Morphological Characteristics and Growth Abilities of Free Living Amoeba Isolated From Domestic Tap Water Samples in Istanbul

Zuhal Zeybek*, Miray Üstüntürk, Ali Rıza Binay
Istanbul University, Faculty of Science, Department of Biology, 34134, Vezneciler, Istanbul-Turkey.

Abstract

Free living amoeba (FLA) have been isolated from water and soil samples throughout the world. Among the many genera of FLA, members of only four genera are responsible for human disease: *Acanthamoeba* spp., *Balamuthia mandrillaris*, *Naegleria fowleri* and *Sappinia diploidea*. *Naegleria* spp. and *Acanthamoeba* spp. can be cultivated on nonnutrient agar (NNA) in the presence of living or dead bacteria. *Balamuthia*, however, will not grow with bacteria as a food source but they can feed upon smaller soil amoeba. In the current study we try to find out the morphological appearance (Trichrome staining) and growth characteristics of free living amoeba in different liquid culture media previously isolated from domestic tap water samples (10 isolates) and a strain of *Acanthamoeba castellanii (A. castellanii)* isolated from a keratitis case. Although *A.castellanii* strain grew on proteose peptone-yeast extract-glucose and proteose peptone-yeast (with or without antibiotics), our isolates from domestic tap water samples did not grow on any tested liquid culture media. It is suggested that investigating both the morphological characteristics and the growth characteristics on liquid culture media are necessary for the identification of free living amoeba.

Keywords: Free living amoeba, liquid culture media, nonnutrient agar (NNA), tap water, trichrome staining.
***Corresponding author:** Zuhal Zeybek (E-mail: zeybek@istanbul.edu.tr)
(Received : 06.02.2009 Accepted : 31.07.2009)

Introduction

Free living amoeba (FLA) have been described as facultative parasites and opportunistic pathogens. They live in soil, fresh water lakes, swimming pools, therapeutic pools, tap water, natural thermal water and air all over the world (Johan and De Jonckheere 1985; Rivera et al. 1989; Rodriguez-Zaragoza and Magana-Becerrs 1997; Schuster and Visvesvara 2004; Jeong and Yu 2005; Gianinazzi et al. 2009). These amoeba can survive in severe conditions by forming resistant cysts. Among the many genera of free-living amoeba, members of only four genera are responsible for human disease: *Acanthamoeba* spp., *Balamuthia mandifloris* (*B. mandrillaris*), *Naegleria fowleri* (*N. fowleri*) and *Sappinia diploidea* (*S. diploidea*). *Acanthamoeba* spp. and *B. mandrillaris* are opportunistic pathogens causing infections of the central nervous system, lungs, sinuses and skin, mostly in immunocompromised humans. *Balamuthia* is also associated with disease in immunocompetent children, and *Acanthamoeba* spp. causes an ocular infection mostly in contact-lens wearers, *Acanthamoeba* keratitis. *N. fowleri* causes an acute and fulminating meningoencephalitis in immunocompetent children and young adults. In addition to human infections, *Acanthamoeba*, *Balamuthia* and *Naegleria* can cause central nervous system infections in animals. There is limited information about *S.diploidea* as an agent of disease. Up to the present, only one human case of encephalitis caused by *S.diploidea* was reported (Tsvetkova et al. 2004; Visvesvara et al. 2007).

Protozoa, especially free living amoeba, can actually be grown on different media. *Naegleria* spp. and *Acanthamoeba* spp. can be cultivated on either nonnutrient agar (NNA) or agar media containing low concentrations of nutrients (e.g., peptone 0.05%, yeast extract 0.05% and glucose 0.1%) in the presence of living or dead bacteria. In general, the bacteria of choice include nonmucoid strains of *Klebsiella pneumoniae*, *Enterobacter* spp. (*Enterobacter aerogenes* and *Enterobacter cloacae*), and *Escherichia coli (E.coli)*. The presence of a mucoid capsule around bacteria appears to impede phagocytosis by amoebas and leads to bacterial overgrowth of the amoeba population. *Balamuthia*, however, could not be grown with bacteria as a food source but they can feed upon smaller soil amoeba or on tissue culture cells (Page 1988; Schuster 2002).

The identification of FLA is based on their trophozoite and cyst morphology. The genus *Acanthamoeba* was divided into three groups based on cyst size and shape. Although this classification scheme has been extensively used by investigators, the differentiation of *Acanthamoeba* at the species level is still problematic. Moreover, species differentiation based on morphology alone may not always be correct, since the morphologies of cysts within a given species may vary according to culture conditions. Therefore, the reliability of morphological characters alone in species identification is of limited value (Page 1988; Visvesvara 1991; Tsvetkova et al. 2004; Shin and Im 2004).

Therefore we try to find out the growth characteristics of free living amoeba in different liquid culture media previously isolated from domestic tap water samples and a strain of *Acanthamoeba castellanii (A. castellanii)* isolated from a keratitis case.

Materials and Methods

To investigate the growth characteristics of FLA in different liquid culture media (given below), one *A. castellanii* strain genotype T4 (isolated from a keratitis case) obtained from Cumhuriyet University Faculty of Medicine and 10 strains (C12, C13, C16, C17, C19, C20, M1, M3, M4, M9) isolated from domestic tap water samples were used (Üstüntürk 2009).

Isolation of FLA from domestic tap water samples

A total of 200 ml of domestic tap water samples (bathroom tap water) were collected in sterile plastic containers from different buildings. They were transferred to the laboratory immediately.

NNA (1.5 %) plates were used for the isolation of FLA from water samples. Before the inoculation of the samples, NNA plates were coated with a dense suspension of heat-inactivated *Escherichia coli* which was prepared in Page Saline. A total of 200 ml of each domestic tap water sample was filtered through a 0.45μm pore size cellulose nitrate membrane filter under a vacuum. The filters were inverted on heat-inactivated *E. coli* treated 1.5% NNA plates. After the inoculation of the samples, all plates were incubated at 28°C and examined daily for up to 10 days using a light microscope (100x) for the presence of FLA (Sanden et al. 1992; Gray et al. 1995; Schuster 2002; Health Protection Agency 2004; Jeong and Yu 2005; Ertabaklar et al. 2007). NNA plates containing FLA isolates were stored at 4-8 °C in a refrigerator. All FLA strains were transferred to a fresh NNA-*E.coli* plate every month to check their viability and each of them were used in the experiments.

Staining of FLA

FLA Isolates were stained with Trichrome staining procedure in order to observe their morphology more detailed. Before the staining process agar pieces, which were marked previously by looking under the light microscope to see where the FLA growth had taken place, were taken out of the plates and placed upside down on a microscobe slide. All preparations were fixed in Schaudinn fixative after 1.5 hours and stained with Trichrome staining procedure (Akın 2001; Daldal et al. 2002). The stained preparations were then examined under the light microscope (500x).

Test Media

Nine liquid media used in the study were proteose peptone-yeast (PPY), proteose peptone-glucose (PPG), proteose peptone-yeast extract-glucose (PPYG), Jones's medium (JONES'S), Chang's serum-casein-glucose-yeast extract medium (CSGYEM), tryptone soya broth (TSB) (Oxoid), M119 (Sigma), RPMI (Sigma) and soil extract with salts (SEWS). All of them were sterilized by autoclave at 121°C for 15 minutes before use. (Page 1988; Akın Polat 2005).

Growth of FLA isolates in test media

Each test media (approx. 3 ml) was added to the NNA plates containing each of the FLA isolates in order to harvest them so these microorganisms were suspended in these tested media. Then 1.5 ml of these suspensions were taken and placed securely in 24 well microplates. All the microplates were incubated at 30°C and examined daily using an inverted microscope (200x).

Results

Isolated FLA

FLA strains which were transferred onto fresh NNA plates are shown in Figure 1.

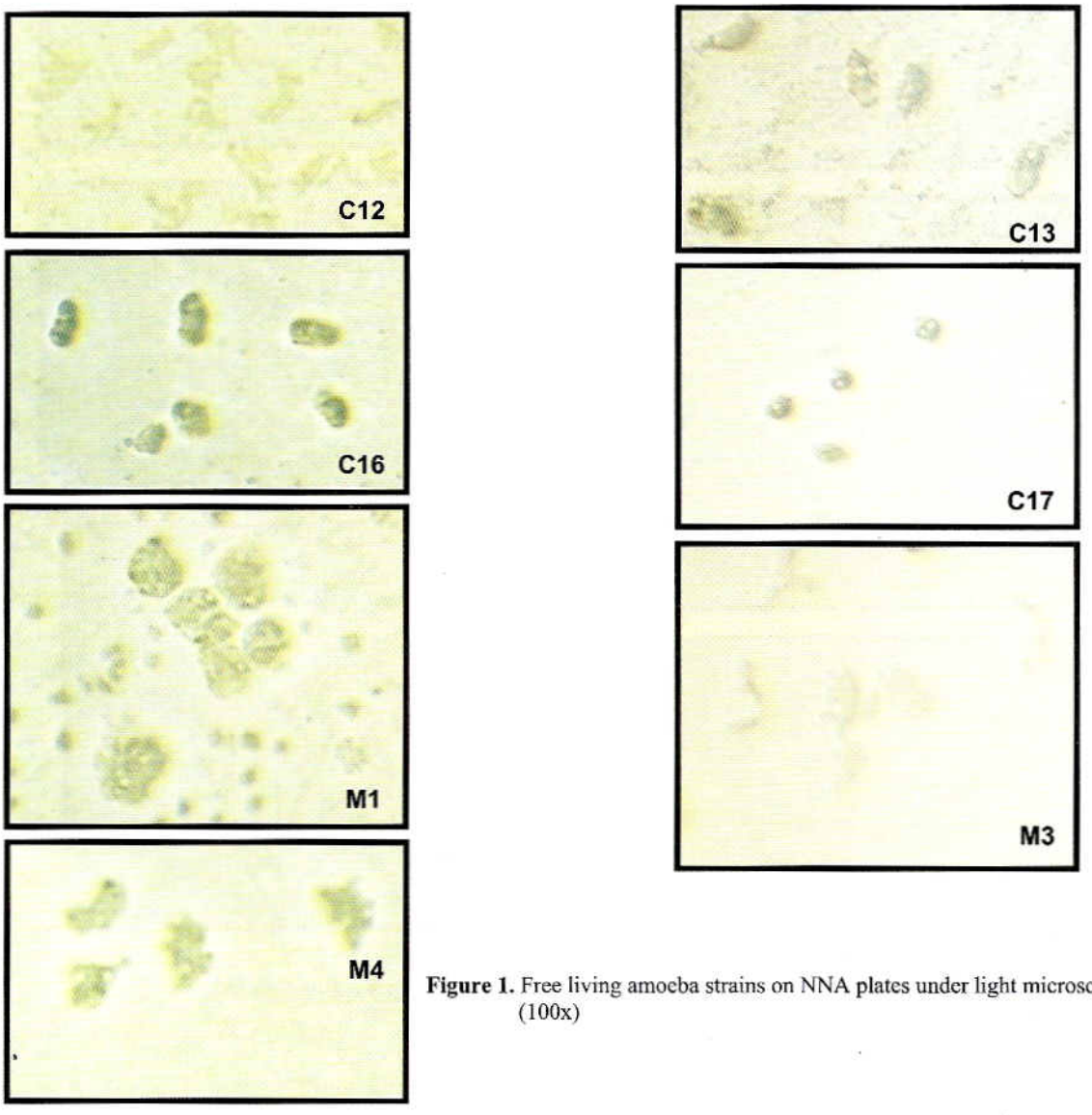

Figure 1. Free living amoeba strains on NNA plates under light microscope (100x)

Stained FLA

The cell structure of FLA such as nucleus, nucleolus, endoplasm, ectoplasm and contrectile vachole could be recognised easily by staining Trichrome method (Fig. 2).

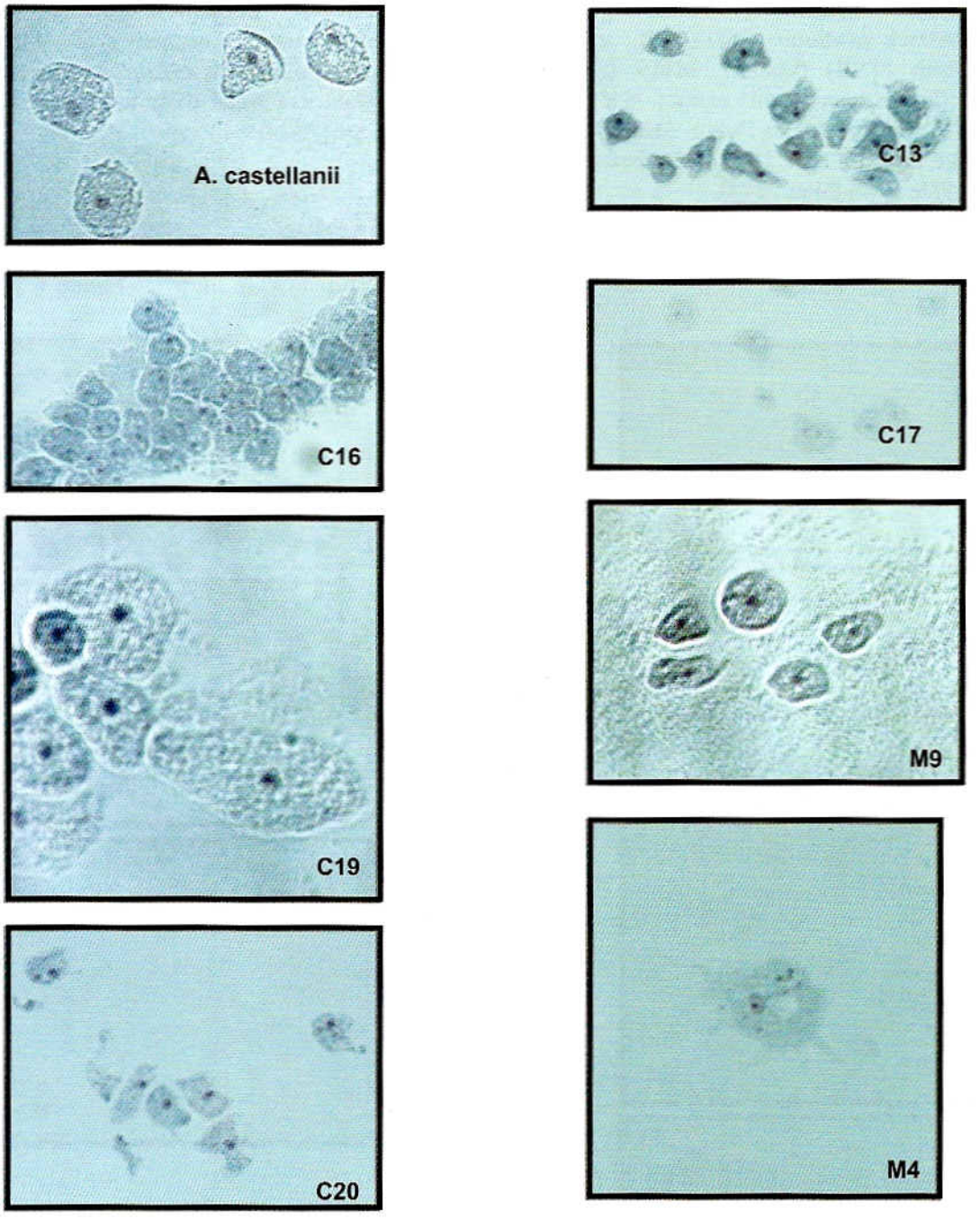

Figure 2. Trichrome stained free living amoeba trophozoites (500x)

Growth on test media

The growth on different liquid culture media of our FLA isolates (C12, C13, C16, C17, C19, C20, M1, M3, M4, M9) and *A. castellanii* is shown in Table 1. Although the *A. castellanii* strain grew on PPYG and PPY (with or without antibiotics), our isolates from domestic tap water samples did not grow on any tested liquid culture media.

Table 1: Recovery of free living amoeba from different liquid culture media

Media / Strains	PPYG	PPG	PPY	RPMI	TSB	M-199	CSGYEM	JONES'S	SEWS	PPYG ANTB.	PPG ANTB.	PPY ANTB.
A. castellanii	+	-	+	-	-	-	-	-	-	+	-	+
C12, C13	-	-	-	-	-	-	-	-	-	-	-	-
C16, C17, C19, C20	-	-	-	-	-	-	-	-	-	*	*	*
M1, M3, M4, M9	-	-	-	-	-	-	-	-	-	*	*	*

- : no growth
+ : growth
* : not tested
ANTB. : antibiotics added

Discussion

The free-living amoeba, especially *Acanthamoeba* and *Naegleria* are widely distributed throughout the world. They have been isolated from a variety of enviroments such as fresh water, soil and dust (Shin and Im 2004).

These microorganisms can easily be isolated from water samples by the membrane filtration method on NNA seeded with *E.coli*. Our FLA isolates cultured on NNA by using this method are seen in Figure 1. Although FLA can be easily isolated by simple methods, identification of most species is a difficult issue and in many cases, cannot be conclusive. Identification of amoeba (based on morphological and ultrastructural data) requires the establishment of cultures and both light and electron microscopy examination, and is highly dependent on the skill and experience of the investigator. In fact, it is seen that the light microscopy of our FLA isolates which were stained by the trichrome method have a similar appearance (Fig. 2). For this reason these microorganisms cannot be identified by morphological characteristics alone.

It is necessary to grow these microorganisms on axenic cultures (without any other organism present) for rapid identification of isolates (Schuster 2002). Many species are poorly or insufficiently described and correct identification of such amoeba requires detailed analysis of the literature, including older reports (Smirnov and Brown 2004). The mtDNA restriction fragment length polymorphisms

(RFLP) typing has been shown to be a powerful technique for differentiating morphologically identical strains of *Acanthamoeba*. However, the technique requires that the isolates be adapted to axenic liquid media, which is not always successful with some strains (Kilvington et al. 2004). As a matter of fact that our isolates did not grow on tested liquid media (Table 1). So, our isolates should be adapted to axenic liquid media before identification.

Test media used in our study for axenic culture were choosen according to the literature to make the identification of the isolates at least to genus level. PPG medium (Page 1988) and PPYG medium (Schuster 2002) have been used for the axenic maintenance of *Acanthamoeba* strains. In this study, our isolates did not grow on these media. We think that these strains do not belong to the genus *Acanthamoeba*. On the other hand, although *A. castellanii* genotype T4 grew on PPYG, they did not grow on PPG. So, we think that the growth of FLA species/strains can vary according to the used media for axenic culture. CSGYEM medium (Page 1988) and liver extract and calf serum added nutrient medium (Schuster 2002) have been used for the axenic culture of *Naegleria*. Because our isolates did not grow on the CSGYEM medium, we think that these strains do not belong to the genus *Naegleria*. Also, our isolates did not grow on Jones's medium which is specific for *Entamoeba moshkovskii* and *Phreatamoeba balamuthi*.

Free-living amoeba can be established in axenic culture from initially bacterized cultures by providing an enriched nutrient medium with antibiotics (penicillin- streptomycin and gentamicin) added to kill off contaminating bacteria (Schuster 2002). We observed bacterial contamination on microplates after 5 days of incubation. This condition worsened the observation of FLA on tested liquid media. Further studies should be done by adding several antibiotics to liquid culture media to avoid bacterial contamination.

In conclusion, it is suggested that morphologic, biochemical and molecular techniques should be used for the complete and correct identification of the FLA. One of the most important steps of the morphological examination is culturing these microorganisms on axenic cultures. Because the nutrient requirement of different genus and species of FLA vary from each other, it is difficult to adapt these microorganisms to axenic cultures successfully. So, further studies should be done by testing another enrichment media .

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