface of the medium and incubated under the necessary conditions, such as a temperature of 35 and 37°C and an atmosphere of 5% CO₂ [13]. If the media is cloudy without any inoculation of the parasite, it indicates that the pH is too high, and the mixture must be discarded [13]. A blind passage should be constructed after 48 hours have passed with no signs of development. Less than 1 ml of the liquid that was above the sediment is then removed from the tube. The debris is redissolved in the remaining liquid and moved to a brand-new culture tube containing a medium, and an antibiotic. The culture is once again inspected as previously mentioned after an additional 48 hours of incubation. Second 48-h incubation is necessary if no amebae are found, and this is followed by a reexamination. If no amebae are discovered, the culture is disregarded as negative [14].

Identification of *E. histolytica* in stool samples

The parasite was diagnosed in the stool samples collected by each patient with amoebic dysentery, was given a sterile plastic cup marked with name, age and asked to provide stool sample each sample was processed using formalin -ether centrifugal sedimentation technique [11]. The following methods as shown in Figure 1.

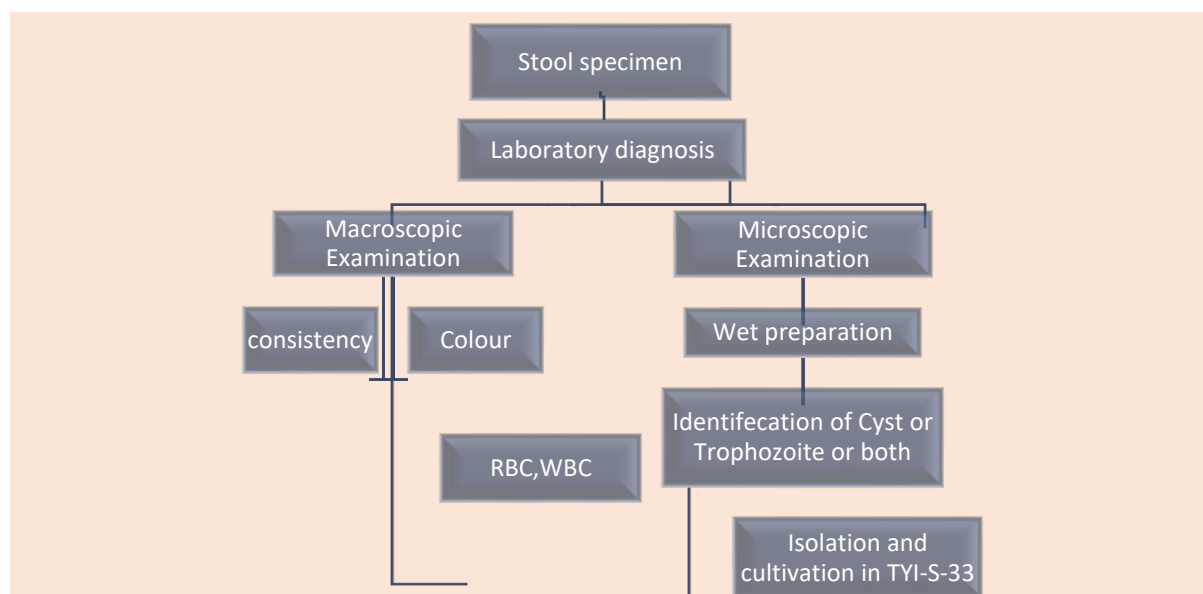


Figure 1. Steps for identification of *E. histolytica* macroscopically and microscopically in stool specimen.

Laboratory diagnosis

A- Macroscopic examination of the stool

Stool samples should be visually inspected for color, consistency, quantity, shape, odor, and presence of mucus. A small amount of mucus in the stool is normal. however, large amounts of mucus or bloody mucus are abnormal. The normal color is brown due to the presence of bilirubin and bile [16].

B- Microscopic examination of stool

The most important stage in identifying abnormalities and gastrointestinal problems is a microscopic examination of the feces. Microscopy is a diagnostic tool that can be used to identify parasitic infection (trophozoite and cyst) of *E. histolytica* [17]. To accomplish this, Emulsify a tiny amount of feces sample with a few drops of saline or Lugol's iodine solution on a glass slide with a thickness of 1 mm. A coverslip was applied on top, and apparent dirt or air bubbles were taken out of the preparation [16]. Stool smears examined at low (10x) and high (40x) magnification with 15 minutes microscopically [18].

Isolation and cultivation of *E. histolytica*: -

For isolation *E. histolytica*, fecal matter can be introduced directly into the medium in pea-sized. the stool sample was obtained with a wooden stick. It is always a good idea to include any parts of the clinical sample's feces that are mucoid or red blood cells [17].

After the incubation period for 48 hours at 37°C was up, the culture media was inspected under a microscope by extracting a drop of the sediment (which is composed of both liquid and solid phases) and placing it on a glass slide using a sterile Pasteur pipette. When the follow-up of the aphid feeding stages was deemed a successful outcome and the culture augmentation was successful, the first microscopic examination was conducted under the lowest magnification power (10x), followed by the highest magnification power (40x), and then the maintenance of the culture medium started. After another 48 hours in the incubator, it is checked once more; if no development is seen, the outcome is deemed negative, and the transplant is abandoned [14,17].

In the early research, it was discovered that without living bacteria, ongoing propagation was impossible and studies of numerous features of the amoeba bacteria host interaction could be conducted thanks to the invention of axenic media which allowed amoebae to grow and divide in the absence of any other living entity or cell. Nevertheless, not all amoebae could develop in axenic circumstances as shown in Figure 2.

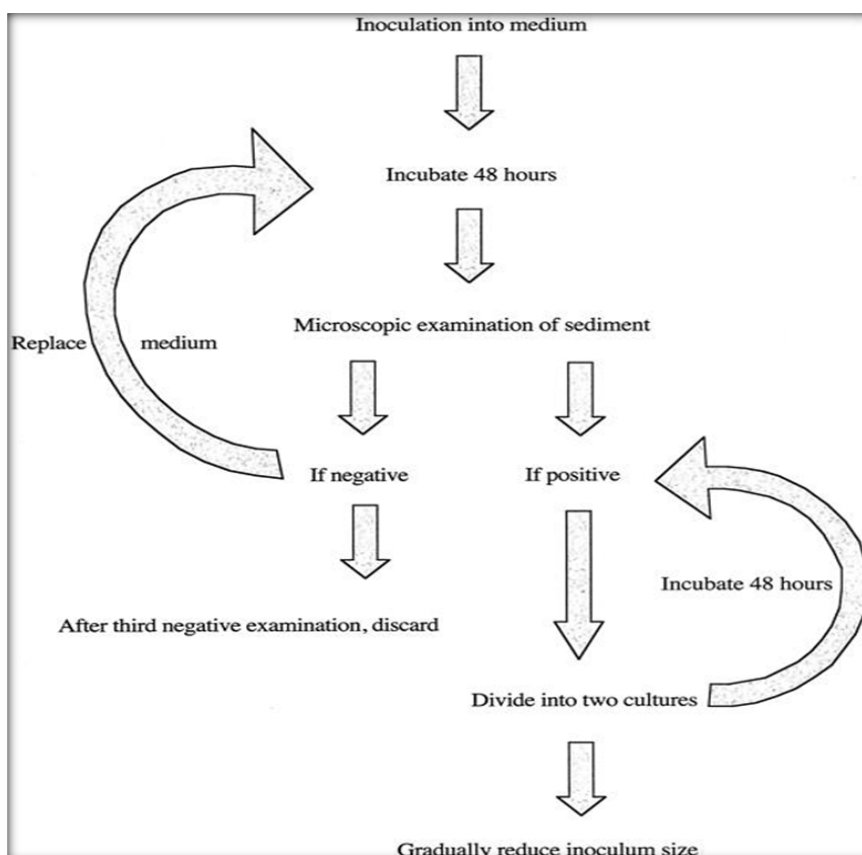


Figure 2. Stages in cultivation of *E. histolytica* in vitro [13].

3-Maintenance of *E. histolytica* in culture media

A drop of *E. histolytica* was removed after 48 hours of incubation at 37°C, and inspected under a microscope, and numerous subcultures were carried out when growth was observed. The tube that contained the parasites, were combined and they were then put into a brand-new, sterile culture tube, which contained the culture medium. For an additional 48 hours, this procedure was repeated to keep the amoeba alive with best PH for survival of trophozoite [17] [18]. For assay, the parasite was maintained in axenic condition at 36.5°C for 30 days by serial sub cultivation in TYI-S-33 [19].

Results

Counting of *E. histolytica* in culture media

At initial counting of the parasite by putting 20 µl of a sample containing *E. histolytica* in 380 µl of TYI-S-33 media and a total count of 44 cell /µl as shown in Figure-3, and counting as this equation:

Total number of parasites = number of parasites in four large squares $\times 25000 \times 2$ [20].

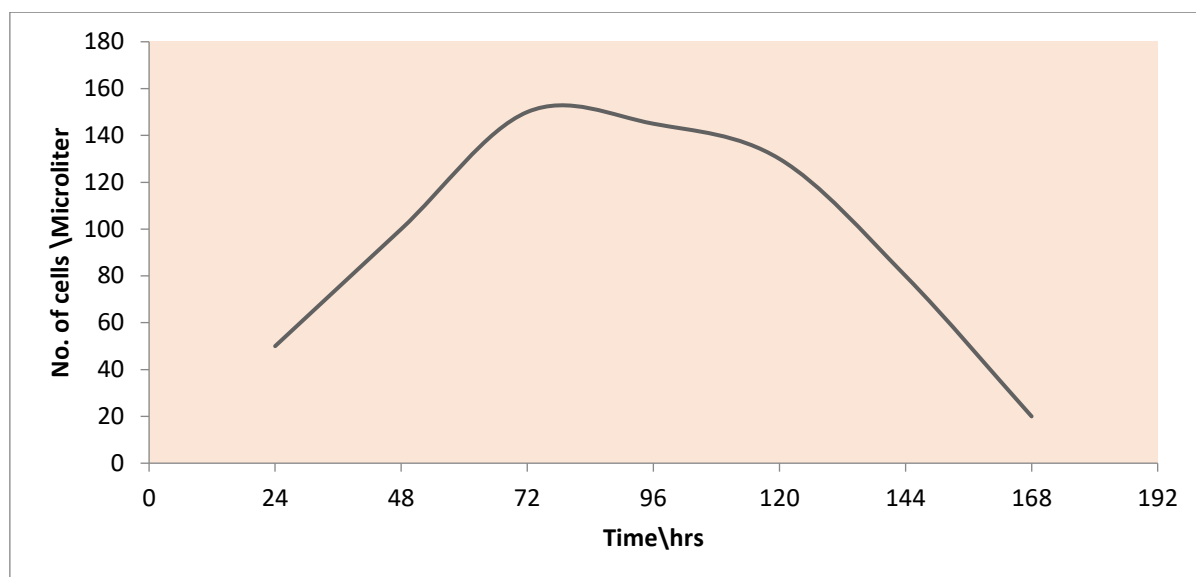


Figure 3. Growth curve represents number of parasites\ ul VS Time\hrs. for *E. histolytica* growth on TYI-S-33 medium at 37°C.

The culture medium (TYI-S-33) contains different components as well as healthy human serum instead of bovine serum [21]. It was used to develop *E. histolytica* outside the living body, as the results showed that only five samples gave growth in it out of a total of thirty-nine samples, with a percentage of 12.82 %. and significant differences were observed among them at level 0.0008 as shown in Table 1.

Table 1. Number and percentages of cultivated samples studied for patients in vitro.

Culture result on TYI-S-33	NO. of sample	Percentage %
Positive result	5	12.82 %
Negative result	34	87.179 %
Total	39	100 %
**		
Chi-Square = 43.128 P-Value = 0.0008		

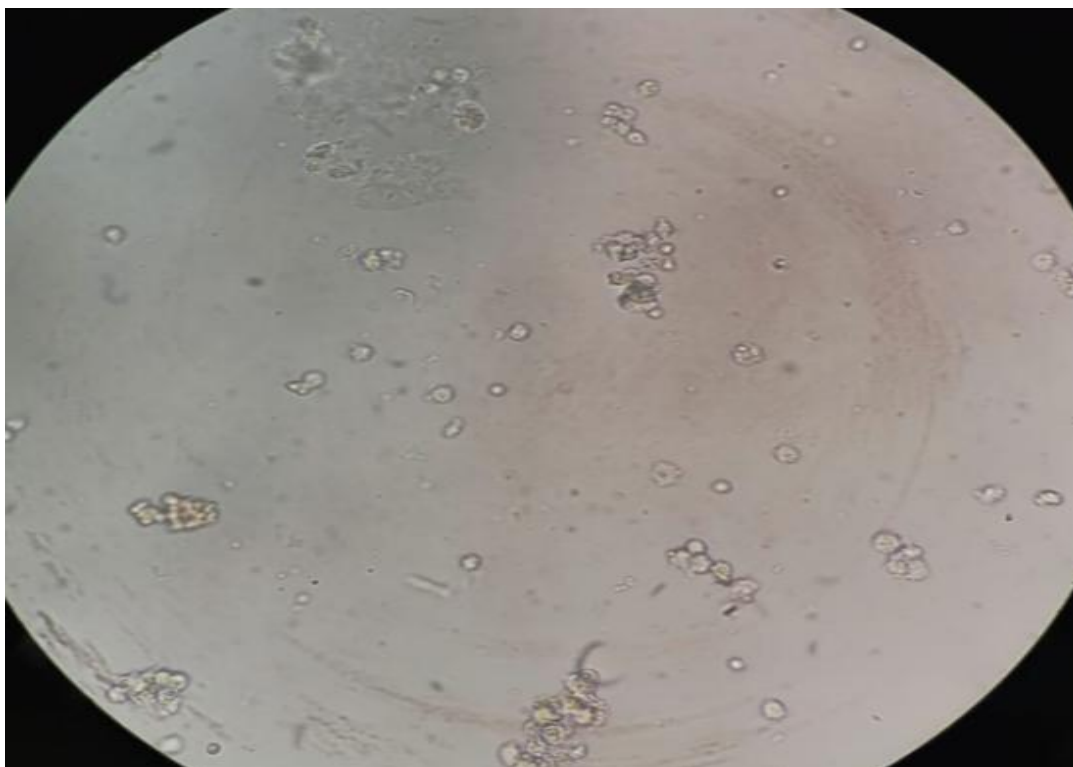


Figure 4. Positive culture of *E. histolytica* on TYI-S-33

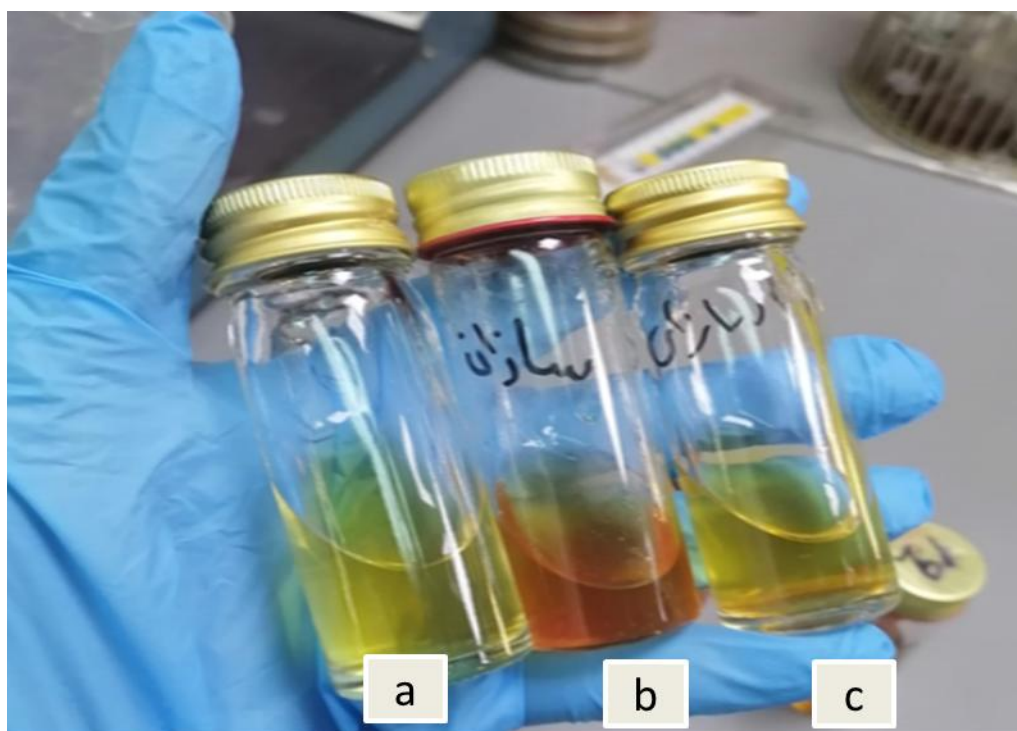


Figure 5. TYI-S-33 media: a. TYI-S-33 media; b. direct culture of stool after 48 hrs. of incubation; c. Subculture after 24 hrs. of incubation

Discussion

In order to distinguish the parasites and study their pathogenesis, the full investigation of the amoeba that causes amoebiasis has become one of the most important goals in medical and research laboratories, and based on it, diagnostic methods have been developed, in order to distinguish the two genetically different types. Therefore, the development of amoeba in culture media is an important step in epidemiological and research

studies to study pathogenesis and biological nature for this parasite [22]. The fact that parasite culture is more sensitive compared to wet microscopic preparation methods reduces the need for many samples in diagnosis [23].

The use of culture media in research laboratories has declined only due to its time consumption and high expense, so it is not used in medical laboratories [24]. The medium was maintained in the culture medium every 48 hours because it was found that the best time to transfer the culture medium is between (48 and 72) hours after the addition of human serum, vitamins, and antibiotics, the rate of parasite multiplication is at its highest level during this time. During this time, there was a significant amount of amoeba destruction in the culture medium due to the delay in maintaining the amoebic culture medium. Additionally, the high amoebic sensitivity to bacterial and fungal development also causes the parasite to die in some cases under study it required adding many antibiotics (Penicillin, Streptomycin, Nystatin) to inhibit bacterial and fungal growth, respectively [25].

E. histolytica parasite was successfully isolated and developed from the feces of patients on the TYI-S-33 culture medium [26]. The medium has a high degree of accuracy in detecting infections with *E. histolytica* in the stool [27], because the composition of TYI-S-33 medium that is a rich source of vitamins, minerals, amino acids and glucose source of energy [13].

Our results compared with other results that are used LEM media and similar in cultivation and isolation *E. histolytica* but LEM media not specific for *E. histolytica* and used for the establishment and maintenance of the following organisms (*Entamoeba histolytica*, *Giardia intestinalis*, *Trichomonas vaginalis*, *Dientamoeba fragilis*, *Blastocystis hominis*, and *Balantidium coli*) [15].

There are other media xenic (parasite growth in the presence of normal and unspecified bacteria) like Modified Boeck and Drbohlav egg diphasic medium, Balamuth's medium, Jones's medium, and TYSGM-9 are examples of xenic medium utilized for culture of *Entamoeba* spp. but TYI-S-33 axenic media that parasite growth without any normal flora [28,29], and this serve our more to detected any contamination.

As shown in Figure-3, the curve represents the number of cells in culture media over a period of time. During initial growth of parasite showed the low number of replications after 24 hours, or the cell not increasing, but protein synthesis was still occurring. After 48 hours, the replication increased and the number of cells increased, but after 72 hours, the replication remained stable, which indicates that the number of living and dead cells had equaled, and then the parasite's growth continued to decline until complete sterility [30]. When grown in TYI-S-33 culture medium at a temperature of 37 °C, the growth stages of the *E. histolytica* were measured from the beginning of the inoculation process (0.1-10 ml trophozoite stage) until the amoeba's eventual destruction. The examination was conducted every 24 hours of incubation without changing the culture medium or maintaining it, a drop of culture medium was taken to count the parasite, and the incubation process was repeated for another day. As a result, the procedure persisted until the amoeba was entirely eliminated. The findings revealed variations in the amoeba's rate of doubling over the course of the observation period, with an increase in growth rate seen after 72 hours (the third day) of incubation as a result of a rise in the cytoplasmic division level. The *E. histolytica* was present in the feeding stages but the parasite's growth rate fell after 96 hours (the fourth day) as a result of a decline in the rate of amoebic division. This trend persisted until the nine days of incubation, when the histolytic amoeba was completely destroyed.

The TYI-S-33 culture media was perpetuated every 48 hours to achieve the best amoebic count after 5 to 6 culture transfers after the effective establishment of *E. histolytica* there. The multiplication rate reaches its peak level after 48 hours after inoculation in the culture media, the multiplication rate peaks at 48–72 hours of incubation, and then it begins to decline [28,29]. The fact of this is due to symbolizes the amoeba's logarithmic phase of growth, during which it is collected. Following this time, nutrients in the medium start to decline as a result of the parasite's feeding stages consuming them, and amoebic growth remnants start to accumulate. Both indicated that this has an impact on the growth of the amoeba's feeding stages [25,30].

Cells became rounder and decreased in size, and by 168 h, no live trophozoites were visible and only rounded cysts and dead cell debris remained. To ascertain that trophozoites had indeed undergone stage conversion to true cysts, we assessed the four hallmarks of encystation: presence of a chitin cell wall, tetra nucleation, decreased cell size and roundedness, and detergent resistance [31], and this agreeing with our result that showed cyst stage after 72hrs. As shown in Figure-4 cyst formation appear clearly and this process decreased the number of trophozoite too [31]. This allows us to study more comprehensively about the parasitic strains and the diseases they cause, as a group of studies was carried out to analyze the identical enzymes (isoenzyme) of the parasite strains developing in the culture media, as well as other more qualitative and sensitive studies such as partial biology, biochemistry, and immunology, as well as microscopy to increase the accuracy and sensitivity of the results. Using the bovine serum as references cause has the component more accurate from healthy serum to cultivation and growing the parasite.

Discussion

Using TYI-S-33 medium supplemented with healthy human serum rather than bovine serum, this study effectively demonstrated the in vitro cultivation and maintenance of *Entamoeba histolytica*. Its suitability for laboratory use was confirmed by the medium's ability to support parasite growth and enable the observation of different developmental stages. Maintaining viable trophozoites and avoiding contamination required routine subculturing every 48 hours. The results demonstrate that TYI-S-33 is a dependable medium for researching the pathogenicity and biology of *E. histolytica*. In order to increase parasite yield and stability for experimental and diagnostic uses, more research may be needed to optimize culture conditions.

References

- [1] Junaidi, J., Cahyaningsih, U., Purnawarman, T., Latif, H., Sudarnika, E., Hayati, Z. and Muslina, M., 2020. *Entamoeba histolytica* neglected tropical diseases (NTDs) agents that infect humans and some other mammals: A review. In E3S Web of Conferences (Vol. 151, p. 01019). EDP Sciences.
- [2] Ankri, S., 2021. *Entamoeba histolytica*—gut microbiota interaction: more than meets the eye. *Microorganisms*, 9(3), p.581.
- [3] Ahmed, A.O., Ali, A.A. and Mardan, H.J., 2023. Hematological Change in Type II Diabetic Mellitus (TIIDM) patient Infected with intestinal parasites in Kirkuk/Iraq. *NTU Journal of Pure Sciences*, 2(1).
- [4] Sultan, N.M., Ali, A.A. and Mardan, H.J., 2023. Hematological changes in IBS patients with intestinal parasites. *NTU Journal of Pure Sciences*, 2(1).
- [5] Varet, H., Shaulov, Y., Sismeiro, O., Trebicz-Geffen, M., Legendre, R., Coppée, J.Y., Ankri, S. and Guillen, N., 2018. Enteric bacteria boost defences against oxidative stress in *Entamoeba histolytica*. *Scientific reports*, 8(1), p.9042.
- [6] Garmie, V., 2016. Prevalence and Intensity of *Entamoeba histolytica* in Patients Attending Health Centres in Mathare Slums, Nairobi County, Kenya (Doctoral dissertation, University of Nairobi).
- [7] Abo-zeid, Y. and Williams, G.R., 2020. The potential anti-infective applications of metal oxide nanoparticles: A systematic review. *Wiley Interdisciplinary Reviews: Nanomedicine and Nanobiotechnology*, 12(2), p.e1592.
- [8] Mathison, B.A. and Pritt, B.S., 2021. Cyclosporiasis—Updates on clinical presentation, pathology, clinical diagnosis, and treatment. *Microorganisms*, 9(9), p.1863.
- [9] Singh, A., Banerjee, T., Khan, U. and Shukla, S.K., 2021. Epidemiology of clinically relevant *Entamoeba* spp. (*E. histolytica*/dispar/moshkovskii/bangladeshi): A cross sectional study from North India. *PLoS neglected tropical diseases*, 15(9), p.e0009762.
- [10] Fotadar, R., Stark, D., Beebe, N., Marriott, D., Ellis, J. and Harkness, J., 2007. Laboratory diagnostic techniques for *Entamoeba* species. *Clinical microbiology reviews*, 20(3), pp.511-532.
- [11] AL-Shaheen, Z., Kassim AL-Maki, A. and Khalaf, K.H., 2007. A STUDY ON PREVALENCE OF ENTAMOEBA HISTOLYTICA & Giardia Lamblia INFECTION. *Basrah Journal of Veterinary Research*, 6(2), pp.30-36.
- [12] Wesel, J., Shuman, J., Bastuzel, I., Dickerson, J. and Ingram-Smith, C., 2021. Encystation
- [13] Carrero, J.C., Reyes-López, M., Serrano-Luna, J., Shibayama, M., Unzueta, J., Leon-Sicairos, N. and de la Garza, M., 2020. Intestinal amoebiasis: 160 years of its first detection and still remains as a health problem in developing countries. *International Journal of Medical Microbiology*, 310(1), p.151358.
- [14] Clark, C.G. and Diamond, L.S., 2002. Methods for cultivation of luminal parasitic protists of clinical importance. *Clinical microbiology reviews*, 15(3), pp.329-341.
- [15] Fotadar, R., Stark, D., Beebe, N., Marriott, D., Ellis, J. and Harkness, J., 2007. Laboratory diagnostic techniques for *Entamoeba* species. *Clinical microbiology reviews*, 20(3), pp.511-532.
- [16] Clark, C.G. and Diamond, L.S., 2002. Methods for cultivation of luminal parasitic protists of clinical importance. *Clinical microbiology reviews*, 15(3), pp.329-341.
- [17] Taylor, A.E. and Baker, J.R., 1968. The cultivation of parasites in vitro. The cultivation of parasites in vitro.
- [18] Thaker, H. M., Tawfeeq A. A, Ali A. A., 2023, Evaluation of *Trichomonas vaginalis* and *Candida albicans* alongside with pathogenic bacteria in Kirkuk females, *NTU Journal of Pure Sciences* 3(1) : 1-10.
- [19] Gonzalez-Salazar, F., Meester, I., Guzmán De La Garza, F.J., La Garza-Salinas, D., Sampayo-Reyes, A., Garza-Gonzalez, J.N., Monsivais-Diaz, O., Barba-Dávila, B.A., Hernández-García, M.E. and Vargas-Villarreal, J., 2018. "CLUPS": A New Culture Medium for the Axenic Growth of *Entamoeba histolytica*. *Journal of parasitology research*, 2018.

- [20] Bartlett, M.S., Harper, K., Smith, N., Verbanac, P. and Smith, J.W., 1978. Comparative evaluation of a modified zinc sulfate flotation technique. *Journal of clinical microbiology*, 7(6), pp.524-528.
- [21] Paniker, C.J. and Ghosh, S., 2017. Paniker's textbook of medical parasitology. JP Medical Ltd.
- [22] Nash, T.E., 2019. Long-term culture of *Giardia lamblia* in cell culture medium requires intimate association with viable mammalian cells. *Infection and Immunity*, 87(11), pp.10-1128.
- [23] Shankar, P., Mishra, J., Bharti, V., Parashar, D. and Singh, S., 2019. Multiplex PCR assay for simultaneous detection and differentiation of *Entamoeba histolytica*, *Giardia lamblia*, and *Salmonella* spp. in the municipality-supplied drinking water. *Journal of Laboratory Physicians*, 11(03), pp.275-280.
- [24] El-Dib, N.A., 2017. *Entamoeba histolytica*: an overview. *Current Tropical Medicine Reports*, 4, pp.11-20.
- [25] Saidin, S., Othman, N. and Noordin, R., 2019. Update on laboratory diagnosis of amoebiasis. *European Journal of Clinical Microbiology & Infectious Diseases*, 38, pp.15-38.
- [26] Naiyer, S., Bhattacharya, A. and Bhattacharya, S., 2019. Advances in *Entamoeba histolytica* biology through transcriptomic analysis. *Frontiers in microbiology*, 10, p.1921.
- [27] König, C., Meyer, M., Lender, C., Nehls, S., Wallaschkowski, T., Holm, T., Matthies, T., Lercher, D., Matthiesen, J., Fehling, H. and Roeder, T., 2020. An alcohol dehydrogenase 3 (ADH3) from *Entamoeba histolytica* is involved in the detoxification of toxic aldehydes. *Microorganisms*, 8(10), p.1608.
- [28] Wesel, J., Shuman, J., Bastuzel, I., Dickerson, J. and Ingram-Smith, C., 2021. Encystation of *Entamoeba histolytica* in axenic culture. *Microorganisms*, 9(4), p.873.
- [29] Burgess, S.L. and Petri, W.A., 2016. The intestinal bacterial microbiome and *E. histolytica* infection. *Current Tropical Medicine Reports*, 3, pp.71-74.
- [30] Jafari, M., Amini-Khoei, H., Cheshmpanam, M. and Abdizadeh, R., 2022. A survey on the anti-*Trichomonas vaginalis* effect of the hydroalcoholic extract of various medicinal plants in vitro. *Journal of Shahrekord University of Medical Sciences*, 25(1), pp.1-6.
- [31] Dimpler, J., Huppertz, T. and Kulozik, U., 2020. Invited review: Heat stability of milk and concentrated milk: Past, present, and future research objectives. *Journal of dairy science*, 103(12), pp.10986-11007.