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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

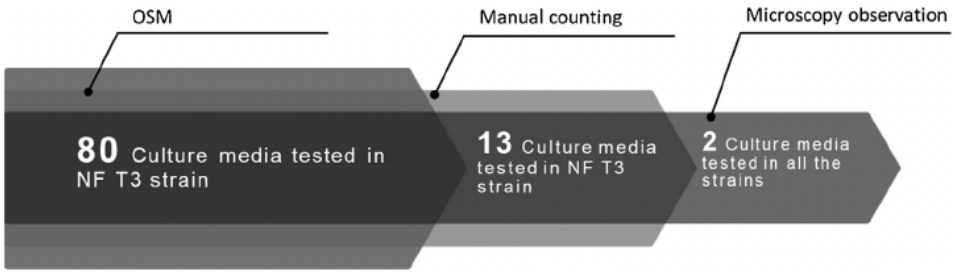

Fig. 1. Flow chart showing the experimental design of screening of *Acanthamoeba* growth media in this study.

combined with manual counting (see below). The hypothesis addressed was, could a unique respiration profile eventually identify a medium with good cultivation performance.

In addition, culture assays using 1X CERVA with 10% synthetic foetal bovine serum (SFBS – Panexin basic® from PAN Biotech, Aidenbach, Germany) was tested as well by OSM and manual counting, in order to ascertain if this economical component could replace the more expensive standard foetal bovine serum (FBS – SIGMA, St. Louis, MI, USA), currently in use in our laboratory. Growth with PBS was always included in assays as a negative control.

Culture media: components and their preparation

Nutritional components used in the development of new media are: glucose (SIGMA), LB medium (Scharlab), malt extract (Scharlab), yeast extract (Scharlab), bactocasitone (Scharlab), tryptone (Scharlab), foetal bovine serum (SIGMA), grape juice, alcohol-free beer, diverse preparations of chicken-bovine-pork-vegetable broths, fish-vegetable broth, *Spirulina* + *Fucus* capsules, yeast scales food supplement, lactose-free UHT skimmed milk, soy drink and hen's egg. The composition of the eighty empirically designed liquid media is presented in Tables S1–S4 as Supplementary material. Some commercial products from local supermarkets (either Auchan-La Dehesa or Mercadona-Juan Ramón Jiménez, both located in Alcalá de Henares, Spain) were used for economic reasons. To ensure reproducibility, two production batches of the same brand (namely lactose-free skimmed milk and commercial meat/vegetable broths) were tested in culture (this was done only for the most performant liquid medium). Medium LBM (2X Luria-Bertani medium supplemented with malt extract) consisted of yeast extract at 10 g/L, triptone at 20 g/L, malt extract at 40 g/L and NaCl at 10 g/L. HEPES buffer was prepared at 0.2 M (20X solution), filtered through a filter syringe (0.22 μm), frozen in aliquots at −20 °C and used in medium at 10 mM (pH = 7.4). Antibiotics (0.1 g/L streptomycin and 0.06 g/L penicillin) were added as a 100X solution.

Since all commercial liquid broths do not indicate the exact amount of components used in industrial cooking, two laboratory-made broths (with weighted ingredients) were prepared as follows:

- Chicken broth: 400 g chicken leg, 75 g carrots, 175 g potato, 130 g onion, 750 mL tap water.
- Mussel broth: 1 kg mussels (with shell), 85 g onion, 500 mL tap water.

These ingredients were purchased in the same local supermarkets mentioned above. Components were added to a three-liter wide mouth, metal screw-cap glass container and autoclaved at 121 °C as usual. All types of broth (commercial and laboratory-made), along with lactose free skimmed milk (LFSM) and soya drink were clarified/filtered (CF) in order to obtain a non-turbid medium. The process of particle elimination was carried out in five steps:

1) Centrifugation at 2000 × *g* for 10 min. The supernatant was recovered with a pipette.
2) The supernatant was centrifuged at 10,000 × *g* for 15 min. The supernatant was recovered as before.
3) The supernatant was filtered through 0.45 μm syringe filter
4) The filtrate was centrifuged again at 10,000 × *g* for 15 min.
5) Finally, the supernatant was filtered through 0.22 μm syringe filter and considered ready for use in culture assays.

Lactose-free skimmed milk and soy drink were processed in a way similar to the broth. The only difference was that they were previously diluted in sterile distilled water to facilitate CF. Skimmed milk was diluted at 15% and soy drink at 45%.

Alcohol-free beer was open in sterile conditions. Beer was transferred to 50 mL Falcon conical centrifuge tubes and left at 4 °C overnight. Tube cap was not tightly closed in order to facilitate depletion of carbonic gas from liquid, that otherwise would acidify the culture medium. *Spirulina* and *Spirulina-Fucus* capsules were opened under sterile conditions and their content was resuspended at either 50 mg/mL (*Spirulina*) or 50 + 4.1 mg/mL (*Spirulina* + *Fucus*) in sterile distilled water, mixed on an orbital shaker at 50 rpm for 10 min at room temperature and then centrifuged 10 min at 10,000 × *g*. The resulting supernatant was recovered and considered sterile, particle-free and transparent enough as to be used directly in cultures. The same procedure was followed to prepare yeast extract from commercial yeast scales used as food supplement. To obtain hen's egg yolk or egg white, egg surface was wiped with 70% ethanol. Once dry, an egg was slightly

Table 2. Ingredients (in μL) used to prepare 1 mL of media 39 and 80 and medium respiration profile.

Ingredients	Media 39	Media 80
Sol. 0.2 M HEPES	50	50
Penicilin + Strept. mix 10 + 6 g/L	10	10
Aneto® chicken broth	350	350
LBM	210	210
15% LF skimmed milk	330	0
50% egg yolk in 0.9% saline sol.	0	30
Spiruline + *Fucus* suppl. Extract/50 + 4.1 g/L	0	25
Sterile water	50	325
Respiration profile	1	1

flamed and then carefully cracked in a sterile Petri dish in a laminar flow hood (to keep the content sterile). Due to the high viscosity of egg's fluids, both yolk and egg white were recovered with a 1 mL automatic pipette using wide orifice filter tips (Finntip®, Thermofisher Scientific, Waltham, MA. USA). Care was taken to avoid pipetting the egg's blastoderm. Yolk could not be totally clarified. It was emulsified at 50% with sterile physiological saline solution (0.9% NaCl) and then centrifuged at 10,000 × *g* for 10 min. The slightly cloudy supernatant was recovered and used in media preparation. Egg white was diluted as well at 50% with sterile physiological saline solution, to facilitate pipetting. Media containing CF components was clear enough to permit microscopic observation of amoebic cultures.

Experimental design for respiration studies in the screening of new media in the model system organism (NF T3 isolate MYP 2004)

The eighty experimental media (Tables S1–S4) were assayed with the NF T3 isolate with the aid of OSM. Only thirteen media out of 80 (showing different respiration profiles – see results) were selected for further evaluation of viability by manual counting and microscopy. In addition, one of these media (number 39) showed good performance in culture of NF genotype T3 and was tested without HEPES, in order to assess the importance of buffering the pH of cultures.

Two media (39 and 80-see Table 2) of the several reliable for growing the NF T3 isolate (MYP 2004) were selected for culture assays in the F T3 isolate (ISC-UAH66/14) and the rest of non-fastidious isolates (Table 1). Amoebic growth was only confirmed by microscopy, without performing any viability analysis. Both PYG and CERVA media were used as negative controls (no-growth) for the F T3 isolate and as positive controls for the non-fastidious strains.

Finally, the effect of refrigeration/freezing on preservation of the reliable new media was studied with OSM in the NF T3 isolate. The performance of two freshly prepared media (39 and 80) was compared with aliquots of the same media kept in the refrigerator for two weeks or kept in the freezer for two weeks and at the same time subjected to six cycles of freezing and thawing.

Studies on respiration analysis (using OSM microplates) in NF genotype T3 *Acanthamoeba* (isolate MYP 2004)

The procedure employed for respiration analysis with OSM microplates has been described in detail elsewhere (Heredero-Bermejo et al. 2015b; Martín-Pérez et al. 2017a). In brief, 10 μL of PBS containing 10^5 amoebae from log phase cultures (grown for 48–72 h) was added to 90 μL of the medium being assayed. Such a high inoculum was necessary to avoid issues of background fluorescence in the control (CERVA medium). The medium with protozoa was loaded into wells and overlaid with 100 μL of mineral oil to avoid oxygen replenishment from air. Media, amoebae and the microplate were maintained on ice during preparation, to ensure assay synchronicity as much as possible. Unless otherwise stated, culture medium was assayed in triplicate in at least two separate experiments. The plate was placed in a fluorescence reader (VICTOR® – PerkinElmer, Waltham, MA, USA), which was programmed to obtain two readings per well and hour (for up to 72 h) at 37 °C. Time-resolved fluorescence (excitation at 340 nm/emission at 642 nm) was measured at delay times of 30 and 70 microseconds. Fluorescence readings were transformed in O_2 concentration using the Stern-Volmer equation (Criado-Fornelio et al. 2017). Respiration plots in this study use the variables $1/[O_2]$ versus time, which provides exponential curves similar to those obtained by measuring trophozoite growth by manual counting.

Viability analysis by manual counting and microscopic observations in *Acanthamoeba* isolates

Media 5, 10, 27, 30, 35, 39, 54, 63, 69, 74, 78, 79 and 80 (Tables S1–S4) were selected for performing viability studies by manual counting in NF genotype T3 isolate. They were chosen based on their different respiration profiles, as explained above. For such purposes, sterile 24-well microtiter plates were seeded with medium (0.6 mL) and trophozoites. Non-fastidious amoebae from log phase cultures were resuspended in PBS, counted and adjusted so that the medium being assayed was inoculated with 10 μL of amoebae suspension, at a final density of 10^5 trophozoites/well. After different periods of incubation (1, 3 and 7 days) at either 37 or 25 °C, depending on the isolate, trophozoites were detached by cold shock and counted in a Fuchs-Rosenthal chamber using 0.2% Congo red to assess viability (Heredero-Bermejo et al. 2013).

For the rest of the NF strains used in this study, amoeba inoculation was done as explained above. No viability analysis was performed in these cases; cultured amoebae status

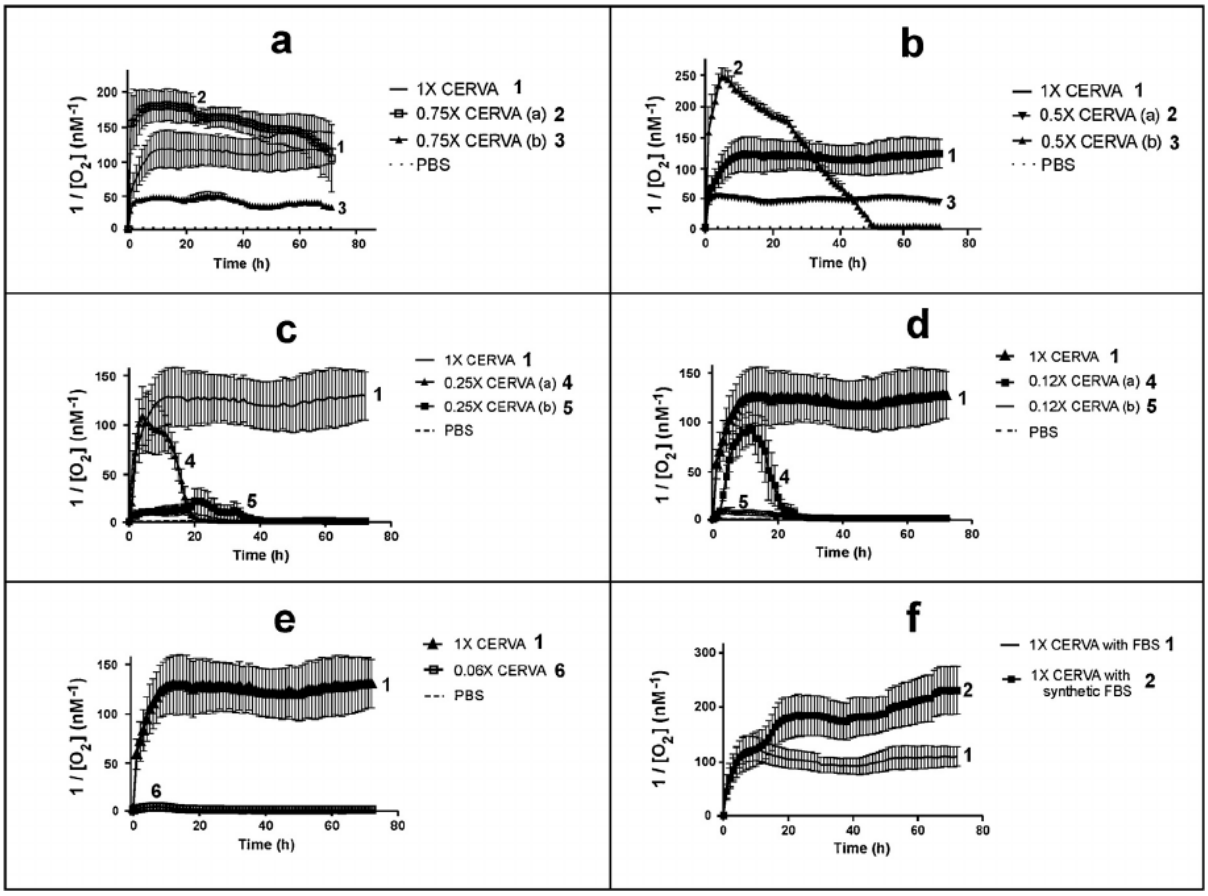

Fig. 2. Respiration plots of NF genotype T3 *Acanthamoeba* obtained in diverse media: PBS, 1X CERVA (optimal concentration), five suboptimal concentrations of CERVA and CERVA medium made with either FBS or SFBS. Fluorescence readings were transformed in $[1/O_2]$. (a) Curves for 0.75X CERVA, (b) curves for 0.5X CERVA, (c) curves for 0.25X CERVA, (d) curves for 0.12X CERVA, (e) curves for 0.06X CERVA, and (f) curves for FBS and SFBS. Data shown are the results obtained in four experiments; in all cases two experiments suited one of the respiration profiles (a) and the remaining two the other respiration profile (b), except in (e), where a unique curve type appeared in the four experiments. Vertical error bars represent SD.

was qualitatively checked using microscopy. The observation period lasted 30 days.

In assays for growing the F T3 isolate, amoebae resistance stage (approximately 1000 cysts in 20 μL of sterile PBS) were inoculated in sterile 24-well microtiter plates as described above. As no trophozoites were available, cysts for inoculation were obtained by PBS washing of non-nutrient agar plates seeded with heat-killed *E. coli* where the amoeba had been grown for 2 weeks. Cultures were examined daily for excystation under an inverted microscope for 30 days.

Statistical analyses

Respiration experiments were run in triplicate wells and in at least two independent experiments. Results of manual counting are provided as mean ± SD of data obtained from at least three independent experiments. The statistical significance of the results on manual counting was determined using the unpaired Student's *t* test. All statistical analyses were performed using the GraphPad Prism 5® software package (GraphPad Software, San Diego CA, USA). The statistical significance was defined at $P < 0.05$.

Results

Respiration profiles of the NF T3 isolate MYP 2004 in standard CERVA medium and its variants

The NF T3 isolate (MYP 2004) cultured in 1X CERVA showed exponential-shaped oxygen consumption graphs (Fig. 2a – profile 1). The use of CERVA at lower concentrations (0.75X and 0.5X) showed either enhanced (Fig. 2a and b – profile 2a) or diminished respiration curves (Fig. 2a and b – profile 2b). It is important to highlight that oxygen consumption decreased always at about 60–65 h (or sooner) in profiles 2a and 2b. As expected, CERVA at 0.75X and 0.5X showed less amoebic growth than 1X CERVA as determined by manual counting (Fig. 3), albeit trophozoite count was similar in 1X and 0.75X CERVA at $t = 7$ days (non-significant

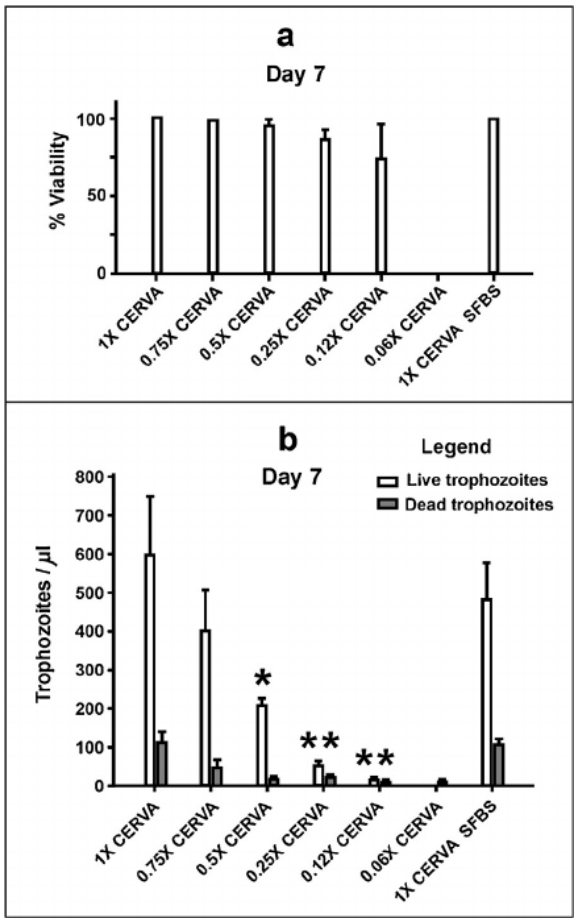

Fig. 3. Barplot charts showing analysis of viability (a) and trophozoite counts (b) in the same media studied by OSM in Fig. 1, obtained after 7 days of incubation. Legend for statistically significant differences in cell counts between 1X CERVA and suboptimal CERVA dilutions: * = $P < 0.05$; ** = $P < 0.005$. Data shown are the results obtained in three experiments. Vertical error bars represent SD.

difference, $P > 0.2$), but significantly lower in 0.5X CERVA ($P < 0.02$). When low-nutrient medium (CERVA at 0.25X or 0.12X) was tested for growth, two types of respiration graphs were observed: (1) a curve with an initial burst of respiratory activity, followed by fast decline to the basal level in about 24–30 h (Fig. 2c and d – profile 4a), or (2) a smooth exponential curve followed by plateau and a decay period, reaching oxygen consumption a basal level in less than 45 h (Fig. 2c and d – profile 5b). Manual counting (Fig. 3) showed significant loss of amoebae viability and trophozoite counts in these media at $t = 7$ days ($P < 0.005$). In 0.06X CERVA, a single type of profile was obtained: respiration was only slightly greater than in PBS at the beginning of the incubation period and decayed quite quickly (Fig. 2e – profile 6). Manual counting (Fig. 3) showed that in 0.06X CERVA there were no live trophozoites following 7 days of culture. Finally, cultures supplemented with synthetic foetal bovine serum (SFBS) returned respiration graphics that were similar to 1X CERVA for the first 12 h of incubation and then respiration was slightly enhanced after such period (Fig. 2f). In this case, manual counting (Fig. 3) revealed that there was no significant difference ($P > 0.3$) in trophozoite viability or cell counts between FBS and SFBS when growing the NF T3 isolate MYP 2004 (Fig. 3).

Preparation of the new media (with emphasis in CF processing)

The procedure developed for CF of medium components was straightforward for diluted LFSM, diluted soy milk, Auchan® cocido broth, Aneto® chicken broth or laboratory-made chicken broth. Other broth preparations were more difficult to clean and the efficiency of CF was lower (the 0.22 µm syringe filter was quickly clogged). This was caused by their high content of tiny particles and fats (the latter remaining on the upper part of the supernatant after centrifugation, hampering the recuperation of clarified broth). Diluted egg yolk could not be totally clarified due to the existence of a large amount of very tiny particles. This fact did not preclude its use in cultivation, as egg yolk was obtained under sterile conditions, and then diluted with sterile saline solution and centrifuged, thus avoiding the sterilization by syringe filter. In spite of the presence of such suspended material, egg yolk at 1.5% (or less) in culture medium was clear enough

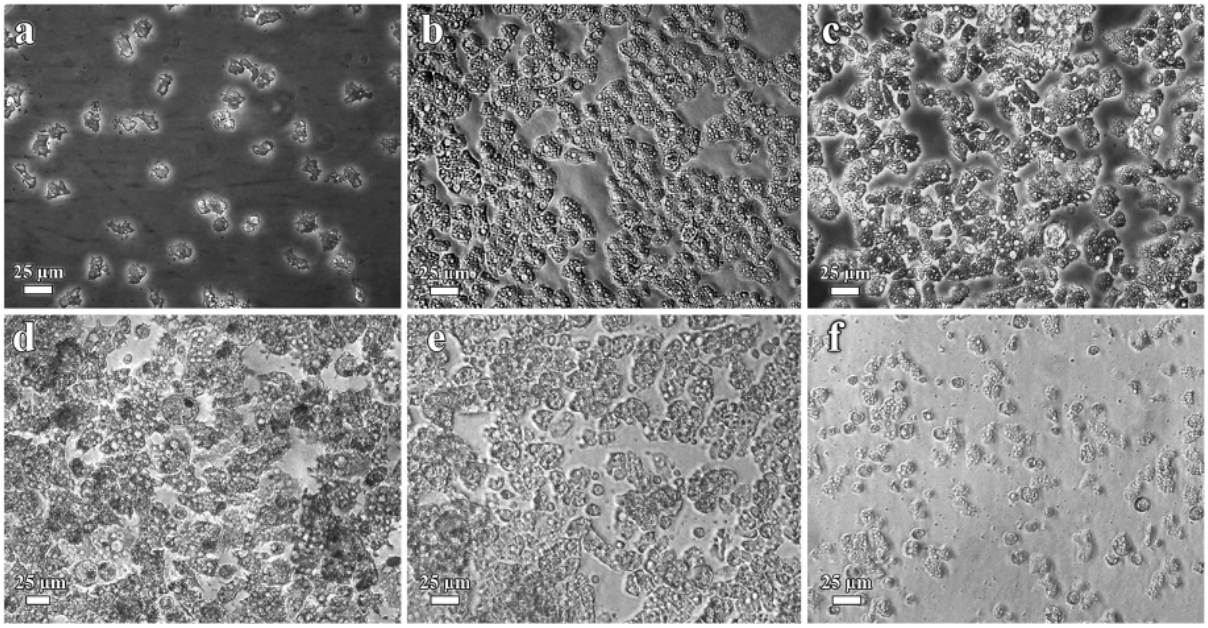

Fig. 4. Phase-contrast micrographs at 200X of diverse isolates of *Acanthamoeba* grown in the new alternative media for FLA (a) trophozoites of the F genotype T3 ISC-UAH66/14 after two days of culture, (b) trophozoites of the NF genotype T4 ATCC 30234 grown in medium 39 after three days of culture, (c) trophozoites of the NF genotype T4 ATCC 30234 grown in medium 80 after three days of culture in 24 well microtiter plate. Note that in spite of the presence of tiny egg yolk particles in medium, these are in suspension and do not interfere with viewing the amoeba or taking the micrograph. (d) Trophozoites of the NF *A. astronyxis* CCAP 1501/9 cultured in medium 80, (e) trophozoites of the NF genotype T2 (BYB2017) cultured in medium 39, and (f) trophozoites of the NF *Acanthamoeba* sp. (EV-UAH-P6) cultured in medium 39.

as to permit good microscopic observation of the amoebae (Fig. 4c).

Performance of the new media in the model system isolate (NF genotype T3 MYP 2014) based on OSM and viability data

Respiration profiles of the NF T3 isolate (MYP2004) cultured in experimental media basically showed similar profiles than those found using 1X CERVA and its dilutions. For simplicity, culture performance for each medium is indicated (in Tables S1–S4) as type of respiration profile (according to graphs shown in Fig. 2). Based on a comparison of respiration profile with manual counting data at day 7 (Fig. 5) in the thirteen selected media, it was concluded that media yielding respiration profile type 1 (27, 30, 35, 39, 69, 78, 79, 80) showed similar viability (and trophozoite counts) than those grown in CERVA 1X ($P > 0.1$, difference non-significant), whereas types 2 and 3 (medium 54 and 63) showed reduced growth compared to control ($P < 0.05$). Profiles 4–6 usually lead to a rapid loss of viability and cell counts (medium 5 and 10, $P < 0.01$; medium 74 presented no live cells at day 7). Amoebae grown in several media containing diverse commercial broth preparations combined with LBM and LFSM, (numbers 26, 27, 29, 30, 35, 37, 38, 39, 40, 42, see Tables S2) showed respiration profiles type 1, the same as 1X CERVA. Therefore, most types of commercial broth at 17–30% were suitable for growing NF T3 isolate (MYP2004). Medium containing chicken broth prepared in the laboratory (e.g., 50, 51 or 52 – Table S3) showed equivalent performance than media containing commercial chicken broth (37–42, Table S2). In contrast, media containing lab-made mussel broth (media 53–58, Table S3) were less performant than other media made with meat/vegetable broth. On the other hand, media 69, 78, 79 and 80 (Table S4), made with combinations of egg yolk and spiruline/fucus extract instead of LFSM, were equally readable for growing the NF T3 isolate.

The use of commercial broth and LFSM from two different production batches did not result in any change in the performance of medium 39 (Fig. 6). On the other hand, medium 39 prepared without HEPES buffer showed only a limited respiration level, which suggests that this component is essential for a reliable amoebic growth (Fig. 6). Repeated freeze/thaw cycles (6X) of medium 39, containing broth, LBM and LFSM, caused no effect on their performance, whereas refrigeration decreased it (Fig. 6). In contrast, medium 80, containing diluted egg yolk and algal extracts, was best conserved when kept refrigerated at 4 °C for two weeks (Fig. 6).

Evaluation of the new media performance with the F Acanthamoeba T3 isolate (ISC-UAH66/14) and the NF isolates, as determined by microscopic observations

The F T3 isolate ISC-UAH66/14 showed no excystation or development whatsoever when maintained in classic culture media (CERVA or PYG) after one month of incubation at either 25, 32 or 37 °C. Media 39 and 80 supported growth

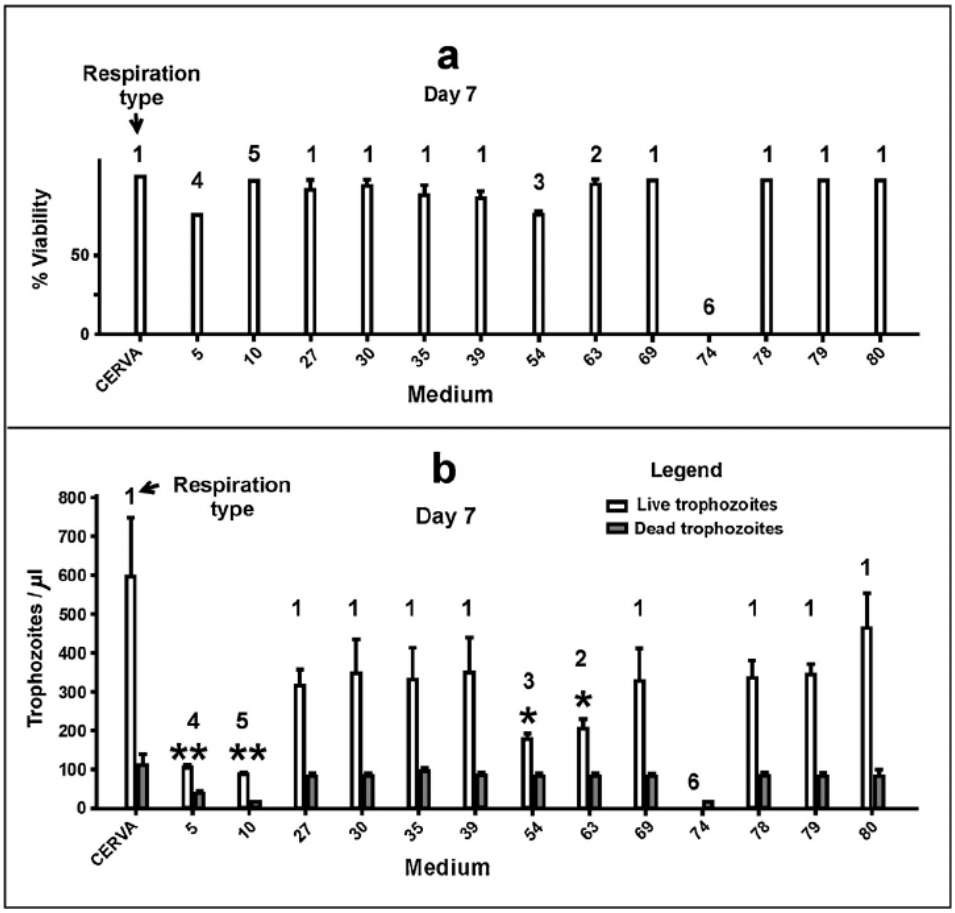

Fig. 5. Barplot charts showing analysis of viability (a) and trophozoite counts (b) in thirteen selected experimental media after 7 days in culture. The type of respiration profile for every medium is on top of the corresponding bar plot. Legend for statistically significant differences of the new medium with rapport to CERVA: * = $P < 0.05$; ** = $P < 0.01$. Data shown are the results obtained in three experiments. Vertical error bars represent SD.

of the hard-to-grow isolate at 32 °C. When cysts of the F genotype T3 isolate were inoculated in any of these media, excystation was evident after one day of culture and noticeable growth was attained after 4–6 days (Fig. 4a). However, a strong tendency towards encystment was observed afterwards, especially if the medium was nor replaced regularly every 2–3 days. Qualitative microscopic observations showed that trophozoites of the fourteen isolates assayed were able to grow both in medium 39 and 80 (Fig. 4). After 7 days of inoculation, amoebic cultures show tendency towards encystment if the medium is not renewed. When the media is changed every three days, almost no encystment was observed in the cultures (the same as for CERVA or PYG medium).

Discussion

CERVA medium and its variants as a primary model for evaluating culture status with OSM

In agreement with previous studies in other *Acanthamoeba* isolates (*A. castellanii* and *A. polyphaga* – Heredero-Bermejo et al. 2015b), respiration curves of NF T3 isolate (MYP2044) grown in 1X CERVA (that is, under optimal conditions) yielded exponential graphs similar to growth curves obtained by manual counting. Amoebae at high inoculum were needed to avoid any problem with background fluorescence. The shape of the respiration graphs obtained suggests that the amoebae survived and multiplied (for a limited time) under such conditions. It is evident from our studies that anaerobiosis (caused by the use of the mineral oil overlay in microplate wells) does not impair amoebic growth. This is consistent with findings by Alves et al. (2017), who pointed out that anaerobic glycolysis and protein catabolism pathways are present in *A. castellanii*.

Amoebic growth monitored with OSM at suboptimal CERVA concentrations showed sometimes divergent results in different experiments. The reason for this might be that amoeba inocula were not exactly in the same growth moment. It is likely that amoebae respiratory response would be variable depending on if they were collected in the middle of the exponential grow phase, in the end of the exponential growth phase or in the stationary phase. On the other hand, cultures performed in suboptimal 0.75X CERVA sometimes

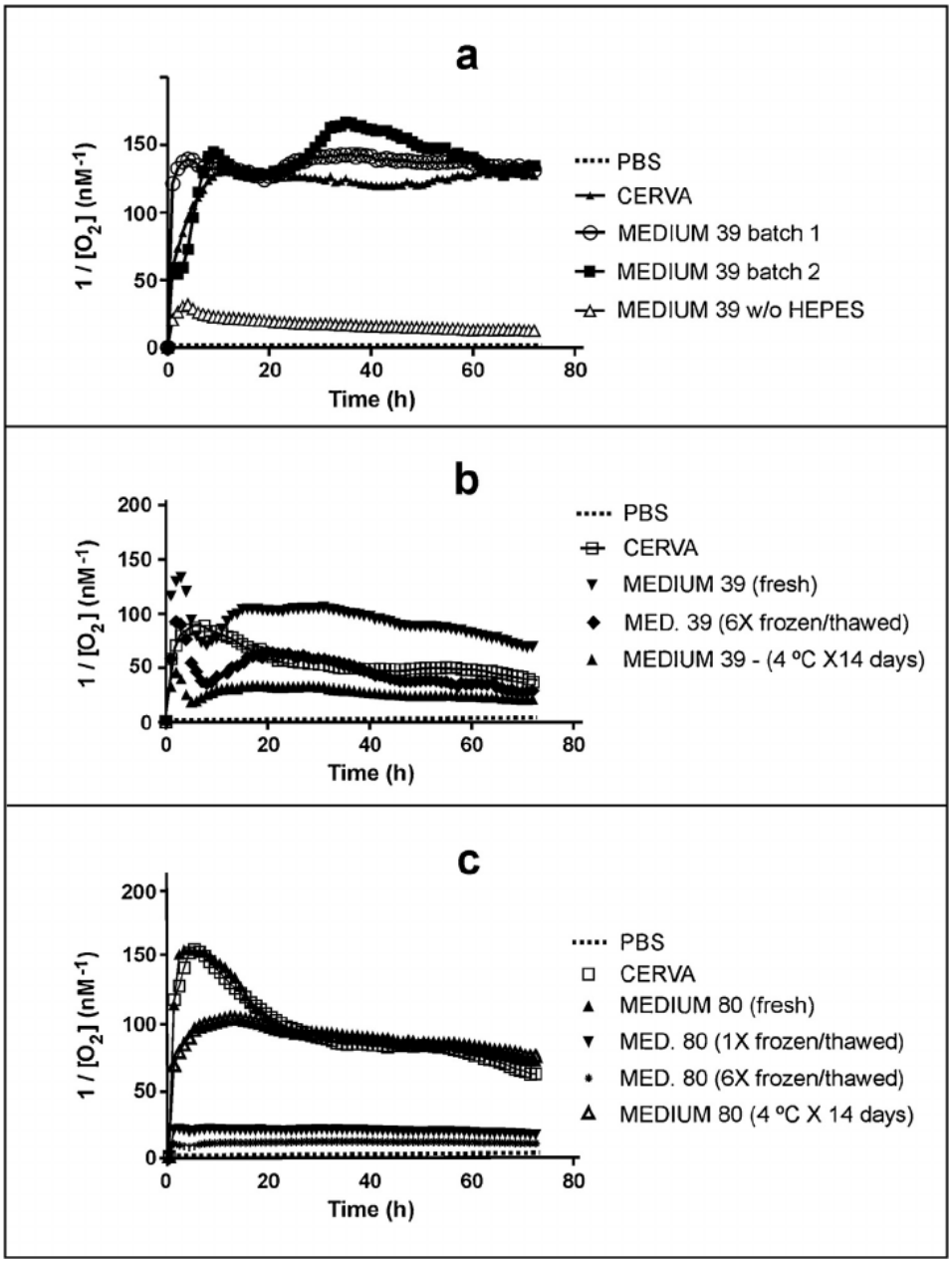

Fig. 6. Respiration plots of NF genotype T3 *Acanthamoeba* obtained in diverse media (see text for explanation). (a) Oxygen consumption plots for PBS, CERVA, medium 39 prepared with different batches of broth and LFSM or without HEPES buffer. (b) Oxygen consumption plots for PBS, CERVA, and medium 39 kept at diverse temperatures. (c) Oxygen consumption plots for PBS, CERVA, and medium 80 kept at diverse temperatures. Data shown are the results obtained in three experiments. Vertical error bars were ommited for clarity, but these can be found in the alternative graph provided in the Supplementary material.

showed higher oxygen consumption than the 1X CERVA control, but this did not correlate with higher viability or cell counts. Such a lack of correspondence between growth and respiration may be caused by oxidative stress, as previously observed in other protozoa (Mastronicola et al. 2011). Moreover, treatment with drugs may induce a similar respiratory burst effect (Martín-Pérez et al. 2017a). Despite the possible drawback of OSM (Criado-Fornelio et al. 2017), the respiratory profile type 1 unequivocally pointed to reliable media, as demonstrated later by viability analysis and cell counts. If none of the assayed medium shows this optimum respiration profile, media showing profile 2 might be tested as well as an alternative. Such a pattern was observed in 0.75X CERVA and CERVA with SFBS, where cell counts was similar to 1X CERVA. Therefore, OSM microplates may serve as a valuable tool in future attempts to develop and optimize culture media for other protozoa or microorganisms. On the other hand, the synthetic alternative to real FBS (namely,

Panexin basic®) was reliable for culture of NF T3 *Acanthamoeba* isolate in CERVA medium, in spite of the fact that this component was designed for use in human cell culture (Mahboudi et al. 2013) rather than for protozoa maintenance. Since SFBS is 32% less expensive than the standard FBS, the present study implies the development of a more economical procedure for cultivation of *Acanthamoeba* in CERVA medium.

Culture media for trophozoites: design, preparation and performance

The use of soil water extracts, skimmed milk and LBM medium in cultures of *Acanthamoeba* has been recently proposed for growing the vegetative stage of *A. griffini* and *A. castellanii* (Martín-Pérez et al. 2017a). However, such an approach was disregarded in the present contribution. Instead, medium design and formulation for NF genotype T3 *Acanthamoeba* culture was carried out on an empirical basis owing to the lack of fundamental knowledge on nutritional needs of this genotype. Since medium optimization using a one-factor-at-a-time approach is time-consuming, expensive, and often leads to misinterpretation of results (Batista and Fernandes, 2015), in the present study a strategy of empirical combination of several nutritional components was followed. Some classical culture components (peptone, yeast extract and milk protein-derived components) were combined with new nutritive ingredients (malt extract, skimmed milk, egg yolk, egg white and algae/meat/vegetable extracts). In fact, egg white has been already used (as egg albumin slope) in slanting cultures of *Entamoeba histolytica* (Moundipa et al. 2005). The use of undefined media for growing *Acanthamoeba* is common in many specialized laboratories (Schuster, 2002). However, low-priced components were used in the present medium screening assay, instead of testing media developed for other organisms, which is the usual strategy (De Jonckheere, 1980; Hiti et al. 2001; Nagyová et al. 2010; Martín-Pérez et al. 2017b). Such an approach has proven successful for growing both F + NF T3 and other. The fastidious T3 isolate ISC-UAH66/14 was finally grown at 32 °C, whereas monoxenic cultures in non-nutrient agar and *E. coli* grew better at 37° C (Martín-Pérez et al. 2017b). The improved growth of pathogenic *Acanthamoeba* isolates at temperatures lower than 37 °C, was remarked on by Schuster (2002) also. There is still a strong tendency towards encystment of the fastidious isolate in the new medium, implying frequent passages are needed to keep the amoebae in the trophozoite stage.

It is important to underline that amoebae isolated from the new media do not contain contamination of foreign DNA, in contrast with previous contributions where xenic cultures with bacteria were developed (Wang and Ahearn, 1997; Pickup et al. 2007; De Moraes and Alfieri, 2008). Particles (<0.22 μm) derived from egg yolk (used for instance in medium 80) are composed of protein (Vadehra et al. 1977), so that uncontaminated DNA may be successfully isolated from amoebic cultures.

One of the possible problems associated with the preparation of the new media reported in this study is the need for CF of some components. Ultracentrifugation is a relatively fast way to eliminate particles in milk and egg yolk (Downey and Andrews, 1969; Vadehra et al. 1977). Nevertheless, such an approach was disregarded in our experimental design, since ultra-centrifuges are expensive instruments, not available in every clinical/research laboratory. The techniques described here may be adapted to most research institutions, since only syringe filters and a mini-centrifuge (reaching 10,000 × *g*) are needed to get rid of particles found in some nutritive components. Although medium clarification is to some extent time-consuming, it should not be forgotten that the alternative media were developed as a last resort solution, destined to maintain slow-growing or fastidious isolates. Furthermore, the reagents needed are generally inexpensive compared to FBS, for instance. The components of the laboratory-made chicken/vegetable broth are available in most parts of the world and are not fraught with any cultural or religious taboos. Hence, cultivation of *Acanthamoeba* isolates may be done almost everywhere, even if the broth brands employed by us in Spain are unavailable. Evidently, local broth brands in other geographic locations may be tested as well for commodity. Batch to batch variations in broth seem not to be a problem, as demonstrated here. Conservation of the new media in the freezer is possible for egg yolk-free medium, whereas refrigeration is recommended for medium containing egg yolk. Such a long-term conservation feature is an advantage compared to media based on soil extracts, which are relatively short-lived (Martín-Pérez et al. 2017a). Although it is unsure which ingredients contribute to the success of the new culture media for FLA, the fact that milk and egg yolk are rich in vitamins A and D (Imaeda et al. 1999; Semba, 2012; Schmid and Walther, 2013), suggests that these may be important to promote *Acanthamoeba* growth. In fact, vitamin supplements have been previously used to grow fastidious and non-fastidious *Acanthamoeba* isolates (Byers et al. 1980; Shukla et al. 1990; Schuster and Visvesvara, 1998). However, further studies using vitamin mixes would be necessary to address such a hypothesis.

Conclusions

The present study has shown that OSM microplates are suitable tools in culture media screening. Some new media are reliable for growing both F and NF T3 isolates along with NF isolates of other genotypes, while these *Acanthamoeba* isolates had to be formerly cultured in different media. The procedures described here are promising, as they can facilitate the task of in vitro drug screening, since a single medium might be used for cultivation of many isolates. Further attempts to grow other *Acanthamoeba* spp. isolates in new media in cooperation with other laboratories are under-

way to address such a hypothesis. Likewise, simplification of the CF procedure for culture medium would be desirable if the method is routinely used.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.ejop.2018.04.002.

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