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Sensitivity of free-living amoeba trophozoites and cysts to water disinfectants

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ABSTRACT

Free-living amoebae are naturally present in water. These protozoa could be pathogenic and could also shelter pathogenic bacteria. Thus, they are described as a potential hazard for health. Also, free-living amoebae have been described to be resistant to biocides, especially under their cyst resistant form. There are several studies on amoeba treatments but none of them compare sensitivity of trophozoites and cysts from different genus to various water disinfectants. In our study, we tested chlorine, monochloramine and chlorine dioxide on both cysts and trophozoites from three strains, belonging to the three main genera of free-living amoebae. The results show that, comparing cysts to trophozoites inactivation, only the *Acanthamoeba* cysts were highly more resistant to treatment than trophozoites. Comparison of the disinfectant efficiency led to conclude that chlorine dioxide was the most efficient treatment in our conditions and was particularly efficient against cysts. In conclusion, our results would help to adapt water treatments in order to target free-living amoebae in water networks.

Introduction

Free-living amoebae (FLA) are a heterogeneous group of protozoa, comprising several genera. They are ubiquitously found in natural environments, such as water and soils (Rodriguez-Zaragoza, 1994), as well as in artificial environments, such as water networks and cooling towers (Berk et al., 2006). Among FLA found in the environment and in water networks, *Acanthamoeba*, *Hartmannella* and *Naegleria* are the most common genera but other genera such as, *Vahlkampfia*, *Balamuthia*, *Willaertia* and *Nuclearia* can be found occasionally (Critchley and Bentham, 2009; Loret and Greub, 2010; Thomas and Ashbolt, 2011). Most FLA can exist in at least two forms: a vegetative trophozoite form, which is able to feed, move and divide, and a dormant cyst form, which is inactive. The differentiation process leading to the cyst, known as encystment, is triggered by nutritional or osmotic stresses (Fouque et al., 2012).

FLA has been described as reservoirs for several pathogenic bacteria, such as *Legionella*, *Mycobacterium* and *Chlamydia* (Corsaro et al., 2010; Molmeret et al., 2005; Thomas et al., 2009). Indeed, FLA ingest bacteria by phagocytosis but some bacteria are able to resist amoebal phagocytosis and, consequently, to survive within FLA. These bacteria are collectively named amoeba-resisting bacteria (ARB) (Greub and Raoult, 2004) and some of them, such as *Legionella pneumophila*, are even able to multiply within FLA (Rowbotham, 1980). For *L. pneumophila*, FLA are likely to be the main reservoirs in the environment (Borella et al., 2005). Furthermore, after intra-amoebal growth, *L. pneumophila* become more virulent than BCYE-grown *L. pneumophila* (Molmeret et al., 2005) and more resistant to treatments (Barker et al., 1992). In addition, *L. pneumophila*, as well as other bacteria, can also be found within FLA cysts (Kilvington and Price, 1990; Skinner et al., 1983) leading to higher resistance, since cysts can provide a physical barrier to treatments. Thus, FLA has been described recently as by-passes because they are able to protect bacteria from water treatments (Loret and Greub, 2010). Besides, some FLA is pathogenic for humans and are responsible for keratitis and infections of the central nervous system (Schuster and Visvesvara, 2004; Visvesvara et al., 2007). *Naegleria fowleri* is responsible for a rare but acute primary amoebic meningoencephalitis and *Acanthamoeba* for keratitis or granulomatous amoebic encephalitis.

As FLA may be pathogenic or able to bear pathogenic bacteria, efforts have been made to find efficient treatments against FLA in water networks (Critchley and Bentham, 2009). In order to prevent the development of pathogenic bacteria, such as *L. pneumophila*, in water networks, many treatments have been evaluated (Kim et al., 2002). Besides, there are few publications dealing with FLA

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treatment (Critchley and Bentham, 2009; Cursons et al., 1980; De Jonckheere and van de Voorde, 1976; Kuchta et al., 1993). These studies indicate that *Acanthamoeba* is often the less sensitive genus (Cursons et al., 1980; De Jonckheere and van de Voorde, 1976) and that environmental strains might be less sensitive than collection strains (Coulon et al., 2010). However, it remains difficult to compare precisely the efficiency of these treatments as experimental procedures are different and Ct values are not always reported (Loret and Greub, 2010).

In our study, we compared the effectiveness of three water disinfectants on trophozoites and cysts of FLA strains belonging to the *Acanthamoeba*, *Naegleria* and *Hartmannella* genera. Treatments were performed and inactivation was reported in terms of Ct values for each condition. Finally, the sensitivity of the cysts and the trophozoites was compared.

Materials and methods

FLA isolation and culture

FLA strains from water samples from different sources were collected by Anjou Recherche (Maisons-Laffitte, France) and Electricité De France (EDF, Chatou, France). A drop of water was placed onto a non-nutrient agar plate (made only with water and agar 15 g/L), seeded with a lawn of *Escherichia coli* XL1 Blue (Stratagene). This medium is referred to as NNA-Eco. Plates were incubated at various temperatures and examined daily for 7–14 days. After FLA growth (indicated by a lysis zone), an isolate was transferred onto a fresh NNA-Eco plate. Strains were axenised by transfer into an axenic broth 1034 medium (peptone 10 g/L, yeast extract 10 g/L, ribonucleic acid 1 g/L, folic acid 15 mg/L, hemin 1 mg/L, KH_2PO_4 0.36 g/L, Na_2HPO_4 0.5 g/L, pH 6.5) containing antibiotics (ampicillin 200 mg/mL and streptomycin 200 mg/mL). For the *Hartmannella 1534* strain, culture in axenic 1034 medium was not possible. This strain was cultivated in amoeba buffer (2.5 mM KH_2PO_4, 4 mM $MgSO_4$, 0.5 mM $CaCl_2$, 2.5 mM Na_2HPO_4, 0.05 mM $(NH_4)_2FeII(SO_4)^2$) supplemented with *E. coli* XL1 blue at a concentration of 5.10^8 bacteria per mL, and incubated at 37 °C.

Cyst production

Cysts were produced from a culture of trophozoites. For the strains of *Acanthamoeba* and *Hartmannella*, the encystment was induced by incubating trophozoites in the encystment buffer (K_2HPO_4 47 mM, KH_2PO_4 à 3 mM, $CaCl_2$ 0,4 mM, $NaHCO_3$ 1 mM, KCl 100 mM, pH 8). Then, the cultures were placed at room temperature for 15 days. For the *Naegleria* strain, axenic 1034 medium have replaced by a buffer composed by 90% of amoeba buffer completed with 10% of axenic 1034 medium. Then, the *Naegleria* trophozoites have been placed at 4 °C for 15 days.

Amoeba identification

DNA was extracted from amoeba cells using the NucleoSpin Tissue kit (Macherey Nagel). An 18S rDNA PCR was performed with primers Ami6F1 and Ami9R, as described previously (Thomas et al., 2006). The amplicons were sequenced with each primer using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and analysed using the 3130 Genetic Analyzer (Applied Biosystems).

Disinfection treatments

Three different disinfectants, commonly used by industries to disinfect their networks and circuits, were used: chlorine, chlorine dioxide and monochloramine. The treatments were performed at 30 °C. For trophozoite disinfection, the initial concentrations were as follows: chlorine 3 mg Cl_2/L (to provide approximately 1 mg Cl_2/L residual after 1 h), chlorine dioxide 0.6 mg/L and monochloramine 0.8 mg Cl_2/L. For cyst disinfection, the doses were adapted to provide a 3-log reduction during the treatment time. For the chlorine treatments, the initials doses applied were 50 mg/L for *Acanthamoeba*, 3 mg/L for *Naegleria* and 10 mg/L for *Hartmannella*. For the chlorine dioxide treatments, the doses applied were 6 mg/L for *Acanthamoeba*, 0.6 mg/L for *Naegleria* and 1 mg/L for *Hartmannella*. Finally, for the monochloramine treatments, the doses applied were 15 mg/L for *Acanthamoeba*, 0.8 mg/L for *Naegleria* and 3 mg/L for *Hartmannella*.

All of the solutions were prepared from reagent grade chemicals and deionised water. Stock solutions were stored at 4 °C. All glassware was cleaned with chlorine (100 mg/L) for at least one hour and carefully rinsed with deionised water. The chlorine solution was freshly prepared by diluting sodium hypochlorite (13%, ACROS Organics). The chlorine concentration was measured using the 4500-Cl G DPD method (APHA, 2005) before and during treatment in order to determine the residual concentration. The stock solution of monochloramine was obtained by adding free chlorine to a solution of ammonium chloride under agitation, with a chlorine-to-nitrogen molar ratio of 0.5 and a pH of 8.5. The final concentration of the stock solution of monochloramine was 2 mM (or approximately 140 mg Cl_2/L). A stock solution of chlorine dioxide was prepared by slowly adding sulphuric acid to a sodium chlorite solution and then by collecting the gaseous chlorine dioxide produced in ultrapure water using the 4500-ClO2 B method (APHA, 2005). The concentration of the stock solution was around 400 mg/L. The DPD method was also used to measure the residual concentration of monochloramine and chlorine dioxide. Treatment with chlorine, monochloramine and chlorine dioxide was stopped by adding sterile sodium thiosulphate (0.1 M).

Before each treatment, 1 mL of FLA suspension was transferred into 100 mL of sterile phosphate buffer (50 mM, pH 8), leading to a concentration of 10^4 cell/mL. The sample was incubated at 30 °C under agitation and disinfectant was added. The concentration of the disinfectant and the survival of the FLA (trophozoites and cysts) were followed after 0, 2, 30 and 60 min of treatment. The disinfectant exposure was quantified by Ct (concentration × time, in mg min/L), which corresponds to the geometric area below the disinfectant decay curve. Microbial inactivation (loss of cultivability) was recorded as a function of Ct, to evaluate the effectiveness of the disinfectants. Ct tables have been developed for some waterborne pathogens to indicate the conditions necessary for a 2-log ($Ct_{99\%}$) or 3-log ($Ct_{99.9\%}$) inactivation (King et al., 1988; Rose et al., 2005).

Survival of amoeba

The survival of FLA (trophozoites and cysts) was determined using the most probable number (MPN) procedure (Beattie et al., 2003). Various volumes (1, 0.1, 0.01 and 0.001 mL) of each sample were inoculated onto NNA-Eco plates. Each inoculation was performed in quintuplicate. Plates were incubated for 15 days at different temperatures depending on the genus of amoeba tested: 25 °C for *Acanthamoeba* V1, 30 °C for *Naegleria* E1 and 37 °C for *Hartmannella 1534*. Each plate was examined for the presence or absence of microbial growth and the results were reported using an MPN table (Beattie et al., 2003). The low and high limits of detection were 1.8×10^2 NPP/L and 1.6×10^6 NPP/L, leading to a maximum amplitude of 3.94 log.