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Development of a real-time PCR assay for quantification of *Acanthamoeba* trophozoites and cysts

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Abstract

Free-living amoebae have been found to be a reservoir for various pathogenic bacteria in aquatic environments. For example, the *Acanthamoeba* genus renders possible the intracellular multiplication of *Legionella pneumophila*, which is responsible for legionellosis. It consequently matters to quantify *Acanthamoeba* cells and thereby enhance our assessment of the risk of contamination. The classical microbiological method of quantification relies on amoebae growth and most probable number calculation.

We have developed a real-time PCR assay based on a TaqMan probe that hybridizes onto 18S rDNA. This probe is specific to the *Acanthamoeba* genus. The assay was successful with both the trophozoite and the cyst forms of *Acanthamoeba*. Highly sensitive, it proved to permit detection of fewer than 10 cells, even those that are not easily cultivable, such as the cyst forms.

Keywords: Real-time PCR; Amoebae; Cyst

1. Introduction

The study of free-living amoebae is worthy of increasing interest insofar as they may cause opportunistic infection, such as keratitis (Marciano-Cabral and Cabral, 2003) and particularly because they are a reservoir for numerous pathogenic bacteria. For example, the genus *Acanthamoeba*, which is the archetype of amoeba–bacteria interaction, is known to be associated with *Mycobacterium*, *Pseudomonas* and *Legionella* (Brown and Barker, 1999). *Acanthamoeba* presents two developmental stages: the trophozoite and the cyst forms. The former is metabolically active while the latter is a dormant form. Amoebae encyst in the event of food depletion or other stress conditions. Cysts have a specific envelope and are more resistant to biocides than trophozoites (Lloyd et

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al., 2001). Trophozoites feed on bacteria by phagocytosis. However, the evolution of some bacteria has rendered them resistant to phagocytosis and entailed their multiplication within the amoeba cell. These bacteria have been collectively named amoeba-resistant bacteria (ARB) (Greub and Raoult, 2004). While they are highly diversified, most are pathogenic or potentially pathogenic. Therefore, detection and quantification of *Acanthamoeba* is crucial when evaluating risk of infection by ARB. Detection of *Acanthamoeba* is assessed mainly by dilution culturing methods and subsequent most probable number (MPN) calculations (Beattie et al., 2003). They can be grown axenically in PYG medium or on non-nutrient agar seeded with bacteria. Such methods are time-consuming and identify no organisms other than those amenable to growth under defined conditions. Cyst forms, which do not excyst, and trophozoites, which are damaged during sample preparation, might not be detected by culture. Moreover, a morphological distinction between amoebae genera is not always easily obtained. Compared with culture, PCR methods are more rapid; in addition, non-cultivable cells might be detected and genus discrimination achieved. PCR methods, based on 18S rRNA genes amplification, have been described as *Acanthamoeba*-specific (Gast, 2001; Khan et al., 2001; Pasricha et al., 2003; Schroeder et al., 2001). However, as of now no real-time PCR has been described for *Acanthamoeba*, even though it permits quantification of cells and is highly sensitive. With the use of this method it has become possible to detect amounts of DNA the equivalent of a single cell in other protozoa including *Giardia*, *Cryptosporidium* and *Entamoeba* (Blessmann et al., 2002; Guy et al., 2003).

In this study, we have developed a real-time PCR assay meant to be used on both trophozoite and cysts.

2. Materials and methods

2.1. Amoebae strains and culture conditions

Acanthamoeba castellanii ATCC 30234 was grown axenically in flasks without shaking during 3 days at 25 °C in PYG medium: 20 g/l proteose peptone, 1 g/l yeast extract, 1 g/l sodium citrate, 4 μM $MgSO_4$, 0.4 μM $CaCl_2$, 2.5 μM Na_2HPO_4, 2.5 μM KH_2PO_4, 5 μM $(NH_4)_2FeII(SO_4)_2$, 0.1 M glucose (Schuster, 2002). Adherent cells were rinsed 3 times with amoeba buffer: PYG medium without proteose peptone, yeast extract and glucose. The washed cells were detached and resuspended in amoeba buffer.

2.2. Encystation conditions

After 3 days of cultures, trophozoites were transferred in encystation medium: 0.1 M KCl, 0.02 M Tris, 8 mM MgSO4, 0.4 mM, $CaCl_2$, 1 mM $NaHCO_3$, to initiate a rapid and fairly synchronous encystation (Neff and Neff, 1969). Flasks were left at 25 °C during 10 days. The cysts were harvested by centrifugation (2000 ×*g*, 5 min) and resuspended in amoeba buffer before use.

2.3. DNA extraction

The number of cells was counted directly by using a Mallassez counting chamber. DNA was extracted from trophozoites and cysts with a Wizard® SV genomic DNA purification system (Promega), using a modified protocol based on the protocol for purification of genomic DNA from tissue culture cells provided by the manufacturer. When necessary, samples were vacuum-filtered through 0.8-μm pore-size cellulose ester membranes on a sterile analytical stainless steel filter holder (Millipore). Cells or filters were vigorously stirred for 30 s in 8 ml of 4 M guanidium thiocyanate, in order to lyse the cells. A vacuum manifold was used to perform several DNA extractions at the same time. The sample lysates were passed through minicolumns and washed 4 times with 800 μl of Wizard® SV Wash solution. Minicolumns were dried during 4 min and transferred into microtubes. DNA was eluted by addition of 200 μl H_2O and 2 μl RNAse before centrifugation (13,000 ×*g*, 1 min). The amount of DNA was measured by using a spectrophotometer.

2.4. Primers and probe design

The complete sequence of twelve 18S rDNA genes, found in the GenBank database, from different amoebae genus (i.e. *Acanthamoeba*, *Hartmanella*, *Naegleria*, *Balamuthia*, *Nuclearia*, *Vahlkampfia*) was fetched. A 18S rDNA gene multiple alignment