Free-living amoebae isolated from a hospital water system in South Africa: A potential source of nosocomial and occupational infection

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This study investigated the occurrence of free-living amoebae (FLA) in a public hospital in South Africa. A total of 97 water and biofilm samples from the municipal water inlet of the hospital, theatres, theatre sterilization service unit, central sterilization service unit, endoscopy/gastroscopy unit, intensive care unit and the renal unit were collected and examined for the presence of FLA using an amoebal co-culture and molecular techniques. Of the 97 samples, 77 (79.4 %), 40 (52%) water and 37 (48.1%) biofilm, contained FLA. The genera *Acanthamoeba*, *Vermamoeba* (formerly *Hartmanella*) and *Naegleria* were detected by morphology, 18S rRNA PCR and sequence analyses. Further sequence analysis of the five *Acanthamoeba* positive isolates revealed a close resemblance with the potentially pathogenic T20 genotype. These results show a potential health risk to immuno-compromised patients and health care workers as some of the species detected are pathogenic and may harbour potential intracellular bacteria responsible for nosocomial infections. To date, this is the first report on the detection of potentially pathogenic amoebae from South African hospital water systems.

Key words: *Acanthamoeba*, amoebal co-culture, nosocomial infections, free-living amoebae, T20 genotype

INTRODUCTION

Free-living amoebae (FLA) are unicellular eukaryotes that are ubiquitous in the environment, mainly in water and soil, where they feed on bacteria and other small microorganisms and play a role in the maintenance of soil fertility and the recycling of nutrients. FLA are present in most natural aquatic environments (rivers, streams, hot springs) as well as in man-made water systems such as domestic tap water, swimming pools and hospital water distribution networks (Thomas & Ashbolt, 2011; Ovrutsky *et al*., 2013). Although mostly beneficial in their natural habitat, *Acanthamoeba*, *Naegleria fowleri*, *Balamuthia mandrillaris* and *Sappinia pedata* are known human pathogens responsible for opportunistic and non-opportunistic infections (Visvesvara *et al*., 2007; Thomas & Ashbolt, 2011). These FLA can cause central nervous system (CNS) infections, amoebic encephalitis, as well as lung and skin infections (Visvesvara, 2013). *Acanthamoeba*, in particular, is also associated with *Acanthamoeba* keratitis (AK), a vision threatening corneal infection that may lead to blindness in healthy individuals following corneal abrasions or corneal trauma due to injury. This infection is common in contact lens wearers often

due to poor care like rinsing of lenses with tap water and swimming in contaminated water (Trabelsi *et al*., 2012). These infections commonly occur in immuno-compromised and immune-suppressed individuals such as cancer patients, HIV/AIDS patients, diabetics and individuals undergoing organ transplants (Visvesvara *et al*., 2007; Visvesvara, 2013). *Balamuthia* CNS infections have also been reported in children and older individuals with no known history of immunodeficiency. *Naegleria* is responsible for primary amoebic meningoencephalitis, another rare but potentially fatal CNS infection which occurs in healthy children and young adults (Lorenzo-Morales *et al*., 2011).

Certain pathogenic and non-pathogenic FLA also serve as a platform for the survival, multiplication and transmission of other bacterial pathogens such as *Legionella pneumophila, Pseudomonas aeruginosa* and methicillin-resistant *Staphylococcus aureus* (MRSA) (Greub & Raoult, 2004; Lone *et al*., 2009; Ben Salah & Drancourt, 2010) and numerous other gastro-intestinal and pulmonary pathogens. These amoeba resistant bacteria (ARB) are able to infect, resist the digestive process of FLA, survive, multiply and exit FLA. The association of these pathogens with FLA protects them from common disinfection measures such as chlorination when amoebae are in cyst form. The resistant nature of FLA cysts provides involuntary protection to the pathogenic intracellular bacteria which are packaged in expelled vesicles or released when during amoebae lysis (Greub & Raoult, 2004; Denocourt *et al.,* 2014).

FLA can colonise most water systems as amoebal cysts are resistant to extremes of temperature, desiccation, pH and even disinfectants such as chlorine and gluteraldehyde (Storey *et al*., 2004; Dillon *et al*., 2014). Therefore, FLA may act as reservoirs and vectors of pathogenic bacteria that are difficult to eradicate, especially in water distribution systems where concentration of disinfectants is low (Evstigneeva *et al.,* 2009). Furthermore, these intracellular bacteria can undergo genetic modifications within FLA becoming more virulent and resistant to antibiotics and biocides like chlorine used in water treatment (Schmitz-Esser *et al.*, 2010). This presents a potential public health concern especially in hospital water distribution system where a large population of immune-compromised patients and medical personnel may be exposed to contaminated water. The purpose of the present study was therefore to investigate the occurrence of FLA and potential intracellular bacteria in wards and sterilization areas of a hospital in Johannesburg, South Africa.

MATERIALS AND METHODS

Sample collection

A total of 97 samples (51 water and 46 biofilm) were collected from a Johannesburg tertiary hospital. Samples were collected from the inlet of hospital (4), theatres (41), theatre sterilization service unit (TSSD) (4), central sterilization service unit (CSSD) (8) and endoscopy/gastroscopy unit (18), intensive care unit (ICU) (10) and the renal unit (12) after consultation with the infection control staff and management of the hospital and a walkthrough investigation of high risk patient areas. Biofilm samples were collected by swabbing 20 cm^2 of surfaces prior to opening taps. Water samples were collected after running the taps/shower for 1-2 minutes in 1 L sampling bottles containing 5 mg/L sodium thiosulfate (Merck, SA) to neutralize the chlorine disinfectant.

Physico-chemical characteristics

At each sampling site, the water temperature, pH, turbidity, conductivity and total organic carbon (TOC) were measured with a portable COMBO TESTER® (Hanna, SA). Free residual chlorine was measured with a chlorine photometer (Hanna, SA). The samples were then collected and transported at ambient temperature in cooler boxes to the laboratory and processed on the day of receipt.

Media preparation

Page's modified Neff's amoebae saline (PAS) was prepared by dissolving 120 mg NaCl, 4 mg $MgSO_4.7H_2O$, 4 mg $CaCl_2.2H_2O$, 142 mg Na_2HPO_4, and 136 mg KH_2PO4 in 1 L of distilled water. Nonnutritive agar (NNA) plates were prepared by adding 15 g agar/L (Merck, SA) of PAS. All media were sterilized by autoclaving for 15 min at 121 °C.

Isolation of free-living amoebae

The amoebal enrichment technique for the detection of FLA was adapted from Thomas *et al.* (2006) and Lamoth & Greub (2010). Five hundred millilitres of water samples were concentrated by filtration using a 0.45 µm pore size cellulose nitrate membrane (Millipore, SA). Swabs were vortexed for 30 s in 10 mL PAS in individual sterile tubes and the suspensions were concentrated by filtration. The filter membranes were placed upside down onto NNA overlaid with a suspension of heat-killed *Escherichia coli* (ATCC 25922) (*E. coli*; 100 µL for each plate). The plate was then incubated aerobically at 32 °C and examined daily for three weeks under a light microscope (Olympus, Japan) with a 10X objective for appearance of amoebal trophozoites and/or cysts. Plates were recorded as negative after three weeks of observation. Plates positive for amoebae were sub-cultured by cutting small agar plugs, placing them upside down onto fresh NNA-*E. coli* plates and incubating as before. This was done three to four times to purify amoebae as much as possible. Once purified, amoebae were harvested by gently scraping the agar surface and re-suspending in 2 mL of PAS. To further remove extracellular bacteria and debris, the suspension was centrifuged three times at 1000 x g for 20 minutes. The washed pellet was then suspended in 1 mL PAS. Nine hundred and fifty microlitres was inoculated in 12 well, flat bottomed microtitre wells (Nunc, USA) and incubated at 32 °C. The plates were checked daily under an inverted microscope (Leica, Germany), with a 40 X objective, for appearance of FLA. Transmission electron microscopy (TEM) was used to determine the presence of intracellular bacteria.

Transmission electron microscopy

Each non-nutrient agar plate with amoebal trophozoites was flooded with fixative and left overnight. The fixative contained 2.5 % glutaraldehyde and 2 % formaldehyde in 0.1 M sodium cacodylate buffer (pH 7.15), osmotically adjusted by the addition of 0.01 M $CaCl_2$, 0.01 M $MgCl_2$, and 0.09 M sucrose. The agar surface was then scraped, and for subsequent steps in the protocol, the sample was pelleted by gentle centrifugation (300 x g for 20 minutes). Buffer rinses (30 minutes each) and post fixation with 1 % osmium tetroxide for one hour preceded graded ethanol dehydration and embedding in a low viscosity resin. 70 nm sections were cut on a Leica EM UC6 ultramicrotome, placed on Formvar®-coated slot grids, double stained with uranyl acetate and lead citrate, and viewed on an FEI BioTwin Spirit transmission electron microscope at 80 kV (FEI, USA). Images were captured with an Olympus Quemesa CCD camera.

PCR and sequence analysis of the positive FLA samples

Amoebal DNA was extracted from 200 µL of the prepared amoebae suspension (from each of the amoebae positive plates) using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. The nucleic acid was eluted in 100 µL elution buffer into a 1.5-mL microcentrifuge tube and stored at −20 °C for subsequent analysis by PCR, agarose gel electrophoresis and sequence analysis. A 18S rRNA PCR was performed using the primers Ami6F1 (5'-CCA GCT CCA ATA GCG TAT ATT-3'), Ami6F2 (5'-CCA GCT CCA AGA GTG TAT ATT-3'), and Ami9R (5'- GTT GAG TCG AAT TAA GCC GC-3') at a concentration of 0.5 µM (Thomas *et al*., 2006) with PCR Master Mix No Rox (Eurogentec, Belgium). After a first step consisting of 94 °C for 5 min, 40 cycles of amplification were performed by using denaturation at 94 °C for 1 min, annealing at 55 °C for 30 s, and elongation at 72 °C for 2 min and a final cycle at 72 °C for 10 min. Sequencing reactions were then performed with each primer. Sample purification was performed by using the Genelute Kit (Sigma-Aldrich, USA) following manufacturer's protocol. Efficiency of PCR has been confirmed by agarose gel electrophoresis with 8 µL of the PCR product on 2 % gel (data not shown). Genome content of the positive samples has been determined by photometric method (Qubit 2.0 Fluorometer, Life Technologies, USA) and subsequently sequenced with 3130xl Genetic Analyzer (Applied Biosystems, USA). In order to determine *Acanthamoeba* genotype, follow-up PCR was performed with the primers set JDP1 (5'-GGC CCA GAT CGT TTA CCG TGA A-3') and JDP2 (5'-TCT CAC AAG CTG CTA GGG GAG TCA- 3') described by Schroeder *et al*. (2001). Cycling conditions were as follows: 95 °C for 5 min for the initial denaturation step, followed by 35 cycles of 15 s at 95 °C for denaturation, 15 s at 62 °C for annealing, 3 s at 72 °C for extension, and a final extension at 72 °C for 10 min. PCR products of *Acanthamoeba* were confirmed like mentioned before via gel electrophoresis on a 2 % agarose gel (data not shown). Phylogenetic construction produced gene trees by using neighbour-joining distance trees with a generation of 1,500 bootstrapped replicates. In order to allow BLAST searching and alignment with the MEGA 6.06 (Mega Software, Tempe, Arizona, USA) software, the 18S rRNA gene sequences were assigned to the GenBank database at the National Center for Biotechnical Information (NCBI). Isolates which have not been identified thus far were deposited in the GenBank under accession numbers KT18374-KT183626. Obtained sequences were aligned with sequences of *Acanthamoeba* genotypes T1-T20 (Corsaro *et al*., 2015). A list of the used reference strains and their GenBank accession numbers can be found in Supplementary Material 1.

RESULTS

Physico-chemical characteristics

Water temperatures recorded ranged between 14.8 °C and 27.3 °C (Mean 23.1 °C), pH ranged between 1.74 and 8.77 (Mean 7.77), TDS ranged between 10-187 mg/L (Mean 126 mg/L) and residual chlorine ranged between 0.01-0.35 mg/L (Mean 0.12 mg/L).

Morphological identification of free-living amoebae

Based on morphological characteristics of trophozoites and cysts, typical *Acanthamoeba* spp. and *Vermamoeba* spp. (formerly *Hartmanella* spp.) were observed suspended in Page amoebal saline under an inverted microscope (Figure 1). Overall, 77 (79.4 %) of the 97 samples (40 water and 37 biofilm) contained amoebae after amoebal enrichment. Amoebae were generally isolated in all sampling sites, with the exception of one sample from the intensive care unit. Of the FLA positive samples, 6 (7.80 %) and 26 (33.8 %) were morphologically identified as *Acanthamoeba* spp. and *Vermamoeba* spp. respectively. The other FLA (39.0 %) could not be identified

morphologically. Three samples isolated from the central sterilization service unit (n=1) and theatres (n=2) contained both *Acanthamoeba* and *Vermamoeba* species (Table 1). From samples positive for FLA, TEM showed the occurrence of potential intracellular bacteria whose identity were not determined in this study (Figure 2).

Table 1: FLA identified by morphological examination from the hospital water distribution system

Sampling site (N)	*Acanthamoeba* spp. (+) (%)	*Vermamoeba* spp. (+) (%)	Other FLA[a] (+) (%)
Inlet (5)	1 (25.0)	0	0
TSSD/CSSD (12)[b]	2 (16.7)	2 (16.7)	4 (33.3)
EB unit (16)	0	5 (31.3)	12 (75.0)
Theatre complex (42)	3 (7.1)	11 (26.2)	26 (61.9)
Intensive care unit (10)	0	1 (10.0)	2 (20.0)
Renal unit (12)	0	7 (58.3)	3 (25.0)
Total	**6 (7.80)**	**26 (33.8)**	**47 (39.0)**

[a] Amoebae not identified as morphologically as *Acanthamoeba* spp. or *Vermamoeba* spp.
[b] Samples with both *Acanthamoeba* spp. and *Vermamoeba* spp.
EB-endoscopy/bronchoscopy
TSSD-theatre sterilization service unit
CSSD-central sterilization service unit

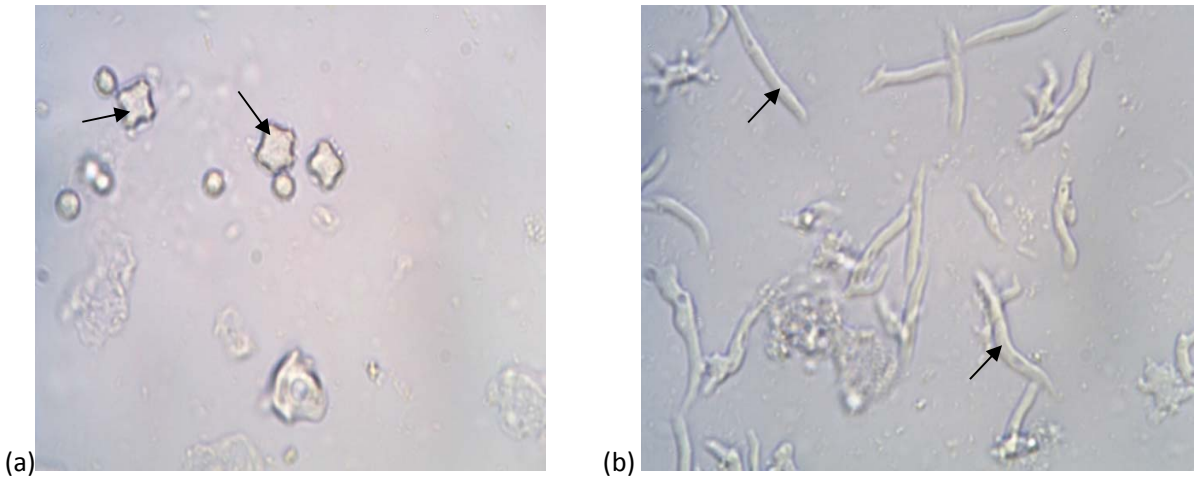

Figure 1 (a) *Acanthamoeba* cysts and (b) *Vermamoeba* trophozoites (arrows), scale bar 100µm

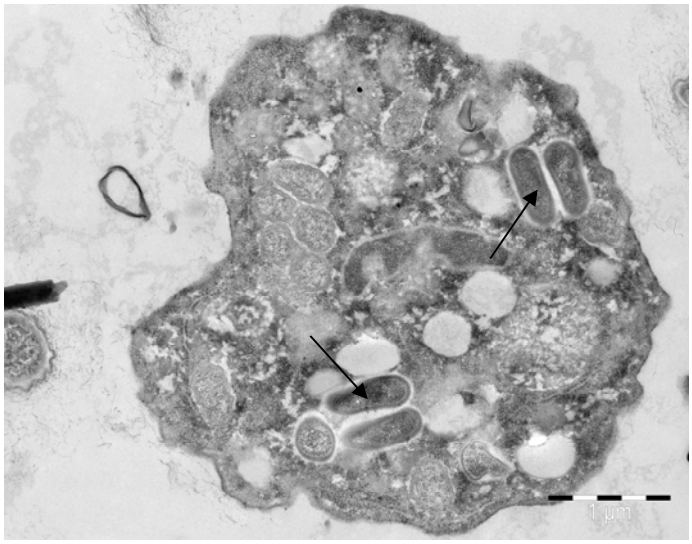

Figure 2: Transmission electron image of a *Acanthamoeba* trophozoite with intracellular bacteria (arrows), scale bar 1µm

Molecular identification of FLA isolates

The 77 samples containing FLA were amplified using the universal primers, Ami6F1, Ami6F2 and Ami9R, for the 18S rRNA PCR and sequenced. Of these samples, 11 (14.3 %) did not show bands on agarose gel and 13 (16.9 %) were negative after sequence analysis. These samples were considered negative for FLA. Overall, FLA were detected in 53 (68.8 %) of the samples analysed by molecular methods. *Vermamoeba vermiformis* (46.4 %) were the most abundant amoebae identified from all the sampling areas, followed by *Acanthamoeba* spp. (33.8 %) and other FLA (24.7 %). A total of 23 *Vermamoeba vermiformis* isolates (GenBank accession no.s shown in table 2), 5 *Acanthamoeba* spp. isolates (GenBank accession no.s: KT183616, KT183582, KT183604, KT183575, KT183576), one isolate each for *Amoebozoa* sp. (GenBank accession no. KT183581) and *Naegleria* sp. (GenBank accession no KT183611) were identified based on 18 rRNA gene regionsfrom different sampling areas of the hospital. Furthermore, other protozoa *Pseudoparamoeba pagei* (GenBank accession no. KT183621) and *Schizoplasmodiopsis amoeboidea* (GenBank accession no. KT183622) were also detected in four samples but were excluded from this study (Table 2). Neighbour joining (NJ) analysis was performed using the genus specific primer set JDP-1 and JDP2 to show relationships between the *Acanthamoeba* positive isolates in this study, and reference strains from NCBI GenBank for the genotypes T1-T20. The five *Acanthamoeba* isolates detected in this study were 91-95 % (mean 93 %) similar to the T20 genotype sequence (GenBank accession no. DQ451162) as evidenced by the NJ tree (Figure 3).

Table 2: Biodiversity of FLA isolated from different sources of a South African hospital

Origin	GenBank Accession No.	Published closest described species
Inlet	KT183594	*Vermamoeba vermiformis*
TSSD/CSSD	KT183577	*Vermamoeba vermiformis*
	KT183616	*Acanthamoeba* sp.
Endoscopy/Bronchoscopy unit	KT183578	*Vermamoeba vermiformis*
	KT183608	*Vermamoeba vermiformis*
	KT183579	*Vermamoeba vermiformis*
	KT183580	*Vermamoeba vermiformis*
	KT183588	*Vermamoeba vermiformis*
	KT183581	*Amoebozoa sp.*
	KT183582	*Acanthamoeba* sp.
Theatre complex	KT183583	*Vermamoeba vermiformis*
	KT183585	*Vermamoeba vermiformis*
	KT183587	*Vermamoeba vermiformis*
	KT183606	*Vermamoeba vermiformis*
	KT183607	*Vermamoeba vermiformis*
	KT183592	*Vermamoeba vermiformis*
	KT183603	*Vermamoeba vermiformis*
	KT183604	*Acanthamoeba* sp.
	KT183575	*Acanthamoeba* sp.
	KT183621	*Pseudoparamoeba pagei*
	KT183611	*Naegleria* sp.
Intensive care unit	KT183618	*Vermamoeba vermiformis*
	KT183574	*Vermamoeba vermiformis*
	KT183593	*Vermamoeba vermiformis*
	KT183591	*Vermamoeba vermiformis*
Renal unit	KT183576	*Acanthamoeba* sp.
	KT183612	*Vermamoeba vermiformis*
	KT183600	*Vermamoeba vermiformis*
	KT183625	*Vermamoeba vermiformis*
	KT183620	*Vermamoeba vermiformis*
	KT183619	*Vermamoeba vermiformis*
	KT183622	*Schizoplasmodiopsis amoeboidea*
	KT183584	*Pseudoparamoeba pagei*

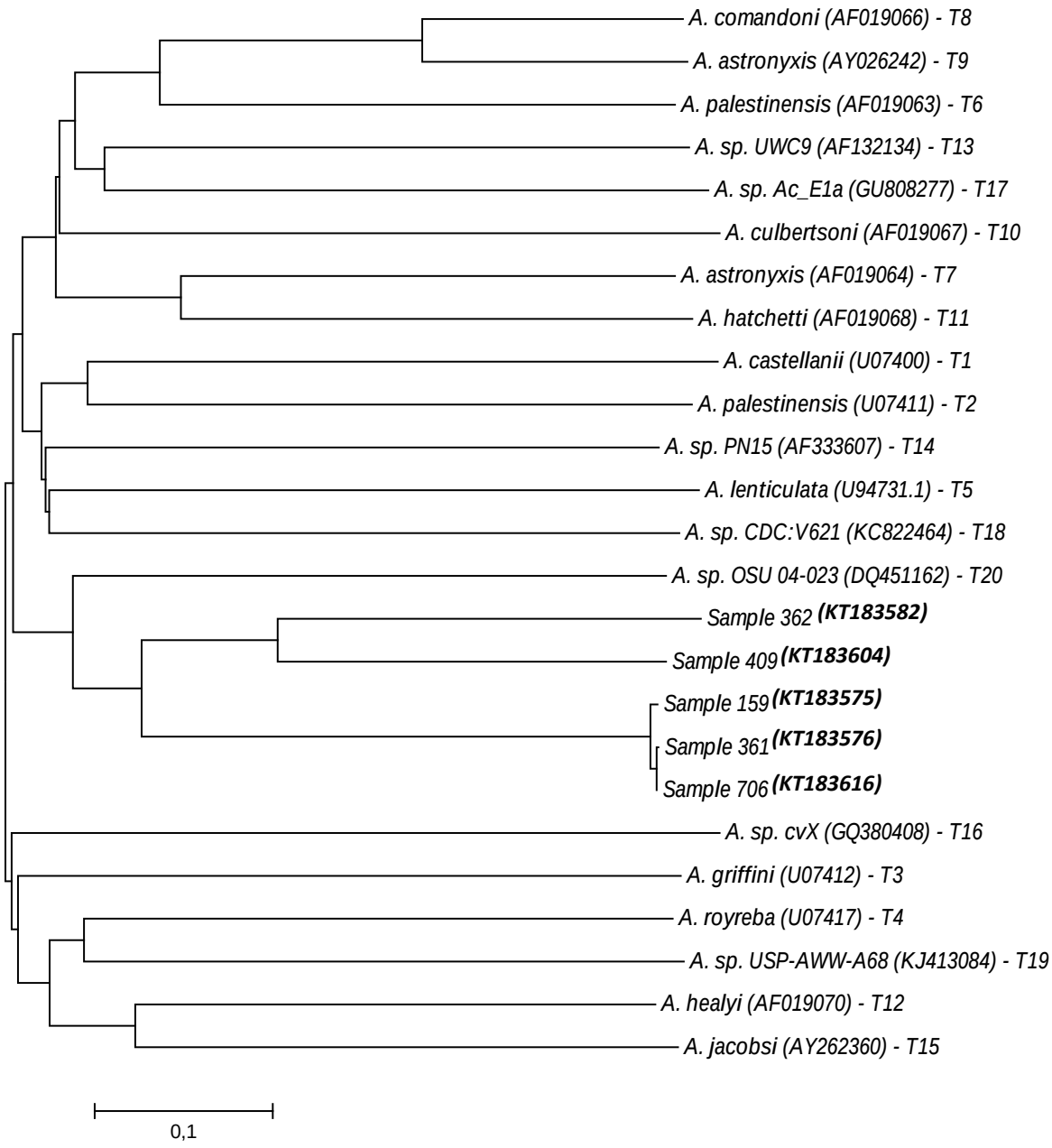

Figure 3: Phylogenetic relationships of the PCR products of *Acanthamoeba* species obtained from environmental samples and reference strains for *Acanthamoeba* subtype T1 – T20 from the NCBI GenBank inferred by neighbour-joining analysis using MEGA 6.06. Sample labeling consists of the Taxa, the accession number (in brackets) and the corresponding subtype. The length of the branch indicates the evolutionary resemblance between each sample

DISCUSSION

The presence of FLA in environmental waters such as rivers, lakes, drinking water purification plants, domestic tap water and marine waters has been well documented worldwide (Loret & Greub, 2010). Therefore, it is important to study their ecology to prevent exposure and outbreaks of FLA associated diseases and the potential spread of amoeba resistant bacteria. This study focused on the occurrence of FLA in a hospital water distribution system, a potential source of nosocomial infections that has been overlooked by many studies, particularly in Africa. To our knowledge this is the first study to investigate the occurrence of FLA in water and biofilm from a South African hospital water system.

FLA were isolated from 77 (79.4 %) of the samples analyzed. The prevalence of FLA in this study is higher than in studies done by Rohr *et al*. (1998) in Germany and Lasheras *et al.* (2006) in France where amoebae were detected in 29 of 56 (52%) and in 73 of 106 (68.9%) hospital water samples respectively. Another study by Thomas *et al*. (2006) also detected amoebae from 15 of 200 samples (7.5%) in a Swiss hospital. These studies analyzed water from hospital hot water systems compared to the current work which analyzed samples from a cold water system where the mean water temperature was 23.1 °C (range, 14.4 to 27.3 °C). Thomas *et al*. (2006) reported a higher prevalence of FLA in cold water compared to hot water, with prevalence of FLA significantly lower for temperatures above 60 °C. This might explain the higher prevalence of FLA in our study compared to those other studies. Furthermore, the variability in the prevalence values of FLA can also be explained by difference in water source, geographical areas and the different methods used in the identification of FLA. The occurrence of biofilms in water systems have also been shown to provide better conditions for FLA multiplication compared to bacteria suspended in water systems (Pickup *et al*., 2007). A recent study by Ovrutsky *et al*. (2013) detected amoebe in 14.8% of hospital samples that were analysed. Of the positive samples, 12 (80.0 %) were biofilm samples while 3 (20.0 %) were water samples. This was in agreement with our study as there was high detection rate in biofilm samples, that is, 37 (80.4 %) compared to water samples in which amoebae were detected in 40 (78.4 %) samples.

Using both culture-based and molecular based methods, genera belonging to *Acanthamoeba* and *Vermamoeba* (formerly *Hartmanella*) and *Naegleria* were detected. The universal primers, Ami6F1, Ami6F2 and Ami9R, used in this study did not specify if the only *Naegleria sp*. isolate belonged to the pathogenic *N. fowleri*. The other medically important FLA, *B. mandrillaris*, and *Sappinia* sp. were also not detected probably because of their low prevalence in the environment, especially in water (Visvesvara, 2013). In addition, the incubation temperature (32 °C) used in this study may have not been adequate to allow the growth of other FLA, particularly *Naegleria* spp. which grow better at higher temperatures. The most prevalent genus in the current study was *Vermamoeba* detected with both culture based (33.8 %) and molecular methods (46.4 %). Our findings are in agreement with a study from Turkey where the genus *Vermamoeba* was detected in 24 out of 33 detected FLA from 150 drinking water samples. Similar results were described by Thomas *et al*. (2006) in a study done in Switzerland to determine the biodiversity of FLA in a hospital water network where *V. vermiformis* had a prevalence of 86.7 %. However, the genus *Vermamoeba* is not necessarily the most prevalent FLA in the environment. Several studies have reported *Acanthamoeba* to be the most common FLA in air, soil and water (Thomas *et al*., 2006; Coşkun *et al*. 2013). The higher prevalence of *Vermamoeba* compared to *Acanthamoeba* in our study can be explained by the fact that we used universal FLA primers which might be more

effective in amplification of *Vermamoeba* than *Acanthamoeba*. The genus *Vermamoeba* is not usually associated with human disease and has been isolated in human tissues without the proof that the isolates are the cause of infection (Cabello-Vílchez *et al*., 2014). However, cytopathogenicity has been observed on keratocytes and has been reported as a causative agent of a form of keratitis which can lead to corneal ulcers (Kennedy *et al.,* 1995; Abedkhojasteh *et al*., 2013). Based on phylogenetic analysis, the five samples positive for *Acanthamoeba* spp. closely resembled the T20 genotype. This is, to our knowledge, the first report of this genotype in Africa. This genotype is has recently been described by Fuerst *et al.* (2015) and contains several *Acanthamoeba* strains previously miss-assigned to genotypesT16 or T4. The T20 genotype contains highly pathogenic strains responsible for *Acanthamoeba* keratitis, granulomatous amoebic encephalitis and respiratory infections in humans. Infections that affect a number of organs and tissues have also been reported in other vertebrates (Visvesvara *et al*., 2007; Visvesvara, 2013). Therefore, the presence of the genera *Vermamoeba* and *Acanthamoeba* is a possible risk factor to a large population of immune-compromised individuals and medical personnel who might be exposed to these opportunistic FLA. Patients and health care personnel exposure can occur during drinking (ingestion), showering (inhalation or aspiration aerosols) or bathing and washing of hands (contact) (Anaissie *et al.,* 2002). The persistent nature of amoebal cysts means that these populations are frequently exposed to FLA. An example of their persistent nature was shown by *Acanthamoeba* cysts reactivation after being stored in a completely dry environment for over 20 years (Visvesvara, 2013). Furthermore, the residual chlorine range of 0.01-0.35 mg/L in water samples analyzed in this study is not sufficient in eliminating FLA as they have been shown to survive concentrations as high as 100 mg/L for 10 minutes of exposure (Storey *et al*., 2004).

In addition to causing opportunistic infections, both *Vermamoeba* and *Acanthamoeba* are known to act as reservoirs and transmitters of nosocomial pathogens including *Legionella*, *Pseudomonas aeruginosa* and *Mycobacteria* (Greub and Raoult, 2004; Lamoth & Greub, 2010) Although this study did not identify specific intracellular bacteria that can be harboured by FLA, TEM showed their presence inside an amoeba host (Figure 2). These FLA are known to protect amoeba resistant pathogens from disinfectants and increase their virulence and resistance to antibiotics compared to microorganisms not propagated inside amoebae. Therefore the occurrence of FLA in a hospital network presents a potential public health risk which should be monitored to limit the risk of nosocomial infection and disease outbreaks.

CONCLUSIONS

FLA belonging to the genera *Acanthamoeba* and *Vermamoeba* were isolated from 77 (79.4 %) of the samples analysed based on morphological caharacteristics. These FLA had potential to contain intracellular bacteria. From positive samples, the FLA *Acanthamoeba*, *Vermamoeba* and *Naegleria* were detected using 18S rRNA PCR and sequence analysis. *Vermamoeba* were the most abundant FLA followed by *Acanthamoeba* and then the only *Naegleria* species. Further sequencing of *Acanthamoeba* with genus-specific closely resembled the T20 pathogenic genotype. Therefore patients and healthcare workers might be exposed to pathogenic amoebae in the hospital water system.

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REFERENCES

Abedkhojasteh, H., Niyyati, M., Firoozeh Rahimi, F., Heidari, M., Shohreh Farnia, S. & Rezaeianr, M. 2013. First Report of *Hartmannella* keratitis in a Cosmetic Soft Contact Lens Wearer in Iran *Iranian J Parasitol.* **8** (3):481–485.

Anaissie, E.J., Penzak, S.R. & Dignani, C. 2002. The hospital water supply or a source of nosocomial infection; a plea for action. *Arch Intern Med*. **162**:1483-1492.

Ben Salah, I. & Drancourt, M. 2010. Surviving within the amoebal exocyst: the *Mycobacterium avium* complex paradigm. *BMC Microbiol*. **10**:99. doi: 10.1186/1471-2180-10–99.

Cabello-Vílchez, A.M., Mena, R., Zuñiga, J., Cermeño, P., Martín-Navarro, C.M., González, A.C., López-Arencibia, A., Reyes-Batlle, M., Piñero, J.E., Valladares, B. & Lorenzo-Morales. J. 2014. Endosymbiotic *Mycobacterium chelonae* in a *Vermamoeba vermiformis* strain isolated from the nasal mucosa of an HIV patient in Lima, Peru. *Exp Parasitol*. **145**:127–130.

Corsaro, D., Walochnik, J., Köhsler, M. & Rott, M.B. 2015. *Acanthamoeba* misidentification and multiple labels:redefining genotypes T16, T19, and T20 and proposal for *Acanthamoeba* micheli sp. nov. (genotype T19). *Parasitol Res.* DOI 10.1007/s00436-015-4445-8.

Coşkun, K.A.,Ozcelik,S.,Tutar, L., Eladi,N. & Tutar,Y. 2013. Isolation and identification of free-living amoebae from tap water in Sivas,Turkey. *Biomed Res Int.* **2013** (2013):675145.

Denoncourt, A.M., Paquet, V.E. & Charette, S.J. 2014. Potential role of bacteria packaging by protozoa in the persistence and transmission of pathogenic bacteria. *Front. Microbiol*. **5**:240. doi: 10.3389/fmicb.2014.0024.

Dillon, A. Undine E.H., Achilles-Day, Sim K. Singhrao, S.K.,Mark Pearce, M. & Morton, L.H.G. 2014. Biocide sensitivity of *Vermamoeba vermiformis* isolated from dental-unit-waterline systems. *Int Biodeter Biodegr*. **88**: 97–105.

Evstigneeva A, Raoult D, Karpachevskiy L & La Scola B. 2009. Amoeba co-culture of soil specimens recovered 33 different bacteria, including four new species and *Streptococcus pneumoniae*. *Microbiol.* **155** (2):657-664.

Fuerst, P.A., Booton, G.C. & Crary, M. 2015. Phylogenetic analysis and the evolution of the 18 S rRNA gene typing system of *Acanthamoeba*. *J Eukaryot Microbiol.* **62**:69–84.

Greub, G. & Raoul, D. 2004. Microorganisms resistant to free living amoebae. *Clin. Microbio Rev*, **17** (2):413–433.
Kennedy, S., Devine, P., Hurley, C., Ooi, Y-S. & Collum, L.M.T. 1995. Corneal infection associated with *Hartmannella vermiformis* in contact-lens wearer. *Lancet*. **346** (8975):637–638.

Lamoth, F.& Greub, G. 2010. Amoebal pathogens as emerging causal agents of pneumonia. *FEMS Microbiol Rev.* **34**:260–280.

Lasheras A., Boulestreau, H., Rogues, A-M, Ohayon-Courtes, C., Labadie, J-C. & Gachie, J-P. 2006. Influence of amoebae and physical and chemical characteristics of water on presence and proliferation of *Legionella* species in hospital water systems. *Am J Infect Control* 2006; **34:**520–525.

Lorenzo-Morales, J., Martín-Navarro, C.M., Enrique Martínez-Carretero, E., José E. Piñero, J.E. & Basilio Valladares. 2011. Encephalitis Due to Free Living Amoebae: An Emerging Issue in Human Health, Non-Flavivirus Encephalitis, Dr. Sergey Tkachev (Ed.), ISBN: 978-953-307-720-8, InTech, Available from: http://www.intechopen.com/books/non-flavivirus-encephalitis/encephalitis-due-to-free-living-amoebae-anemerging-issue-in-human-health

Ovrutsky, A. R., Chan, E. D., Kartalija, M., Bai, X., Jackson, M., Gibbs, S., Falkinham, J. O,. Iseman, M. D., Reynolds, P. R. & McDonnell, G. 2013. Cooccurrence of Free-Living Amoebae and Nontuberculous *Mycobacteria* in Hospital Water Networks, and Preferential Growth of *Mycobacterium avium* in *Acanthamoeba lenticulata*. *Appl and Environ Microbiol*. **79** (10):3185–3192.

Pickup, Z.L., Pickup, R. & Parry, J.D. 2007. Growth of *Acanthamoeba castellanii* and *Hartmannella vermiformis* on live, heat-killed and DTAF-stained bacterial prey. *FEMS Microbiol Ecol.* **61**: 264–272.

Rohr, U., Weber, S., Michel, R., Selenka, F. & Wilhelm, M. 1998. Comparison of free-living amoebae in hot water systems of hospitals with isolates from moist sanitary areas by identifying genera and determining temperature tolerance. *Appl Environ Microbiol.* **64**:1822–1824.

Schmitz-Esser, S., P. Tischler, R. Arnold, J. Montanaro, M. Wagner, T. Rattei & M. Horn. 2010. The genome of the amoeba symbiont "*Candidatus Amoebophilus asiaticus*" reveals common mechanisms for host cell interaction among amoeba-associated bacteria. *J Bacteriol*. **192** (4): 1045–1057.

Schroeder, J.M., Booton, G.C., Hay, J., Niszl, I.A., Seal, D.V., Markus, M.B., Fuerst, P.A., Byers, T.J. 2001. Use of subgenic 18S ribosomal DNA PCR and sequencing for genus and genotype identification of Acanthamoebae from humans with keratitis and from sewage sludge. *J. Clin. Microbiol*. **39**: 1903–1911.

Storey, M.V., Winiecka-KrusnellI, J., Ashbolt, N.J. &. Stenstorm, T.A. 2004. The efficacy of heat and chlorine treatment against thermotolerant *Acanthamoebae* and *Legionellae. Scand J Infect Dis.* **36**: 656–662.

Thomas, V., Herrera-Rimann, K., Blannci, D.S. & Greub, G. 2006. Biodiversity of amoebae and amoeba-resisting bacteria in a hospital water network. *Appl Environ Microbiol*. **72**:2428–2438.

Thomas, J.M. & Ashbolt, N.J., 2011. Do free-living amoebae in treated drinking waters ystems present an emerging health risk? *Environ. Sci. Technol*. **45**: 860–869.

Trabelsi, H., Dendana, F., Sellami, A., Sellami, H., Cheikhrouhou, F., Neji, S.,. Makni, F. & Ayadi, A. 2012. Pathogenic free-living amoebae: Epidemiology and clinical review. *Pathologie Biologie.* **60**:399–405.

Visvesvara, G.S., Moura, H. & Shuster, F.L. 2007. Pathogenic and opportunistic free-living amoebae: *Acanthamoeba spp., Balamuthia mandrillaris, Naegleria fowleri* and *Sappinia diploidea*. *FEMS Immunol Med Microbiol*. **50**:1–26.

Visvesvara, G.S. 2013. Infections with free-living amoebae. *Handbook of Clinical Neurology*. **114** (3): 153-168.