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RESEARCH

Detection of amoeba-associated *Legionella pneumophila* in hospital water networks of Johannesburg

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The prevalence of free-living amoeba and associated *Legionella* spp. in hospital water systems may pose a risk of Legionnaires' disease to immuno-compromised patients. This study investigated the occurrence of amoeba-associated *Legionella pneumophila* in three South African hospital water systems. A total of 98 water and/or biofilm samples were collected from the sterilisation unit, theatres, neonatal ward and intensive care units. Amoebae were isolated from 71 (72.4%) samples. Isolated amoebae were analysed using qPCR and culture methods to test for the presence of Legionella. *L. pneumophila* did not grow on selective media in any of the samples. A total of 7 out of the 71 (9.9%) amoeba-positive samples showed a positive reaction for *L. pneumophila* using qPCR. Although relatively few samples were positive for *Legionella* in this preliminary study, the association with amoeba still presents a potential public health risk to immuno-compromised patients when exposed to contaminated water.

Keywords: amoeba, *Legionella pneumophila*, Legionnaires disease

Introduction

Legionella species are gram-negative, non-spore-forming, rod-shaped or filamentous fastidious aerobic bacteria. They have been isolated from man-made aquatic environments such as cooling towers, hot tubs, air-conditioning systems and potable water systems where they can proliferate at temperatures between 20° and 50°C.[1,2] Among the 58 described *Legionella* spp., *Legionella pneumophila* serogroup 1 is the most common serotype responsible for at least 84% of infections in humans. Inhalation of aerosols containing *Legionella* spp. may result in two kinds of infections, the mild, non-fatal, influenza-like illness Pontiac fever and the severe form of pneumonia and potentially fatal Legionnaires' disease (LD) in both community and health-care settings. Aspiration of contaminated water or direct contact with surgical wounds are the other less common modes of transmission.[3] Worldwide, there are few LD cases where the environmental source of *Legionella* infection is determined successfully. Studies have shown that contaminated potable water supplies within hospitals could be responsible for hospital-acquired LD cases.[4,5]

The discovery by Rowbotham[6] that *L. pneumophila* in aquatic environments can exist as an intracellular parasite of amoebae has provided a link between bacterial interactions in the environment and human disease. *Legionella* spp. are known to naturally infect and survive within the amoebae genera that include *Acanthamoeba*, *Vermamoeba* and *Naegleria* in the environment.[7–10] Internalised *Legionella* spp. are protected inside amoeba-resistant cysts and can survive adverse aquatic environment conditions like the presence of chlorine, commonly used to treat water in engineered water systems. In addition to survival, *Legionella* spp. can multiply inside the amoeba before being released into the environment in vesicles or by lysis.[11,12] *Legionella* spp. released from amoebae have been reported to increase in virulence, biocide and antibiotic resistance as a result of horizontal gene transfer between several intracellular bacteria and their amoebae hosts.[13–15] This can have remarkable public health implications if immunocompromised patients are exposed to water systems in hospitals contaminated with amoeba and *Legionella*. Our previous studies have shown a high amoeba prevalence and coexistence of other clinically important gram-negative bacteria in public hospitals of Johannesburg.[16] The current study is preliminary work to establish potential sources of hospital-acquired LD by screening hospital waters and/or biofilm for the presence of amoeba-associated *L. pneumophila*.

Methods

Sample collection

A walk-through assessment of the water systems of three Johannesburg hospital facilities was conducted to identify areas of high-risk exposure to waterborne pathogens. A systematic sampling strategy was followed to collect and analyse water and/or swab samples of every second water tap. Water samples were collected from the cold-water system. A total of 98 samples (51 water and 47 swab) were collected from Hospital A: the sterilisation unit ($n = 8$) and theatres ($n = 42$); Hospital B: neonatal ward ($n = 13$); and Hospital C: intensive care units ($n = 35$). The samples were analysed within 24 h of collection. Swab samples were collected by swabbing the inside surfaces of the taps prior to opening them. Water samples (500 ml) were collected after running the taps for 1– 2 min in 1 litre of sterile sampling bottles containing 5 mg/l sodium thiosulfate (Merk, Modderfontein, South Africa). At each sampling site, water temperature was measured with a portable COMBO TESTER® (Hanna Instruments, Bedfordview, South Africa) according to the manufacturer's instructions. Residual chlorine was measured using a chlorine photometer (Hanna Instruments, Bedfordview, South Africa) according to the manufacturer's instructions.

Sample analysis

As the rationale for this work is the detection and quantification of amoeba-associated *Legionella*, an enrichment technique used to detect amoeba was adapted from a previous study.[16] Briefly, 500 ml of water sample and 10 ml of Page's amoebal saline buffer (PAS) swab suspension was concentrated by filtration using a

0.45 µm nitrocellulose membrane (Merk, Modderfontein, South Africa). The membrane was placed upside down onto a non-nutrient agar (NNA) plate overlaid with heat-killed *E. coli* (NNA-HKEC plates). The plates were incubated at 32 °C and checked daily under light or inverted microscope for the appearance of amoebal trophozoites and cysts for up to 21 days. Plates with amoebal growth were purified by aseptically cutting small agar plugs, placing them upside down onto fresh NNA-HKEC plates, and incubating as before. Once purified, amoeba were re-suspended in 1 ml sterile PAS, inoculated into a sterile 24-well flat-bottomed microtiter plate (Life Technologies, Randburg, South Africa), and again incubated at 32°C. The plates were then observed for the morphological appearance of amoebae trophozoites and/or cysts under an inverted microscope using a 40-x objective (SMM Instruments, Johannesburg, South Africa).

Amoeba from the amoeba-positive samples were lysed by passing amoeba cells suspended in 300 µl Page amoebal saline through a 27-gauge syringe filter with a pore size of 0.45 µm three times to release any potential *Legionella* species. One hundred microlitres of the resulting suspension were inoculated on non-selective buffered charcoal yeast extract (BCYE) and selective Glycine-Vancomycin-Polymyxin-Cycloheximide (GVCP) *Legionella* agar (Quantum Biotechnologies, Randburg, South Africa) and incubated aerobically at 37°C for up to 10 days to culture *Legionella*. DNA was extracted from cultures from remaining 100 µl amoeba suspension for PCR identification and/or screening for *L. pneumophila* using the QIAmp DNA Blood Mini Kit (Qiagen, Hilden, Germany) as per the manufacturer's instructions and amplified according to Myamoto *et al*.,[17] with minor adaptions to increase the specificity and sensitivity of the assay according to Omiccioli *et al*.[18] The DNA Blood Mini extraction kit was chosen due to its high DNA recovery rate and low susceptibility to the presumed co-concentration of organic and inorganic polymerase inhibitory substances in the samples as demonstrated by a previous study.[19] Both quantitative and qualitative detection of *L. pneumophila* was performed using PCR with primers LpneuF (5′-CCGATGCCACATCATTAGC-3′) and LpneuR (5′-CCAATTGAGCGCCACTCATAG-3′) and for the quantification of *L. pneumophila* after the samples were shown to contain *Legionella* DNA by gel electrophoresis, a TaqMan probe, LpneuP (5′-6-carboxyfluorescein [FAM]-TGCCTTTAGCCATTGCTTCCG-BHQ1–3′). As stated above, adapted amplification cycle conditions are listed in Miyamoto *et al*. and Omiccioli *et al*. [17, 18] The amplification mixture consisted of 25 µl of iQ supermix (Life Science, Veenendaal, the Netherlands), 0.4 mg/ml of bovine serum albumin (Roche Diagnostics, Almere, The Netherlands), 0.2 µM each primer and probe, 0.2 µM each primer, and 10 µl of DNA template in a total reaction volume of 50 µl. To detect *Legionella* spp., the polymerase in the reaction tubes was initially activated at 95°C for 90 s, followed by 43 cycles of amplification using denaturation at 95°C for 3 min followed by annealing at 55°C for 30 s and extension at 72°C for 1 min.

As it had to be expected that not all samples would be positive for *Legionella*, a two-tier approach was chosen to conserve resources: in a first step, the efficiency of PCR was confirmed qualitatively by agarose gel electrophoresis with 8 µl of the PCR product on 2% gel as shown in Figure 1. Quantification of amplicons in GU/l (genomic units per litre) as well as data analysis was performed with a real-time PCR Rotor-Gene 6000 Cycler (Corbett Life Science, Mortlake, Australia) on samples positive for *Legionella* according to ISO-TS 12869:2012 and the TaqMan Probe. In brief, a calibration range that comprised four serial dilutions of 25 to 25 000 GU of *L. pneumophila* (ATCC 33152) per well using a working calibration solution (Corbett Life Science, Mortlake, Australia).[20] This was used to interpolate the concentration of DNA amplicons of the samples under investigation within the linear response range of the qPCR method.

Statistical analysis

Statistical analysis was used to determine whether *L. pneumophila* co-occurred with amoebae. The collected data were analysed with SPSS®, version 20 (SPSS Inc., Chicago, IL, USA), using crossing tables and a chi-square test (asymptotic significance, 2-tailed). Significance was set at $p < 0.05$. Pearson's chi-square test was used to test for association between amoeba and *L. pneumophila*. The interpretation was performed at 95% confidence limit.

Results

The water temperature of the three hospitals at the time of sampling ranged between 20.7° and 27.3°C (mean 22.5°C) at hospital A, 20.7° and 27.3°C (mean 22.5°C) at hospital B and 20.0° and 23.7°C (mean 21.6°C) for hospital C. The chlorine concentrations of the individual hospitals were 0.01–0.17 mg/l (mean 0.09 mg/l) for hospital A, 0.21–0.28 mg/l (mean 0.23 mg/l) for hospital B and 0.01–0.32 mg/l (mean 0.23 mg/l). Amoebae were isolated from 72.4% (n = 71) of the 98 water and biofilm

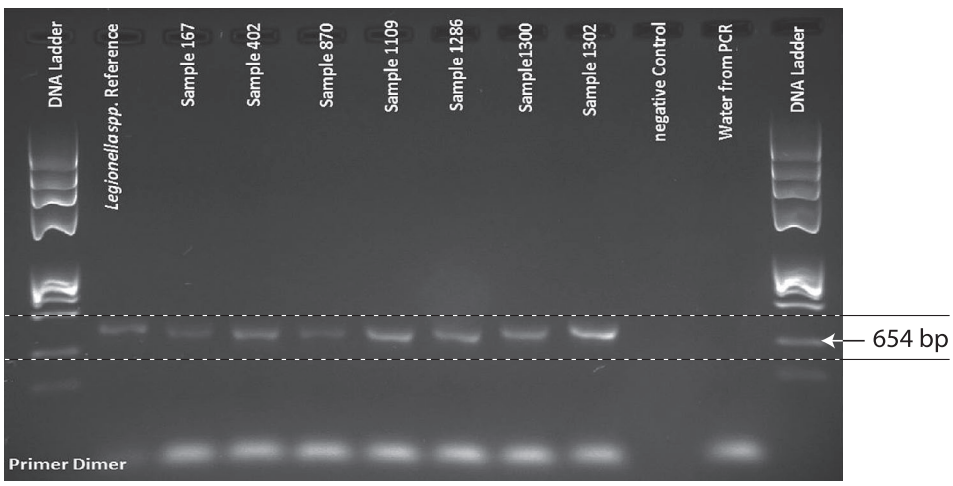

Figure 1: Agarose gel (2%) electrophoresis of the first-step PCR products of water samples from three hospitals performed with primers LEG 225 and LEG 858, which amplifies approximately 654 bp (arrow) of the 16S rRNA gene and a 100 bp ladder.

samples, of which 69.4% ($n = 68$) were microscopically identified as *Vermamoeba vermiformis* and 30.6% ($n = 30$) identified as *Acanthamoeba* spp.

All samples cultured on BYCE and GVCP agar were negative for *Legionella* species. However, a total of 7 (six water and one swab) out of 71 (9.9%) amoeba-positive samples showed a positive reaction for *L. pneumophila* using the q-PCR. The positive samples were isolated from the central sterilisation service department (CSSD) (sample 167); theatre tap (sample 402); neonatal ward cubicle tap (sample 870); from the cardiothoracic intensive care unit (ICU) (sample 1109) and trauma ICU (samples 1130; 1302; 1286) (see Figure 1). *Legionella* co-occurred with the amoeba *V. vermiformis* in all samples and this coexistence between the two species was statistically significant ($p < 0.05$). Using qPCR, the positive samples were quantified to determine the GU/l. Sample 1302 showed the highest concentration with 3.8×10^2 GU/l, corresponding to the strongest band signal on the agarose gel (see Figure 1). Sample 870 had the lowest concentration (2.7×10^0 GU/l), corresponding to the weakest band signal on the agarose gel (Table 1).

Discussion

Generally, all the measured water temperature and residual chlorine for all three hospitals were within the limits prescribed by the South African National Standard for Drinking Water Systems.[21] In this study, amoeba-associated *Legionella* spp. did not grow on BCYE and GVCP agar. However, *L. pneumophila* were detected in 9.9% (7/71) of samples using conventional and quantitative PCR. Since they were positive in the ISO certified qPCR, it is likely that there were residues of amoeba-resistant *L. pneumophila* present, which entered a viable but non-culturable (VBNC) state. Similar studies conducted in Italy detected *L. pneumophila* in 33.3% (22/66) of hospital water systems,[10] while in Greece *Legionella* spp. were detected from 16.9% (22/130) of hospital water samples.[22] Another study of hospital water systems in Taiwan reported 62.5% (10/16) of the samples to be positive for *L. pneumophila*.[23] The highest concentration obtained in this study of 3.8×10^2 GU/l for *L. pneumophila* was quantified from trauma ICU. This is lower than the concentration peak of 4.0×10^4 GU/l of *L. pneumophila* that was quantified in the otorhinolaryngology, pathologic anatomy, and paediatrics and surgery wards of an Italian hospital.[10] Several factors such as different geographical locations, different molecular detection methods used as well as matrix features of the water source might explain the variability in the prevalence and concentration values.

Table 1: Quantitative and qualitative results of PCR for *Legionella pneumophila* and free-living amoebae isolated from hospital water

Hospital amoebae		Sample source	*L. pneumophila* (GU/l)
Hospital A	*V. vermiformis*	CSSD (S)	$2.7x10^0$
	V. vermiformis	Theatre (W)	$1.6x10^1$
Hospital B	*V. vermiformis*	Neonatal ward (W)	$2.9x10^0$
Hospital C	*V. vermiformis*	Cardiothoracic ICU (W)	$4.7x10^1$
	V. vermiformis	Trauma ICU (W)	$1.4x10^1$
	V. vermiformis	Trauma ICU (W)	$1.6x10^1$
	V. vermiformis	Trauma ICU (W)	$3.8x10^2$

Notes: S = swab; W = water; GU/l = genetic units per litre; CSSD = central service sterilisation unit; ICU = intensive care unit.

Although relatively few samples were positive for *Legionella* in the current study, which according to its rationale did not account for the *Legionella* eventually present in the water phase, the coexistence between *Legionella* and *V. vermiformis* was statistically significant ($p < 0.05$). This suggests that these FLA may serve as reservoirs of *Legionella*, thus contributing to the environmental survival of *Legionella* as well as transmission vectors in hospital settings by releasing them into the water stream. Therefore, their detection may still indicate a risk to hospitalised patients as *Legionella* spp. are known to replicate rapidly intracellularly within protozoan hosts for prolonged periods of time forming amoebic vesicles that can contain hundreds of *Legionella* cells.[6,24] The relatively few positive *Legionella* detected may be attributed to the exclusion of free-living *Legionella* detection in the method.

Health-care-associated Legionnaires' disease (LD) has been reported worldwide.[25,26] In South Africa, the first investigation of an outbreak of LD in a Johannesburg teaching hospital in 1985 reported 12 cases in hospitalised patients, with two patients confirmed to have acquired the disease in the hospital.[27] A more recent surveillance study of two South African hospitals by Wolter *et al.*[28] reported *Legionella* in 21 (1.2%) cases of patients diagnosed with HIV or tuberculosis infections. However, this study did not aim to prove if the infections were acquired from the hospital environment. Hospital-associated LD cases in South Africa may be under-reported due to the lack of robust and reliable surveillance mechanisms and lack of accurate diagnosis. Routine sampling of hospital water supplies with the application of amoebal enrichment and co-culture techniques to resuscitate bacteria in the VBNC state can be effective strategies to prevent and manage hospital-acquired LD.[5,29] Therefore, future work will focus on the detection of *Legionella* spp. from hospital water supplies and hospital surfaces, and comparing them with clinical samples. This will establish any link between occurrence of the organisms in the hospital environment with LD infection in patients using amoeba culture and molecular techniques. Knowledge on the occurrence of amoebae-associated *L. pneumophila* in the hospital water systems will also provide baseline information to monitor potential outbreaks that may be facilitated by their presence.

Conclusions

Free-living amoebae were detected in 71 (72.4%) of the 98 water and biofilm samples collected. From the amoebae-positive samples, *Legionella* did not grow on BCYE and GVCP agar. However, *L. pneumophila* were detected in 9.9% (7/71) of samples using conventional and real-time PCR. The seven *L. pneumophila* positive samples were from trauma ICU, cardiothoracic ICU, a neonatal ward and a central service sterilisation unit. Patients and health-care workers might be exposed to waterborne amoeba-associated *L. pneumophila*

Compliance with ethical standards – The manuscript does not contain clinical studies or patient data.

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