Limited presence of *Waddlia chondrophila* in drinking water systems in the Netherlands

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Abstract

Waddlia chondrophila is an emerging pathogen belonging to the order of *Chlamydiales*. This obligate intracellular bacterium was initially isolated from an aborted bovine fetus and is associated with adverse pregnancy outcomes in women. The ability of *W. chondrophila* to reside and replicate within a range of free-living amoebae implies a possible widespread environmental presence. Potential hosts of *W. chondrophila* are present in Dutch drinking water. This study therefore investigated the presence of *W. chondrophila* DNA in drinking water by analysing 59 samples from ten drinking water systems throughout the Netherlands. Samples were taken at three distances from the treatment plant, during both summer and winter. Twelve of the samples were positive, originating from two of the treatment plants, of which three samples were quantifiable.

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Introduction

Waddlia chondrophila is an emerging pathogen belonging to the order of *Chlamydiales*. It is an obligate intracellular bacterium that was initially isolated from an aborted bovine fetus [1]. Later, a second case of *W. chondrophila* was found in a septic stillborn calf [2]. *Waddlia chondrophila* is a zoonotic bacterium and has been associated with bovine abortion [3], as well as adverse pregnancy outcomes and infertility problems in women, such as tubal factor infertility [4–6]. Furthermore, *W. chondrophila* has been detected in samples from children with respiratory infections and in individuals with community-acquired pneumonia [7,8]. The ability of *W. chondrophila* to induce respiratory infections was demonstrated in an experimental animal model [9].

Unlike the well-known *Chlamydia trachomatis*, which mainly spreads through sexual contact, the transmission routes of *W. chondrophila* have not yet been fully elucidated. Potential routes of infection include the consumption of milk and uncooked meat, as well as contact with animals [10]. Sexual transmission of *W. chondrophila* is unlikely given the low numbers of patients being positive for both *C. trachomatis* (a typical agent of sexually transmitted infections) and *W. chondrophila* [5]. Its ability to reside and replicate within a range of free-living amoebae (FLA) implies a possible widespread environmental occurrence of *W. chondrophila* [11,12].

The ability of amoebae-resistant microbes (ARM) to infect FLA provides them with the advantage of transportation within the environment. By forming persistent cysts, FLA provide protection for ARM against water disinfectants such as chlorine, and other stresses [13,14]. The ubiquitous presence of FLA in

soil, air, animals, plants and water facilitates transport into drinking water systems. FLA have been reported to break through the treatment barrier and enter water distribution systems, where they can colonize and regrow [15,16]. The colonization of pathogenic ARM in drinking water systems might pose a clinical risk, as has been observed in the case of *Legionella pneumophila* [17].

Waddlia chondrophila can infect, among others, *Acanthamoeba* spp. and *Vermamoeba vermiformis* (formerly *Hartmannella vermiformis*) [11], both of which have been identified in drinking water distribution systems and in treated drinking water in many countries worldwide [15]. Moreover, *W. chondrophila* DNA has been identified in drinking water sources in various European countries, such as France, Spain and Switzerland [18–20]. Although no *Acanthamoeba* spp. were detected in Dutch drinking water systems [21], the presence of *V. vermiformis* has been confirmed in distributed drinking water [16]. As a possible protozoan host for *W. chondrophila* is present in Dutch drinking water, this study was performed to investigate the presence of *W. chondrophila* DNA in drinking water systems in the Netherlands.

Materials and methods

Sample selection

In total, 59 drinking water samples were measured, obtained from the distribution systems of ten treatment plants throughout the Netherlands (plants A–J). Treatment plants A–E use surface water, which is treated with a multiple barrier approach, involving pre-treatment (e.g. rapid sand filtration, coagulation/sedimentation), disinfection process (e.g. dune infiltration, ozonation, UV, or UV/H_2O_2) and post-treatment (e.g. active carbon filtration, slow sand filtration). Plants F–J use groundwater, which is treated with aeration followed by rapid media filtration. From each treatment plant, samples were taken during summer and winter at three distances from the treatment plants (proximal, central and distal location). This provided six samples per treatment plant, except for plant F, for which no sample was available from the central location in summer. The kitchen water tap was flushed for 4 minutes before sampling, to make sure that microorganisms present in the premise's plumbing system were flushed out and the results displayed microorganisms from the distribution systems. Table 1 shows more details of the different treatment plants, including water temperature, total organic carbon and adenosine triphosphate levels.

DNA extraction and analysis

DNA extraction was performed by the KWR Water Research Institute [21] In short, 1 L of each drinking water sample was filtered through a 25-mm polycarbonate filter (0.22-μm pore size). DNA extraction was performed following the protocol of the PowerBiofilm™ DNA Isolation kit (MoBio, Carlsbad, CA, USA).

Quantitative PCR analysis for *W. chondrophila*, *Acanthamoeba* spp. and *V. vermiformis*

DNA was analysed for the presence of *W. chondrophila*-specific DNA using quantitative PCR as previously developed by Goy et al. [7]. A calibration curve was used as a positive control and for quantification, consisting of the *W. chondrophila*-specific 16S rRNA gene plasmid containing a 100-bp fragment. Gene copy numbers were calculated by comparing the threshold cycle (CT) values of the samples with those of the calibration curve.

TABLE 1. Detailed information on treatment plants and drinking water samples

Treatment plant	Region in the Netherlands	Water source[a]	TOC (mg C/L)[b]	Season	Date samples taken	Temperature (°C)	ATP (ng ATP/L)
A	West	SW	1.9	Summer	10-09-2012	19.4 ± 1.0	4.8 ± 4.3
				Winter	13-02-2013	6.0 ± 0.6	1.3 ± 0.3
B	West	SW	2.1	Summer	27-08-2012	20.5 ± 0.8	4.7 ± 0.7
				Winter	09-01-2013	8.9 ± 0.9	2.1 ± 0.1
C	West	SW	2.1	Summer	29-08-2012	18.6 ± 0.8	5.4 ± 1.8
				Winter	14-01-2013	9.0 ± 0.6	1.0 ± 0.0
D	West	SW	3.4	Summer	03-09-2012	20.0 ± 0.6	3.9 ± 0.3
				Winter	16-01-2013	7.2 ± 0.1	3.1 ± 0.5
E	West	SW[c]	2.2	Summer	04-09-2012	18.4 ± 1.3	1.9 ± 0.9
				Winter	28-01-2013	7.5 ± 0.5	1.5 ± 0.2
F	North	GW	8.0	Summer	22-08-2012	19.3 ± 1.1	6.8 ± 1.9
				Winter	30-01-2013	5.4 ± 2.9	4.7 ± 0.1
G	East	GW	0.3	Summer	11-09-2012	19.8 ± 0.6	0.2 ± 0.1
				Winter	04-02-2013	7.2 ± 0.8	2.5 ± 2.8
H	South	GW	3.4	Summer	18-09-2012	16.1 ± 1.0	4.0 ± 1.7
				Winter	06-02-2013	9.4 ± 1.5	3.2 ± 1.4
I	North	GW	4.3	Summer	20-09-2012	15.2 ± 0.6	5.4 ± 2.0
				Winter	11-02-2013	6.9 ± 0.3	5.1 ± 1.5
J	South	GW	2.0	Summer	24-09-2012	16.7 ± 0.8	2.4 ± 0.3
				Winter	07-01-2013	10.9 ± 2.0	2.9 ± 2.4

[a]SW, surface water; GW, groundwater.
[b]Total organic carbon (TOC) concentrations as measured in the treated water of the treatment plant.
[c]At treatment plant E surface water is infiltrated into the dunes before being abstracted for the production of drinking water.

TABLE 2. *Waddlia chondrophila* and *Vermamoeba vermiformis* DNA analysis in distributed drinking water from treatment plants A and C, the two plants that are positive for *W. chondrophila*

Treatment plant	Season	Distance	*W. chondrophila* (copies/L)	*V. vermiformis* (copies/L)
A	Summer	Proximal	<10 (+)[a]	126.8 (+)
		Central	<10 (+)	<100 (+)
		Distal	<10 (+)	<100 (+)
	Winter	Proximal	<10 (+)	<100 (+)
		Central	<10 (+)	<100 (+)
		Distal	<10 (+)	<100 (+)
C	Summer	Proximal	<10 (+)	<100 (+)
		Central	<10 (+)	<100 (+)
		Distal	<10 (+)	<100 (−)
	Winter	Proximal	569 (+)	<100 (+)
		Central	43 (+)	<100 (+)
		Distal	25 (+)	<100 (+)

Samples were taken in summer and winter at three different distances from the treatment plants.
[a](+), above detection limit; (−), below detection limit.

Sensitivity of the quantitative PCR was ten gene copies, based on the lowest detected dilution of the positive plasmid control (quantification limit).

DNA isolated from the water samples of plants A to D was analysed for the presence of *Acanthamoeba* spp. and *V. vermiformis*-specific DNA using the quantitative PCR analyses as previously described [21,22]. Quantifications were based on comparison of the sample CT value with the CT values of a calibration curve based on known copy numbers of the respective gene from *Acanthamoeba* or *V. vermiformis*.

Results

The analysis of the 59 samples taken from ten water treatment plants throughout the Netherlands showed a low number of positive samples for *W. chondrophila* DNA in drinking water derived from treatment plants A and C (Table 2). Three of these samples, all from treatment plant C and taken during winter, were quantifiable. The sample that was taken at a proximal location from the treatment plant showed the highest copy number of *W. chondrophila* DNA. All other samples from treatment plants A and C, with copy numbers of <10 per litre (Table 2), showed a *W. chondrophila*-specific amplification curve below the quantification limit. In contrast to the samples from treatment plants A and C, no *W. chondrophila* DNA was detected in the samples from the remaining eight treatment plants. In these samples no *W. chondrophila*-specific amplification curve was observed, indicating that these 47 samples were all negative for the organism.

The presence of DNA from *Acanthamoeba* spp. and *V. vermiformis* was also examined on the samples from plants A to D. *Acanthamoeba* spp. could not be detected in any of these samples. *Vermamoeba vermiformis* DNA was detected in 11 of the 12 samples taken from the distribution systems of plants A and C, with one sample having levels above the quantification limit (Table 2). In addition, *V. vermiformis* DNA was detected in all samples taken from plants B and D with levels above and below the quantification limits (data not shown).

Discussion

The current study shows the presence of *W. chondrophila* DNA in Dutch drinking water. This is in concordance with three other European studies that investigated its occurrence in (drinking) water systems. In Spain, Codony et al. detected *W. chondrophila* DNA in 10 of the 40 analysed well water sources, but all 30 drinking water samples were negative [19]. In France, Agusti et al. detected low *W. chondrophila* DNA levels in 12 of the 59 investigated samples from non-domestic hot water systems [18]. Three of the twelve positive samples could be quantified, nine samples showed a qualitative positive detection but were below the quantification limit. In addition, they observed that more water systems were positive for *W. chondrophila* than for *Legionella* spp., respectively nine versus four. In contrast to our study, they sampled hot water (average temperature 57.3°C, ranging from 28.0°C to 65.3°C), whereas we sampled cold water (ranging from 5.4°C to 20.5°C). In Switzerland, *W. chondrophila* DNA was detected in one of the 48 domestic drinking water samples and one biofilm sample [20]. Although biofilms were not investigated in our study, the study by Lienard et al. indicates that biofilms could form a possible niche for *W. chondrophila*, as well as for various other *Chlamydiales* [20].

In order to determine whether drinking water provides a possible transfer route of *W. chondrophila*, it is important to know which infected FLA hosts carry *W. chondrophila* in the water distribution system. *Acanthamoeba* spp. and *V. vermiformis* were found to be the most suitable hosts, but also *Vahlkampfia ovis* and *Dictyostelium discoideum* could be infected with *W. chondrophila* [11]. We could not detect *Acanthamoeba* spp. in the samples from the two plants that were positive for *W. chondrophila* DNA, but *V. vermiformis* was detected at low levels in most of the samples from the two plants that were positive for *W. chondrophila* DNA. Therefore, this amoeba might serve as a host for *W. chondrophila*. However, samples from two *W. chondrophila*-negative plants (plants B and D), were also positive for *V. vermiformis* DNA. This indicates that the presence of *V. vermiformis* is not a reliable indicator for the presence of *W. chondrophila*. The two other reported host protozoans (*Vahlkampfia ovis* and *D. discoideum*) were not

detected in an extensive 18S RNA gene analysis of drinking water sampled from two groundwater treatment plants in the Netherlands [16], but it remains uncertain if these two hosts were also absent in drinking water from plants A and C.

In contrast to the Netherlands, where *Acanthamoeba* spp. were not detected, samples from Spanish, French and Swiss water sources were positive for *Acanthamoeba* and/or *V. vermiformis* [21,23–25]. However, the higher prevalence of *Acanthamoeba* spp. in water sources in Spain, France and Switzerland does not seem to influence the presence of *W. chondrophila*, as the current study showed its presence in Dutch drinking water despite the absence of *Acanthamoeba* spp. In the Swiss study, Lienard et al. also detected *V. vermiformis* in some of the drinking water and biofilm samples. However, the samples that were positive for *W. chondrophila* were negative for *V. vermiformis* [20]. It is possible that *W. chondrophila* uses *V. vermiformis* as a host in drinking water, but that its main source is another FLA host that is as yet unknown.

Our results showed a higher presence of *W. chondrophila* DNA in samples taken during winter, than in those taken during summer at the same treatment plant. This is in contrast to most findings, where higher numbers of various microbes are found in the summer season due to higher water temperatures [16,21]. To our knowledge, we are the first to investigate seasonal associations with *W. chondrophila* specifically. It can be hypothesized that *W. chondrophila* or its hosts have lower optimum temperatures, leading to the higher numbers during winter. Alternatively, the higher temperature might be favourable to some bacterial species that overgrow and are detrimental to *W. chondrophila* or its eukaryotic hosts.

As mentioned before, higher numbers of *W. chondrophila* DNA were detected in the drinking water samples proximal to the treatment plant than in the more distal parts of the distribution system. The highest numbers of *V. vermiformis* in the summer were also observed at the proximal location of treatment plant A (Table 2). At treatment plants B and D, the highest *V. vermiformis* numbers were again observed at the proximal site, either in the winter (plant B) or summer and winter (plant D). The concurrent occurrence of *W. chondrophila* and *V. vermiformis* at the proximal sites of the distribution system suggests that *W. chondrophila* might use *V. vermiformis* as a protozoan host.

A question that remains unanswered in this study is whether there is a relation between drinking *W. chondrophila*-containing water and human infection. The clinical impact and risk of the presence of *W. chondrophila* in drinking water has yet to be examined. First, it remains unknown whether the presence of *W. chondrophila* DNA in the samples indicates the presence of live *W. chondrophila* that is capable of infecting another host. As *W. chondrophila* is an obligate intracellular bacterium, it cannot be cultured on an agar plate and live bacteria cannot be easily quantified. A possible direction for their study might be a co-culture of the drinking water samples with amoebae. Second, it is unknown what transmission route would be used by *W. chondrophila* to infect hosts via drinking water. The association between drinking *W. chondrophila*-containing water and infection has never been made. It is however known that the cysts of FLA, possibly containing ARM, can travel into the human respiratory tract via aerosols [15]. Increased exposure to aerosols through, for example, air conditioning systems could therefore possibly lead to an increased infection rate [20], as it does for the intracellular bacterium *Legionella pneumophila* [17]. The effects of exposure to *W. chondrophila*-containing drinking water or aerosols may be more likely to occur in the respiratory tract than in the reproductive system.

In summary, this study showed that in eight of the ten analysed Dutch treatment plants, *W. chondrophila* could not be detected in the distributed drinking water. Nonetheless, drinking water from two treatment plants showed positive samples for *W. chondrophila*. As only DNA was detected and gene copy numbers were low, it remains unlikely that these two drinking water types are an important source for infection with *W. chondrophila*. In conclusion, drinking water from the Netherlands does not seem to be a likely infection route for *W. chondrophila*. However, future studies are needed to investigate whether low concentrations of *W. chondrophila* could lead to infection, and whether this might result in infected tissues and related clinical implications. As *W. chondrophila* is a zoonotic pathogen, it would furthermore be interesting to investigate its occurrence in water sources around farms, to obtain more knowledge of the environmental presence of *W. chondrophila* and its implications from a One Health approach.

Conflicts of interest

None declared.

Acknowledgements

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