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The effect of different environmental conditions on the encystation of *Acanthamoeba castellanii* belonging to the T4 genotype

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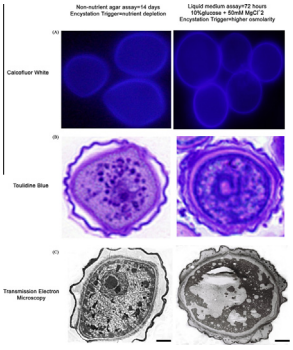
HIGHLIGHTS

- Osmolarity is an important trigger to induce *Acanthamoeba castellanii* encystation.
- Cyst prepared from agar or liquid medium are similar in morphological and histological properties.
- Temperature over 37 °C or pH 9 adversely effected encystation.
- Light-dark cycles, 5% CO_2 and microaerophilic conditions had no effect on encystation of *A. castellanii*.

GRAPHICAL ABSTRACT



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ABSTRACT

In this study, *Acanthamoeba castellanii* was cultivated under different stress conditions to induce possible encystation. The morphological and histological properties were analysed by light and electron microscopy as well as cyst-specific staining. The findings revealed that cysts prepared through liquid medium using higher osmolarity as a trigger (10% glucose with 50 mM magnesium chloride for 72 h) are similar to cysts prepared using non-nutrient agar (nutrient deprivation as a trigger in plating assays for 14 days), as determined by SDS-resistance, cyst-specific Calcofluor white staining and transmission electron microscopy. Using liquid medium assay, *A. castellanii* encystation was studied by exposing trophozoites to media lacking growth ingredients (phosphate buffered saline or distilled water), inappropriate temperatures (4–45 °C), pH (3–9), artificial light–dark cycles, 5% CO_2, and microaerophilic conditions. Optimal encystation was observed when cells were incubated in PBS with 50 mM $MgCl_2$ and 10% glucose at 24–30 °C at pH 7. Increasing temperature over 37 °C or pH 9 adversely affected encystation, while light-dark cycles, 5% CO_2 and microaerophilic conditions had no effect on encystation of *A. castellanii*. None of the aforementioned conditions had any effect on the viability of *A. castellanii*, as determined by Trypan blue exclusion assay. A complete knowledge of encystation in *A. castellanii* is crucial to our understanding of the biology of these ecologically and medically important organisms.

1. Introduction

Acanthamoeba castellanii is a unicellular free-living protist pathogen. The genus *Acanthamoeba* consists of 17 genotypes (T1–T17) (reviewed in Khan, 2012; Marciano-Cabral and Cabral, 2003), but the T4 genotype has been most frequently associated with blinding keratitis and fatal granulomatous amoebic encephalitis (GAE) which occurs mostly in immunocompromised individuals (Martinez and Visvesvara, 1997). *Acanthamoeba* keratitis is a painful sight-threatening ulceration of the cornea and is most likely associated with the improper use of contact lenses (Marciano-Cabral and Cabral, 2003). The sequence of events in *Acanthamoeba* keratitis involves the breakdown of the epithelial barrier, stromal

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invasion by *Acanthamoeba*, keratocyte depletion, induction of an intense inflammatory response and finally stromal necrosis (Garner, 1993; Vemuganti et al., 2004). Treatment is problematic and consists predominantly of hourly topical application of a mixture of drugs including polyhexamethylene biguanide or chlorhexidine digluconate together with propamidine isethionate or hexamidine. Moreover chloramphenicol or neomycin is also given to prevent mixed bacterial infection (Perez-Santonja et al., 2003). The treatment lasts for several months and even then, recurrence can occur (Ficker et al., 1990). This is due to the ability of *Acanthamoeba* to rapidly adapt to extracellular changes and differentiate into a resistant cyst form under harsh conditions (Byers, 1979; Hirukawa et al., 1998; Khunkitti et al., 1998; Turner et al., 2000; Weisman, 1976). The Cysts are double-walled, metabolically inactive structures that pose a major challenge in the successful treatment of infection. In the present study, we investigated encystation in *A. castellanii* belonging to the T4 genotype, *in vitro* by exposing trophozoites to various environmental and physiological adverse conditions.

2. Materials and methods

2.1. A. castellanii cultures

All chemicals were purchased from Sigma Labs (Poole, Dorset, England), unless otherwise stated. *A. castellanii* belonging to the T4 genotype (ATCC 50492) was originally isolated from a keratitis patient and was grown in 75 cm^2 tissue culture flasks in the presence of 10 mL PYG medium [proteose peptone 0.75% (w/v), yeast extract 0.75% (w/v) and glucose 1.5% (w/v)] without shaking at 37 °C as described previously (Aqeel et al., 2012). Media were refreshed 15–20 h prior to experiments. *A. castellanii* adherent to flasks represented the trophozoite form and were used in all subsequent assays.

2.2. Encystation assays

Encystation assays were performed as described previously (Dudley et al., 2009) but with slight modifications. Briefly, 2×10^6 *A. castellanii* were inoculated in phosphate buffered saline (PBS; containing 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4) in the presence of 50 mM $MgCl_2$ and 10% glucose (i.e., encystation trigger is higher osmolarity) in 24 well-tissue culture plates at 37 °C. After 72 h, *A. castellanii* viability was quantified using a haemocytometer, by Trypan blue exclusion assay. Next, sodium dodecyl sulfate (SDS, 0.5% final concentration) was added and placed on a shaker for 10 min to solubilize all the trophozoites and cysts were enumerated using a haemocytometer (cysts are resistant to 0.5% SDS). The percentage encystation was determined as follows; No. of *A. castellanii* post-SDS treatment/total number of *A. castellanii* × 100 = % encystation. Data are represented as the mean ± standard error of three independent experiments.

2.3. Environmental conditions

To study the effects of different stress conditions on the induction of encystation in *A. castellanii*, assays were performed in the presence of PBS and distilled water (nutrient deprivation), variable temperatures (4–45 °C), different pH (3–9), artificial light and dark cycles, 5% CO_2 and microaerophilic conditions.

To identify the optimum liquid medium and the role of nutrient deprivation on the encystation of *A. castellanii*, 2×10^6 amoebae were inoculated in the presence or absence of 10% glucose and 50 mM $MgCl_2$ in PBS or distilled water at 30 °C for 72 h. After this incubation trophozoites were lysed with 0.5% SDS treatment and cysts were enumerated by haemocytometer counting.

To determine the optimum temperature for *A. castellanii* differentiation, encystation assays were performed at variable temperatures (4–45 °C) in the absence and presence of 50 mM $MgCl_2$ and 10% glucose. After this incubation trophozoites were lysed with 0.5% SDS treatment and cysts counts were determined by haemocytometer counting.

To study the effects of pH on *A. castellanii*, encystation assays were performed using different pH buffers (3.0, 5.0, 7.0 and 9.0). The pH 3 and 5 were obtained using citrate-phosphate buffer [0.2 M sodium phosphate (Na_2HPO_4) and 0.1 M citrate buffer and pH adjusted to 3.0 or 5.0]; pH 7 was obtained using PBS buffer and pH adjusted to 7.0; and pH 9 was obtained using glycine–sodium hydroxide buffer (0.2 M glycine and 0.2 M sodium hydroxide buffer and pH adjusted to 9.0). Plates were then incubated at 30 °C in the absence and presence of 50 mM $MgCl_2$ and 10% glucose. After 72 h, 0.5% SDS was added to lyse trophozoites and cysts counts determined using a haemocytometer.

The effect of complete light, complete dark and 12 h light/dark cycle on the encystation of *A. castellanii* was studied by inoculating 2×10^6 amoebae in 24 well tissue culture plates in the presence and absence of 10% glucose and 50 mM $MgCl_2$ at 37 °C incubator under complete dark or under complete light (visible light). For light/dark cycles, plates were incubated at 37 °C incubator set with automatic Panasonic timer (TB2118) and Philips bulb (visible light). After 72 h, cells were treated with 0.5% SDS to lyse trophozoites and cysts were enumerated using a haemocytometer.

To study the effect of 5% CO_2 on encystation, 2×10^6 cells of *A. castellanii* were inoculated in PBS. Amoebae were then dispensed in 24 well tissue culture plates in the absence or presence of 10% glucose and 50 mM $MgCl_2$. Plates were then incubated at 37 °C in a shel lab water jacketed tissue culture incubator which maintains 5% CO_2 level. After this incubation trophozoites were lysed with 0.5% SDS treatment and cysts were enumerated by haemocytometer counting.

The role of microaerophilic conditions on *A. castellanii* encystation were determined by inoculating 2×10^6 amoebae in the presence and absence of 10% glucose and 50 mM $MgCl_2$ in 35 mm tissue culture dishes. Amoebae were then incubated in oxoid anaerobic jars loaded with campy gen bags (absorbs all the free oxygen) at 37 °C. After 72 h, 0.5% SDS was added and amoeba cysts were enumerated using a haemocytometer.

2.4. Calcofluor white staining

A. castellanii trophozoites were inoculated on 3% non-nutrient agar plates and incubated at 37 °C for 14 days (encystation trigger was nutrient deprivation). Cysts were collected by scraping off the agar surface using a rubber policeman and then resuspended in PBS. Simultaneously, *A. castellanii* cysts were also prepared by inoculating trophozoites at 37 °C for 72 h with 50 mM $MgCl_2$ and 10% glucose in PBS (encystation trigger was higher osmolarity). Cysts were centrifuged at 900*g* for 20 min. Following this, cysts were resuspended in 2.5% Calcofluor white stain and incubated at 30 °C for 120 min. Next, cysts were collected by centrifugation at 900*g* and resuspended in PBS. This process was repeated twice to remove excess Calcofluor white stain, and finally cysts resuspended in PBS. Finally, 30 μL suspension was applied to glass slides and wet mounts of each slide were examined under Olympus, BX-41 fluorescent microscope (excitation 405 nm and emission band pass 420–480 nm). Our previous studies have shown that Calcofluor white does not stain trophozoite stage of amoebae (Dudley et al., 2007), hence *A. castellanii* trophozoites were used as negative controls. Similarly, previous studies have shown that Calcofluor white