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# Development and optimization of new culture media for *Acanthamoeba* spp. (Protozoa: Amoebozoa)

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## Abstract

The isolation and growth in axenic liquid media of *Acanthamoeba* strains is necessary in order to carry out primary in vitro drug screening. Amoebic isolates which are hard to grow in the current liquid media have been reported. Such circumstances hampers the ability of conducting drug sensitivity tests. Therefore, finding suitable universal growth media for *Acanthamoeba* species is required. The present study was aimed at the development of liquid medium suitable for growing a fastidious (F) genotype T3 *Acanthamoeba* isolate, and eventually for other genotypes of this genus as well. Trophozoite growth was indirectly monitored by respiration analysis with oxygen-sensitive microplates (OSM) and further confirmed by manual counting. Media were empirically designed and tested first in a non-fastidious (NF) T3 isolate and then tested with 14 different strains, including the fastidious one. Combinations of nutritive components such as meat/vegetable broth, LB medium, malt and skimmed milk led to the design of new media suitable for culturing all the isolates tested, in conditions similar to those obtained in standard culture media such as PYG or CERVA.


***Keywords:*** *Acanthamoeba*; Axenic liquid medium; Culture; Oxygen-sensitive microplates; Respiration; Trophozoite

## Introduction

Protozoa of the genus *Acanthamoeba* are mainly non-parasitic organisms found in soil, water and dust (Khan, 2009). These free-living amoebae (FLA) sporadically infect humans, causing *Acanthamoeba* keratitis (AK) or granulomatous *Acanthamoeba* encephalitis (GAE) (Marciano-Cabral and Cabral, 2003). There is no standard drug for treatment for AK, and the search for chemicals with anti-*Acanthamoeba* properties is currently ongoing in many laboratories worldwide. In vitro drug screening of new amoebicides requires both cysts and trophozoites, since the resistance stage is far less sensitive to them than the vegetative form.

Isolation of amoebae trophozoites in patients with AK is performed by inoculating corneal scraps samples in monoxenic culture. Such a cultivation system is based on non-nutritive agar plates seeded with inactivated (heat-killed) bacteria, as these protozoa optimally feed on particulate material (De-Moraes and Alfieri, 2008). However, routine culture is conducted without bacteria, in axenic liquid media. Many cultivation procedures for FLA trophozoites have

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**Table 1.** *Acanthamoeba* isolates used in the present study.

| Isolate ID | Genotype and species/Genbank accession number | Origin | Growth in standard liquid medium/optimal growth temperature | Reference |
|---|---|---|---|---|
| ATCC – 30234 | T4 – *A. castellanii* | Culture type collection | PYG + 2% Bactositone/25 °C | Not applicable |
| CCAP – 1501/1A | T4 – *A. castellanii* | Culture type collection | PYG + 2% Bactositone/25 °C | Not applicable |
| ATCC – 30171 | A. culbertsoni | Culture type collection | CERVA/25 °C | Not applicable |
| CCAP – 1501/9 | *A. astronyxis* | Culture type collection | PYG + 2% Bactositone/32 °C | Not applicable |
| 2961 | *A. polyphaga* | Isolate donated by Dr. Hadas[a] | PYG + 2% Bactositone/32 °C | Unpublished data |
| BYB2017 | T2/MF113385 | Cat with keratitis | CERVA/25 °C | Martín-Pérez et al. (2017a) |
| MYP 2004 | T3 – *A. griffin*/KF010846 | Patient with keratitis | CERVA/37 °C | Heredero-Bermejo et al. (2015b) |
| ISCIII-UAH 64/13 | T4B/KY072778 | Patient with keratitis | CERVA/32 °C | Martín-Pérez et al. (2017b) |
| ISCIII-UAH 85/13 | T4A/KY072780 | Patient with keratitis | CERVA/32 °C | Martín-Pérez et al. (2017b) |
| ISCIII-UAH 66/14 | T3 – *A. griffini*/KY072779 | Patient with keratitis | No/37 °C | Martín-Pérez et al. (2017b) |
| ISCIII-UAH 161/15 | T4/KY072781 | Patient with keratitis | CERVA/32 °C | Martín-Pérez et al. (2017b) |
| EV-UAH-P6 | *Acanthamoeba* sp./MH087090 | Environmental | PYG + 2% Bactositone/32 °C | Unpublished data |
| EV-UAH-V7 | *Acanthamoeba* sp./MH087091 | Environmental | PYG + 2% Bactositone/32 °C | Unpublished data |
| P-UAH-O3 | T2/MH087093 | Environmental | PYG + 2% Bactositone/32 °C | Unpublished data |
| PV-UAH-V2 | T2/MH087092 | Environmental | CERVA/32 °C | Unpublished data |

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been designed since the pioneering works by Adam (1959), Adam and Blewett (1967), Cerva (1969) and Visvesvara and Balamuth (1975). Axenic liquid culture of amoebae is usually straightforward and trophozoites of most isolates grow well in either PYG or CERVA (Schuster, 2002; Martín-Pérez et al. 2017b). Diverse alternative media, such as BSC, BHI, PPG, *Giardia* medium, etc., have been used with relative success to grow *Acanthamoeba* sp., according to the specialized literature (see Martín-Pérez et al. 2017a, for a detailed review). Interestingly, some *Acanthamoeba* isolates fail to grow in axenic liquid media (De Jonckheere, 1980; Hiti et al. 2001; Nagyová et al. 2010), a problem also observed in our laboratory with a genotype T3 isolate (Martín-Pérez et al. 2017b).

The present work was aimed at the development and optimization of an alternative liquid medium capable of supporting growth of the F genotype T3 isolate available in our laboratory (Martín-Pérez et al. 2017b) and eventually of isolates of other genotypes. Oxygen-sensitive microplates (OSM) were employed for screening media destined to grow trophozoites of NF T3 genotype, used as model system. This system provides indirect information on amoebic growth and metabolism (Heredero-Bermejo et al. 2015a). Finally, performance of reliable media was confirmed by viability analysis and microscopic observations in fourteen *Acanthamoeba* isolates.

## Material and Methods

### Amoeba isolates and culture conditions

The isolates used in this study (along with their biological features) are shown in Table 1. The protocol followed in assays for evaluation of amoebic growth in new media is shown in Fig. 1.

### Experimental design for respiration studies in low-nutrient media

In order to determine the respiration profiles occurring in optimal or suboptimal culture conditions by means of OSM (see below), the NF T3 isolate (MYP 2004) was grown in CERVA medium at 1X, 0.75X, 0.5X, 0.25X, 0.125X and 0.06X. Media dilutions were made in sterile distilled water. These experiments were repeated four times. This study was