Heliyon 6 (2020) e04805

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

Growth comparison of *Acanthamoeba* genotypes T3 and T4 in several culture media

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ARTICLE INFO

Keywords:
Cell biology
Microbiology
Microorganism
Protozoa
Acanthamoeba castellanii
Acanthamoeba griffin
Culture media

ABSTRACT

Acanthamoeba causes severe diseases such as Granulomatous Amebic Encephalitis (GAE) and *Acanthamoeba* keratitis (AK). Improving the culture media classically used for this amoeba could help to identify it quickly and facilitate its study as a biological model. The purpose of this study was to compare the growth of two *Acanthamoeba* genotypes (T3 and T4) in several culture media. *Acanthamoeba griffini* (T3 genotype) and *Acanthamoeba castellanii* (T4 genotype) were cultured in PYG, TSY, TYI-S-33, RPMI, and RPMI-FBS medium. The number of amoebas grown in different culture media was counted and compared to each other for 14 days. Findings in this research revealed the highest growth in RPMI-FBS medium. For this reason, we can recommend this culture medium to promote the growth of *Acanthamoeba* in its biological studies.

1. Introduction

Acanthamoeba is one of the most common opportunistic free-living amoebae with ubiquitous presence in various environmental sources such as air, soil, and water. *Acanthamoeba* causes severe diseases such as Granulomatous Amebic Encephalitis (GAE), cutaneous lesions, and *Acanthamoeba* keratitis (AK) that is associated with contact lens use or corneal trauma (Marciano Cabral and Cabral, 2003). This amoeba is also used for biological studies, and there are many studies about the interaction of *Acanthamoeba* with other microorganisms (Siddiqui and Khan, 2012; Axelsson-Olsson et al., 2005; Abrahão; et al., 2014). Currently, there is no fully efficient treatment scheme to treat Acanthamebiasis, more research is needed to understand its molecular and biochemical processes and thus efficiently control and treat infections. It is necessary to improve the culture media to grow faster and therefore to carry out biochemical and molecular studies in less time (Baig, 2018; Khan, 2003; De Jonckheere, 1980). The non-nutrient agar (NNA) and Peptone-yeast-glucose (PYG) media have been introduced as standard culture media for *Acanthamoeba* (Garcia, 2007; Axelsson-Olsson et al., 2009; Sheng et al. 2009; Panjwani, 2010). In this study, *Acanthamoeba griffini* (T3 genotype) and *Acanthamoeba castellanii* (T4 genotype) were cultured in PYG, Trypticase Soy Broth with Yeast Extract (TSY), Trypticase-Yeast Extract-Iron-Serum (TYI-S-33), Roswell Park Memorial Institute Media (RPMI) and RPMI-FBS medium. Then the number of amoebas in different culture media was counted and compared to each other for 14 days. TYI-S-33 culture media is used for the axenic culture of *Entamoeba histolytica* (Diamond, 1982). It should be noted that a modified form of this culture medium is also used for the cultivation of *Giardia* and *Trichomonas* parasites (Clark and Diamond, 2002). TSY, in addition to being used for the growth of *Acanthamoeba*, is widely used to study symbiotic interactions between this organism and bacteria (Lagkouvardos et al. 2014). RPMI 1640, also known as RPMI medium, is a growth medium used in cell culture. It can also be used to culture *leishmania* and *malaria* parasites. RPMI-FBS provides a very rich medium for the growth of parasites and cells. This culture medium is more accessible compared to others and is commercially available (Schuster, 2002; Lemesre et al., 1988). Additionally, the growth of both *Acanthamoeba* genotypes on non-nutrient agar (NNA) was evaluated by replacing the use of the bacterial lawn with a certain amount of each described culture medium and the growth was quantified on specific days.

2. Material and methods

2.1. Preparation of Acanthamoeba for cultivation

Both *Acanthamoeba griffini* (genotype T3) (MH938695) and *Acanthamoeba castellanii* (genotype T4) (MH938701) were used during the experiments. They were preserved and grown in nutrient-free agar (NNA) in the facilities of the Parasitology Laboratory, University of Tehran. They were harvested from the plate surface using Cell Scrapers and then,

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https://doi.org/10.1016/j.heliyon.2020.e04805
Received 9 January 2019; Received in revised form 7 May 2019; Accepted 24 August 2020

Table 1. T-test for equality of means.

T	DF	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference		N	Mean	Std. Deviation	Name of amoeba
					Lower	Upper				
0.510	78	0.612	0.52500	1.02984	1.52524	2.57524	40	8.775	4.92241	*Acanthamoeba castellanii* (T4 genotype)
							40	8.250	4.26524	*Acanthamoeba griffini* (T3 genotype)

Table 2. One-way analysis of variance (One-way ANOVA) to study the growth of *Acanthamoeba* trophozoite at different time points.

Day	N	Mean	Std. Deviation	95% Confidence Interval for Mean		Minimum	Maximum	F	SIG
				Lower Bound	Upper Bound				
1.00	10	5.0000	.00000	5.0000	5.0000	5.00	5.00	25.008	0.000
2.00	10	7.7000	1.88856	6.3490	9.0510	5.00	11.00		
4.00	10	12.2000	3.55278	9.6585	14.7415	8.00	19.00		
6.00	10	13.6000	3.97772	10.7545	16.4455	8.00	21.00		
8.00	10	12.7000	3.16403	10.4366	14.9634	9.00	17.00		
10.00	10	9.1000	2.42441	7.3657	10.8343	6.00	13.00		
12.00	10	5.5000	1.71594	4.2725	6.7275	3.00	8.00		
14.00	10	2.3000	1.56702	1.1790	3.4210	1.00	5.00		
Total	80	8.5125	4.58394	7.4924	9.5326	1.00	21.00		

washed by centrifugation three times with amoeba saline (ATCC 1323 Page's amoeba saline) and counted in the Neubauer chamber.

2.2. Preparation of culture media

Culture media were prepared according to protocols (PYG) (ATCC 712), (TSY) (ATCC 993), (TYI-S-33) (Diamond, 1982). The RPMI 1640 medium and Fetal Bovine Serum was purchased from Sigma-Aldrich. One ml of prepared culture media were added to each of three wells of a 24 well plate (SPL Cell Culture Plate). Finally, 0.1 mg/mL penicillin (Sigma-Aldrich), 0.1 mg/mL streptomycin (Sigma-Aldrich), were added to all wells. The amount of FBS added to the RPMI medium was twelve percent. Finally, 5×10^4 of each amoeba in 100 µl and added to each well. Plates were incubated at 28 °C for 2–14 days.

2.3. Qualitative study of amoeba growth in non-nutrient agar (NNA) culture media

Fifty µl of each culture medium (PYG, TSY, TYI-S-33, RPMI and RPMI-FBS) and 5 x 10^4 trophozoites were added in five plates with NNA medium. later the growth was evaluated using an inverted microscope (AE31 Elite Binocular) every day for 14 days. As a control, NNA standard medium supplemented to an *Escherichia coli* lawn was used.

3. Results

3.1. Effect of culture media on amoeba growth

After counting the number of amoebae recovered every 24 h for 14 days, the results based on the collected data are shown in Tables 1 and 2. The mean for *Acanthamoeba castellanii* (genotype T4) was 8.77, and for *Acanthamoeba griffini* (genotype T3) it was 8.25. Based on the independent t-test, the p-value was $0.612 > 0.05$ with $T = 0.510$. This result indicates that there was no significant difference between the two groups (Table 1).

Based on one-way analysis of variance (One-way ANOVA) to study the difference in the number of amoeba on different days and according to the amount of $F = 25.008$ and p-value $0.000 < 0.05$, there is a significant difference in amoebic growth on different days. This amount was higher on the sixth day (Table 2). When evaluating whether there is a difference in the growth of both *Acanthamoeba* genotypes using different culture media. The value of F was 2,879 while the value of P was $0.013 < 0.05$, demonstrating that there is indeed a difference in growth when using different culture media. The highest growth was obtained in RPMI supplemented with FBS (Table 3).

3.2. Effect of culture media on amoeba growth in NNA culture media

In the study where amoebas were inoculated into added NNA plates from each culture medium, the highest growth was observed on the plate containing RPMI-FBS, PYG, TSY, TYI-S-33, and RPMI Media. This result

Table 3. One-way analysis of variance (One-way ANOVA) to study the differences in numbers of *Acanthamoeba* trophozoite at (1–14) days of culturing in different media.

culture media	N	Mean	Std. Deviation	95% Confidence Interval for Mean		Minimum	Maximum	F	SIG
				Lower Bound	Upper Bound				
PYG	16	10.0625	4.37369	7.7319	12.3931	4.00	17.00	2.879	0.028
TSY	16	9.3125	4.04506	7.1570	11.4680	3.00	15.00		
TYI-S-33	16	7.8750	4.12916	5.6747	10.0753	1.00	14.00		
RPMI	16	5.8750	2.65518	4.4602	7.2898	1.00	9.00		
RPMI + FCS	16	10.5625	6.33476	7.1869	13.9381	1.00	21.00		
Total	80	8.8250	4.61910	7.7971	9.8529	1.00	21.00		

was consistent with the results of amoeba cultivation in the axenic culture media. There was no difference in growth between the *Acanthamoeba griffini* (T3 genotype) and *Acanthamoeba castellanii* (T4 genotype).

4. Discussion

Acanthamoeba is one of the free-living amoebas that cause severe diseases and used for biological studies. Currently, more research is needed to control this opportunistic amoeba, in addition to culture media that accelerate growth when it is first isolated, thus allowing faster detection and biochemical and molecular analysis in less time (Baig, 2018; Khan, 2003; De Jonckheere, 1980). In this study, it was observed that *Acanthamoeba* spp. have the highest growth in the early days in RPMI-FBS medium, but in other culture media, more days are needed to increase the amoeba growth. On the other hand, growth in NNA medium enriched with RPMI-FBS was the highest compared to the other compound media and the control of NNA with *Escherichia coli*. This result can be useful in the diagnosis of AK, promoting the growth of trophozoites and/or cysts found in corneal scraping samples. In the study of Axelsson-Olsson et al. (2009), they proposed a novel and simple technique based on the use of PYG medium for long-term storage of *Acanthamoeba*, resulting in survival rates of at least four years for *Acanthamoeba polyphaga* and three years for *Acanthamoeba castellanii* (Axelsson-Olsson et al., 2009). The results of the present study were consistent with the results of Eroğlu; et al., they observed the highest growth peak in RPMI-FBS, however, this finding agrees with the increase in growth in the other culture media. Also, there were no signficant differences in growth in PYG culture medium as compared to our study (Eroğlu et al., 2015). In another study by Peretz, it was observed that culture in PYG and TYI-S-33 medium could help to diagnose *Acanthamoe*ba keratitis alongside the calcofluor white staining and fluorescence microscopy. PYG culture media was reliable, cost-effective, efficient, and straightforward method for the detection and diagnosis of *Acanthamoeba* keratitis (AK) (Peretz et al., 2015). Heredero-Bermejo; et al., observed that both the BHI medium (brain-heart infusion, standard medium in microbiology, rarely used in amoeba culture) and the PYG prepared with Pancreatic Digest of Casein (Bacto casitone) are the most appropriate for growing and maintaining the *Acanthamoeba castellanii* strain. Also, PYG–Bacto casitone is the most suitable for conducting prolonged pharamacological screening assays prolonged (Heredero-Bermejo et al., 2012). In a study by Rahdar et al., while amoeba growth in PYG medium was not successful, efficient culturing in TYI-S-33 could be achieved, Therefore, they conclude that TYI-S-33 could be a substitute for PYG medium (Rahdar et al., 2012). PYG and NNA media are used daily by researchers working with *Acanthamoeba*, both for the isolation and identification of *Acanthamoeba spp*., in addition to its maintenance over many years (Milanez et al., 2020; Wopereis et al., 2020; Retana et al., 2020). Few studies have been conducted comparing *Acanthamoeba* growth in different culture media. As discussed above, newer and more accessible culture media can be tested for this amoeba.

5. Conclusion

The present investigation showed the advantages of using RPMI-FBS medium to increase the growth of *Acanthamoeba* in axenic culture media. Furthermore, *Acanthamoeba* was observed to grow faster in nutrient-free agar (NNA) culture medium supplemented with RPMI-FBS compared to the other media and the usual method of adding bacteria.

Declarations

Author contribution statement

Alireza Latifi: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Mahboobeh salami: Performed the experiments.

Funding statement

This work was supported by the Deptartment of Medical Parasitology and Mycology, School of Public Health, Tehran University of Medical Sciences.

Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

Acknowledgements

We wish to thank for valuable guidance Dr. Elham Kazemirad, Dr. Mostafa Rezaeian, Dr.Saeedeh Shojaee and Amezola Rivera Jesús Antonio.

References

Abrahão, J.S., Dornas, F.P., Silva, L.C., Almeida, G.M., Boratto, P.V., Colson, P., La Scola, B., Kroon, E.G., 2014. *Acanthamoeba polyphaga* mimivirus and other giant viruses: an open field to outstanding discoveries. Virol. J. 30 (11), 120.

Axelsson-Olsson, D., Waldenstrom, J., Broman, T., Olsen, B., Holmberg, M., 2005. Protozoan *Acanthamoeba polyphaga* as a potential reservoir for Campylobacter jejuni. Appl. Environ. Microbiol. 71, 987–992.

Axelsson-Olsson, D., Olofsson, J., Ellström, P., Waldenström, J., Olsen, B., 2009. A simple method for long-term storage of *Acanthamoeba* species. Parasitol. Res. 104 (4), 935–937.

Baig, A.M., 2018. Innovative methodology in the discovery of novel drug targets in the free-living amoebae. Curr. Drug Targets, 2018 Apr 25.

Clark, C.G., Diamond, L.S., 2002. Methods for cultivation of luminal parasitic protists of clinical importance. Clin. Microbiol. Rev. 15, 329–341.

De Jonckheere, J., 1980. Growth characteristics, cytopathic effect in cell culture, and virulence in mice of 36 type strains belonging to 19 different *Acanthamoeba* spp. Appl. Environ. Microbiol. 39 (4), 681–685.

Diamond, L.S., 1982. A new liquid Media for xenic cultivation of *Entamoeba histolytica* and other lumen dwelling protozoa. J. Parasitol. 68, 958–959.

Eroğlu, F., Evyapan, G., Koltaş, İ.S., 2015. The cultivation of Acanthamoeba using with different axenic and monoxenic media. Middle Black Sea J. Health Sci. 1 (3), 13–17.

Garcia, L.S., 2007. Diagnostic Medical Parasitology, fifth ed. American Society for Microbiology, Washington, D.C.

Heredero-Bermejo, I., San Juan Martin, C., Soliveri de Carranza, J., Copa-Patiño, J.L., Pérez-Serrano, J., 2012. *Acanthamoeba castellanii*: in vitro UAH-T17c3 trophozoite growth study in different culture media. Parasitol. Res. 110 (6), 2563–2567.

Khan, N.A., 2003. Pathogenesis of *Acanthamoeba* infections. Microb. Pathog. 6, 277–285.

Lagkouvardos, I.1, Shen, J., Horn, M., 2014. Improved axenization method reveals complexity of symbiotic associations between bacteria and *Acanthamoebae*. Environ. Microbio. Rep. 6 (4), 383–388.

Lemesre, J.L., Kweider, M., Darcy, F., Santoro, F., 1988. Requirement of defined cultivation conditions for standard growth of *Leishmania* promastigote in vitro. Acta Trop. 45, 99–108.

Marciano Cabral, F., Cabral, G., 2003. *Acanthamoeba* spp. as agents of disease in humans. Clin. Microbiol. Rev. 16, 273–307.

Milanez, G., Masangkay, F., Hapan, F., Bencito, T., Lopez, M., Soriano, J., Ascaño, A., Lizarondo, L., Santiago, J., Somsak, V., Kotepui, M., Tsiami, A., Tangpong, J., Karanis, P., 2020. Detection of *Acanthamoeba* spp. in two major water reservoirs in the Philippines. J. Water Health 18 (2), 118–126.

Panjwani, N., 2010. Pathogenesis of *Acanthamoeba* keratitis. Ocul. Surf. 8 (2), 70–79.

Peretz, A., Geffen, Y., Socea, S.D., Pastukh, N., Graffi, S., 2015. Comparison of fluorescence microscopy and different growth media culture methods for *Acanthamoeba* keratitis diagnosis. Am. J. Trop. Med. Hyg. 93 (2), 316–318.

Rahdar, M., Niyyati, M., Salehi, M., Feghhi, M., Makvandi, M., Pourmehdi, M., Farnia, S., 2012. Isolation and genotyping of *Acanthamoeba* strains from environmental sources in ahvaz city, khuzestan province, southern Iran. Iran. J. Parasitol. 7 (4), 22–26.

Retana, Moreira L., Vargas Ramírez, D., Linares, F., Prescilla Ledezma, A., Vaglio Garro, A., Osuna, A., Lorenzo Morales, J., Abrahams Sandí, E., 2020. Isolation of *Acanthamoeba* T5 from water: characterization of its pathogenic potential, including the production of extracellular vesicles. Pathogens (2), 9. Feb 21.

Schuster, F.L., 2002. Cultivation of *plasmodium* spp. Clin. Microbiol. Rev. 15 (3), 355–364.

Sheng, W.H., Hung, C.C., Huang, H.H., Liang, S.Y., Cheng, Y.J., Ji, D.D., Chang, S.C., 2009. First case of granulomatous amebic encephalitis caused by *Acanthamoeba castellanii* in Taiwan. Am. J. Trop. Med. Hyg. 81 (2), 277–279.

Siddiqui, R., Khan, N.A., 2012. Biology and pathogenesis of *Acanthamoeba*. Parasites Vectors 10, 5–6.

Wopereis, D.B., Bazzo, M.L., de Macedo, J.P., Casara, F., Golfeto, L., Venancio, E., de Oliveira, J.G., Rott, M.B., Caumo, K.S., 2020. Free-living amoebae and their relationship to air quality in hospital environments: characterization of *Acanthamoeba* spp. obtained from air-conditioning systems. Parasitol. 18, 1–9.