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ORIGINAL ARTICLE

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

J. Lienard[1], A. Croxatto[1], A. Gervaix[3], Y. Lévi[4], J.-F. Loret[5], K. M. Posfay-Barbe[3] and G. Greub[1,2]

1) *Center for Research on Intracellular Bacteria, Institute of Microbiology, Lausanne University Hospital and University of Lausanne,* 2) *Infectious Diseases Service, Lausanne University Hospital, Lausanne, Switzerland,* 3) *Children's Hospital of Geneva, University Hospitals of Geneva and Medical School of the University of Geneva, Geneva, Switzerland,* 4) *University of Paris-Sud XI, Faculty of Pharmacy, Paris and* 5) *Suez Environnement CIRSEE, Le Pecq, France*

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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Corresponding author: G. Greub, MD, PhD, Center for Research on Intracellular Bacteria (CRIB), Institute of Microbiology, Lausanne University Hospital and University of Lausanne, 1011 Lausanne, Switzerland
E-mail: gilbert.greub@chuv.ch

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

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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, biofilms 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 CO_2, in 75 cm^2 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 (Milian, Wohlen, Switzerland) at 5 × 10^5 amoebal 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 CO_2. 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 amoebae-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 I (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. (TB285F/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/

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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

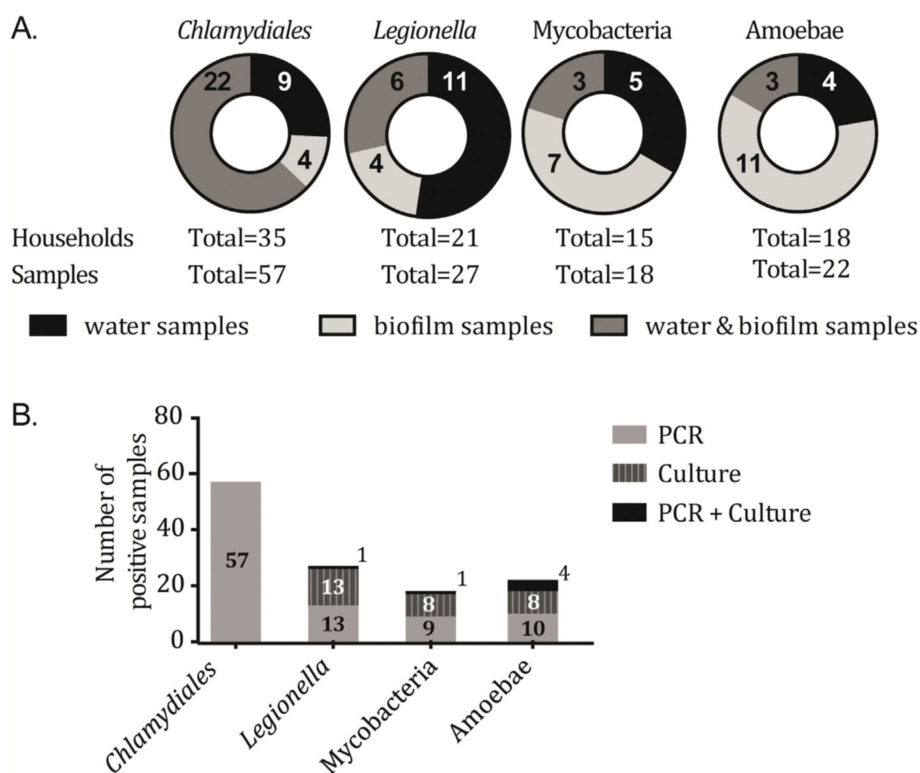

FIG. 1. Distribution of type of samples and detection methods for each bacterial groups and amoebae detected. (A) Parts of whole representing number of positive households for *Chlamydiales, Legionella, Mycobacterium* or amoeba, detected in water, biofilm or both in water and biofilm samples. Corresponding number of positive samples is also indicated. (B) Distribution of detection methods among positive samples (water and biofilm) by PCR only, culture only or both PCR and culture.

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TABLE 1. Summary of all *Chlamydiales* spp, *Legionella* spp., *Mycobacterium* spp. and amoebae detected in each water system of 48 households investigated

	Water		Biofilm	
Household ID	**Species**	**Detection**	**Species**	**Detection**
GE10016	*Criblamydiaceae* putative species 1 [Chlam]	qP	*Criblamydiaceae* putative species 2 [Chlam]	qP
	100% *L. waltersii* [Legio]	P		
GE10027	*Criblamydiaceae* [Chlam]	qP		
	Parachlamydiaceae [Chlam]	qP		
GE10028	*Parachlamydiaceae* [Chlam]	qP	*Parachlamydiaceae* [Chlam]	qP
	99% uncultured bacterium clone ncd843d07c1 [Legio]	C	95% *M. moriokaense* or *M. barrassiae*[a] [Myco]	P
GE10032	Unclassified *Chlamydiales* [Chlam]	qP		
GE10037	100% *Mycobacterium iranicum strain CCUG 52297*[a] [Myco]	C	100% *M. gilvum* [Myco]	C
	99% *H. vermiformis* [Amoeba]	C	100% *M. phocaicum* strain MBWY-1[b] [Myco]	C
GE10044	*Criblamydiaceae* [Chlam]	qP		
	100% *L. anisa* [Legio]	C		
GE10049			*Parachlamydiaceae* [Chlam]	qP
			98% uncultured *Legionella* sp. [Legio]	C
GE10056	*Chlamydiales* (failed sequencing)	qP	*Parachlamydiaceae* [Chlam]	qP
GE10061	*Criblamydiaceae* [Chlam]	qP	97% *M. tusciae*[a] [Myco]	P
	100% *Mycobacterium* sp. FI-10135[a] [Myco]	C	100% *H. vermiformis*	P+C
	97% *M. tusciae*[a] [Myco]	P		
GE10062			*Parachlamydiaceae* [Chlam]	qP
			100% *M. gilvum* [Myco]	P
GE10064	Unclassified *Chlamydiales* [Chlam]	qP	Unclassified *Chlamydiales* [Chlam]	qP
	100% *L. taurinensis* [Legio]	C	100% *L. taurinensis* [Legio]	C
	99% *H. vermiformis* [Amoeba]	P+C	99% *H. vermiformis* [Amoeba]	P
GE10068	*Criblamydiaceae* putative species 1 [Chlam]	qP	*Criblamydiaceae* putative species 2 [Chlam]	qP
	98% uncultured bacterium clone F20 [Legio]	P		
GE10088	*Criblamydiaceae* [Chlam]	qP	Unclassified *Chlamydiales* [Chlam]	qP
GE10096			*Parachlamydiaceae* [Chlam]	qP
			99% *Mycobacterium* sp. [Myco]	C
GE10143	*Parachlamydiaceae* [Chlam]	qP		
	Criblamydiaceae [Chlam]	qP		
	98% *L. longbeachae* [Legio]	C		
GE10148	*Criblamydiaceae* putative species 1 [Chlam]	qP	*Criblamydiaceae* putative species 2 [Chlam]	qP
	100% *L. waltersii* [Legio]	P		
GE10150	*Parachlamydiaceae* putative species 1 [Chlam]	qP	100% *L. waltersii* [Legio]	P
	Parachlamydiaceae putative species 2 [Chlam]	qP		
	99% *M. gordonae*[a] [Myco]	P		
GE10159	*Parachlamydiaceae* [Chlam]	qP	*Parachlamydiaceae* [Chlam]	qP
	100% *H. vermiformis* [Amoeba]	P	100% *H. vermiformis* [Amoeba]	P
GE10160	100% uncultured *Legionella* sp. [Legio]	P	99% *H. vermiformis* [Amoeba]	P
GE10170	*Parachlamydiaceae* [Chlam]	qP	*Parachlamydiaceae* [Chlam]	q P
	100% *L. pneumophila* [Legio]	C	100% *H. vermiformis* [Amoeba]	P+C
	97% *M. tusciae*[a] [Myco]	C		
GE10174	Failed sequencing [Chlam]	qP	*Parachlamydiaceae* [Chlam]	qP
	100% *L. waltersii* [Legio]	C	99% *L. waltersii* [Legio]	P
			99% uncultured eukaryote clone TKR07M.106 [Amoeba]	C
			100% *H. vermiformis* [Amoeba]	P
GE10175	98% *L. gratiana* [Legio]	C	99% *L. beliardensis* [Legio]	P
			97% *M. tusciae*[a] [Myco]	P+C
			100% *H. vermiformis* [Amoeba]	P
GE10179	99% *H. vermiformis* [Amoeba]	P		
GE11050	*Chlamydiales* (failed sequencing) [Chlam]	qP		
	94% *M. neoaurum*[a] [Myco]	P		
	100% *H. vermiformis* [Amoeba]	P+C		
GE11064	*Criblamydiaceae* [Chlam]	qP		
	98% uncultured bacterium clone 1C227246 [Legio]	P		
GE11093	*Parachlamydiaceae* [Chlam]	qP	*Chlamydiales* (failed sequencing) [Chlam]	qP
	98% *L. fallonii* strain LLAP10 [Legio]	P	99% *Stenamoeba* CRIB 68 [Amoeba]	P+C
	99% *M. abscessus* subsp. *bolletii* 50594 [Myco]	C		
GE11103	*Parachlamydiaceae* putative species 1 [Chlam]	qP	*Parachlamydiaceae* putative species 1 [Chlam]	qP
	100% *L. pneumophila* [Legio]	C	*Parachlamydiaceae* putative species 2 [Chlam]	qP
	98% uncultured bacterium clone nbu179b03c1 [Legio]	P		
	99% *H. vermiformis* [Amoeba]	P+C		
GE11112			100% *L. waltersii* [Legio]	P
			100% *M. chelonae*[a] [Myco]	P
HE20032	*Parachlamydiaceae* species 1 [Chlam]	qP	*Parachlamydiaceae* species 2 [Chlam]	qP
	100% *L. pneumophila* [Legio]	C	100% *L. pneumophila* [Legio]	C
	99% *Mycobacterium* sp. [Myco]	C	100% *H. vermiformis* [Amoeba]	P+C
HE20036	*Chlamydiales* (failed sequencing) [Chlam]	qP	*Criblamydiaceae* [Chlam]	qP
	99% *M. senegalense* strain MF-417 or *M. conceptionense* strain PCH-033[a] [Myco]	C	100% *L. waltersii* [Legio]	P
	100% *H. vermiformis* [Amoeba]	C	100% *H. vermiformis* [Amoeba]	P+C
HE21001	*Parachlamydiaceae* [Chlam]	qP	*Waddliaceae* [Chlam]	qP
HE21011	*Criblamydiaceae* [Chlam]	qP	*Criblamydiaceae* [Chlam]	qP
HE21012	*Parachlamydiaceae* [Chlam]	qP	*Criblamydiaceae* [Chlam]	qP
	Waddliaceae [Chlam]	qP	88% *Stenamoeba amazonica* strain P119 [Amoeba]	C
HE21023	*Criblamydiaceae* [Chlam]	qP	*Simkaniaceae* [Chlam]	qP
HE21032	*Parachlamydiaceae* [Chlam]	qP	*Parachlamydiaceae* [Chlam]	qP
VS30003	100% *L. waltersii* [Legio]	C	*Chlamydiales* (failed sequencing) [Chlam]	qP
VS30013	*Parachlamydiaceae* putative species 1 [Chlam]	qP	*Parachlamydiaceae* putative species 2 [Chlam]	qP
			100% *Mycobacterium* sp. [Myco]	C
VS30044	*Criblamydiaceae* [Chlam]	qP		
VS30055	*Parachlamydiaceae* putative species 1 [Chlam]	qP	*Parachlamydiaceae* putative species 2 [Chlam]	qP

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TABLE 1. Continued

Household ID	Water Species	Water Detection	Biofilm Species	Biofilm Detection
VS31006	*Parachlamydiaceae* [Chlam]	qP	100% *H. vermiformis* [Amoeba] *Chlamydiales* (failed sequencing) [Chlam]	C qP

Percentages of sequence identity with most similar GenBank sequence (for *legionella*, mycobacteria or amoebae) or classification at the family or family-level lineage (for *Chlamydiales*) are indicated.
C, culture; P, PCR; qP, quantitative real-time PCR; [Chlam], *Chlamydiales;* [Legio], *Legionella;* [Myco], *Mycobacterium*.
[a]Species further identified by sequencing *rpoB* gene.
[b]Species found within amoeba by nonnutrient agar screening.

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 of 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).

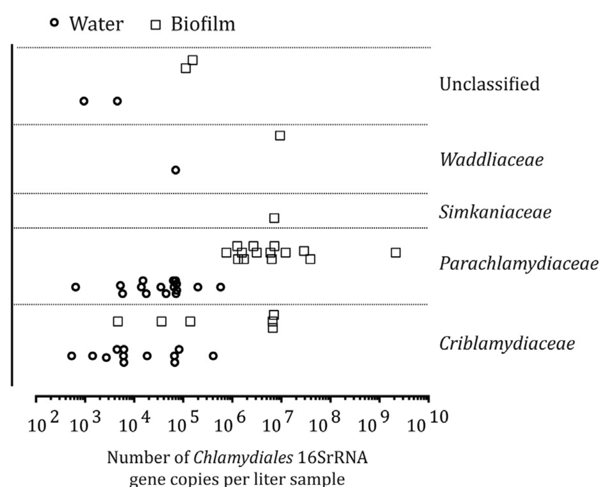

FIG. 2. *Chlamydiales* 16S ribosomal RNA gene copy number detected by *Chlamydiales*-specific real-time PCR in water and biofilm samples. Each symbol represents sample positive for *Chlamydiales* detected by specific quantitative PCR and its corresponding gene copy number expressed per litre of sample.

Legionella species

In total, the presence of *Legionella* was found in 21 (43.8%) drinking water systems. *Legionella* was detected by PCR and/or by 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*

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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 *Chlamydiales*, 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 *Chlamydiales*-specific rtPCR was used and allowed for the first time to observe such a high prevalence and diversity of *Chlamydiales* in domestic drinking water. The high sensitivity of the rtPCR allowed the detection of a *Chlamydiales* in 35 (72.9%) different domestic water systems, corresponding to members of at least four different family-level lineages of the *Chlamydiales* 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 *Chlamydiales*-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

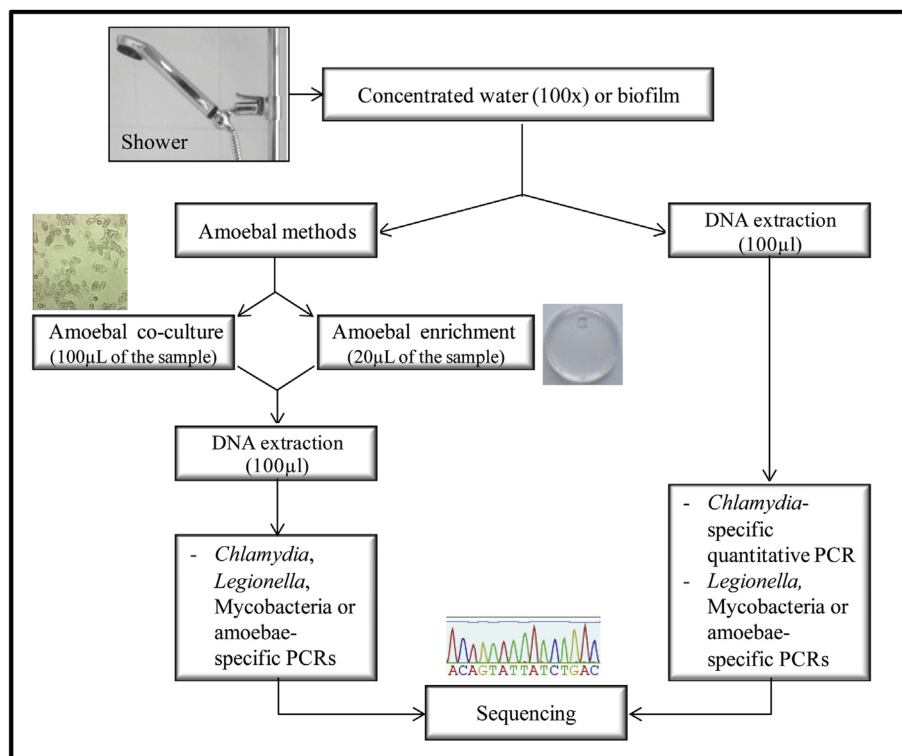

FIG. 3. Protocol of collection and processing of water and biofilm samples. Samples were collected from distal water conduit after removal of shower head. Cold water was concentrated 100× by filtration, and biofilms swabs were resuspended in 3 mL of collected shower water. Samples were then directly 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 *Naegleria* 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 waltersii, 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 acanthamoebae* is associated with respiratory infections [17], the common occurrence of *Parachlamydiaceae* observed here is important in terms of public health. Larger prospective studies including different settings are needed to better investigate the role of domestic water systems or other systems generating aerosols, such as cooling towers, in the transmission of *Chlamydiales* to humans and other susceptible hosts.

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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.

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