Surveillance of parasitic *Legionella* in surface waters by using immunomagnetic separation and amoebae enrichment

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Free-living amoebae (FLA) are potential reservoirs of *Legionella* in aquatic environments. However, the parasitic relationship between various *Legionella* and amoebae remains unclear. In this study, surface water samples were gathered from two rivers for evaluating parasitic *Legionella*. Warmer water temperature is critical to the existence of *Legionella*. This result suggests that amoebae may be helpful in maintaining *Legionella* in natural environments because warmer temperatures could enhance parasitisation of *Legionella* in amoebae. We next used immunomagnetic separation (IMS) to identify extracellular *Legionella* and remove most free *Legionella* before detecting the parasitic ones in selectively enriched amoebae. *Legionella pneumophila* was detected in all the approaches, confirming that the pathogen is a facultative amoebae parasite. By contrast, two obligate amoebae parasites, *Legionella*-like amoebal pathogens (LLAPs) 8 and 9, were detected only in enriched amoebae. However, several uncultured *Legionella* were detected only in the extracellular samples. Because the presence of potential hosts, namely *Veramoeba vermiformis*, *Acanthamoeba* spp. and *Naegleria gruberi*, was confirmed in the samples that contained intracellular *Legionella*, uncultured *Legionella* may survive independently of amoebae. Immunomagnetic separation and amoebae enrichment may have referential value for detecting parasitic *Legionella* in surface waters.

Keywords: Immunomagnetic separation (IMS), Intracellular *Legionella*, Extracellular *Legionella*, Free-living amoebae

Introduction

The genus *Legionella* is a pathogenic group of Gram-negative bacteria and is currently classified into 52 species and 71 serogroups, with at least 20 species associated with human diseases.^{1–4} First identified during a severe outbreak of pneumonia in Philadelphia, *Legionella pneumophila* can cause the mild Pontiac fever and the deadly Legionnaire's disease, which has a 20% fatality rate.⁵ *L. pneumophila* causes more than 90% of Legionnaire's disease cases worldwide.⁶ Highly adaptive, *Legionella* can survive in a wide range of temperature (5–65°C) and are commonly found in surface waters and wetlands, as well as in

inadequately maintained water distribution systems.⁷ Inhaling contaminated water aerosols is a major route to *Legionella* infection.^{8,9}

Free-living amoebae (FLA) are an important reservoir of *Legionella* in natural environments and support its multiplication. Several species of *Legionella* have been confirmed to be parasites of *Acanthamoeba* spp., including *L. pneumophila*, *Legionella anisa*, *Legionella lytica*, *Legionella fallonii*, *Legionella rowbothamii*, *Legionella drozanskii* and *Legionella drancourtii*.^{10–14} *Legionella* harboured in amoebae are more resistant to various environmental stresses, such as disinfectants; therefore, they are more likely to survive the water treatment process and migrate through pipelines.¹⁵ In addition, amoebae may enhance the invasion of *Legionella* into epithelial cells or macrophages.¹⁶ Several *Legionella*-like amoebal pathogens (LLAPs), which are obligate amoebae parasites, are easily isolated from sources associated

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with confirmed cases or outbreaks of Legionnaires' disease.^{15,17} According to these reports, amoebae may be an important mediator for *Legionella* infection. However, the interaction between various *Legionella* and amoebae in natural circumstances remains unclear. In general, selection for resistance to grazing by amoebae has contributed to the evolution of *L. pneumophila* as a pathogen. Some *Legionella* grazers are closely related to species that are known hosts for *L. pneumophila*, indicating the presence of unknown specificity determinants for this interaction to occur.¹⁸

Immunomagnetic separation (IMS) technology uses antibody-coated magnetic microbeads to target specific biological substances. It is widely applied for separating pathogens in foods, clinical specimens and environmental samples.^{19–21} Several studies have independently confirmed the efficiency of IMS in recovering *Legionella* spp. from different aquatic environments.^{20–22}

In this study, we utilised IMS to pinpoint extracellular *Legionella* or to remove most free organisms to facilitate detecting parasitic *Legionella* after selective amoebae enrichment. By comparing the detection rates of different approaches, we determined the presence and species of parasitic *Legionella*.

Materials and Methods

Sample collection and parameters analysis

Water samples were taken from two watersheds in southern Taiwan, namely, those of the Puzih River (23°28'N, 120°13'E) and the Kaoping River (22°37'N, 120°18'E). Samples were taken between January 2009 and September 2009. For each sample, 2000 ml of water was stored in two sterile 1 l bottles and transported to the laboratory at room temperature within 24 hours. In addition, 300 ml of water samples were also collected in a sampling bag (Nasco Whirl-Pak, Salina, USA) and stored at 4°C for heterotrophic bacteria and total coliforms analyses. Heterotrophic bacteria were measured using the spread method, and total coliforms were measured using membrane filtration and incubation with a differential medium, as prescribed in the Standard Method for the Examination of Water and Wastewater (Methods 9222 B and D).²³ In addition, *in situ* measurements of various water quality parameters, including water temperature, taken with a thermometer; pH level, measured using a portable pH metre (D-24E, Horiba Co, Fukuoka, Japan); and turbidity, determined with a turbidimeter (HACH Co, Loveland, CO, USA), were recorded at each sampling location. The relationship between *Legionella* population and five water quality parameters was determined using STATISTICA software (StatSoft, Inc., Tulsa, USA).

Sample preparation and experimental design

One litre of each water sample was filtered through cellulose nitrate membranes (pore size: 0.22 µm, diameter: 45mm; Pall, New York, USA). Each membrane was swirled with 100 ml of sterile phosphate-buffered saline (PBS; 7.5 mM Na₂HPO₄, 3.3 mM NaH₂PO₄, 108 mM NaCl, pH 7.2) to elute the microbes from the membranes. The solution was transferred into two conical centrifuge tubes (50 ml of each) and centrifuged at 5800 × *g* for 30 minutes. For each centrifuged solution, the top supernatant fluid (approximately 47.5 ml) was aspirated and discarded. The pellet in the remaining 2.5 ml of solution was resuspended by being vortexed in a disinfected tube.

Each of the post-concentrated water samples was subjected to five parallel procedures to determine the presence of extracellular and intracellular *Legionella*. Fig. 1 shows a schematic diagram of the experimental design. In short, a portion of each sample eluent was sent directly for DNA extraction and polymerase chain reaction (PCR) (denoted as 'direct extraction'). The rest of the samples were processed using IMS, some in combination with selective incubation, prior to DNA extraction (M1–M4). For M2, a *Legionella*-selective medium was used to determine the presence of viable, extracellular *Legionella*. In M4, a selective medium for FLA was used to confirm the presence of intracellular *Legionella*. After incubation, DNA extraction was performed using candidate colonies and all DNA extracts were subject to PCR for species confirmation.

IMS procedure

For each water sample, 1 ml of eluate was added to a 1.5 ml microfuge tube containing aliquots of 20 µl anti-*Legionella* Dynabeads (Life Technologies, Grand Island, NY, USA). Each microfuge tube was affixed to a rotating mixer and rotated at approximately 18 rpm for 1 hour at room temperature. After rotation, the microfuge tube was placed in a magnetic particle concentrator (MPC-S) for 3 minutes. The supernatant was then decanted without removing the tube from the MPC-S. Bead-*Legionella* complex was added with 1 ml of PBS-Tween 20 (0.1M PBS, 0.5% Tween 20, pH 7.4), after which the microfuge tube was placed in the MPC-S for 2 minutes. The supernatant was collected for analysis of uncaptured *Legionella* (M3) or for amoebae enrichment (M4). The bead-*Legionella* complex was washed three times with 1 ml of PBS-Tween 20 solution. Subsequently, the bead-*Legionella* complex was resuspended in 100 µl of PBS-Tween 20 solution for DNA extraction (M1) or *Legionella* culture (M2). The waste washing solution was collected for M3 and M4.

Figure 1 Schematic diagram of experimental setup.

Culture confirmation with selective media

Two selective media were used to enrich *Legionella* and the FLA hosts in the water samples. For *Legionella* enrichment, a 1-ml sample of eluate was pre-treated using heat (50°C for 30 minutes) and acid (0.2M HCl, 0.2M KCl, pH 2, for 5 minutes) to

reduce non-*Legionella* microbes.²⁴ The heated eluate was then transferred onto a buffered charcoal yeast extract (BCYE) agar plate (with alpha-ketoglutarate, L-cysteine and ferric pyrophosphate). The inoculated plates were then incubated in a 5% CO₂ incubator at 37°C for 5 days. After

incubation, the presumptive colonies were selected for DNA extraction and PCR analysis. In M4, the FLA-selective enrichment procedure was performed using the amoeba-*Legionella* culture method. The sample eluates (1 ml) were filtered through cellulose nitrate membranes (Pall, New York, USA, 0.45 µm pore size), each of which was placed upside-down on a non-nutrient agar and Page's amoebae saline (PAS, ATCC recipe: www.lgcstandards-atcc.org) plate seeded with heat-killed *E. coli*, according to the methods described by Greub and Raoult.²⁵ The inoculated plate was incubated at 30°C for 4 days. Subsequently, the filters were removed and the plates were incubated for an additional 3–4 days. In the next step, potential plaques were removed to the glass tube, resuspended in the PAS, and then placed in a refrigerator at 4°C overnight for encystment. The remaining trophozoites and extracellular bacteria were removed by 3% HCl (v/v in H₂O) treatment for 2 hours followed by washing and centrifugation with the PAS (425 × g, 6 minutes, 4°C). Finally, the adherent cysts were scraped from the bottom of the glass tubes and reserved for DNA extraction.

DNA extraction and PCR analysis

Two methods were used to extract DNA. In most approaches, DNA was extracted using direct heating at 95°C for 10 minutes, followed by centrifuging at 9700 × g for 10 minutes. For M4, DNA was extracted using Nucleospin Tissue (Macherey-Nagel Inc., Düren, Germany), which is recommended for the purification of total DNA from clinical samples and was deemed suitable for FLA detection in our previous studies.^{26–29}

The 50-µl of PCR mixture contained 5 µl of the derived DNA templates, 5 µl of 10 × PCR buffer (20 mM MgCl₂), 1 µl of dNTP mix (10 mM of each dNTP), 200 pmol each of the oligonucleotide primers, 0.3 µl of VioTaq™ DNA Polymerase (Vio-gene, Taipei, Taiwan, 5 U/µl) and DNase-free deionised water. Table 1 presents a summary of the target gene sequences and reaction settings. Subsequently, 5 µl of PCR products were run on 2% agarose gels (Bio-basic Inc., Markham, Canada). The BigDye Terminator Cycle

Sequencing Kit (Applied Biosystems, Foster, USA) was used to sequence the M1–M4 DNA fragments and the DNAMAN software (Lynnon Biosoft, Vaudreuil, Canada) was used to align the gene sequences. Positive controls in this study were *Acanthamoeba* isolated from a clinical sample from National Cheng Kung University Hospital, *Vermamoeba* ATCC 50237, *Naegleria* ATCC 22758 and *L. pneumophila* ATCC 33823. Negative DNA controls were also analysed by replacing the DNA template with distilled water. All samples and positive and negative DNA controls were analysed in triplicate in each PCR run. According to one of our previous study and unpublished data, copy number higher than hundreds could be easily detected.³⁰

Results and Discussion

Legionella detection by using various approaches

Water samples from two rivers in southern Taiwan were analysed to determine the relationship between amoebae and different species of *Legionella*. One was the Puzih River, a moderately polluted stream and an important water source for local agriculture, fish farming and industry. The other was the Kaoping River, the main water source for Kaohsiung City and its 2.8 million inhabitants. As shown in Table 2, testing the directly concentrated samples by PCR determined that 41 samples (56.9%) and 4 samples (28.6%) from the Puzih River and the Kaoping River, respectively, contained *Legionella*. Overall, more than half of all samples (52.3%) contained *Legionella*. These results suggest *Legionella* are nearly ubiquitous in surface waters in Taiwan. Significant differences were observed in the Puzih River samples' total coliforms, pH level and temperature between the samples with and without *Legionella* (Mann–Whitney *U* test, *P* < 0.05). By contrast, none of the Kaoping River samples exhibited significant differences (Table 3). According to a previous study, pH value and temperature changes could regulate the encystation of amoebae.³⁵ The parasitic relationship between *Legionella* and amoebae was reported to be terminated at lower temperatures (< 20°C) because of the exclusion of *Legionella* by

Table 1 Primer sequences and PCR conditions for *Legionella*, *Acanthamoeba*, *Naegleria*, and *Vermamoeba*

Pathogen	Target gene	Primer names and sequences (5'–3')	Annealing Temp. (°C)	Cycling No.	Amplicon size (bp)	Reference
<i>Legionella</i>	16S rDNA	LEG 225 5'-AAGATTAGCCTGCGTCCGAT-3' LEG 858 5'-GTCAACTTATCGCGTTTGCT-3'	62	40	654	31
<i>Acanthamoeba</i>	18S rDNA	JDP1 5'-GGCCCAGATCGTTTACCGTGAA-3' JDP2 5'-TCTCACAAGCTGCTAGGGGAGTCA-3'	62	35	450	32
<i>Naegleria</i>	5.8S rDNA	ITS1 5'-GAACCTGCGTAGGGATCATTT-3' ITS2 5'-TTTCTTTTCTCCCTTATTA-3'	53.5	35	400	33
<i>Vermamoeba</i>	18S rDNA	Hv1227F 5'-TTACGAGGTCAGGACACTGT-3' Hv1728R 5'-GACCATCCGGAGTTCTCG-3'	56	40	502	34

Table 2 Detection of *Legionella* using different methods. The locations of studied areas were also shown

Study areas	Puzih River 2328'N, 12013'E	Kaoping River 2237'N, 12018'E	Total
Sample number	72	14	86
Direct extraction	41 (56.9%)	4 (28.6%)	45 (52.3%)
Method 1	4 (5.6%)	1 (7.1%)	5 (5.8%)
Method 2	5 (6.9%)	1 (7.1%)	6 (7.0%)
Method 3	6 (8.3%)	3 (21.4%)	9 (10.5%)
Method 4	4 (5.6%)	3 (21.4%)	7 (8.1%)

Table 3 Non-parametric differences (Mann–Whitney *U* test, *P* Levels) in the presence or absence of *Legionella* species in terms of five water quality parameters (Sample number: 86). Value range of each parameter was also shown

Water quality parameter	Puzih River <i>P</i> level of Mann–Whitney <i>U</i> test	Range	Kaoping River <i>P</i> level of Mann–Whitney <i>U</i> test	Range
Heterotrophic plate counts (CFU/ml)	0.644	135 ~ 6.2×10^5	0.257	500 ~ 3.4×10^4
Total coliforms (CFU/100ml)	0.046*	22 ~ 7.4×10^4	0.386	400 ~ 1.6×10^4
Turbidity (NTU)	0.077	25 ~ 471	0.947	25 ~ 5.0×10^3
Temperature (°C)	0.001*	18.4 ~ 25.3	0.549	26.3 ~ 31.5
pH value	0.027*	7.7 ~ 8.7	0.790	7.2 ~ 8.2

* Significant difference at $P=0.05$.

encystation or digestion.³⁶ Because the Puzih River is located farther north and has a cooler water temperature, interaction between *Legionella* and amoebae may be influenced by the shifting of water temperature to approximately 20°C (Table 3). Moreover, the significance of temperature in the existence of *Legionella* increases the likelihood that parasitisation inside amoebae is beneficial to the maintenance of *Legionella* in surface waters.

To further evaluate the existence of extracellular and parasitic *Legionella* in surface waters, IMS was used to capture free *Legionella* in water samples (Fig. 1). The derived beads-attached *Legionella* were then directly lysed (M1) or alternatively enriched by BCYE culture before DNA extraction to verify viable organisms (M2). To identify intracellular *Legionella*, IMS was used to remove most extracellular *Legionella* and the remaining amoebae were selectively enriched by NNA-PAS-*E. coli* culture prior to DNA extraction (M4). The flow-through from IMS was harvested simultaneously for evaluating unbound *Legionella* and the ones harboured in other protozoa (M3). For the results of M1 and M2, five samples (5.8%) and six samples (7.0%), respectively, were determined to contain *Legionella* (Table 2). In contrast to the sensitivity of the traditional method (52.3%), lower M1 and M2 detection rates were observed in this study. Immunomagnetic separation can remove the objects interfering in PCR and *Legionella* cultivation, such as inhibitors, impurities and microbiota.^{37–42} It should be regarded as having a positive function in improving recovery. However, the result (lower M1 and M2 detection rates) observed in the study is controversial. It may

be because of the diversity of *Legionella* species in environmental water, and most of them do not belong to *L. pneumophila*. In addition, the anti-*Legionella* Dynabeads are designed to capture pathogenic *Legionella*, not to capture all *Legionella* species. An explanation to the higher detection rate in M2 than in M1 is the increase of trace amount of *Legionella* or the dilution of PCR inhibitors during culture process. On the other hand, PCR does not differentiate between live and dead *Legionella*, which could have greatly affected the results. It may lead to an overestimation of the actual quantity and sanitary risk. However, this method contributes to a better knowledge about the distribution and abundance of *Legionella* spp. in surface waters.

Some free *Legionella* were believed to escape from the magnetic separation, although the organisms detected in M3 might include parasitic *Legionella*. Nevertheless, selective enrichment of amoebae in M4 still guaranteed the isolation of parasitic *Legionella*. In contrast to M3, which may identify both extracellular and intracellular *Legionella*, M4 is specific for identifying amoebae-harboured organisms. Of the eight samples with successfully cultured amoebae colonies, seven were proven to contain intracellular *Legionella*. The M4 approach is inconsistent with the results of *Legionella* prevalence in the two watersheds (56.9 vs 28.6%), suggesting that the Kaoping River has a higher ratio of parasitic *Legionella* regardless of the sample size (5.6 vs 21.4%). These results further suggest that warmer temperatures may enhance interaction between amoebae and *Legionella*, although larger-scale research may be required to evaluate the effects of

various environmental factors on the occurrence of parasitic *Legionella*. Despite the water quality of the Kaoping River meeting the national standard for household usage, the clearance efficiency of *Legionella*, particularly parasitic *Legionella*, in distribution systems warrants more attention.

Extracellular and intracellular *Legionella* and FLA hosts

Although the existence of *Legionella* in aquatic environments appears correlated with amoebae, it remains unclear which *Legionella* species most heavily rely on amoebae for survival and pathogenesis. After the 16S rRNA amplicons in positive samples were sequenced, more than six species of *Legionella* were identified (Table 4). We next compared the extracellular-intracellular ratio among various *Legionella*. As a reference, *L. pneumophila* was detected by all approaches, including four samples by M1, one by M2, one by M3 and four by M4. After growing in an amoebae, *Legionella* lyse the cell, escape and swim around to find another amoebae. So, the other species were not observed in the original sample; they were observed only in the processed approaches. Another possibility is that *L. pneumophila* is more abundant than the other species, and the other species might become detectable only after some type of selection. The results suggest that *L. pneumophila* may be a facultative amoebae parasite. Unexpectedly, all the species grouped into uncultured *Legionella* were found only in M1–M3, suggesting that most uncultured species may survive in free forms or live with protozoa other than amoebae.

Among the six classified species of *Legionella*, three species (namely LLAP 8, LLAP 9 and *L. sainthelensi*) were found only in M4. Because LLAPs 8 and 9 are obligate amoebae parasites, these results increase the validity of M4 in specifying the amoebae-harboured *Legionella*. Arguably, the detection of *Legionella* in M4 could be attributed to bacteria attaching onto amoebae rather than parasitisation; however, this explanation may be ruled out because, theoretically, the extracellular

Legionella could be detected by M1–M3. However, based on our results, the detected species in M1–M3 were nearly exclusive from that of M4, further suggesting independence between M1–M3 and M4. Jointly, these results confirm that M4 is useful in pinpointing parasitic *Legionella*.

According to the description of Amaro *et al.*,¹⁸ some of the *Legionella* grazers were closely related to species that are known hosts for *L. pneumophila*, indicating the presence of unknown specificity determinants for this interaction. Several unrelated organisms were able to graze efficiently on *L. pneumophila*. The protist grazers exert selective pressure on *Legionella* to acquire and retain adaptations that contribute to survival, and that these properties are relevant to the ability of the bacteria to cause disease in people. They also report a novel mechanism of killing of amoebae by one *Legionella* spp. that requires an intact Type IV secretion system but does not involve intracellular replication. Finally, we would like to characterise the potential *Legionella* hosts. In the seven samples that tested positive for intracellular *Legionella*, the enriched amoebae were harvested for identification of *Acanthamoeba* spp., *Vermamoeba vermiformis* and *Naegleria* spp., three known *Legionella* hosts.^{16,29,43} We discovered that three samples contained two types of FLA hosts simultaneously. Two of these samples contained *V. vermiformis* and *Acanthamoeba* (*Acanthamoeba* spp. and *Acanthamoeba hatchetti*) and one sample contained *H. vermiformis* and *Naegleria gruberi*. In three other samples, the only FLA host found was *V. vermiformis*. Intriguingly, LLAP 9 was discovered in the last sample, in which the species of the FLA host was not identified by the PCR specific to *Acanthamoeba*, *Naegleria* or *Vermamoeba*. This sample indicated that LLAP 9 may be harboured by another amoebae or protozoa, such as *Tetrahymena thermophila*.⁴⁴ Overall, six of the seven samples (86%) with intracellular *Legionella* were tested positive for *V. vermiformis*. This finding is in agreement with a previous study in which *V. vermiformis* and cultivable *L. pneumophila* were both found in the

Table 4 Specification of *Legionella* and FLA hosts

<i>Legionella</i> species	Detection methods				FLA hosts in M4*
	M1	M2	M3	M4	
Uncultured <i>Legionella</i>	1	4	4	0	
<i>L. pneumophila</i>	4	1	1	2	<i>Vermamoeba vermiformis</i> (2)
<i>L. fairfieldensis</i>	0	1	3	0	
<i>L. feeleyi</i>	0	0	1	0	
<i>L. amoebal pathogen 8</i>	0	0	0	1	<i>V. vermiformis</i> , <i>Acanthamoeba</i> spp. T4
<i>L. amoebal pathogen 9</i>	0	0	0	3	<i>A. hatchetti</i> (1), <i>V. vermiformis</i> (2)
<i>L. sainthelensi</i>	0	0	0	1	<i>V. vermiformis</i> , <i>Naegleria gruberi</i>
Total	5	6	9	7	

*Numbers in parentheses indicate the number of samples positive with the specified FLA host.

drinking water of three Caribbean islands.⁴⁵ Because *V. vermiformis*, *Acanthamoeba* and *Naegleria* are frequently found in streams, water treatment plants, tap water and hot springs, the presence of such amoebae may present an additional threat to human health because of the dispersal of facultative intracellular bacteria, such as *Legionella*.^{46–49}

Undeniably, the lower recovery efficiency in M1–M4 may influence final conclusion. It is also impossible to maintain equal recovery efficiencies among the methods. Modification of experimental procedures, e.g., using another filters or smaller beads, may increase recovery efficiency and facilitate similar studies in the future. However, an advantage of this study is to allow assessment of parasitisation by comparing the relative proportions of a unique *Legionella* species in approaches especially if the sample number is big enough. According to the result in Table 4, unequal distribution of different *Legionella* species in each approach suggests that IMS combined with amoebae enrichment may have referential value for identifying parasitic *Legionella*.

In this study, we utilised IMS and amoebae enrichment to identify extracellular and parasitic *Legionella* in surface waters. In contrast to the high prevalence of *Legionella* in the Puzih River, parasitic organisms were more frequently found in the Kaoping River, implying that a warmer water temperature may enhance interaction between amoebae and *Legionella*. Among the identified species, intracellular forms LLAPs 8 and 9 were found, and *L. fairfieldensis*, *L. feeleii*, and several uncultured *Legionella* were detected only outside amoebae. However, the most clinically associated species, *L. pneumophila*, may exist in both free and parasitic forms in the related watersheds. Three reported protozoa hosts, namely *A. hatchetti*, *N. gruberi* and *V. vermiformis*, were also discovered in the positive samples. These results add to concern regarding *Legionella* residence in insufficiently disinfected water distribution systems. This study suggests that IMS combined with amoebae enrichment may have referential value for detecting parasitic *Legionella* in receiving waters.

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Disclaimer Statements

Contributors

TKH, SFW, BMH, WTJ, PMK, CWT, SMS and WCH contributed to the study concept, design, data collection, analysis, manuscript preparation and approval. WTJ and CWF contributed to data collection, analysis, and manuscript preparation and approval.

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Conflicts of interest All authors have agreed to publish the results.

Ethics approval None.

References

- 1 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.
- 2 Doleans A, Aurell H, Reyrolle M, Lina G, Freney J, Vandenesch F, et al. Clinical and environmental distributions of *Legionella* strains in France are different. *J Clin Microbiol*. 2004;42:458–60.
- 3 Den Boer JW, Bruin JP, Verhoef LP, Van der Zwaluw K, Jansen R, Yzerman EP. Genotypic comparison of clinical *Legionella* isolates and patient-related environmental isolates in the Netherlands, 2002–2006. *Clin Microbiol Infect*. 2008;14:459–66.
- 4 Bargellini A, Marchesi I, Righi E, Ferrari A, Cencetti S, Borella P, et al. Parameters predictive of *Legionella* contamination in hot water systems: association with trace elements and heterotrophic plate counts. *Water Res*. 2011;45:2315–21.
- 5 Nazarian EJ, Bopp DJ, Saylor A, Limberger RJ, Musser KA. Design and implementation of a protocol for the detection of *Legionella* in clinical and environmental samples. *Diagn Microbiol Infect Dis*. 2008;62:125–32.
- 6 Phin N, Parry-Ford F, Harrison T, Stagg HR, Zhang N, Kumar K, et al. Epidemiology and clinical management of Legionnaires' disease. *Lancet Infect Dis*. 2014;14:1011–21.
- 7 Guillemet TA, Levesque B, Gauvin D, Brousseau N, Giroux JP, Cantin P. Assessment of real-time PCR for quantification of *Legionella* spp. in spa water. *Lett Appl Microbiol*. 2010;51:639–44.
- 8 Hsu SC, Martin R, Wentworth BB. Isolation of *Legionella* species from drinking water. *Appl Environ Microbiol*. 1984;48:830–2.
- 9 Steinert M, Hentschel U, Hacker J. *Legionella pneumophila*: an aquatic microbe goes astray. *FEMS Microbiol Rev*. 2002;26:149–62.
- 10 Drozanski W. Fatal bacterial infection in soil amoebae. *Acta Microbiol Pol*. 1956;5:315–7.
- 11 Rowbotham TJ. Current views on the relationships between amoebae. *Legionellae* and man. *Isr J Med Sci*. 1986;22:678–89.
- 12 Drozanski WJ. *Sacrobium lyticum* gen. nov. sp. nov., an obligate intracellular bacterial parasite of small free-living amoebae. *Int J Syst Bacteriol*. 1991;41:82–7.
- 13 Adeleke AA, Fields BS, Benson RF, Daneshvar MI, Pruckler JM, Ratcliff RM, et al. *Legionella drozanskii* sp. nov. *Legionella rowbothamii* sp. nov. and *Legionella fallonii* sp. nov.: three unusual new *Legionella* species. *Int J Syst Evol Microbiol*. 2001;51:1151–60.
- 14 La Scola B, Mezi L, Weiller PJ, Raoult D. Isolation of *Legionella anisa* using an amoebic coculture procedure. *J Clin Microbiol*. 2001;39:365–6.
- 15 Abu Kwaik Y, Gao LY, Stone BJ, Venkataraman C, Harb OS. Invasion of protozoa by *Legionella pneumophila* and its role in bacterial ecology and pathogenesis. *Appl Environ Microbiol*. 1998;64:3127–33.
- 16 Cirillo JD, Falkow S, Tompkins LS. Growth of *Legionella pneumophila* in *Acanthamoeba castellanii* enhances invasion. *Infect Immun*. 1994;62:3254–61.
- 17 Lamoth F, Greub G. Amoebal pathogens as emerging causal agents of pneumonia. *FEMS Microbiol Rev*. 2010;34:260–80.
- 18 Amaro F, Wang W, Gilbert JA, Roger Anderson O, Shuman HA. Diverse protist grazers select for virulence-related traits in *Legionella*. *ISME Journal*. 2015;9(7):1607–18.

- 19 Cudjoe KS, Krona R, Olsen E. IMS: a new selective enrichment technique for detection of *Salmonella* in foods. *Int J Food Microbiol.* 1994;23:159–65.
- 20 Yanez MA, Carrasco-Serrano C, Barbera VM, Catalan V. Quantitative detection of *Legionella pneumophila* in water samples by immunomagnetic purification and real-time PCR amplification of the *dotA* gene. *Appl Environ Microbiol.* 2005;71:3433–41.
- 21 Fuchslin HP, Kotsch S, Keserue HA, Egli T. Rapid and quantitative detection of *Legionella pneumophila* applying immunomagnetic separation and flow cytometry. *Cytometry A.* 2010;77:264–74.
- 22 Sethi S, Gore MT, Sethi KK. Increased sensitivity of a direct fluorescent antibody test for *Legionella pneumophila* in bronchoalveolar lavage samples by immunomagnetic separation based on BioMags. *J Microbiol Methods.* 2007;70:328–35.
- 23 American Public Health Association. Standard method for the examination of water and wastewater. 21st edn. Washington, DC: APHA, WEF and AWWA; 2005.
- 24 De Luca G, Stampi S, Lezzi L, Zanetti F. Effect of heat and acid decontamination treatments on the recovery of *Legionella pneumophila* from drinking water using two selective media. *New Microbiol.* 1999;22:203–8.
- 25 Greub G, Raoult D. Crescent bodies of *Parachlamydia acanthamoeba* and its life cycle within *Acanthamoeba polyphaga*: an electron micrograph study. *Appl Environ Microbiol.* 2002;68:3076–84.
- 26 Hsu BM, Lin CL, Shih FC. Survey of pathogenic free-living amoebae and *Legionella* spp. in mud spring recreation area. *Water Res.* 2009;43:2817–28.
- 27 Hsu BM, Ma PH, Liou TS, Chen JS, Shih FC. Identification of 18S ribosomal DNA genotype of *Acanthamoeba* from hot spring recreation areas in the central range, Taiwan. *J Hydrol.* 2009;367:249–54.
- 28 Huang SW, Hsu BM. Isolation and identification of *Acanthamoeba* from Taiwan spring recreation areas using culture enrichment combined with PCR. *Acta Tropica.* 2010;115:282–87.
- 29 Huang SW, Hsu BM. Survey of *Naegleria* and its resisting bacteria-*Legionella* in hot spring water of Taiwan using molecular method. *Parasitol Res.* 2010;106:1395–02.
- 30 Ji WT, Hsu BM, Chang TY, Hsu TK, Kao PM, Huang KH, et al. Surveillance and evaluation of the infection risk of free-living amoebae and *Legionella* in different aquatic environments. *Sci Total Env.* 2014;499:212–19.
- 31 Miyamoto H, Yamamoto H, Arima K, Fujii J, Maruta K, et al. Development of a new seminested PCR method for detection of *Legionella* species and its application to surveillance of *Legionellae* in hospital cooling tower water. *Appl Environ Microbiol.* 1997;63:2489–94.
- 32 Dykova I, Lom J, Schroeder-Diedrich JM, Booton GC, Byers TJ. *Acanthamoeba* strains isolated from organs of freshwater fishes. *J Parasitol.* 1999;85:1106–13.
- 33 Sheehan KB, Fagg JA, Ferris MJ, Henson JM. PCR detection and analysis of the free-living amoeba *Naegleria* in hot springs in Yellowstone and Grand Teton National Parks. *Appl Environ Microbiol.* 2003;69:5914–8.
- 34 Kuiper MW, Valster RM, Wullings BA, Boonstra H, Smidt H, van der Kooij D. Quantitative detection of the free-living amoeba *Hartmannella vermiformis* in surface water by using real-time PCR. *Appl Environ Microbiol.* 2006;72:5750–6.
- 35 Greub G, Raoult D. Microorganisms resistant to free-living amoebae. *Clin Microbiol Rev.* 2004;17:413–33.
- 36 Ohno A, Kato N, Sakamoto R, Kimura S, Yamaguchi K. Temperature-dependent parasitic relationship between *Legionella pneumophila* and a free-living amoeba (*Acanthamoeba castellanii*). *Appl Environ Microbiol.* 2008;74:4585–8.
- 37 Rossen L, Norskov P, Holmstrom K, Rasmussen OF. Inhibition of PCR by components of food samples, microbial diagnostic assays and DNA-extraction solutions. *Int J Food Microbiol.* 1992;17:37–45.
- 38 Kreader CA. Relief of amplification inhibition in PCR with bovine serum albumin or T4 gene 32 protein. *Appl Environ Microbiol.* 1996;62:1102–6.
- 39 Wilson IG. Inhibition and facilitation of nucleic acid amplification. *Appl Environ Microbiol.* 1997;63:3741–51.
- 40 Burtcher C, Wuertz S. Evaluation of the use of PCR and reverse transcriptase PCR for detection of pathogenic bacteria in biosolids from anaerobic digestors and aerobic composters. *Appl Environ Microbiol.* 2003;69:4618–27.
- 41 Kermekchiev MB, Kirilova LI, Vail EE, Barnes WM. Mutants of Taq DNA polymerase resistant to PCR inhibitors allow DNA amplification from whole blood and crude soil samples. *Nucleic Acids Res.* 2009;37(1–14):e40.
- 42 Diaz-Flores A, Montero JC, Castro FJ, Alejandres EM, Bayón C, Solís I, et al. Comparing methods of determining *Legionella* spp. in complex water matrices. *BMC Microbiol.* 2015;15(1–9):91.
- 43 Brieland JK, Fantone JC, Remick DG, LeGendre M, McClain M, Engleberg NC. The role of *Legionella pneumophila*-infected *Hartmannella vermiformis* as an infectious particle in a murine model of Legionnaire's disease. *Infect Immun.* 1997;65:5330–3.
- 44 Kikuhara H, Ogawa M, Miyamoto H, Nikaido Y, Yoshida S. Intracellular multiplication of *Legionella pneumophila* in *Tetrahymena thermophila*. *J UOEH.* 1994;16:263–75.
- 45 Valster RM, Wullings BA, van den Berg R, van der Kooij D. Relationships between free-living protozoa, cultivable *Legionella* spp., and water quality characteristics in three drinking water supplies in the Caribbean. *Appl Environ Microbiol.* 2011;77:7321–8.
- 46 Thomas V, Loret JF, Jousset M, Greub G. Biodiversity of amoebae and amoebae-resisting bacteria in a drinking water treatment plant. *Environ Microbiol.* 2008;10:2728–45.
- 47 Edagawa A, Kimura A, Kawabuchi-Kurata T, Kusuhara Y, Karanis P. Isolation and genotyping of potentially pathogenic *Acanthamoeba* and *Naegleria* species from tap-water sources in Osaka, Japan. *Parasitol Res.* 2009;105:1109–17.
- 48 Hsu BM, Huang CC, Chen JS, Chen NH, Huang JT. Comparison of potentially pathogenic free-living amoeba hosts by *Legionella* spp. in substrate-associated biofilms and floating biofilms from spring environments. *Water Res.* 2011;45:5171–83.
- 49 Huang SW, Hsu BM, Chen NH, Huang CC, Huang KH, Chen JS, et al. Isolation and identification of *Legionella* and their host amoebae from weak alkaline carbonate spring water using a culture method combined with PCR. *Parasitol Res.* 2011;109:1233–41.