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Biodiversity of amoebae and amoeba-associated bacteria in water treatment plants

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ABSTRACT

In this study, we enlarged our previous investigation focusing on the biodiversity of chlamydiae and amoebae in a drinking water treatment plant, by the inclusion of two additional plants and by searching also for the presence of legionellae and mycobacteria. Autochthonous amoebae were recovered onto non-nutritive agar, identified by 18S rRNA gene sequencing, and screened for the presence of bacterial endosymbionts. Bacteria were also searched for by *Acanthamoeba* co-culture.

From a total of 125 samples, we recovered 38 amoebae, among which six harboured endosymbionts (three chlamydiae and three legionellae). In addition, we recovered by amoebal co-culture 11 chlamydiae, 36 legionellae (no *L. pneumophila*), and 24 mycobacteria (all rapid-growers).

Two plants presented a similar percentage of samples positive for chlamydiae (11%), mycobacteria (20%) and amoebae (27%), whereas in the third plant the number of recovered bacteria was almost twice higher. Each plant exhibited a relatively high specific microbiota. Amoebae were mainly represented by various *Naegleria* species, *Acanthamoeba* species and *Hartmannella vermiformis*. *Parachlamydiaceae* were the most abundant chlamydiae (8 strains in total), and in this study we recovered a new genus-level strain, along with new chlamydiae previously reported. Similarly, about 66% of the recovered legionellae and 47% of the isolated mycobacteria could represent new species. Our work highlighted a high species diversity among legionellae and mycobacteria, dominated by putative new species, and it confirmed the presence of chlamydiae in these artificial water systems.

Introduction

Within the water distribution systems, a peculiar microbial ecology is to be defined, taking into account the interplay of different physical, chemical and biotic variables, like temperature, type of pipe surface, nutrient levels, presence of disinfectant chemicals, biofilms, predatory protists and their endosymbionts (Berry et al., 2006). Among the protists, free-living amoebae have gained growing interest, as it has been shown that they are widespread in aquatic habitats, and successfully colonize many man-made water systems, like cooling towers, humidifiers, hospital water networks, or drinking water production plants (Corsaro et al., 2009; Hoffmann and Michel, 2001; Thomas et al., 2006b, 2008). Amoebal species developing cysts are generally resistant to treatment processes (Gerba et al., 2003; Hijnen et al., 2006; Loret et al., 2008a). Some species and strains of amoebae are opportunistic parasites (amphizoic amoebae) of vertebrates, including humans, in which they cause mainly keratitis and meningo-encephalitis (Visvesvara et al., 2007). In addition, several microorganisms, including established pathogens, are able to infect and survive within different amoebae, and thus may by-pass disinfection treatment (Greub and Raoult, 2004; Loret et al., 2008b). Due to this peculiar lifestyle, laboratory strains of amoebae, mainly *Acanthamoeba* sp., have been used as host cells to isolate amoeba-resisting bacteria from either environmental (Collingro et al., 2005a; Pagnier et al., 2008; Thomas et al., 2006b, 2008) and clinical (Rowbotham, 1998; Greub et al., 2004) samples.

Legionellae and mycobacteria are well known inhabitants of aquatic biofilms. Various species within both groups are recognized pathogens (*Legionella pneumophila*, *Mycobacterium avium* complex) or highly suspected pathogens (Fields et al., 2002; Greub and Raoult, 2004; Primm et al., 2004), and the elimination of pathogenic microorganisms should be performed prior to the entry in the final

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distribution systems (Council Directive 98/83/EC). Our interest for the chlamydiae, comes from the evidences of their huge and unexplored diversity in the environment (Corsaro et al., 2003, 2009), and from the possibility that novel chlamydial pathogens may occur in the environment (Corsaro and Venditti, 2004; Corsaro and Greub, 2006).

In this study we used *Acanthamoeba* co-culture to isolate three main groups of amoeba-associated bacteria, i.e. *Chlamydiales*, *Legionella* spp., and *Mycobacterium* spp., from three water treatment plants producing drinking water in Spain. This work extended our recent investigation focusing on the biodiversity of chlamydiae and amoebae in one of these three plants (plant C) (Corsaro et al., 2009).

Materials and methods

Samples

A total of 125 samples was collected from August, 2006 to July, 2007, from three treatment plants (A, B and C) producing drinking water, located in Spain. Samples originated from different points upstream and downstream the major steps of the industrial processes (Table 1). Samples of 1 litre were filtered through a 0.2-μm polycarbonate membrane, and the filter was resuspended in 50 ml of sterile distilled water in Falcon tubes, and sent to the laboratory at room temperature. Prior to any inoculation assay onto agar plates or amoeba co-culture, tubes were vortexed for 15–30 s in order to resuspend the microorganisms present on the filters.

Temperature, turbidity (Nephelometric Formazine Units, NFU) and quantitative microbiological analyses per litre for total amoebae (most probable number, MPN), and aerobic bacteria (colony-forming unit, CFU) were performed, as described previously (Corsaro et al., 2009).

Recovery of bacteria by amoeba co-culture

Chlamydiae, legionellae and mycobacteria potentially present in the samples were recovered by amoeba co-culture in 24-well microplates (Costar, Corning, NY), using *Acanthamoeba* sp. (strain ATCC 30010), as described previously (Corsaro et al., 2009). Briefly, 100 μl of each sample were inoculated in serial dilution onto *Acanthamoeba* monolayers, centrifuged (1500 × *g* for 30 min), and incubated at 32 °C in a humidified atmosphere in the dark. At 6 days post-inoculation, subcultures were performed on fresh *Acanthamoeba* (second co-culture). Along the first and the second co-culture, amoebae were screened for by using specific PCR (see below), and bacterial strains were identified by sequencing a portion of the 16S rRNA gene (see below).

Recovery of autochthonous amoebae and screening for bacterial endosymbionts

Autochthonous amoebae from each sample were isolated onto bacterized non-nutritive agar (NNA) at 32 °C in the dark, as described previously (Page, 1967; Rowbotham, 1980; Corsaro et al., 2009). Distinct morphotypes were subcultured onto NNA to obtain clonal amoebae, which were identified by sequencing portion of the 18S rRNA gene (see below). Naturally harboured bacteria were searched for by applying the specific 16S rRNA gene PCR.

DNA extraction, gene amplifications and sequencing

Total DNA was extracted from infected *Acanthamoeba* co-cultures, and from clonal amoebae recovered directly from the NNA, with the AquaPure Genomic DNA extraction kit (Bio-Rad). PCR were performed in 50 μl reaction tubes containing specific primer sets. To detect chlamydiae, the almost complete 16S rRNA gene was amplified with the primers 16SIGF (5′-CGGCGTGGATGAGGCAT-

Table 1
Types of samples and summary of results.

	Samples	*n*	Samples positive for[a]			
			Chlamydiae	Legionellae	Mycobacteria	Amoebae
Plant A	Raw surface water	2		1	1	1
	Settled water	4				
	Sand-filtered water	8	1	1		
	Finished water	2				1
	Sludge from clarifier	2		2		1
	Biofilm from filter	3			1	
	Total (%)	21	1 (4.7)	4 (19.0)	2 (9.5)	3 (14.2)
Plant B	Raw surface water	2	1[b]			2
	Settled water	5			1	
	Sand-filtered water	9			3	
	Finished water	2				
	Sludge from clarifier	5	1	1	1	4
	Biofilm from clarifier	2	1			1
	Total (%)	25	3 (12)	1 (4)	5 (20)	7 (28)
Plant C	Raw groundwater	8		3		
	Raw surface water	8	4	5[d]	4	7
	Sand-filtered water	8		5	1	2
	Ozonated water	1		1		
	GAC-filtered water	8	1	6		1
	Finished water	8				1
	Biofilm from distribution system	11	2	2[d]	3	2
	Sediment from distribution system	12	2[c]	5	6	7
	Water from dead leg	5		3	1	2
	Water from distribution system	10		1	2	
	Total (%)	79	9 (11.4)	33 (41.7)	17 (21.5)	22 (27.8)

[a] Bacteria were recovered by *Acanthamoeba* co-culture, autochthonous amoebae onto bacterized NNA.
[b] A *Protochlamydia* strain was recovered within its natural host, an *Acanthamoeba* T4.
[c] A *Neochlamydia* strain was recovered within its natural host, an *Acanthamoeba* T4.
[d] Two *Legionella* were recovered within their natural hosts, a *Naegleria australiensis* (raw water), and an *Acanthamoeba* T4 (biofilm).