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Prevalence and diversity of *Chlamydiales* and other amoeba-resisting bacteria in domestic drinking water systems

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Abstract

A growing number of human infections incriminate environmental bacteria that have evolved virulent mechanisms to resist amoebae and use them as a replicative niche. These bacteria are designated amoeba-resisting bacteria (ARB). Despite the isolation of these ARB in various human clinical samples, the possible source of infection remains undetermined in most cases. However, it is known that the ARB *Legionella pneumophila*, for instance, causes a respiratory infection in susceptible hosts after inhalation of contaminated water aerosols from various sources. The *Chlamydiales* order contains many ARB, such as *Parachlamydia acanthamoebae* or *Simkania negevensis*, previously implicated in human respiratory infections with no identified contamination sources. We thus investigated whether domestic water systems are a potential source of transmission of these *Chlamydiales* to humans by using amoebal culture and molecular methods. Other important ARB such as mycobacteria and *Legionella* were also investigated, as were their possible amoebal hosts. This work reports for the first time a very high prevalence and diversity of *Chlamydiales* in drinking water, being detected in 35 (72.9%) of 48 investigated domestic water systems, with members of the *Parachlamydiaceae* family being dominantly detected. Furthermore, various *Legionella* and mycobacteria species were also recovered, some species of which are known to be causal agents of human infections.

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Keywords: Amoebal co-culture, amoebal enrichment, biofilm, *Criblamydiaceae*, *Parachlamydiaceae*

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Introduction

Free-living amoebae are ubiquitous in the environment, especially water. In case of unfavourable growth conditions, such as starvation or desiccation, these protists can exhibit a resistant form, termed cysts. The cyst structure helps the amoebae to survive various disinfection treatments [1–3]. Thus, amoebae may bypass all the barriers present in drinking water treatment plants [4] and may reach the water distribution system, where they may colonize biofilms and sediments.

Amoebae have been shown to be natural hosts of different bacteria that can resist intracellular killing through several mechanisms [5]. Some of these amoeba-resisting bacteria (ARB) have been shown to reside in the amoebal cyst, where they are protected from biocides and disinfection treatments [6–8]. The evolution of traits that result in bacterial resistance to amoebae may explain the ability of some ARB to also resist other phagocytic cells, such as macrophages [9–12]. The observation that some ARB are able to infect both amoebae and macrophages supports this hypothesis [13,14].

Humans may be exposed to these ARB through various water systems such as cooling towers, humidifier aerosols, drinking water, spas or swimming pools, all of which have previously been shown to be reservoirs of ARB. For instance, the
ARB Legionella pneumophila was discovered after an outbreak of pneumonia in 1976 in Philadelphia in which dozens of people were infected by a contaminated air-conditioning system [15]. Breiman et al. [16] later showed a correlation between Legionnaire’s disease due to Legionella pneumophila and the use of showers. Newly discovered ARB are emerging as potential respiratory pathogens, such as Parachlamydia acanthamoebae [17] and Simkania negevensis [18], both able to replicate in amoebae [7,19,20]. However, the mode of transmission of these Chlamydia-related bacteria remains to be determined.

Recently a Chlamydiales-specific quantitative PCR was developed and was applied to 422 nasopharyngeal swabs from patients [21]. This study showed that 48 patients were positive for a member of the Chlamydiales order, among which 38 corresponded to Chlamydia-related bacteria, demonstrating that these bacteria can reach the human respiratory tract.

Thus, in the present work, domestic drinking waters and biofilms from plumbing systems were investigated for the presence of Chlamydiales by PCR and culture methods. These samples were also screened for other ARB belonging to the families Legionellaceae and Mycobacteriaceae, from which several members are established as human pathogens. Finally, the screening of potential amoebal hosts was also performed.

**Materials and Methods**

**Sample**

Water (n = 48) and biofilm (n = 48) samples were collected from 48 different domestic water systems in the regions of Geneva (n = 37), Lausanne (n = 7) and Sion (n = 4), Switzerland. Sampling was performed from September 2010 to August 2011. One litre of first-flow water was first sampled from the shower, filtered through a 0.22 μm membrane, which was then resuspended in 10 mL of filtrated water. The mean temperature of the water was 20.6 ± 3.8°C. Then, using a sterile swab, biofilm samples were collected from the flexible pipe connected to the shower head (after unscrewing the shower head) and was then resuspended on site in about 3 mL of shower water. Aliquots of 100 μL of concentrated water and 100 μL of resuspended biofilm were kept at −20°C for DNA extraction (Fig. 3) while the samples were processed immediately for analyses.

**Screening of ARB with amoebal co-culture**

*Acanthamoeba castellanii* ATCC 30010 was used to cultivate ARB. *A. castellanii* was grown in the rich peptone yeast-extract glucose (PYG) medium [22,23], at 28°C without CO₂, in 75 cm² surface cell culture flasks (Becton Dickinson, Allschwil, Switzerland). Amoebae were collected by centrifugation (1500 × g, 10 minutes) and washed with phosphate-buffered saline and finally resuspended in poor medium Page amoeba saline (PAS) [22,23] to avoid extracellular overgrowth of bacteria. Amoebae were seeded in a 24-well culture microplate (Milan, Wohlen, Switzerland) at 5 × 10⁵ amoeba cells/mL. An aliquot (100 μL) of biofilm or concentrated water sample was then inoculated, and tenfold serial dilutions were performed. The microplates were immediately centrifuged at 1790 × g for 15 minutes, and the cells were incubated for 1 hour at 28°C. Cells were gently washed once with PAS and incubated at 32°C in a humidified atmosphere without CO₂. Amoebae were observed daily for amoebal lysis, and the co-cultures were reseeded on fresh confluent amoebae in PAS after 7 and 14 days [24]. At day 7 and day 14, 100 μL of each amoeba-containing well was collected and stored at −20°C until DNA extraction.

**Screening of amoebae with amoebal enrichment**

Nonnutrient agar plates were covered with a solution of live *Escherichia coli* ATCC 25922. About 20 μL of concentrated water or biofilm samples was seeded onto the agar and incubated at 28°C in a humidified atmosphere. Plates were observed daily under an optical microscope for the presence of amoebae. When positive, subcultures were performed [24], and amoebae were collected and frozen at −20°C until DNA extraction.

**PCR on water samples and biofilms**

DNAs were automatically extracted by the LC automated system (Roche, Rotkreuz, Switzerland) and the MagNA Pure LC DNA isolation kit 1 (Roche) using 100 μL of water and 100 μL of biofilm sample. For each run of extraction, a negative extraction control was included. Water samples (n = 48) and biofilm samples (n = 48) were analysed by 16S rRNA gene-directed PCR for the presence of DNA from Legionella spp. (Leg225/Leg858 primers [25]), *Mycobacterium* spp. (TB264R primers [26]) and *Chlamydiales* (panCh16F2/panCh16R2 primers and panCH16S probe [21]). Finally, amoebae were identified by sequencing a part of the 18S rRNA gene, amplified using the Ami6F1/Ami9R primers [43]. The *Chlamydiales*-specific real-time PCR targeting the 16S rRNA gene was performed as previously described [21]. Briefly, using the primers panCh16F2, panCh16R2 and a probe panCh16S, 5 μL of DNA was analysed in duplicate with 50 cycles consisting of denaturing for 15 seconds at 95°C, annealing for 15 seconds at 67°C and amplification for 15 seconds at 72°C.

When the PCR or quantitative real-time PCR was positive, the PCR product was purified with the MSB Spin PCRapace kit and sequenced with the same primers. In the case of positive samples for mycobacteria with the 16S rRNA PCR, a second PCR targeting the rpoB gene and using the primers MycoF/
MycoR was used for precise identification by sequencing [28]. Concerning the PCR products obtained with the Chlamydiales-specific real-time PCR, they were purified using the GenElute PCR Clean-Up Kit (Sigma, Buchs, Switzerland), and sequencing was performed with inner primers as described elsewhere [21]. All newly generated nucleotide sequences were submitted to GenBank; the accession numbers may be found in the Supplementary Tables.

PCR on amoebal culture and amoebal enrichment
Amoebal co-culture wells were screened by PCR for the presence of Legionella spp., Mycobacterium spp. and Chlamydia-related bacteria after 1 and 2 weeks of incubation. DNA was extracted from 100 μL of the culture using the Wizard genomic DNA purification kit (Promega, Duebendorf, Switzerland) in the presence of proteinase K (20 mg/mL) following the manufacturer’s protocol for animal tissues. For each run of extraction, a negative extraction control was included. Detection by PCR and sequencing of mycobacteria, Legionella and amoebae was performed as described above. For the Chlamydiales, the 16SigF/Rp2Chlam primers were used, as described elsewhere [29].

Results

ARB documented in water and biofilm samples
The number of bacteria and amoebae detected in this study are represented in Fig. 1. In addition, for each domestic water system, all bacterial and amoebal species identified by sequencing are presented in Table 1.

Chlamydiales species
Among the 48 domestic water systems investigated, 35 (72.9%) were positive for Chlamydiales detected by specific real-time PCR (rtPCR) in the water, the biofilm or both samples (Fig. 1 and Table 1). Sequencing of the rtPCR products gave a sequence of about 200 bp that was used to classify the bacteria at the family level following the criteria of Everett et al. [29]. A total of 55 Chlamydiales sequences could be obtained for 33 of these 35 positive households. The classification could be achieved for 51 DNA sequences (Table 1 and Supplementary Table S1), and four remained unclassified. Among these 55 sequences, 28 (50.9%) may correspond to new species-level
### TABLE 1: Summary of all *Chlamydiaceae* spp., *Legionella* spp., *Mycobacterium* spp. and amoebae detected in each water system of 48 households investigated

| Household ID | Water Species | Detection | Biofilm Species | Detection |
|--------------|---------------|-----------|----------------|----------|
| GE0016       | *Criblamydiaceae* putative species 1 [Chlam] | qP         | *Criblamydiaceae* putative species 2 [Chlam] | qP       |
| GE0027       | 100% *L. waltersii* [Legio] | P          | 99% *M. tusciae* sp. [Myco] | P+P+C    |
| GE0028       | *Parachlamydiaceae* [Chlam] | qP         | 95% *M. mononuclei* or *M. baratrise* [Myco] | P        |
| GE0032       | Unclassified *Chlamydiaceae* [Chlam] | C          | 100% *M. phocaicum* [Legio] | C        |
| GE0037       | 100% *Mycobacterium* trancium strain CCUG 52297 [Myco] | C          | 100% *M. phocatum* strain MBY1-1 [Myco] | C        |
| GE0044       | *Criblamydiaceae* [Chlam] | qP         | *Criblamydiaceae* putative species 2 [Chlam] | qP       |
| GE0049       | 100% *L. anisa* [Legio] | C          | 98% *M. tusciae* [Myco] | P        |
| GE0056       | *Chlamydiaceae* (failed sequencing) | qP         | 97% *M. tusciae* [Legio] | P        |
| GE0061       | *Criblamydiaceae* [Chlam] | qP         | 100% *H. vermiformis* [Legio] | P+P+C    |
| GE0062       | 100% *L. pneumophila* sp. Fl-10135 [Myco] | C          | 97% *M. tusciae* [Legio] | P        |
| GE0064       | Unclassified *Chlamydiaceae* [Chlam] | C          | 99% *H. vermiformis* [Myco] | P        |
| GE0068       | *Criblamydiaceae* putative species 1 [Chlam] | qP         | *Criblamydiaceae* putative species 2 [Chlam] | qP       |
| GE0088       | 98% uncultured bacterium clone F20 [Legio] | P          | 100% *M. phocaicum* [Legio] | C        |
| GE0096       | *Criblamydiaceae* [Chlam] | qP         | 99% *Mycobacterium* sp. [Myco] | C        |
| GE0143       | *Parachlamydiaceae* [Chlam] | qP         | 98% *L. longbeachae* [Legio] | C        |
| GE0148       | *Criblamydiaceae* putative species 1 [Chlam] | C          | *Criblamydiaceae* putative species 2 [Chlam] | qP       |
| GE0150       | 100% *L. waltersii* [Legio] | P          | 100% *L. waltersii* [Legio] | P        |
| GE0159       | *Parachlamydiaceae* putative species 1 [Chlam] | qP         | 99% *M. porcina* [Myco] | P        |
| GE0160       | *Parachlamydiaceae* putative species 2 [Chlam] | qP         | 100% *H. vermiformis* [Myco] | P        |
| GE0170       | *Parachlamydiaceae* [Chlam] | qP         | 100% *H. vermiformis* [Myco] | P        |
| GE0174       | 100% *L. pneumophila* [Legio] | C          | 100% *H. vermiformis* [Myco] | P+P+C    |
| GE0175       | 98% *L. taurinensis* [Legio] | C          | 99% *L. waltersii* [Myco] | P        |
| GE0177       | 99% *H. vermiformis* [Myco] | P          | 99% *uncultured eukaryote clone TKR07M.106 [Amoeba] | C        |
| GE0194       | *Chlamydiaceae* (failed sequencing) [Chlam] | qP         | 99% *L. waltersii* [Myco] | P        |
| GE0195       | 94% *M. neoaurum* [Myco] | P          | 99% *uncultured eukaryote clone CRIB 68.106 [Amoeba] | C        |
| GE0196       | 100% *H. vermiformis* [Myco] | P          | 100% *H. vermiformis* [Myco] | P+P+C    |
| GE0197       | 97% *M. tuscarii* [Legio] | C          | 100% *H. vermiformis* [Myco] | P        |
| GE0198       | 99% *M. porcina* [Myco] | P          | 99% *uncultured eukaryote clone TKR07M.106 [Amoeba] | C        |
| GE0199       | 99% *H. vermiformis* [Myco] | P          | 99% *uncultured eukaryote clone TKR07M.106 [Amoeba] | C        |
| GE0200       | 99% *M. phocaicum* [Legio] | P          | 99% *uncultured eukaryote clone CRIB 68.106 [Amoeba] | C        |
| GE0201       | *Criblamydiaceae* putative species 1 [Chlam] | qP         | *Criblamydiaceae* putative species 2 [Chlam] | qP       |
| GE0202       | *Criblamydiaceae* putative species 2 [Chlam] | qP         | 98% *uncultured bacterium clone ncd843d07c1 [Legio] | C        |
| GE0203       | *Criblamydiaceae* putative species 3 [Chlam] | qP         | 99% *H. vermiformis* [Legio] | P        |
| HE2002       | *Parachlamydiaceae* species 1 [Chlam] | qP         | 99% *M. chelonae* sp. [Myco] | P        |
| HE2003       | 100% *M. phocaicum* [Legio] | C          | 100% *L. pneumophila* [Legio] | C        |
| HE2004       | *Criblamydiaceae* putative species 2 [Chlam] | qP         | 99% *M. abscessus* subsp. bolleti 50574 [Myco] | P+P+C    |
| HE2100       | *Parachlamydiaceae* [Chlam] | qP         | *Parachlamydiaceae* putative species 2 [Chlam] | qP       |
| HE2101       | 99% *M. senegalense* [Myco] | P          | *Parachlamydiaceae* putative species 2 [Chlam] | qP       |
| HE2102       | *Parachlamydiaceae* [Chlam] | qP         | *Parachlamydiaceae* putative species 2 [Chlam] | qP       |
| HE2103       | *Parachlamydiaceae* [Chlam] | qP         | *Parachlamydiaceae* putative species 2 [Chlam] | qP       |
| HS0003       | *Criblamydiaceae* [Chlam] | qP         | *Parachlamydiaceae* putative species 2 [Chlam] | qP       |
| HK0004       | *Criblamydiaceae* putative species 1 [Chlam] | qP         | *Parachlamydiaceae* putative species 2 [Chlam] | qP       |
lineages if fully characterized because the sequences exhibit a similarity with a previously reported species below 97% [17]. Figure 2 illustrates the number of bacteria detected in biofilm or water samples, based on the number of 16S rRNA gene copies quantified by the Chlamydiales-specific rtPCR. The majority of the sequences corresponded to members of the Parachlamydiaceae family (n = 30 sequences), which were detected in 20 different water systems. Criblamydiaceae DNAs were also amplified (18 sequences from 14 different domestic water systems) as well as two sequences from the Waddliaceae family and one sequence from the Simkaniaceae family. The highest number of bacteria was detected in biofilms and corresponded to members of the Parachlamydiaceae family (Fig. 2).

**Legionella species**

In total, the presence of Legionella was found in 21 (43.8%) drinking water systems. Legionella was detected by PCR and/or amoebal co-culture (but never as an amoebal endosymbiont of amoebae grown using the amoebal enrichment method). The results are shown in Table 1, and the identification of Legionella species is detailed in Supplementary Table S2. By PCR and/or amoebal co-culture, Legionella was detected in 29 samples (ten biofilms and 19 waters); it corresponded to 15 different species (Table 1 and Supplementary Table S2). The most common species were Legionella waltersii (present in eight water systems) and L. pneumophila (present in three water systems).

**Mycobacterium species**

Using PCR and amoebal methods, 15 (31.3%) domestic water systems were positive for Mycobacterium species such as Mycobacterium gordonae, chelonae or mucogenicum. The results are summarized in Table 1, and complete identification can be found in Supplementary Table S3. Of particular note, two different mycobacteria (M. iranicum strain CCUG52297 and M. phocaicum) were found within the amoeba Hartmannella vermiformis, recovered from water and biofilm samples of the same domestic water system (GE10037).

**Amoebae isolated by amoebal enrichment and/or detected by PCR**

Using both PCR and amoebal enrichment, the presence of amoebae was documented in 18 (37.5%) domestic water systems (Fig. 1). Amoebae were present in water and/or biofilm samples (Fig. 1), with Hartmannella vermiformis being predominantly detected in 16 water systems (Table 1). Two Stenamoeba species were also isolated from two different biofilms, one being a potential new amoebal species. Finally, in a biofilm already positive by PCR for H. vermiformis, an uncultured eukaryote strain related to the Prostelium nocturnum amoeba was isolated by culture (water system GE10174). The complete identification of amoebae per type of sample can be found in Supplementary Table S4.

**Discussion**

In this study, the presence of ARB belonging to the Chlamydiales order as well as to the Legionellaceae and Mycobacteriaceae...
families was investigated using amoebal culture methods and PCR on water and biofilm samples collected from domestic water systems of 48 different households. Overall, 39 (81.3%) of the investigated domestic water systems were positive for the presence of a Chlamydiaceae, a Legionellaceae and/or a Mycobacteriaceae. In 18 (46.2%) of these systems, the bacterium was detected by culture. In the other systems, the bacteria were only detected by PCR.

A Chlamydiaceae-specific rtPCR was used and allowed for the first time to observe such a high prevalence and diversity of Chlamydiaceae in domestic drinking water. The high sensitivity of the rtPCR allowed the detection of a Chlamydiaceae in 35 (72.9%) different domestic water systems, corresponding to members of at least four different family-level lineages of the Chlamydiaceae order. The dominant family-level lineage was the Parachlamydiaceae family. Members of the Parachlamydiaceae family have been frequently isolated from environmental samples [30,32,33]. The high prevalence of strains belonging to this family compared to other Chlamydia-related bacteria was also previously observed when using the same Chlamydiaceae-specific rtPCR on nasopharyngeal swabs taken from children [21]. The second family detected in 14 water systems was the Criblamydiaceae. The presence of Criblamydiaceae species in water and biofilm samples was not surprising because these bacteria have been previously isolated from water and/or sediment samples [33–35]. This result is particularly interesting because serologic evidence indicates that Criblamydiaceae may be associated with cases of pneumonia (Lienard et al., personal communication).

We also detected two members of the Waddliaceae family. To our knowledge, this is the first documentation of Waddliaceae in drinking water systems. Although the bacterium Waddlia chondrophila was previously associated with human and bovine hosts [36–39], its potential presence in water was suggested by its ability to also grow and survive in amoebae [40,41]. Only one sequence corresponding to the Simkaniaceae family was detected, which did not correspond to the species Simkania negevensis. This result contrasts with a previous work where S. negevensis was detected by PCR in the majority of tap water samples [42]. However, this latter study was performed

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**FIG. 3.** Protocol of collection and processing of water and biofilm samples. Samples were collected from distal water conduit after removal of shower head. Cold water was concentrated 100× by filtration, and biofilms swabs were resuspended in 3 mL of collected shower water. Samples were then directly inoculated in culture, or DNA was extracted for direct PCR approaches. After amoebal co-culture or enrichment methods, total DNA was extracted from culture well and PCR performed. For positive results, bacterial or amoebal strains were identified by sequencing.

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in Israel, where the microbial ecology and drinking water treatment processes may be different from those in Switzerland.

Among the 55 sequences of Chlamydiales bacteria obtained in this work, only two corresponded to bacteria currently grown in our laboratory, which indicated that the sequences obtained here did not result from a PCR contamination. Overall, 28 different sequences showed less than 97% similarity with a previously reported species. Considering this 97% cutoff [17, 29], these latter 28 sequences may correspond to putative new species, highlighting the broad and underestimated biodiversity of the Chlamydiales order [21]. This report suggests that man-made drinking water could represent an important ecological niche for Chlamydiales bacteria.

No Chlamydiales bacterium was recovered by amoebal co-culture in this study. Another study on drinking water failed to detect any Chlamydiales, either by amoebal co-culture with A. castellanii or by classical PCR [43]. Kahane et al. [42] detected Simkania negevensis in tap water but only by PCR and membrane immunoassay. In the present work, the Chlamydiales-specific quantitative PCR, which is more sensitive than regular PCR, revealed the common occurrence of Chlamydiales DNA in domestic drinking water systems. The growth of Chlamydiales bacteria from environmental samples could have been restricted here by the overgrowth of other environmental bacteria within the co-cultures in A. castellanii. Furthermore, in some cases, Chlamydiales bacteria were probably initially dead or not cultivable. The amoebal co-culture using A. castellanii was previously shown to be effective to recover Chlamydiales, including Criblamydiaceae and Parachlamydiaceae [33, 34], but is clearly inadequate to grow all Chlamydiales. Indeed, considering the large biodiversity of the Chlamydiales order highlighted in the present study, only a few members have been isolated by amoebal co-culture [27, 30, 33, 34, 44]. In addition, a restricted amoebal host spectrum has already been shown for several Chlamydiales bacteria [20, 40, 45, 46], which suggests that multiple amoebal strains should ideally be used to recover a higher biodiversity of these strictly intracellular bacteria in culture. In this work, an Acanthamoeba species was used, which is more suitable for the amoebal co-culture method, as it is less prone to encystment compared to Hartmannella spp. Furthermore, Acanthamoeba spp. are known to be permissive to a large number of bacteria [7, 8, 43, 44, 47, 48]. Thus, other amoebae such as Hartmannella and Naoegleria should also be included in future studies. Finally, several growth parameters such as temperature and media can also be optimized to increase the number of recovered ARB.

Legionella waltersi, which was previously associated with severe pneumonia [49], was the most prevalent species, followed by L. pneumophila, among all Legionella found in this study. In addition, Legionella species considered as potential respiratory pathogens such as L. anisa [50–52], L. longbeachae [51, 53, 54] or L. fallonii [50] were also recovered. In all water systems positive for L. pneumophila, the amoeba H. vermiformis was systematically isolated by amoebal enrichment, supporting the importance of this amoeba as a reservoir for L. pneumophila.

In addition, various nontuberculous mycobacteria have been recovered using amoebal co-culture and amoebal enrichment, including several human pathogens, such as M. mucogenicum [55] and M. chelonae, which have mainly been shown to cause respiratory [56, 57] and soft tissue [58] infection. M. gordonae, which is also sometimes considered pathogenic [59–63], has been previously isolated from drinking water [43, 64] and was isolated in our study from water and biofilm samples. Other nontuberculous mycobacteria were also recovered in the present work, including M. conceptionense [65–68], M. barrassiae [69] and M. neoaurum [70–72]. Finally, one of the two mycobacteria recovered within the amoeba H. vermiformis was M. iranicum. This species was recently described as a new human pathogen; it was isolated from clinical samples such as cerebrospinal fluid and sputum samples from patients from different continents [73, 74]. However, the source of infection has not been determined for these previously reported cases; drinking water should thus be considered.

Using amoebal enrichment and PCR, amoebae were documented in 18 systems (37.5%). Although the number of recovered amoebae is particularly variable between studies [75], the number of amoebae cultivated in this study (n = 15) is higher compared to a previous study using the same culture method [43]. However, the difference of water temperatures between the present and the previous study, with mean temperatures of 20.6°C and 56°C, respectively, may explain these results. Most of the amoebae isolated in this work corresponded to H. vermiformis, which is congruent with a previous investigation of drinking water by amoebal enrichment [43].

In conclusion, the current study highlighted the large colonization of drinking water points of use by ARB and amoebae. This work also demonstrated the common occurrence and large biodiversity of Chlamydiales bacteria in drinking water. Thus, drinking water represents a potential infection source for some Chlamydia-related bacteria. Because Parachlamydia aca

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.nmni.2016.10.003.

Conflict of Interest

None declared.

References

[1] Coulon C, Collignon A, McDonnell G, Thomas V. Resistance of Acanthamoeba cysts to disinfection treatments used in health care settings. J Clin Microbiol 2010;48:2689–97.
[2] Lloyd D, Turner NA, Khunkitti W, Hann AC, Furr JR, Russell AD. Encystation in Acanthamoeba castellanii: development of biocide resistance. J Eukaryot Microbiol 2001;48:1–6.
[3] Mogoa E, Bodet C, Morel F, Rodier MH, Legube B, Hechard Y. Cellular response of the amoeba Acanthamoeba castellanii to chlorine, chlorine dioxide, and monochloramine treatments. Appl Environ Microbiol 2011;77:4974–80.
[4] Lorent JF, Greub G. Free-living amoebae: biological by-passes in water treatment. Int J Hyg Environ Health 2010;213:167–75.
[5] Greub G, Raoult D. Microorganisms resistant to free-living amoebae. Clin Microbiol Rev 2004;17:413–33.
[6] Kilvington S, Price J. Survival of Legionella pneumophila within cysts of Acanthamoeba polyphaga following chlorine exposure. J Appl Bacteriol 1990;68:519–25.
[7] Kahane S, Dvoskin B, Mathias M, Friedman MG. Infection of Acanthamoeba polyphaga with Simkania negevensis and S. negevensis survival within amoebal cysts. Appl Environ Microbiol 2001;67:4789–95.
[8] Steinetz M, Burke K, White E, Fields B, Quinn F. Mycobacterium avium bacilli grow saprozoically in coculture with Acanthamoeba polyphaga and survive within cyst walls. Appl Environ Microbiol 1998;64:2256–61.
[9] Cirillo JD, Falkow S, Tompkins LS. Growth of Legionella pneumophila in Acanthamoeba castellanii enhances invasion. Infect Immun 1999;64:3254–61.
[10] Cirillo JD, Falkow S, Tompkins LS, Bermudez LE. Interaction of Mycobacterium avium with environmental amoeba enhances virulence. Infect Immun 1997;65:3759–67.
[11] Molmeret M, Horn M, Wagner M, Santic M, Abu Kwaik Y. Amoebae as training grounds for intracellular bacterial pathogens. Appl Environ Microbiol 2005;71:20–8.
[12] Salah IB, Ghigo E, Drancourt M. Free-living amoebae, a training field for macrophage resistance of mycobacteria. Clin Microbiol Infect 2009;15:894–905.
[13] Guy G, Croxatto A, Greub G. Wadidia chondrophila enters and multiplies within human macrophages. Microbes Infect 2008;10:556–62.
[14] Horwitz MA, Silverstein SC. Legionnaires’ disease bacterium (Legionella pneumophila) multiplies intracellularly in human monocytes. J Clin Invest 1980;66:441–50.
[15] Fraser DW, Tsai TR, Orenstein W, Parkin WE, Beecham HJ, Sharrar RG, et al. Legionnaires’ disease: description of an epidemic of pneumonia. N Engl J Med 1977;297:1189–97.
[16] Breiman RF, Fields BS, Sanden GN, Volmer L, Meier A, Spika JS. Association of shower use with Legionnaires’ disease. Possible role of amoebae. JAMA 1990;263:2924–6.
[17] Greub G. Parachlamydia acanthamoebae, an emerging agent of pneumonia. Clin Microbiol Infect 2009;15:18–28.
[18] Friedman MG, Dvoskin B, Kahane S. Infections with the Chlamydia-like microorganism Simkania negevensis, a possible emerging pathogen. Microbes Infect 2003;5:1013–21.
[19] Greub G, La Scolla B, Raoult D. Parachlamydia acanthamoebae is endosymbiotic or lytic for Acanthamoeba polyphaga depending on the incubation temperature. Ann N Y Acad Sci 2003;990:628–34.
[20] Hayashi Y, Nakamura S, Matsuo J, Fukumoto T, Yoshida M, Takahashi K, et al. Host range of obligate intracellular bacterium Parachlamydia acanthamoebae. Microbiol Immunol 2010;54:707–13.
[21] Lienard J, Croxatto A, Aeby S, Jaton K, Posfay-Barbe K, Gervaix A, et al. Development of a new Chlamydioides-specific real-time PCR and its application to respiratory clinical samples. J Clin Microbiol 2011;49:2637–42.
[22] Greub G, Raoult D. Crescent bodies of Parachlamydia acanthamoebae and its life cycle within Acanthamoeba polyphaga: an electron micrograph study. Appl Environ Microbiol 2002;68:3076–84.
[23] Rowbotham TJ. Isolation of Legionella pneumophila from clinical specimens via amoebae, and the interaction of those and other isolates with amoebae. J Clin Pathol 1983;36:976–86.
[24] Lienard J, Greub G. Discovering new pathogens: amoebae as tools to isolate amoeba-resistant microorganisms from environmental samples. In: Sen K, Ashbolt NJ, editors. Environmental microbiology: current technology and water applications. Norfolk, UK: Caister Academic Press; 2011. p. 143–62.
[25] Miyamoto H, Yamamoto H, Arima K, Fujii J, Maruta K, Izu K, et al. Development of a new seminested PCR method for detection of Legionella species and its application to surveillance of legionellosis in hospital cooling tower water. Appl Environ Microbiol 1997;63:2489–94.
[26] Kirschner P, Springer B, Vogel U, Meier A, Wrede A, Kiekenbeck M, et al. Genotypic identification of mycobacteria by nucleic acid sequence determination: report of a 2-year experience in a clinical laboratory. J Clin Microbiol 1993;31:2882–9.
[27] Lienard J, Croxatto A, Prod’hom G, Greub G. Estrella lasiannensis, a new star in the Chlamydioides order. Microbes Infect 2011;13:1232–41.
[28] Ben Salah I, Adelkambi T, Raoult D, Drancourt M. rpoB sequence-based identification of Mycobacterium avium complex species. Microbiology 2008;154(Pt. 12):3715–23.
[29] Everett KD, Bush RM, Andersen AA. Emended description of the order Chlamydioides, proposal of Parachlamydiaceae fam. nov. and Simkanioceae fam. nov., each containing one monotypic genus, revised taxonomy of the family Chlamydiaceae, including a new genus and five new species, and standards for the identification of organisms. Int J Syst Bacteriol 1999;49:415–40.
[30] Birdles RJ, Rowbotham TJ, Storey C, Marrie TJ, Raoult D. Chlamydioides-like obligate parasite of free-living amoebae. Lancet 1997;349(9056):925–6.
[32] Fritsche TR, Gautam RK, Seyedi-Rashti S, Bergeron DL, Lindquist TD. Occurrence of bacterial endosymbionts in Acanthamoeba spp. isolated from cornal and environmental specimens and contact lenses. J Clin Microbiol 1993;31:1122–6.

[33] Corsaro D, Feroldi V, Saucedo G, Ribas F, Loret JF, Greub G. Novel Chlamydiales strains isolated from a water treatment plant. Environ Microbiol 2009;11:188–200.

[34] Thomas V, Casson N, Greub G. Criblamydia sequanensis, a new intra-cellular Chlamydiales isolated from Seine river water using amoebal co-culture. Environ Microbiol 2006;8:2125–35.

[35] Corsaro D, Venditti D. Detection of Chlamydiae from freshwater environments by PCR, amoeba co-culture and mixed co-culture. Res Microbiol 2009;160:547–52.

[36] Dilbeck PM, Evermann JF, Crawford TB, Ward AC, Leathers CW, Corsaro D, Venditti D. Detection of Legionella spp. in drinking water and in clinical samples. Zentralbl Hyg Umweltmed 1993;192:154–6.

[37] Rurangirwa FR, Dilbeck PM, Crawford TB, McGuire TC, ElMwaiin TF. Analysis of the 16S rRNA gene of micro-organism WSU 86-1044 from an aboroned bovine foetus reveals that it is a member of the order Chlamydiales: proposal of Waddlaecae fam. nov., Waddla chondrophilia gen. nov., sp. nov. Int J Syst Bacteriol 1999;49:577–81.

[38] Haider S, Collingro A, Walochnik J, Wagner M, Horn M. Chlamydia-like bacteria in respiratory samples of community-acquired pneumonia patients. FEMS Microbiol Lett 2008;281:198–202.

[39] Goy G, Croxatto A, Posfay-Barbe KM, Gervaix A, Greub G. Development of a real-time PCR for the specific detection of Waddlia chondrophila in clinical samples. Eur J Clin Microbiol Infect Dis 2009;28:483–6.

[40] Michel R, Steiner M, Zoller L, Hauröder B, Henning K. Free-living amoebae may serve as hosts for the Chlamydia-like bacterium Waddlia chondrophila isolated from an aboroned bovine foetus. Acta Protozool 2006;43:37–42.

[41] Goy G, Greub G. Diagnostic susceptibility of Waddlia chondrophila in Acanthamoeba castellani amoebae. Antimicrob Agents Chemother 2009;53:2663–6.

[42] Kahane S, Platner N, Dvoskin B, Itzhaki A, Friedman MG. Evidence for the presence of Simkania negevensis in drinking water and in reclaimed wastewater in Israel. Appl Environ Microbiol 2004;70:3346–51.

[43] Thomas V, Herrera-Rimann K, Blanc DS, Greub G. Biodiversity of amoebae and amoeba-resisting bacteria in a hospital water network. Appi Environ Microbiol 2006;72:2428–38.

[44] Collinge AJ, Popper S, Heinz E, Schmitz-Esser E, Euzig A, Schweikert M, et al. Recovery of an environmental Chlamydia strain from activated sludge by co-cultivation with Acanthamoeba sp. Microbiology 2005;151(Pt 1):301–9.

[45] Michel R, Müller KD, Zoller L, Walochnik J, Hartmann M, Schmid EN. Free-living amoebae serve as a host for the Chlamydia-like bacterium Simkania negevensis. Acta Protozoologica 2005;44:113–21.

[46] Coulon C, Eterpi M, Greub G, Collingro A, McDonnell G, Thomas V. Ameoba host range, host-free survival and disinfection susceptibility of environmental Chlamydiae as compared to Chlamydia trachomatis. FEMS Immunol Med Microbiol 2012;64:364–73.

[47] Thomas V, Loret JF, Jousset M, Greub G. Biodiversity of amoebae and amoeba-resisting bacteria in a drinking water treatment plant. Environ Microbiol 2008;10:2728–45.

[48] Corsaro D, Pagès GS, Catalan V, Loret JF, Greub G. Biodiversity of amoebae and amoeba-associated bacteria in water treatment plants. Int J Hyg Environ Health 2010;213:158–66.

[49] Konig C, Hebestreit H, Valenza G, Abele-Horn M, Speer CP. Legionella waltersii—a novel cause of pneumonia? Acta Paediatr 2005;94:1505–7.

[50] McNally C, Hackman B, Fields BS, Plouffe JF. Potential importance of Legionella species as etiologies in community acquired pneumonia (CAP). Diagn Microbiol Infect Dis 2000;38:79–82.

[51] Yu VL, Plouffe JF, Pastoris MC, Stout JE, Schousboe M, Widmer A, et al. Distribution of Legionella species and serogroups isolated by culture in patients with sporadic community-acquired legionellosis: an international collaborative survey. J Infect Dis 2002;186:127–8.

[52] Fenstersheib MD, Miller M, Diggins C, Liska S, Deswiler L, Werner SB, et al. Outbreak of Pontiac fever due to Legionella anisa. Lancet 1990;336(8706):35–7.

[53] Grove DI, Lawson PJ, Burgess JS, Moran JL, O’Fahrataigh MS, Winslow WE. An outbreak of Legionella longbeachae infection in an intensive care unit. J Hosp Infect 2002;52:250–8.

[54] Phares CR, Wangoongsarb P, Chantra S, Paveenkitporn W, Tondella ML, Benson RF, et al. Epidemiology of severe pneumonia caused by Legionella longbeachae, Mycoplasma pneumoniae, and Chlamydia pneumoniae: 1-year, population-based surveillance for severe pneumonia in Thailand. Clin Infect Dis 2007;45:e147–55.

[55] Adebambi T. Mycobacterium mucogenicum group infections: a review. Clin Microbiol Infect 2009;15:911–8.

[56] Goto T, Hamaguchi R, Masahima A, Oyamada Y, Kato R. Pulmonary resection for Mycobacterium chelonae infection. Ann Thorac Cardiovasc Surg 2012;18:128–31.

[57] Singh N, Yu VL. Successful treatment of pulmonary infection due to Mycobacterium chelonae: case report and review. Clin Infect Dis 1992;14:156–61.

[58] Mullin D, Jothi S, Healy D. Mycobacterium chelonae infections involving the head and neck. Ann Otol Rhinol Laryngol 2009;118:714–20.

[59] Weinberger M, Berg SL, Feuerstein IM, Pizzo PA, Witebsky FG. Disseminated infection with Mycobacterium gordonae: report of a case and critical review of the literature. Clin Infect Dis 1992;14:1229–39.

[60] Jankrik LN. Case report: disseminated Mycobacterium gordonae infection in a nonimmunocompromised host. Am J Med Sci 1991;302:382–4.

[61] Asija A, Prasad A, Eskridge E. Disseminated Mycobacterium gordonae infection in an immunocompromised host. Am J Ther 2011;18:e75–7.

[62] Pinho L, Santos J, Oliveira G, Pestana M. Mycobacterium gordonae urinary infection in a renal transplant recipient. Transpl Infect Dis 2009;11:253–6.

[63] den Broeder AA, Vervoort G, van Assen S, Verduyn Lunel F, de Lange WC, de Sevaux RG. Disseminated Mycobacterium gordonae infection in a renal transplant recipient. Transpl Infect Dis 2003;5:151–5.

[64] Fischeder R, Schulze-Robbecke R, Weber A. Occurrence of mycobacteria in drinking water samples. Zentralbl Hyg Umweltmed 1991;119:154–8.

[65] Adebambi T, Stein A, Carvalj J, Rasoul D, Drancourt M. Description of Mycobacterium conceptense sp. nov., a Mycobacterium fortuitum group organism isolated from a posttraumatic osteitis infection. J Clin Microbiol 2006;44:1268–73.

[66] Shoijaei H, Hashemi A, Heidarih P, Ataei B, Naser AD. Pulmonary and extrapulmonary infection caused by Mycobacterium conceptense: the first report from Iran. JRSM Short Rep 2011;2:31.

[67] Thibeaute S, Levy PY, Pelletier ML, Drancourt M. Mycobacterium conceptense infection after breast implant surgery, France. Emerg Infect Dis 2010;16:1180–1.

[68] Liao CH, Lai CC, Huang YT, Chou CH, Hsu HL, Hsueh PR. Subcutaneous abscess caused by Mycobacterium conceptense in an immunocompetent patient. J Infect 2009;58:308–9.

[69] Adebambi T, Rasoul D, Drancourt M, Mycobacterium harrassiae sp. nov., a Mycobacterium marisicola group species associated with chronic pneumonia. J Clin Microbiol 2006;44:3493–8.

[70] Moriimoto Y, Chan ED, Heifets L, Routes JM. Pulmonary infection with Mycobacterium neoaurum identified by 16S ribosomal DNA sequence. J Infect 2007;54:e227–31.

[71] Zanetti S, Faedda R, Fadda G, Dupre I, Molinetti P, Ortu S, et al. Isolation and identification of Mycobacterium neoaurum from a patient with urinary infection. New Microbiol 2001;24:189–92.
[72] Washer LL, Riddell Jr, Rider J, Chenoweth CE. Mycobacterium neoaurum bloodstream infection: report of 4 cases and review of the literature. Clin Infect Dis 2007;45:e10–3.

[73] Shojaei H, Daley C, Gitti Z, Hashemi A, Heidarieh P, Moore ER, et al. Mycobacterium iranicum sp. nov., a rapidly growing scotochromogenic species isolated from clinical specimens on three different continents. Int J Syst Evol Microbiol 2013;63(Pt 4):1383–9.

[74] Balakrishnan N, Tortoli E, Engel SL, Breitschwerdt EB. Isolation of a novel strain of Mycobacterium iranicum from a woman in the United States. J Clin Microbiol 2013;51:705–7.

[75] Thomas JM, Ashbolt NJ. Do free-living amoebae in treated drinking water systems present an emerging health risk? Environ Sci Technol 2011;45:860–9.