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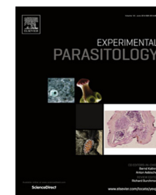

Encystment of *Vermamoeba* (*Hartmannella*) *vermiformis*: Effects of environmental conditions and cell concentration

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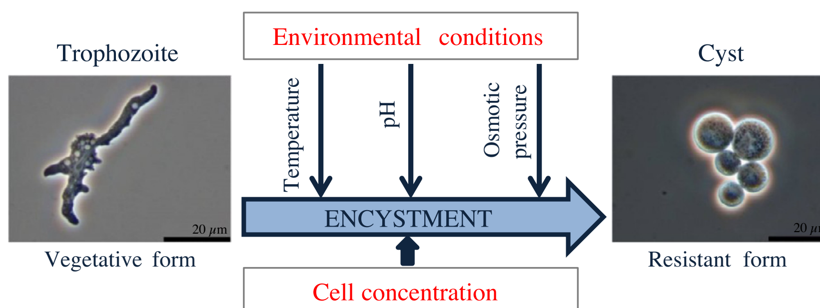
HIGHLIGHTS

- The encystment of *Vermamoeba vermiformis* took around 9 h.
- The encystment rate was favored at basic pH (8–9).
- The encystment rate was favored at moderate osmotic pressure (0.1 mol L^{-1} KCl).
- The encystment rate increased with cell concentration.

GRAPHICAL ABSTRACT



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ABSTRACT

Vermamoeba vermiformis is a free-living amoeba (FLA) which is widely distributed in the environment. It is known to colonize water systems and to be a reservoir of pathogenic bacteria, such as *Legionella pneumophila*. For these reasons the control of *V. vermiformis* represents an important health issue. However, FLA may be resistant to disinfection treatments due to the process of encystment. Thereby, it is important to better understand factors influencing this process. In this aim, we investigated the effect of temperature, pH, osmotic pressure and cell concentration on the encystment of two *V. vermiformis* strains. Encystment was quite fast, with a 100% encystment rate being observed after 9 h of incubation. For the two strains, an optimal encystment was obtained at 25 and 37 °C. Concerning pH and osmotic pressure, there were different effects on the encystment according to the tested strains. For the reference strain (ATCC 50237), the patterns of encystment were similar for pH comprised between 5 and 9 and for KCl concentrations ranging from 0.05 to 0.2 mol L^{-1}. For the environmental strain (172A) an optimal encystment was obtained for basic pH (8 and 9) and for a concentration in KCl of 0.1 mol L^{-1}. The results also clearly demonstrated that the encystment rate increased with cell concentration, suggesting that there is an inter-amoebal communication. The present study establish for the first time environmental conditions favoring encystment and would lay the foundations to better control the encystment of *V. vermiformis*.

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1. Introduction

Free-living amoebae (FLA) are protozoa widely distributed in the environment and isolated from water, soil, air, compost and sediments (Rodriguez-Zaragoza, 1994). They also colonize water

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treatment plants (Thomas et al., 2008), water distribution networks (Thomas et al., 2006) and cooling towers (Srikanth and Berk, 1993; Sykora et al., 1983). FLA are mainly encountered in biofilms where they feed by grazing on microorganisms (Rodriguez-Zaragoza, 1994). *Acanthamoeba*, the best described genus, and *Vermamoeba vermiformis*, which has been classified until 2011 in the *Hartmannella* genus (Smirnov et al., 2011), are predominant in hot water systems (Buse et al., 2013; Rohr et al., 1998; Thomas et al., 2006, 2008).

FLA act as reservoirs of pathogenic bacteria, resulting in protection from hostile conditions and promoting bacterial dissemination in the environment (Greub and Raoult, 2004; Thomas et al., 2010). Indeed, several pathogenic bacteria, like *Legionella pneumophila*, *Listeria monocytogenes*, *Pseudomonas aeruginosa* and *Mycobacterium* spp., may resist amoebal phagocytosis (Greub and Raoult, 2004). Also, FLA themselves might be pathogenic, those belonging to *Acanthamoeba*, *Balamuthia mandrillaris* and *Naegleria fowleri* can be responsible for severe brain pathologies (Marciano-Cabral and Cabral, 2003; Visvesvara et al., 2007) and *Acanthamoeba* may also be responsible for keratitis (Visvesvara et al., 2007). *V. vermiformis* has been poorly studied likely because it is not described as pathogenic for humans. However, it is well recognized that *V. vermiformis* acts as a reservoir of pathogenic bacteria, such as *L. pneumophila*, in water networks and cooling towers (Donlan et al., 2005; Fields et al., 1990; Kuiper et al., 2004).

FLA have two stages of development: a vegetative form, named trophozoite, and a dormant form, named cyst. The differentiation process is called encystment and has been mainly studied in *Acanthamoeba*, for a review see (Fouque et al., 2012). Encystment occurs when environmental conditions become unfavorable such as nutrient starvation or osmotic stress (Byers, 1979; Neff et al., 1964; Weisman, 1976). Encystment induces extensive morphological changes: FLA cell become spherical and a cyst wall, containing from one to three layers depending on FLA genus, is formed. Also, as a dormant cell, the cyst has a reduced metabolism. The cyst wall leads to increased resistance to treatments and harsh conditions (Critchley and Bentham, 2009; Dupuy et al., 2014; Khunkitti et al., 1998). *V. vermiformis* cysts are more resistant than trophozoites, however their resistance to disinfection treatments is poorly documented (Critchley and Bentham, 2009; Dupuy et al., 2014; Kuchta et al., 1993). Finally, encystment of *V. vermiformis* has been rarely described (Smirnov and Michel, 1999) and to our knowledge, the environmental conditions triggering encystment of *V. vermiformis* have never been reported.

In the present study, we evaluated the effects of temperature, pH, osmotic pressure and cell concentration on the encystment of two strains of *V. vermiformis*.

2. Material and methods

2.1. Amoebal strains

Two strains of *V. vermiformis* were used in this study: *V. vermiformis* Page ATCC 50237, a reference strain isolated from a hospital cooling tower in South Dakota (USA), and *V. vermiformis* 172A, an environmental strain recently isolated from a hospital water network (Lausanne, Switzerland) by Thomas et al. (2006) where it was referenced as *Hartmannella vermiformis* strain 2.

2.2. Cultivation

The two strains of *V. vermiformis* were grown axenically in 15 mL of modified PYNFH medium (1% bacto-peptone, 1% yeast extract, 0.1% RNA of torula yeast type VI, 33 μmol L^{-1} folic acid, 1.5 μmol L^{-1} hemin, 3.6 mmol L^{-1} Na_2HPO_4, 26 mmol L^{-1} KH_2PO_4, 10% fetal bovine serum, pH 6.5), in a 75 cm^2 tissue culture flask at 28 °C. Every 3–5 days of culture, the cells were subcultured.

2.3. Encystment

After 3 days of cultivation, cells were harvested by centrifugation (500×*g* for 7 min), washed twice in the Neff's encystment medium (0.1 mol L^{-1} KCl, 8 mmol L^{-1} $MgSO_4$, 0.4 mmol L^{-1} $CaCl_2$, 20 mmol L^{-1} Tris (2-amino-2-hydroxymethyl-1.3-propanediol), $NaHCO_3$ 1 mmol L^{-1}, pH 8.8) and suspended in the same medium at 5×10^5 trophozoites mL^{-1}. The cell concentration was estimated by enumeration in counting chamber (Fast-Read 102®, Biosigma). Then, trophozoites were distributed (1 mL per well) in 12-well microtiter plates and incubated at 25 °C. Encystment rates were measured every 3 h. These kinetics were performed in three independent replicates.

To estimate the effect of the various considered parameters, changes were applied to the above described conditions. The influence of temperature was tested by incubating microtiter plates at four different temperatures: 4, 25, 37 and 50 °C. The effects of pH and osmotic pressure were tested by adjusting pH and KCl concentrations of the Neff's encystment medium. The pH tested values were as follows: 5, 6, 7, 8 and 9. The KCl concentrations were as follows: 0, 0.05, 0.1, 0.2 and 0.5 mol L^{-1}. Assays on cell concentration were performed by suspending cells in the Neff's encystment medium at various cell concentrations: 1×10^4, 1×10^5, 1×10^6 trophozoites mL^{-1}. These tests were performed in three independent replicates.

2.4. Measurement of the encystment rate

Cells were harvested by centrifugation (500×*g* for 7 min) to eliminate the encystment medium and suspended in phosphate buffer (50 mmol L^{-1}, pH 7). The total number of cells (trophozoites, immature cysts and cysts) was enumerated using a counting chamber (Fast-read 102®, Biosigma). The cells were treated with 0.5% of sodium dodecyl sulfate (SDS) in order to lyse trophozoites and immature cysts, as previously described (Dudley et al., 2005). Finally, the number of remaining mature cysts was enumerated. Encystment rate was calculated by the following formula: (Number of mature cysts × 100)/Number of total amoebae.

2.5. Light microscopy

An upright microscope (Olympus BX51) with phase contrast equipped with camera (Olympus DP20-5E Microscope digital camera) was used for pictures. An inverted confocal microscope with rotary disk (Revolution/Andor; Olympus IX81-ZDC) was used for time-lapse studies. The pictures were taken every 30 s during 11 h. The pictures were assembled (15 pictures by second) using FluoView software (Olympus).

3. Results

3.1. Kinetic of encystment

In order to estimate the speed of encystment, we conducted kinetic studies on two strains: *V. vermiformis* 50237 and 172A. For this purpose, the strains were incubated in Neff's encystment medium and the encystment rates were measured every 3 h. Neff's encystment medium was selected because it allows quick and synchronous encystment in *Acanthamoeba* (Neff et al., 1964) and because its pH and osmotic pressure could be easily modified. After 3 h of incubation in the Neff's encystment medium, the cells of the two strains were not encysted (Fig. 1). After 6 h of incubation, 56%

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