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ORIGINAL ARTICLE

The Cultivation of *Acanthamoeba* Using Different Axenic and Monoxenic Media

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Abstract

Objective: *Acanthamoeba* species are the ubiquities free-living amoebae and can infect humans, causing diseases such as keratitis and encephalitis. *Acanthamoeba* species are often grown on non-nutrient agar spread with *Escherichia coli* or peptone-yeast extract-glucose. We investigated the amount of growth of *Acanthamoeba* in different axenic and monoxenic media.

Methods: The non-nutrient agar with *Pseudomonas aeuroginosa*, *Enterobacter aerogenes*, *Staphylococcus aeurous*, *Escherichia coli*, and *Klebsiella pneumoniae* were used as monoxenic media. The encystation, mycological peptone-maltose, peptone yeast extract glucose, roswell park memorial institute 1640 and trypticase beef hemoglobine media were used as axenic media.

Results: We compared the growth of *Acanthamoeba* species in different axenic and monoxenic media in this study. In relation to the growth rate, the non-nutrient agar with *Pseudomonas aeuroginosa* had the highest values achieved among monoxenic media and roswell park memorial institute 1640 media was the highest value among axenic media.

Conclusion: In view of the results, we can affirm that these monoxenic media are adequate to grow of *Acanthamoeba* species. In addition, a classic and basic medium that supports the growth of *Acanthamoeba* species consists of peptone yeast extract glucose. However, the roswell park memorial institute 1640 media was an excellent commercially available media for the growth of *Acanthamoeba* and it was able to keep *Acanthamoeba* by long periods of time.

Key words: *Acanthamoeba*, Axenic, Monoxenic, Media

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Introduction

Acanthamoeba species are the ubiquitous free-living amoebae that cause keratitis, encephalitis, cutaneous and other lesions (Penland and Wilhelmus, 1997; Khan, 2006). The life cycle takes place in two stages are trophozoite (active form) and cyst (inactive form) (Heredero et al., 2012). Their survival depends on their morphological and physiological adaptation to varying and potentially lethal environmental conditions (Heredero et al., 2012). *Acanthamoeba* strains can show different pathogenic ability

(Niyyati et al., 2013). Research on the pathogenic potential of *Acanthamoeba* is complicated by the changes induced by prolong axenix laboratory culture, such as the down regulation of its virulence and encystment capacity and its altered sensitivity to drugs (Verissimo et al., 2013).

Acanthamoeba able to tolerate a range of growth conditions and appear to be prepared to go from bacterized to axenic media without adaptation or selection (Schuster, 2002). A classic and basic media that supports the growth of *Acanthamoeba* consists of axenic and monoxenic media (Penland and Wilhelmus, 1997). Bacteria-free media have been developed for axenic media and include peptone yeast extract glucose (Penland and Wilhelmus, 1997; Heredero et al., 2012). The peptone yeast extract glucose media is the most commonly used for the growth of *Acanthamoeba* (Ahmed, 2009; Axelsson et al., 2009). Acanthamoebic trails have been detected on blood agar, non-nutrient agar and *Acanthamoeba* has been shown to preferentially phagocytize erytrocytes of different animal species (Penland and Wilhelmus, 1998). It has been reported that non-nutrient agar is very rapidly, inexpensive and easy to handle experimentally for growth of *Acanthamoeba* (Axelsson et al., 2009; Borin et al., 2013).

In this study, it was evaluated the efficiency of axenic and monoxenic media in its growth, with the aim of selecting one which allowed better growth, was easier to prepare, and was able to keep *Acanthamoeba* by long periods of time.

Materials and Methods

Acanthamoeba strains *and sources*

Strains of *Acanthamoeba* isolated in the Department of Parasitology, Faculty of Medicine, Cukurova University were used to compare recover on various media. It was shown that GenBank accession number, species, genotype and isolation sources of *Acanthamoeba* strains in table 1.

Table 1 GenBank accession number, species, genotype and isolation sources of *Acanthamoeba* strains.

Acession No	Species	Genotype	Isolation sources
KJ446982	*A. castellanii*	T4	Adana-water and soil samples
KJ446976	*A. castellanii*	T4	Kutahya-water and soil samples
KJ446979	*A. griffinii*	T3	Adana-water and soil samples
KJ446980	*A. griffinii*	T3	Kutahya-water and soil samples
KJ446983	*A. jacobsi*	T15	Adana-corneal scraping
KJ446981	*A. jacobsi*	T15	Kutahya-water and soil samples

Axenic media

Encystation media (EM): EM media consisted of the following ingredients; 1 g KCl, 0.25 g Tris, 8 g $MgSO_4$, 0.75 g $CaCl_2$, 1 g $NaHCO_3$, and 100 mL distilled water. Also, 0.5 mg/mL penicillin (Sigma-Aldrich, Chemical, France), and 0.5 mg/mL streptomycin (Sigma-Aldrich, Chemical, France) were added (Riviere et al., 2006).

Mycological peptone-maltose media (MPM): MPM media was prepared by mixing 10 g mycological peptone and 5 g maltose, 100 mL distilled water, and supplemented with 0.5 mg/mL penicillin (Sigma-Aldrich, Chemical, France), 0.5 mg/mL streptomycin (Sigma-Aldrich, Chemical, France) (Henriquez et al., 2009).

Peptone yeast extract glucose media (PYG): 2 g peptone, 1 g yeast extract, and 1 g glucose, pH 7.2 in 100 mL distilled water and autoclaved at 121ºC for 15 min. In addition, 0.5 mg/mL penicillin (Sigma-Aldrich, Chemical, France), and 0.5 mg/mL streptomycin (Sigma-Aldrich, Chemical, France) were added (Khan et al., 2002).

Roswell Park Memorial Institute Media 1640 (RPMI): The culture flask containing 15 mL RPMI 1640 media (Sigma-Aldrich, Chemical, France) was supplemented with 12% heat-inactivated calf serum (FCS) (Sigma-Aldrich, Chemical, France), 0.5 mg/mL penicillin (Sigma-Aldrich, Chemical, France), 0.5 mg/mL streptomycin (Sigma-Aldrich, Chemical, France), (Sharief et al., 2008).

Trypticase beef hemoglobine media (TBH): The media was prepared by dissolving 1 g trypticase, 1 g beef extract, 1 g yeast extract, 1 g peptone, 1 g liver extract, 0.5 g glucose, 0.8 g NaCl, 0.5 g L-Proline, 0.13 g $NaHCO_3$, 0.26 g Na_2HPO_4, 0.65 g KH_2PO_4, 0.2 g $FeNH_4$, 1 g bovine hemoglobine of 100 mL distilled water. Finally, 0.5 mg/mL penicillin (Sigma-Aldrich, Chemical, France), and 0.5 mg/mL streptomycin (Sigma-Aldrich, Chemical, France) solution were added (Limoncu et al., 2004).

Monoxenic media

The non-nutrient agar (Becton Dickinson Microbiology Systems, Cockeysville, Maryland) was used for growth of *Acanthamoeba*. *Escherichia coli* (*E. coli*) (ATCC 25922), *Enterobacter aerogenes (E. aerogenes)* (ATCC13048), *Klebsiella pneumoniae (K. pneumoniae)* (ATCC 13883), *Pseudomonas aeruginosa (P. aeruginosa)* (ATCC 27853), and *Staphylococcus aeureus (S. aeureus)* (ATCC 25923) were used for monoxenic media. The agar plates were prepared by adding 0.5 mL of the standardized bacterial suspension and *Acanthamoeba* strains.

Incubation and temperature tolerance: The axenic media was autoclaved at 121ºC for 15 min and the pH values of axenic media were 7.2. After cooling, 5 ml of the extract was filtered in each of the 25 cm^3 flasks. The axenic and monoxenic media were incubated at 27ºC under standard atmospheric conditions and examined ever day under an inverted microscope (Leica DM3000, Houston, Texas) with a 10X ocular lens and a 20X objective. The cysts and trophozoites of *Acanthamoeba* from two days media were harvested and counted with a Thoma hemocytometer (Hirschmann Laborgerae, Eberstadt, Germany).

Results

The reproduction of *Acanthamoeba* was increased on 2th day in all axenic media. Furthermore, the growth of value of the RPMI 1640 media was higher than the other media. In TBH, EM, MPM, PYG the number of *Acanthamoeba* were high between the days 6th and 8th day. The number of *Acanthamoeba* in axenic media except the RPMI 1640 media was lower after the 10th day (Table 2, Figure 1). When looking at sustain of growth rates of long time, the best growth rates were achieved with RPMI 1640 media in first experience.

The results of non-nutrient agar spread with different bacteria were compared. The number of *Acanthamoeba* were obtained different level in diagnosis of *Acanthamoeba* from highest to lowest in the order of *P. aeruginosa* > *E. aerogenes* > *S. aeureus* > *E. coli* > *K. pneumoniae*. The highest values achieved between the 4th and 8th day in non-nutrient agar with all bacteria in relation to the growth rate. After 8th day, reproduction of *Acanthamoeba* was decreased day by day. The rank order of non-nutrient agar with bacteria for the recovery of *Acanthamoeba* is presented in Table 3 and Figure 2.

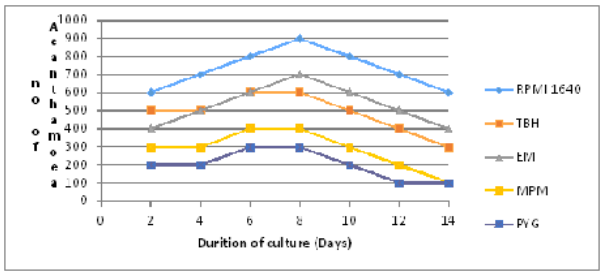

Figure 1 The reproduction of *Acanthamoeba* in different axenic media; Roswell park memorial institute (RPMI-1640), Trypticase beef hemoglobin media (TBH), Encystation media (EM), Mycological peptone-maltose (MPM), and Peptone yeast extract glucose (PYG) axenic media. The figure was shown comparing the number of *Acanthamoeba* different media for each time

Table 2 Reproduction of *Acanthamoeba* in Roswell park memorial institute (RPMI-1640), Trypticase beef hemoglobin media (TBH), Encystation media (EM), Mycological peptone-maltose (MPM), and Peptone yeast extract glucose (PYG) axenic media.

Medium	Number of *Acanthamoeba* reproduced on day						
	2	4	6	8	10	12	14
RPMI 1640	6 X 10^2	7 X 10^2	8 X 10^2	9 X 10^2	8 X 10^2	7 X 10^2	6 X 10^2
TBH	5 X 10^2	5 X 10^2	6 X 10^2	6 X 10^2	5 X 10^2	4 X 10^2	3 X 10^2
EM	4 X 10^2	5 X 10^2	6 X 10^2	7 X 10^2	6 X 10^2	5 X 10^2	4 X 10^2
MPM	3 X 10^2	3 X 10^2	4 X 10^2	4 X 10^2	3 X 10^2	2 X 10^2	1 X 10^2
PYG	2 X 10^2	2 X 10^2	3 X 10^2	3 X 10^2	2 X 10^2	1 X 10^2	1 X 10^2

*The initial inoculation for all of strains is 2 X 10^2 and reproduction of all strains is similar in axenic media.

Table3 Reproduction of *Acanthamoeba* in non-nutrient with *Pseudomonas aeruginosa* (*P. aeruginosa*), *Enterobacter aerogenes* (*E. aerogenes*), *Staphylococcus aeureus* (*S. aeureus*), *Escherichia coli (E. coli*) and *Klebsiella pneumoniae* (*K. pneumonia*) monoxenic media.

Media	Number of *Acanthamoeba* reproduced on day						
	2	4	6	8	10	12	14
P.aeruginosa	5 X 10^2	6 X 10^2	7 X 10^2	7 X 10^2	6X 10^2	5 X 10^2	4 X 10^2
E.aerogenes	4 X 10^2	5 X 10^2	6 X 10^2	6 X 10^2	5 X 10^2	4 X 10^2	3 X 10^2
S.aureu	3 X 10^2	4 X 10^2	5 X 10^2	5 X 10^2	4 X 10^2	3 X 10^2	2 X 10^2
E.coli	2 X 10^2	3 X 10^2	4 X 10^2	4 X 10^2	3 X 10^2	2 X 10^2	1 X 10^2
K.pneumoniae	2 X 10^2	2 X 10^2	3 X 10^2	3 X 10^2	2 X 10^2	1 X 10^2	1 X 10^2

*The initial inoculation for all of strains is 2 X10^2 and reproduction of all strains is similar in monoxenic media.

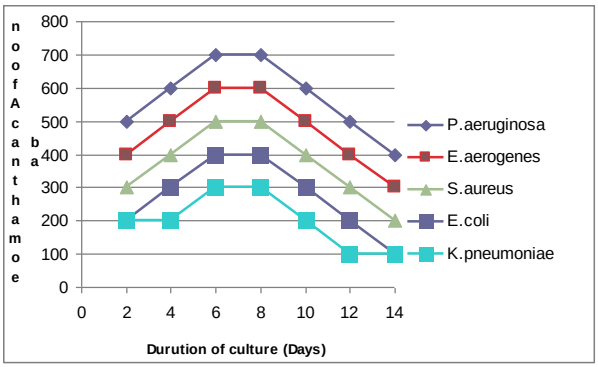

Figure 2 The reproduction of *Acanthamoeba* in non-nutrient with *Pseudomonas aeruginosa* (*P. aeruginosa*), *Enterobacter aerogenes* (*E. aerogenes*), *Staphylococcus aeureus* (*S. aeureus*), *Escherichia coli (E. coli*) and *Klebsiella pneumoniae* (*K. pneumonia*) monoxenic media. The figure was performed comparing the values of different media for each time.

Discussion

The cultivation of *Acanthamoeba* is a useful approach for yielding an amount of parasites appropriate for various diagnostic purposes, for improving our knowledge of host parasite relationships and for determining of molecular, biological and immunological characteristics of the parasite (Borin et al., 2013; Verissimo et al., 2013; Jain et al., 2015). Although, a great number of studies reported that the present *Acanthamoeba* in worldwide, we could find very few studies providing satisfactory information about the media of *Acanthamoeba*. Therefore, we used different media and compared these media for *Acanthamoeba* in this study.

To various degrees, *Acanthamoeba* can be readily established in axenic media from initially bacterized cultures by providing an enriched nutrient media with antibiotics added to kill off contaminating bacteria (Schuster, 2002). The basic nutrient media that is used for *Acanthamoeba* typically contains peptone, yeast extract, and glucose, in concentrations generally higher than those used for growth in bacterized cultures (Schuster, 2002). The PYG media is recommended for growth of *Acanthamoeba* parasite (Peretz et al. 2015). The RPMI 1640 and TBH media the most widely were used media for *Leishmania* parasite (Limoncu et al., 1997; Sharief et al., 2008). As far as we know, these media have not been used before to growth *Acanthamoeba*. However, we used these media and in view of the results these media were effective in the growth of *Acanthamoeba*. When large amounts of *Acanthamoeba* in short periods of time will be required, it would be convenient to use RPMI 1640 media.

Acanthamoeba can be readily cultivated on either non-nutrient agar or agar media containing low concentrations of nutrients in the presence of living or killed bacteria. In general, the bacteria of choice include non-mucoid strains of *K. pneumoniae*, *E. aerognes*, and *E. coli* (Schuster, 2002). We used non-nutrient agar with living *P. aeruginosa*, *E. aerogenes*, *S. aurous*, *E. coli* and *K. pneumoniae* and we found that *P. aeuroginosa* most effective of growth *Acanthamoeba*. Non-nutrient agar with an overlay of live *E. coli* is recommended for the recovery of *Acanthamoeba* but is not readily available (Penland and Wilhelmus, 1997). Although the non-nutrient agar with *E. coli*, the species widely recommended for use in the isolation of *Acanthamoeba*, was significantly lower than those on media with all other live bacterial species except *K. pneumoniae* in this study (Penland and Wilhelmus, 1998).

In cultivations carried out to produce of *Acanthamoeba* species outside natural media in the best possible way, care should be taken to make the media compatible with nutritional and environmental conditions of the natural media. We found that RPMI 1640 media is the most rapid and easy for growth of *Acanthamoeba*, it should be employ as the standard method for growth *Acanthamoeba*.

Conclusion

Acanthamoeba species are able to growth different conditions and different media. The studies about growth of *Acanthamoeba* are important for developing vaccine and drug for *Acanthamoeba* infections. Our results provide further evidence for the effect of prolonged axenic and monoxenic media on the growth of *Acanthamoeba*. As a result of this study, the RPMI 1640 media and non-nutrient agar with *K. pneumoniae* can be used in studies about vaccine, drug and pathogeniticity of *Acanthamoeba*. However, future studies should be carried out to further explore this issue.

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