AN INVESTIGATION INTO THE PRESENCE OF FREE LIVING AMOEBAE AND AMOEBA RESISTANT BACTERIA IN DRINKING WATER DISTRIBUTION SYSTEMS OF HEALTH CARE INSTITUTIONS IN JOHANNESBURG, SOUTH AFRICA

Report to the
Water Research Commission

by

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WRC Report No. 2138/1/16
ISBN 978-1-4312-0764-0

April 2016

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EXECUTIVE SUMMARY

BACKGROUND

Contamination of hospital water with potentially pathogenic microorganisms is often reported in scientific literature. A wide range of bacteria, viruses, fungi and protozoa may be present in the water supply and may cause a substantial increase in the risk of nosocomial infections among patients and in medical personnel. In many instances the etiologic agents of disease or the source of infection are not identified. Free living amoebae (FLA) are ubiquitous in natural and man-made water systems. Certain FLA, including *Acanthamoeba* and *Balamuthia* species, are known human pathogens and are carriers of amoeba resistant bacteria (ARB) known to cause serious, potentially fatal infectious diseases. Members of the genera *Legionella*, *Mycobacterium*, *Vibrio*, *Staphylococcus*, *Pseudomonas* and others are known ARB often implicated in nosocomial infections. Certain non-pathogenic FLA, for example *Vermamoeba vermiformis*, are also known carriers of pathogenic ARB. The importance of drinking water quality supplied to and distributed to public health care facilities, has not been studied previously. This study attempted to provide a general overview on the presence of these organisms within water distribution systems of three public health care institutions in Johannesburg, South Africa, and also briefly highlight the potential human health risk implications.

AIMS AND OBJECTIVES

The project was aimed at determining the presence of free living amoebae and amoeba resistant bacteria in water and biofilm samples at selected high risk areas within the facilities of three hospitals, as determined during walkthrough assessment by the study team and infection control staff at the hospitals. The study objectives were to:

- Obtain permission and access to three large health care institutions in Gauteng
- Perform walkthrough assessments to establish high risk areas of infection by waterborne microorganisms
- Determine which drinking water production plants supply these institutions and obtain information on the production and treatment processes employed at these plants
- Test the water effluent from the production plants as well as the drinking water in high risk areas of the hospitals for the presence of FLA and ARB
- Provide a comprehensive report of the findings to inform collaborating institutions of the occurrence of FLA and ARB in their water distribution systems
- Recommend water treatment strategies to improve water quality if necessary
- Publish the findings in peer reviewed journals and present at national and international conferences

METHOD

This was an observational descriptive study of the distribution and diversity of free living amoebae and potentially pathogenic amoeba resistant bacteria in the water distribution systems of three public hospitals in Johannesburg. Walkthrough assessments of the water distribution systems of the three hospitals were conducted. This was then used to determine a sampling strategy and a systematic random sampling technique was used. On the basis of the number of high risk areas identified during the assessments, a pilot study was conducted.

Water and biofilm samples were collected according to the predetermined sampling strategy and processed within 24 hours of collection. Water temperature, pH, total dissolved solids and residual chlorine levels were determined at the time of sampling. The water samples were also tested for the presence of microbial indicators using standard methodology. All the water and biofilm samples were tested for the presence of free living amoebae using an amoebal enrichment technique. Autochthonous amoebae were then purified

and examined for the presence of intracellular bacteria by light- and inverted microscopy and Gimenez staining. The purified amoebae were ruptured to free the intracellular bacteria, which were then further identified using conventional culture and staining and molecular methods. The free living amoebae were identified by PCR and sequencing. The bacteria were identified using the Vitek® system. *Legionella* species were confirmed by PCR.

RESULTS

The physicochemical parameters of the water samples conformed to the South African Standard for Drinking Water Quality (SANS 241:2015) except at the reverse osmosis plants feeding the renal units of Hospital A and Hospital C where the pH ranged from very low to normal. The microbial quality also conformed to the SANS 241:2015; no coliforms, faecal coliforms or *E. coli* were present in any of the water samples. Free living amoebae were isolated from water and biofilm samples collected at all the sampling areas except the municipal water inlet of Hospital C and the milk room of Hospital B. *Vermamoeba vermiformis* was isolated from the majority of samples from all three hospitals. *Acanthamoeba*, *Naegleria* and *Amoebozoa* species, as well as *Pseudoparamoeba pagei* and *Schizoplasmodiopsis amoeboideae* were also isolated in smaller numbers.

Thirty eight bacterial species belonging to 29 genera were identified from samples that were positive for FLA. The most representative isolates from Hospital A belonged to the genera *Pseudomonas*, *Staphylococcus*, *Moraxella* and *Brevundimonas*. The most representative species were *Aeromonas salmonicida*, *Sphingomonas paucimobilis*, *Delftia acidovorans* and *Comamonas testosteroni*. In Hospital B, *Serratia marcescens* and *Stenotrophomonas maltophilia* were the most representative bacterial species isolated and in Hospital C, *Serratia marcescens, Stenotrophomonas maltophilia* and *Delftia acidovorans*. *Vermamoeba vermiformis* (previously known as *Hartmanella vermiformis*) is not a human pathogen per se but serves as a host for a number of amoeba resistant bacteria and yeasts.

CONCLUSION

Health care institutions use municipal water which is tested for certain indicator organisms before leaving the treatment plant, but not again upon entering the facility or at any point after entry. This study reported the presence of potentially pathogenic free living amoebae and amoeba resistant pathogens in the vast majority of drinking water and biofilm samples collected from the municipal water inlet points and high risk areas of the three hospitals. FLA and ARB were also present in the water and biofilm samples collected after reverse osmosis, which is an additional treatment method used at the renal units of two of the hospitals.

RECOMMENDATIONS

The results obtained suggest that drinking water supplied to and distributed through hospitals and used for patient treatment should be considered as a possible source of nosocomial and occupational infections. For this reason, it is necessary to establish a routine water quality monitoring programme including monitoring for the presence of FLA should amoebae be isolated. Biofilm as well as water should be tested as free living amoebae are more prevalent in biofilm than water samples. In cases of nosocomial infection the water distribution system should be considered as a possible route of infection. Information and training on the importance of the quality of the water used for patient care should be provided to hospital staff.

OUTPUTS FROM THE PROJECT

So far two conference presentations, two peer reviewed articles and several presentations at scientific meetings emanated from the project.

Presentations at conferences and scientific meetings

1. Muchesa, P., Barnard, T.G., Bartie, C. (2014). Free-living amoebae isolated from a hospital water system in South Africa: A potential source of nosocomial and occupational infection. Platform presentation at the IWA 7th International Young Water Professionals Conference, Taiwan, 5-12 December.

2. Bartie, C. (2015). Attendance and discussions at the 16th Free Living Amoeba Meeting, Alghero, Italy, 18-22 May.

3. Muchesa, P., Leifels, M., Jurzik, L., Barnard, T.G., Bartie, C. (2015). Free-living amoebae isolated from a hospital water system in South Africa: A potential source of nosocomial and occupational infection. Platform presentation at the NIOH Research Day, Johannesburg, South Africa, 3 September.

4. Muchesa, P., Leifels, M., Jurzik, L., Barnard, T.G., Bartie, C. (2015). Potentially pathogenic free-living amoebae isolated from a South African hospital water network. Platform presentation at the 4th Young Water Professionals – South Africa Biennial Conference and First Africa-wide YWP Conference, South Africa, 16-18 November.

Journal articles

1. Muchesa, P., Barnard, T.G., Bartie, C. (2015). The prevalence of free-living amoebae in a South African hospital water distribution system. *South African Journal of Science.* http://dx.doi.org/10.17159/sajs.2015/20140278
2. Muchesa, P., Leifels, M., Jurzik, L., Barnard, T.G., Bartie, C. (2015). Free-living amoebae isolated from a hospital water system in South Africa: A potential source of nosocomial and occupational infection. *Water Science & Technology: Water Supply*. doi:10.2166/ws.2015.106, in press.

ACKNOWLEDGEMENTS

The project team wishes to thank the following people for their contributions to the project.

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ACRONYMS & ABBREVIATIONS

AK	Acanthamoeba keratitis
ARB	Amoeba resistant bacteria
BAE	Balamuthia amoebic encephalitis
CA	Cutaneous acanthamoebiasis
CFU	Colony forming units
CFU/mL	Colony forming units per millilitre
CNS	Central nervous system
CSSU	Central sterilization service unit
EPA	Environmental Protection Agency
FLA	Free living amoebae
GAC	Granulated activated carbon
GAE	Granulomatous amoebic encephalitis
GU/L	Genomin units per litre
HAI	Healthcare associated infection
HCI	Healthcare institution
ICU	Intensive care unit
MPN	Most probable number
MRSA	Methicillin resistant *Staphylococcus aureus*
NI	Nosocomial infection
NIPCPS	National Infection Prevention and Control Policy and Strategy
NJ	Neighbour joining analysis
NTM	Nontuberculous mycobacteria
PAM	Primary amoebic meningoencephalitis
PCR	Polymerase chain reaction
PHI	Public healthcare institution
POU	Point of use
RO	Reverse osmosis
SANS	South African National Standard
SARS	Severe acute respiratory syndrome
TDS	Total dissolved solids
TOC	Total organic carbon

TSSU	Theatre sterilization service unit
UTI	Urinary tract infection
UV	Ultraviolet
VAP	Ventilator associated pneumonia
VBNC	Viable but non-culturable

CHAPTER 1: BACKGROUND

1.1 INTRODUCTION

Providing drinking water of good microbial quality requires adequate treatment and management of water distribution systems, particularly in health care institutions (HCIs) where immuno-compromised patients are housed. Health care institutions in Johannesburg, South Africa, use municipal drinking water supplied from water treatment plants situated around the city. The water quality is determined against the South African National Standard for the Quality of Drinking Water (SANS 241:2015), which requires monitoring for certain indicator organisms, chemical quality and physico-chemical parameters. It is not routine to test the water again upon entry into buildings and during distribution through health care facilities. Biofilm and sediment are also not routinely tested for indicator or any other microorganisms before or during distribution.

Sub-Saharan Africa has a high prevalence of HIV/AIDS and tuberculosis. In 2007, Connelly and coworkers reported that the overall prevalence of HIV (CD4 counts < 350 cells/μL) in 2032 hospital and support staff members employed by two large public hospitals in Gauteng, who were exposed to patients daily, was 11.5%. These immuno-compromised patients and workers become targets for infection by microorganisms that may not be pathogenic to healthy individuals. This reduces their productivity and overall work experience, and increases labour turnover and costs for public sector health budgets significantly. Healthcare associated infection (HAI) is an important cause of morbidity and mortality but in many cases, the etiologic agent cannot be identified. Approximately 14% of hospital patients in South Africa are at high risk of developing at least one HAI during a hospital stay compared to < 5% in developed countries (Evidence Medical, 2015). This increases the length and cost of hospitalization substantially.

The study team thus identified a need to study the occurrence of FLA and ARB in the drinking water supplied to health care facilities and throughout their distribution systems. The potential benefits of the study are threefold: (i) assisting water quality managers to obtain important information on points in the distribution systems of their facilities where the potential health risk of nosocomial infection is high; (ii) assisting in the monitoring of drinking water quality in health care institutions in South Africa where large number of immuno-compromised individuals and health care workers use tap water; and (iii) assisting in the development and implementation of efficient water treatment programs specific to FLA and ARB in these institutions.

1.2 PROJECT AIMS

The overall objectives of the project were to establish the occurrence of free living amoebae and amoeba resistant bacteria within drinking water distribution system in health care facilities in Johannesburg and also highlight the potential human health risk implication thereof. In addition, the aim is to use this information to

assist the institutions under study to establish appropriate water quality management programs in areas where patients and personnel are at risk of nosocomial infection caused by these microorganisms.

The aims of the study were:

- To obtain permission and access to three large health care institutions in Gauteng
- To perform walkthrough assessments to establish high risk areas of infection by waterborne microorganisms
- To determine which drinking water production plants supply these institutions and obtain information on the production and treatment processes employed at these plants
- To test the water effluent from the production plants as well as the drinking water in high risk areas of the hospitals for the presence of FLA and ARB
- To provide a comprehensive report of the findings to inform collaborating institutions of the occurrence of FLA and ARB in their water distribution systems
- To recommend water treatment strategies to improve water quality if necessary
- To publish the findings in peer reviewed journals and present at national and international conferences

1.3 SCOPE AND LIMITATIONS

This project was designed to provide information with regards to the presence and possible impact of FLA and ARB in the drinking water of the institutions under study and the potential health implications thereof. The scope of the study did not cover the implementation of appropriate water treatment processes to remediate any of the observed water quality deficiencies in the water distribution systems, but did include the provision of feedback and reports to all concerned.

1.4 ETHICAL AND LEGAL CONSIDERATIONS

Permission for sampling in the areas of the institutions included in the study was obtained from senior management from each of the health care institutions under study. All the sampling was conducted in collaboration with occupational health and infection control staff at the respective hospitals. Sampling records and results will also be kept at the National Institute for Occupational Health and in the Water and Health Research Centre, University of Johannesburg for future research. All co-workers were requested to sign a confidentiality agreement as required by the National Institute for Occupational Health (NIOH), affiliated to the National Health Laboratory Services (NHLS) and the Water Research Commission (WRC).

CHAPTER 2: LITERATURE REVIEW

2.1 INTRODUCTION

Free living amoebae (FLA) are ubiquitous in ground and surface waters used for drinking water production and are common inhabitants of various modern man-made water systems like cooling towers, swimming pools, hospital water networks, wastewater and drinking water plants (Greub and Raoult, 2004; Loret et al., 2008; Pagnier et al., 2009). They feed on smaller microorganisms like bacteria, fungi and algae. Although mostly non-pathogenic, some FLA, particularly *Acanthamoeba* and *Balamuthia* species and *Naegleria fowlerii* are known human pathogens which may cause life threatening disease in both healthy and immuno-compromised individuals. Most FLA have two developmental stages, an active trophozoite during favourable conditions, and a dormant cyst stage during which it is resistant to disinfection and other unfavourable environmental conditions (Visvesvara et al., 2007; Zeybek et al., 2010). FLA can survive in this dormant stage for long periods of time, only to excyst and become active again when conditions return to normal.

FLA feed by phagocytosis and digest their prey in phagolysosomes. However many bacteria, including well-known, newly emerging and re-emerging pathogens, have evolved to resist this process of phagocytosis in various ways. These amoeba resistant bacteria (ARB) are known to survive inside amoebal cysts. Some species continue to proliferate intracellularly until they reach sufficient numbers to lyse the amoebal host cell when it returns to the trophozoite stage. This cycle can continue indefinitely; the ARB may infect new amoebal hosts, and/or may spread through water distribution systems and cause infection in humans. Intra-amoebal replication is known to increase the virulence of some ARB, especially *Legionella pneumophila* and *Mycobacterium avium* (Cirillo et al., 1994; 1997). There are currently well over 100 known ARB, including members of the *Legionellaceae*, *Mycobacteriaceae*, *Enterobacteriaceae*, *Vibrionaceae*, *Chlamydiaceae*, methicillin-resistant *Staphylococcus aureus* and many others (Greub and Raoult, 2004; Thomas et al., 2010; Winiecka-Krusnell and Linder, 2010).

International studies continue to highlight the potential of FLA containing ARB to survive routine drinking water production and treatment processes. Studies conducted in Germany, France and Spain indicated that FLA withstand filtration and clarification processes (Hoffman and Michel, 2001; Loret et al., 2008; Thomas et al., 2008; Corsaro et al., 2009) and multiply in sand and granulated activated carbon (GAC) filters (Thomas et al., 2008). It was also recently shown that pathogenic ARB such as *Legionella, Chlamydia* and *Mycobacteria* can survive the drinking water treatment process and proliferate in the water distribution systems of health care facilities. Treatment with biocides (mainly chlorine), ultraviolet light, heat and the use of point of use (POU) filtration have shown variable success rates in the elimination of FLA in these water systems (Thomas et al., 2010). A study recently conducted at the National Institute for Occupational Health (NIOH) and the University of Johannesburg (UJ) isolated FLA potentially containing coliforms, *E. coli*, *M. avium*, *L. pneumophila* and *V. cholerae* from > 90% of drinking water samples collected from four residential

areas in Johannesburg (publication in preparation). These samples all conformed to the South African Drinking Water Quality Standard (SANS 241:2015) when tested using conventional techniques. These results are in agreement with studies by Hoffman and Michel (2001) and Loret and Greub (2010), who both reported an increase in the prevalence of FLA in drinking water at increasing distances from drinking water treatment plants. The question now arises whether the drinking water supplied to health care institutions in Gauteng contain FLA and potentially also ARB, and whether these organisms enter and are distributed through the water distribution systems of these institutions. If this is the case, patients and staff may be at increased risk of contracting nosocomial infections, especially given the high incidence of HIV/AIDS, tuberculosis and other immuno-compromising conditions in South Africa.

2.2 PUBLIC HEALTH CARE IN SOUTH AFRICA

Public health care institutions (PHCIs) in South Africa house large numbers of immunocompromised patients under conditions that are often far from ideal. The facilities are mostly old, poorly maintained and were not initially designed with infection control in mind. Overcrowding, shortages of well-trained medical personnel, constant equipment breakdowns, water and electricity shortages and funding restrictions are experienced at the majority of PHCIs throughout the country (Dusé, 2005; Hlope et al*.,* 2014; Nejad et al*.,* 2011). Poor quality municipal water delivered to and distributed through the facility may increase risk of waterborne nosocomial infections in patients and staff substantially. Regular monitoring of microbial water quality in health care institutions is thus essential not only for the wellbeing of patients and staff but also for members of the public who visit these institutions. Infection control strategies in PHCIs are often not up to standard due to financial constraints (Dusé, 2005; Nejad et al*.,* 2011). This increases the risk of waterborne infection in patients and decreases the capacity of health care workers to implement sufficient isolation and infection control procedures. Other challenges are the lack of isolation units, insufficient hand basins and taps, wrongly designed taps and a lack of waste management facilities. This is of vital importance, especially in South Africa, given the high incidence of HIV/AIDS and tuberculosis in the general population.

2.2.1 Hospital-acquired (nosocomial) infections

Health care facilities in Johannesburg use municipal drinking water supplied from treatment plants and reservoirs situated around the city. The water enters the facility at a central point from where it is distributed through a complicated plumbing system. In very old buildings like those included in this study, the layout of the distribution system was not mapped out at the time of commissioning hence it is nearly impossible to determine all the potential high risk areas for water contamination in the system. Nevertheless, it is clear from the literature that hospital water distribution systems are a potential source of nosocomial infections. Municipal water is tested for indicator organisms (mainly coliforms and *E.coli*) and disinfected at the treatment plant. It then travels through a system of pipes before reaching the facility. These pipes may contain biofilms which harbour microorganisms and thus increase the likelihood of contamination by providing nutrients for a host of potentially pathogenic microorganisms not included in traditional drinking water quality monitoring regimes and decreases the quality of the drinking water when it reaches the point of

entry to the facility. Hospital water distribution systems are ideal environments for microorganisms to proliferate and spread to patients and staff. Factors like the age and corrosion level of storage tanks, poor water system design, water stagnation and dislodging of biofilms from pipe surfaces in the distribution system further decrease the quality of water reaching patients and staff through sources including taps, showers, contaminated medical equipment and contamination of surfaces cleaned with tap water. As a result, waterborne nosocomial infections have are often been reported internationally (Anaissie et al*.,* 2002; Tai et al*.,* 2012; Lee and Greig, 2013).

Hospital acquired (nosocomial) infections (HAIs) is a major global safety concern for patients as well as healthcare professionals. HAIs can be defined as infections, often caused by multiresistant pathogens, occurring in patients during the process of care in a hospital or other healthcare facility, that were not diagnosed or incubating at the time of admission (Nejad et al*.,* 2011). This definition includes infections acquired in hospital but appearing after discharge and also occupational infections in healthcare personnel (McQuoid-Mason, 2012). In most instances, infections diagnosed after 48 hours of hospitalization are considered to be HAIs. Outbreaks of HAIs are often reported but in many instances the etiologic agents are not isolated and identified. Feldman (2005) published a useful overview of the causes of HAI and provided a list of microorganisms considered to be important causes of nosocomial pneumonia (Table 2.1). In many instances the causative agents grow poorly or not at all on laboratory media due to their fastidious nature and preference for intracellular growth conditions. The majority of amoeba resistant bacteria fall into this category.

Table 2.1: Common causes of nosocomial pneumonia (Feldman, 2005)

Microorganisms	*Approximate frequency*
Gram negative bacilli · *Pseudomonas aeroginosa* · *Acenitobacter species* · *Klebsiella species* · *Enterobacter species* · *Proteus species* · *Escherichia coli* · *Serratia species* · *Haemophilus influenzae*	40-75%
Gram positive cocci · *Staphylococcus species* · *Streptococcus pneumoniae* · *Enterococcus faecalis*	5-30%
Anaerobes	1-5%
Fungi · *Candida* species · *Aspergillus* species	1-5%
Other · *Legionella* species · *Moraxella* species	0-5%

Populations at risk in health care institutions include organ transplant patients (Zhou et al., 2013), patients undergoing renal dialysis (Chertow, 2000; Hassan et al., 2012), patients with chronic lung disease (Sabria and Campins, 2003) and neonates with immature immune systems (Vochem and Doring, 2001; Lee and Greig, 2013). Inhaled amoebae may serve as co-factors in the pathogenesis of pulmonary disease induced by inhaled respiratory pathogens (Brieland et al., 1997). HAIs increase the morbidity, mortality, length of hospital stay and health care costs significantly. According to the World Health Organization (WHO), 7% of patients in developed countries and 10% of patients in developing countries develop at least one HAI at any given time. In high-income countries approximately 30% of patients in intensive care units (ICUs) contract at least one HAI during hospitalisation. However in low- to middle income countries the frequency of ICU-acquired HAIs is at least 2-3 times higher (Nejad et al., 2011).

The 2015 Evidence Medical factsheet (http://www.evidencemedical.co.za) states that approximately 1.1 million South Africans contract nosocomial infections in private health care institutions annually, resulting in a 5-30 day increase in hospital stay at an average cost of R57 000, excluding indirect costs related to blood testing, x-rays and cultures, per infection. This figure is likely to be much higher if nosocomial infections in the public health care sector are taken into consideration. In an article in The Star newspaper, dated 13 August 2015, it was stated that the number of patients contracting HAIs in Gauteng hospitals increased fourfold within a space of three years, from 236 recorded cases in 2012 to 975 in 2014. During 2014, 294 cases of HAI were caused by *Klebsiella pneumoniae* and 226 cases by *Acenitobacter baumannii* (Mkize, The Star Early Edition, August 13, 2015).

2.2.2 Legislation, Policies and Guidelines for public health care institutions

Health care institutions may be held liable to patients who acquire HAIs through negligence by management and employees of the institution, even if best practices were introduced at the facility (McQuoid-Mason, 2012). However waterborne HAIs are often not considered when developing best practice guidelines. According to Anaissie et al. (2001), microbiologically contaminated drinking water is a well-known cause of community-acquired pneumonia (CAP) and although guidelines for the prevention of CAP have been established worldwide, similar guidelines for the prevention of nosocomial infections are scarce.

Locally, the National Infection Prevention and Control Policy and Strategy (NIPCPS) of the South African Department of Health (April 2007) was developed to address the increasing difficulties experienced worldwide to improve the safety and efficacy of health care and on strategies to reduce the risks to patients. The NIPCPS states: “The devastating effect international outbreaks, such as the SARS, Marburg hemorrhagic fever and the numerous *Klebsiella pneumoniae* outbreaks in neonatal units have had in terms of morbidity and mortality and the accompanying financial burden placed on individuals, families and society at large, has served as a reminder of the need for urgent action to ensure efficient management in infectious diseases. Furthermore, the several outbreaks in neonatal units have also highlighted the need for effective environmental cleaning”. The objectives of the NIPCPS were thus to encourage and improve effective prevention and management of health care associated infections for the public health care sector, to prevent

and minimize environmental hazards associated with microbes for all in- and outpatients, health care workers and visitors to health care institutions, to optimize infection prevention and control programmes and resources in health care settings, to control and minimize transmission of and colonization by resistant organisms and to improve infection control surveillance. The NIPCPS recommends certain actions to achieve this, but the recommendations do not include the routine monitoring and general maintenance of the drinking water quality distributed through healthcare facilities.

2.3 FREE-LIVING AMOEBAE

Free-living amoebae (FLA) are unicellular, heterotrophic protozoa that feed on bacteria, fungi, algae, viruses and other protozoa to contribute to nutrient recycling in the environment. FLA are ubiquitous in air, soil and water where their proliferation and diversity depend on temperature, pH, moisture and the availability of nutrients (Rodriguez-Zaragoza, 1994; Thomas et al., 2006). In aquatic environments, FLA are found in natural environments such as lakes and hot springs (Gianiazzi et al., 2010; Loret and Greub, 2010) and in man-made water systems including tap water (Bonilla-Lemus et al., 2010; Edagawa et al., 2009), cooling towers, swimming pools (Barbaree et al., 1986; Caumo et al., 2009), drinking water plants (Thomas et al., 2008) and hospital water networks (Ovrutsky et al., 2013; Thomas et al., 2006) where human exposure is common. Although mostly non-pathogenic, some genera or species of FLA such as *Acanthamoeba* spp., *Naegleria fowleri*, *Balamuthia mandrillaris* and *Sappinia* species cause opportunistic and non-opportunistic infections in humans. Table 2.2 summarizes the most common environmental source and human health risks associated with pathogenic FLA.

Table 2.2: Opportunistic and pathogenic FLA (Adapted from Visvesvara et al. (2007)

FLA	Life cycle	Disease	Groups at risk	Environmental source
Acanthamoeba species	Two stages: trophozoite and cyst	Granulomatous amebic encephalitis, Amoebic keratitis; cutaneous acanthamoebiasis	Immuno-competent (for keratitis and Immuno-compromised (for encephalitis)	Soil, air, lakes and hospital water (taps, hydrotherapy pools, cooling towers)
Naegleria fowleri	Three stages: trophozoite, cyst and flagellate	Primary amoebic meningoencephalitis	Immuno-competent children and young adults	Lakes, hot springs, rivers, swimming pools
Balamuthia mandrillaris	Two stages: trophozoite and cyst	*Balamuthia* amoebic encephalitis	Immuno-competent children or elderly and immuno-compromised individuals	Soil, air and stagnant recreational water
Sappinia species	Two stages: trophozoite and cyst	*Sapinia* amoebic encephalitis	Isolated once from an immuno-competent adult	Soil, rivers, herbivore faeces

2.3.1 Pathogenic free living amoebae and associated human health implications

2.3.1.1 *Acanthamoeba* species

Acanthamoeba species are ubiquitous in natural environments including soil, air and water. In aquatic environments, they occur naturally in rivers and marine water, fresh- and salt water lakes, and are often present in the treated water used in hospitals, dental units and dialysis units (Schuster and Visvesvara, 2004; Thomas et al., 2006; Coskun et al., 2013). *Acanthamoeba* can survive temperatures ranges of 12ºC to 45ºC and the majority of pathogenic species can grow at 37ºC. This explains why they have been recovered from skin lesions, lung tissues, human nasal cavities, cerebrospinal fluid (CSF) and brain necropsies (Khan, 2003; Marciano-Cabral and Cabral, 2003; Schuster and Visvesvara, 2004).

Acanthamoeba species have two developmental stages: a metabolically active trophozoite stage and a dormant cyst stage. *Acanthamoeba* trophozoites (8-40 µm in size) have a single nucleus and contain the typical cellular organelles – Golgi complex, smooth and rough endoplasmic reticulum, free ribosomes, digestive vacuoles as well as finger-like projections of the cell membrane (acanthapodia) which provide adhesion to surfaces, facilitate cellular movements and capture prey. Trophozoites actively feed through phagocytosis and pinocytosis on bacteria, fungi, viruses, yeast, algae and small organic particles in the environment and reproduces by binary fission (Gonzalez-Robles et al., 2001; Marciano-Cabral and Cabral, 2003; Khan, 2006). Adverse environmental conditions such as extremes of pH and temperature, increased osmolarity and lack of nutrients cause trophozoites to encyst into inactive but viable cysts.

The cyst (10-25 µm in size) has a single nucleus and a double-walled structure which consists of an outer wall (ectocyst) and an inner wall (endocyst). The ectocyst is usually thin, wrinked and made up of protein and lipid whereas the endocyst usually thick is star-shaped, hexagonal, polygonal, round or oval and made up of cellulose with two or more pores, known as astioles, which monitor environmental conditions. *Acanthamoeba* cysts are resistant to environmental pressures that includes desiccation, drying, freezing to -20ºC, biocides and can survive up to 20 years in vitro. Cysts revert to trophozoites (excyst) when environmental conditions are favourable again (Akzozek et al., 2002; Visvesvara et al., 2007; Sriram et al., 2008; Fouque et al., 2012).

The majority of *Acanthamoeba* species, including but not limited to *A. polyphaga, A. castellanii, A. culberstoni, A. hatchetii, A. healyi* and *A. divionesis* are associated with infection in immunocompromised and immunocompetent individuals (Schuster and Visvesvara, 2004; Sheng et al., 2009; Trabelsi et al., 2012). The most common infections are granulomatous amoebic encephalitis, *Acanthamoeba* keratitis and cutaneous acanthamoebiasis. Granulomatous amoebic encephalitis (GAE) affects the central nervous system (CNS) of immunocompromised individuals (Visvesvara et al. 2007). Individuals in health care environments with suppressed immunity, such as chemotherapy patients and transplant recipients, are especially vulnerable to GAE. For example, a fatal case of *Acanthamoeba lenticulata* GAE in a heart transplant patient has been reported in France (Barete et al*.,* 2007). Approximately 150 cases of GAE have been recorded globally to date and over 50 of these were found in AIDS patients (Martinez and Visvesvara,

2001; Trabelsi et al., 2012). Cases of GAE have also been reported in previously healthy individuals (Visvesvara et al. 2007; Sheng et al., 2009). The amoebae enter the central nervous system through the lower respiratory tract or skin breaks, reaching the CNS through blood vessels (Khan, 2006). Clinical symptoms of GAE include fever, stiff neck, headache, seizures, dizziness meningitis, lethargy, visual disturbances, vomiting, coma and visual abnormalities. GAE in the later stage may lead to seizures, coma and death if not diagnosed and treated properly (Environmental Protection Agency, 2003; Visvesvara and Maguire, 2006; Khan, 2006).

Acanthamoeba keratitis (AK) is a painful, vision threatening corneal infection that may cause blindness in healthy individuals following corneal abrasions and/or corneal trauma due to injury. AK is often in individuals who wear contact lenses (Chong and Dana, 2007; Visvesvara et al., 2007). Improper maintenance and poor sanitary conditions, like rinsing and storing of lenses in tap water or non-sterile saline solutions and swimming in contaminated water increase the risk of AK in these individuals (Seal, 2003; Trabelsi et al., 2012). Although the incidence of AK in contact lens wearers in the USA (0.15 / million) and the UK (1.4 / million) is high (Cheng et al., 2009; Dart et al., 2009) the incidence of AK in South Africa and other African countries is unknown. Symptoms include eye pain, redness and tearing, epiphora and photophobia which may develop into corneal epithelium ulceration and blindness if not diagnosed and treated timely (Wilhelmus et al., 2008; Szaflik et al., 2012; Trabelsi et al., 2012).

Cutaneous acanthamoebiasis (CA) is a potentially fatal infection which occurs mainly in immunocompromised individuals, particularly in HIV infected patients, but has been reported in healthy individuals (Visvesvara et al., 2007). CA is characterized by lesions, pustules, papules and skin ulcerations. The route of exposure is the skin, before entry into the blood stream from where it spreads to tissue and the central nervous system (CNS). CA may be fatal if not diagnosed and treated within two weeks from onset of symptoms (Torno et al., 2000; Marciano-Cabral and Cabral, 2003).

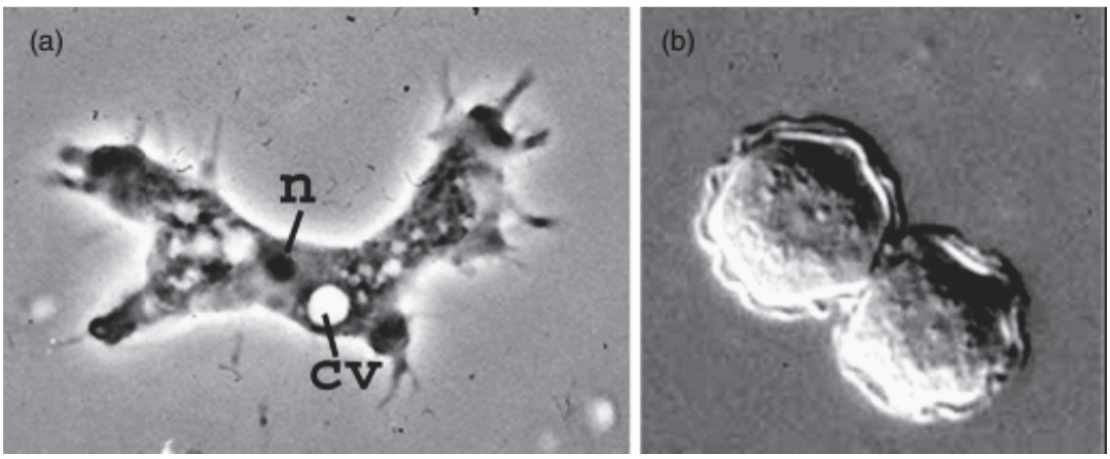

Figure 2.1: *Acanthamoeba castellanii*, trophozoite; (b) cysts; n: nucleus; CV: contractile vacuole. Both images at X1000 (Visvesvara et al., 2007).

2.3.1.2 Naegleria fowleri

Naegleria fowleri is a thermophilic amoebo-flagellate often found in lakes, fresh water, swimming pools, aquaria, sewage, irrigation canals, ponds, hot springs, thermally polluted streams and rivers (Lares-Villa and Hernández-Peña, 2010; Schuster and Visvesvara, 2004;). In humans, *N. fowleri* has been isolated from healthy children and the nasal passages of immunocompromised adults (John and John, 1989; Kemble et al*.,* 2012). Although *N. fowleri* can grow abundantly at temperatures above 30ºC and tolerate temperatures of up to 46ºC it is more sensitive to extreme pH and the elevated levels of osmolarity than *Acanthamoeba* species (Martinez et al., 1997; De Jonckheere, 2012). There are three stages in the life cycle of *Naegleria* species: the trophozoite (10-25 µm), the transitory flagellate stage (10-16 µm) and the cyst stage (8-20 µm) (Figure 2.2a). The trophozoite has a single nucleus with a prominent nucleolus (n), with anteriorly hemispherical bulges called lobopodia and reproduces by binary fission. Changes in the ionic concentrations of the environment transform the trophozoites into the temporary flagellate stage that revert back to the trophozoite stage when conditions are favourable (Visvesvara et al., 2007). The flagellate has two or three flagella and a single nucleus with no cytostome (Figure 2.2b) *Naegleria* cysts are generally smooth and spherical in shape, and contain a thick endocyst and a thin ectocyst in their double-walled structure with fine pores (Figure 2.2c). Like the trophozoite, both the flagellate and the cyst stages contain a single vesicular nucleus with a prominent nucleolus (Schuster and Visvesvara, 2004; Visvesvara, 2013).

The genus *Naegleria* contains over 40 species of which only *N. fowleri* is a known human pathogen that causes primary amoeboic meningoencephalitis (PAM) in immunocompetent individuals (Visvesvara and Maguire, 2006). PAM is a rapidly fatal disease of the central nervous system which occurs in healthy children or young adults with a recent history of contact with naturally warm or thermally polluted waters. *N. fowleri* enters the body through the nasal passages where it attaches to the nasal mucosa from where it migrates to the brain, where it divides rapidly causing death within a week to 10 days following exposure (Didier et al., 2009; Rojas-Hernández et al., 2004; Visvesvara and Maguire, 2006). Initial symptoms include nausea, vomiting, stiff neck, behavioral abnormalities, fever and headache. However, these symptoms are not distinctive and have led to misdiagnosis of PAM, usually mistakenly them for bacterial meningitis. Diagnosis in most cases has only been done at autopsy (Guarner et al., 2007; Visvesvara, 2007).

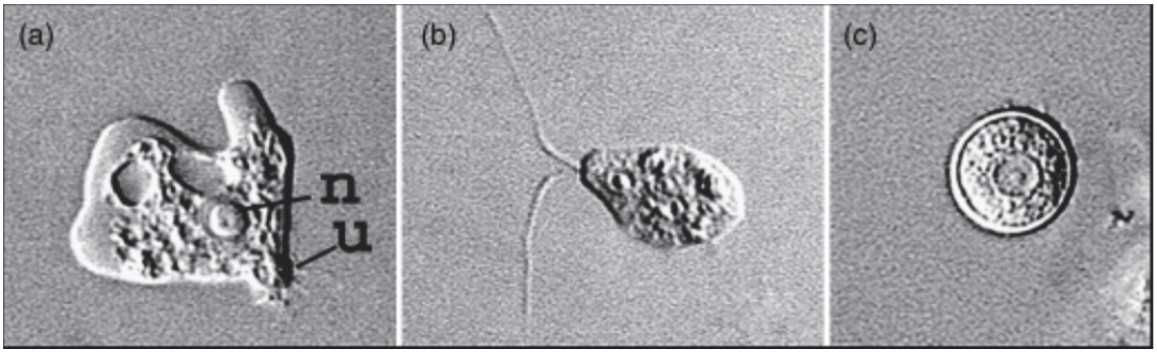

Figure 2.2: *Naegleria fowleri* (a) trophozoite, (b) a flagellate, and (c) a cyst. All images at X1000 (Visvesvara et al., 2007).

2.3.1.3 *Balamuthia mandrillaris*

Balamuthia mandrillaris is the only known pathogenic species of the genus *Balamuthia.* Although not as widely distributed in the environment as *Acanthamoeba* and *Naegleria*, *Balamuthia* species are often present in soil and in stagnant water (Visvesvara et al., 2007; Ahmad et al., 2011; Gianinazzi et al., 2010). Like Acanthamoeba, it has two stages in its life cycle: a trophozoite, measuring 15-60 µm in diameter (Figure 2.3a) and a cyst, measuring 12-30 µm in diameter (Figure 2.3b). The trophozoite has the ability to alter its shape or size in response to changes in environmental conditions (Visvesvara et al., 1997). Although some trophic forms have two cellular nuclei, most trophozoies have a single nucleus that contains a large and centrally placed nucleolus. *B. mandrillaris* cysts also have a single nucleus though they are more spherical in shape than trophozoites and consist of three layers: an outer thin and irregular ectocyst, an inner thick endocyst, and a central, amorphous, fibrillar, mesocyst (Schuster et al., 2004; Dunnebacke et al., 2004).

Balamuthia amoebic encephalitis (BAE) is a rare CNS infection that is similar to GAE. The disease occurs particularly, in immunocompromised individuals such as cancer and HIV patients. However, unlike most cases of *Acanthamoeba* GAE, BAE has been reported in children and older individuals with no known history of immunodeficiency (Schuster et al., 2004; Visvesvara and Maguire, 2006; Tavares et al., 2006). In addition, BAE has also been diagnosed in animals that include dogs and sheep. *Balamuthia* enter the body via skin lesions, nasal passages or via inhalation of cysts carried in air currents to the respiratory tract before being spread to the CNS and other organs (Schuster and Visvesvara, 2008). Clinical symptoms of BAE include weight loss, fever, vomiting, nausea and skin granulomatous lesions (Da Rocha-Azevedo et al., 2009; Bravo et al., 2011).

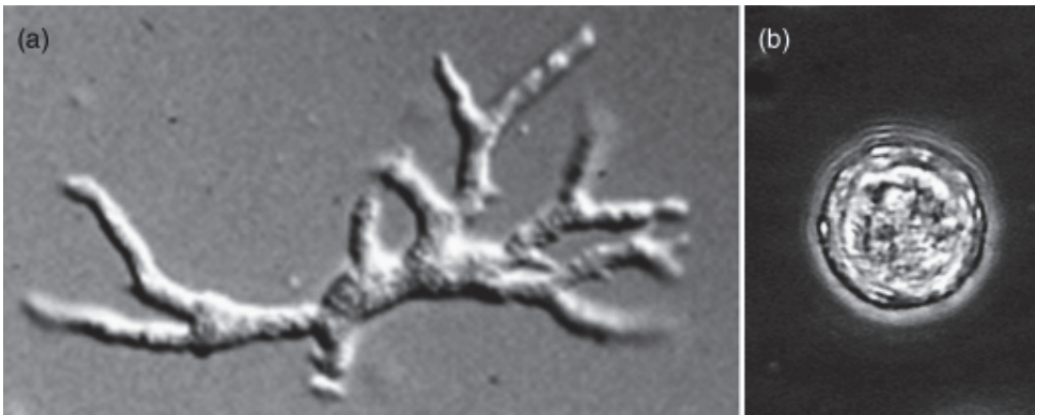

Figure 2.3: *Balamuthia mandrillaris* (a) trophozoite and (b) cyst (X 850) (Visvesvara et al., 2007).

2.3.1.4 *Sappinia* species

The genus *Sappinia* contains two species, *S. pedata* and *S. diploidea*. Sappiniae are widely distributed in the environment where they have been isolated from soil, decaying forest litter and fresh water. The faeces of cattle, buffalo and bison is a known source of *S. pedata* and *S. diploidea* (Visvesvara et al., 2007). The life

cycle of *Sappinia* consists of two stages, a trophozoite (40-80 µm) and a cyst (18-25 µm). Unlike the other pathogenic FLA, both the trophozoite and the cyst stage of *Sappinia* have two nuclei (Figure 2.4). The trophozoite form has a flatterned appearance with occasional wrinkles on the surface that forms an ovoid or oblong shape. It is also characterized by a cytoplasm contains a contractile vacuole and food vacuoles. The cyst form has nuclear pores and a round double-walled structure that can survive gastric fluid of stomach (Shuster and Visvesvara, 2004; Brown et al., 2007 Walochnik et al., 2010).

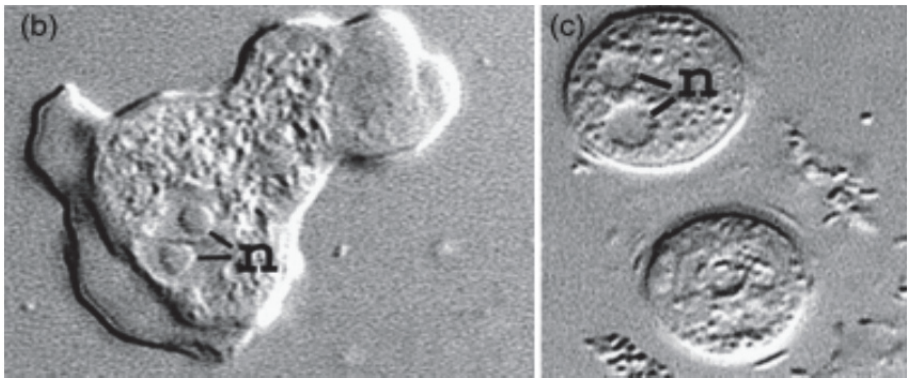

Figure 2.4: *Sappinia diploideae* trophozoite: (n) closely opposed nuclei, each with a prominent nucleolus (left); Micrograph of two cysts, one with two nuclei (n), (right). (Both images at X1000) (Visvesvara et al., 2007).

2.3.2 Non-pathogenic FLA of importance in hospital water distribution systems

2.3.2.1 Vermamoeba vermiformis

Vermamoeba vermiformis (previously known as *Hartmanella vermiformis*) is not widely accepted as a human pathogen of its own accord but serves as a host for a number of amoeba resistant bacteria (ARB) and yeasts and has been isolated from hospital water distribution systems (Cateau et al*.,* 2014; Pagnier et al*.,* 2015; Rohr et al*.,* 1998). Pagnier and coworkers (2015) studied the prevalence of FLA in hospital water in France and concluded that is more commonly associated with amoeba resistant microorganisms than *Acanthamoeba* species. They proposed the use of *V. vermiformis* in conjunction with *Acanthamoeba* species in co-culture experiments when testing water for the presence of potential ARB.

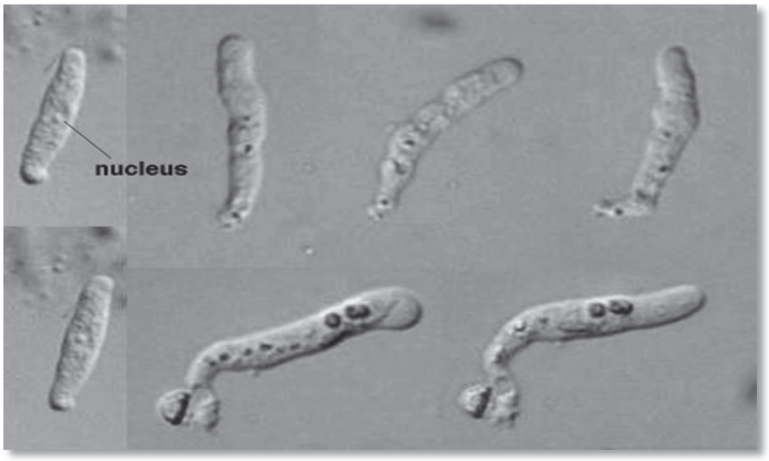

Figure 2.5: *Vermamoeba vermiformis* trophozoites

2.3.2.2 Tetrahymena pyriformis

Tetrahymena pyriformis is a freshwater ciliate, approximately 40-60 µm in length. It feeds on bacteria which it captures by means of four ciliated “membranelles” that form a set of three combs that brush particles into a curved buccal cavity rimmed by a fourth undulant membrane. Ciliates are characterized by three unique features, (i) ciliature that can be specialized for movement or food capture, (ii) alveolar membranes that lie just beneath the plasma membrane forming a set of flattened sacks, and (iii) “nuclear duality” (the presence of a somatic, transcriptionally active macronucleus, as well as a germinal, transcriptionally silent micronucleus). *T. pyriformis* has been implicated as a host for a number of ARB but is not regularly used as a host in co-culture experiments.

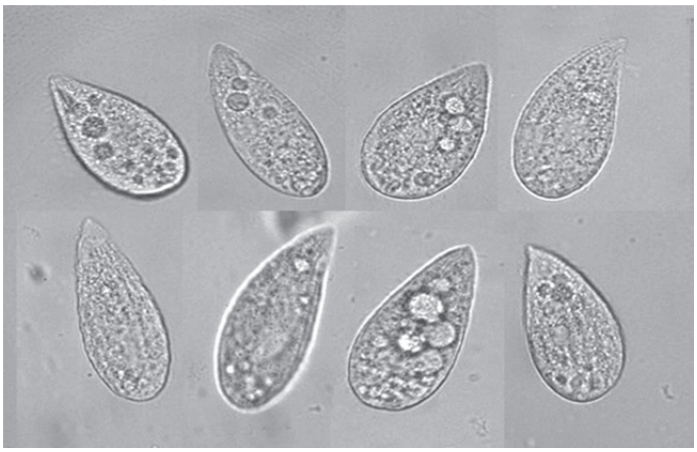

Figure 2.6: *Tetrahymena pyriformis* trophozoites

2.3.3 Amoeba Resistant bacteria

The fact that FLA feed on bacteria has had major evolutionary consequences, allowing some species to genetically adapt to life in protozoa (Greub and Raoult, 2004). These genetic adaptations took place over millions of years and led to the expression of symbiotic or pathogenic phenotypes which can be distinguished by their effect on their host cell. It is believed that FLA provided pathogenic bacteria with a powerful “evolutionary crib” for the development of resistance to FLA as well as to human macrophages. This theory is explained in detail by Greub and Raoult (2004). Over time it allowed the predator (the amoeba) to refine its microbicidal machinery and also allowed the prey (the bacteria) to develop strategies to survive in amoebae. These strategies include (i) increased size to prevent engulfment, (ii) increased multiplication rate to prevent extinction of the species, (iii) colonisation of new environments, (iv) increased resistance to the microbicidal effects of amoebae and macrophages, and (v) increased virulence which explains their adaptation to macrophages after passage through amoebae (Harb et al., 2000; Albert-Weissenberger et al., 2007; Moliner et al., 2010).

Free living amoebae feed by phagocytosis and pinocytosis followed by digestion in phagolysosomes. However, some microorganisms have evolved to become resistant to internalization and digestion strategies such that they survive and even multiply inside FLA. Bacteria in this category are collectively known as “amoebae resistant bacteria” (ARB) (Greub and Raoult, 2004). Some of the ARB and the diseases they cause are listed in Table 2.3. Interactions between bacteria and FLA were first reported by Jeon and Lorch in 1967. They isolated “an unidentified bacterial species” as a cytoplasmic endosymbiont of *Acanthamoeba castellanii* (Jeon and Lorch, 1967; Proca-Ciobanu et al., 1975) which was later confirmed as a member of the order *Legionella* (Park et al*.,* 2004). Rowbotham (1980) was first to show that *L*. *pneumophila* can multiply within FLA. His work led to the discovery of similar interactions among a host of other pathogenic bacteria and amoebae. Several established pathogenic bacteria including *Vibrio cholerae* (Abd et al., 2007), *Shigella dysenteriae* (Saeed et al., 2009) and *Salmonella* spp. (Tezcan-Merdol et al., 2004) were shown to persist in FLA. By 2010 there were >150 bacterial pathogens known to interact with free living amoebae, and many more species suspected to resist digestion and survive, or grow, within FLA. Several of these species can survive and proliferate in more than one amoebal species (Thomas et al., 2010).

In the South African context, methicillin-resistant *Staphylococcus aureus* (MRSA) (Boucher et al*.,* 2008; Communicable Diseases Communique, 2010; Huws et al., 2006, Klein et al., 2009; Navarro et al., 2008; Zinn et al*.,* 2004,), members of the *Mycobacterium avium* complex (Falkinham, 2015; Fields et al*.,* 2002, Greub and Raoult, 2004; Primm et al*.,* 2004; Torvinen et al*.,* 2004), *Vibrio cholerae* (Abd et al*.,* 2007), *Legionella* species (Maartens et al*.,* 1996; Bartie and Klugman, 1997) *Chlamydophila pneumoniae* (Maartens et al*.,* 1996; Bartie and Klugman, 1997) and *Parachlamydia acanthamoebae* (Hall’s coccus) (Greub et al*.,* 2004; Thomas et al*.,* 2008; Lamoth et al*.,* 2010) are particularly important causes of nosocomial and occupational infectious disease. Several other known agents of nosocomial infection are known to interact with FLA in hospital water distribution systems. *Acenitobacter baumanii, Aeromonas* species, *Klebsiella pneumoniae, Pseudomonas* species*, Stenotrophomonas maltophilia,* and *Serratia marcescens* are a few of

the well-known agents of nosocomial infection known to interact with FLA (Corbin et al*.,* 2007; Corsaro et al*.,* 2013; Evstigneeva et al*.,* 2009; La Scola et al*.,* 2012; Maschio et al., 2015).

Table 2.3: Pathogenic bacteria commonly associated with FLA

ARB	Disease/symptoms	Amoebal hosts	References
Acenitobacter species	Septicaemia Urinary tract infections Wound infection Endocarditis Meningitis Osteomyelitis	*Acanthamoeba* species *Vermamoeba* species	eMedMD.com, 2015 Pagnier et al*.,* 2008
Burkholderia species	Bacteraemia Urinary tract infections Wound infection Endocarditis Septic arthritis	*Acanthamoeba* species *Vermamoeba* species	eMedMD.com, 2015 Maroldal et al*.,* 1999 Inglis et al*.,* 2000
Chlamydia pneumoniae	COPD Chronic bronchitis Common colds Persistent cough Pharyngitis	*Acanthamoeba castellanii*	Corsaro and Greub, 2006
Delftia acidovorans	Bacteraemia Endocarditis	*Acanthamoeba* species *Vermamoeba* species	eMedMD.com, 2015 Pagnier et al., 2008
Enterobacter species	Bacteraemia Respiratory tract infection Urinary tract infection	*Acanthamoeba* species	eMedMD.com, 2015 Pagnier et al*.,* 2008
Helicobacter pylori	Gastritis	Unknown	eMedMD.com, 2015 O'Toole et al*.,* 2000 Snelling et al., 2005
Klebsiella pneumoniae	Wound infection Respiratory tract infection	*Acanthamoeba* species	eMedMD.com, 2015 Pagnier et al., 2008
Legionella species	Legionnaires' disease Pontiac fever	*Acanthamoeba* species *Vermamoeba* species *Naegleria gruberi*	Bichai et al*.,* 2008
Mycobacterium avium complex	MAC lung disease	*Acanthamoeba* species	Whan et al., 2006
Mycobacterium species	Respiratory infection Pyelonephritis Myocarditis Meningitis	*Acanthamoeba* species *Vermamoeba* species	Adekambi et al*.,* 2006 eMedMD.com, 2015 Pagnier et al*.,* 2008
Parachlamydia acanthamoebae	Bronchitis Bronchiolitis Pneumonia	*Acanthamoeba* species	Greub et al., 2003; 2006
Salmonella typhimurium	Salmonellosis	*Acanthamoeba castellanii*	Gaze et al., 2003
Shigella dysenteriae	Shigellosis	*Acanthamoeba castellanii*	Saeed et al*.,* 2009
Methicillin-resistant *Staphylococcus aureus*	Pneumonia Urinary tract infection Bacteraemia Wound infections	*Acanthamoeba polyphaga*	Lone et al., 2009; Huws et al., 2006
Vibrio cholerae	Cholera	*Acanthamoeba castellanii*	Abd et al., 2005,2007; Sandstrom et al*.,* 2010
Pseudomonas aeruginosa	Pneumonia Septic shock Hermorrhage	*Acanthamoeba polyphyga*	Michel et al., 1995; Sharbati-Tehrani, *et.al*., 2005
Listeria monocytogenes	Listeriosis Septicaemia	*Tetrahymena pyriformis,* *Acanthamoeba castellanii*	Ly and Muller, 1990; Akya et al., 2009

2.4 OCCURRENCE OF FREE LIVING AMOEBA IN DRINKING WATER

2.4.1 Occurrence of FLA in source waters

The presence of FLA in environmental waters such as rivers, lakes, ponds, ground water and marine waters is well documented (Gianinazzi et al. 2009; Loret and Greub, 2010; Liu et al., 2006). Physicochemical parameters such as temperature fluctuation, dissolved organic matter and pH determine the occurrence and composition of FLA in these environments (Loret et al., 2008; Valster et al., 2009). Seasonal differences in the types and numbers of FLA have been reported. For example, *Naegleria* and *Acanthamoeba* species are more prevalent in spring and early to mid-summer, whereas *Vannella*, *Vahlkampfia* and *Vermamoeba* are prevalent in late summer and spring (Ettinger et al., 2003). Since FLA are widely distributed in environmental waters, they are likely to be transported to drinking water treatment plants and from there through the water supply system as illustrated in Figure 2.7.

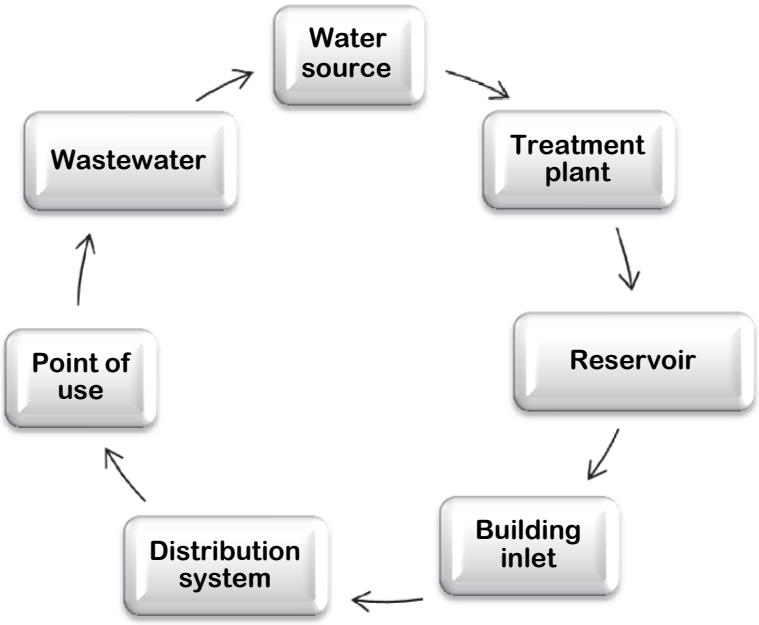

Figure 2.7: Drinking water supply chain

2.4.2 FLA removal in drinking water treatment plants

The occurrence, density and diversity of FLA in different water treatment systems have not been studied extensively to date. A few groups compared the efficiency of water treatment plants in the removal of FLA from raw (ground and surface) waters by clarification/filtration and by ultrafiltration/disinfection (Table 2.4). Studies conducted in France, Spain and Germany reported a 0-4.6 log reduction of FLA during clarification/ filtration (Corsaro et al., 2009; Loret et al., 2008; Thomas et al., 2008). A similar study in South Korea did not report any reduction during this treatment step (Jeong and Yu, 2005). However, despite the reduction in FLA

numbers after clarification and filtration reported in some studies, Hoffmann and Michel (2001) and Thomas et al. (2008) reported that the filters used in these steps are often colonized with high numbers of FLA that may be released into the treated water. Baudin and coworkers (2008) reported the colonization of a new drinking water treatment plant with FLA within a few weeks.

Similarly, although studies by Hoffmann and Michel (2001) and Corsaro *et al*. (2010) reported log removals of 1-2.6 during ultrafiltration and disinfection, Loret et al. (2008) observed a presence of FLA in approximately one third of the treated water. In addition, the isolation of FLA belonging to genera such as *Acanthamoeba, Naegleria*, *Hartmannella* and *Vannella* along the treatment lines confirmed their ability to withstand drinking water treatment and presence at the outlet of the treatment plant. This increases the risk of regrowth when the water enters distribution systems (Loret and Greub, 2010). There is therefore an urgent need for more effective treatment methods used in water production to minimize the number of FLA entering water distribution systems.

Table 2.4: Prevalence of FLA in drinking water treatment plants

Country	Sample source	Log removal	Main genera identified	Reference
France	River	-	*Acanthamoeba, Naegleria, *Vermamoeba, Vannella, Echinamoeba, Glaeseria, Platyamoeba*	Thomas et al., 2008
	Clarification and Sand filtration	0-2	**Vermamoeba*	
	GAC filtration	0-2	*Echinamoeba, *Vermamoeba*	
	Chlorinated water	-		
France & Spain	Surface source water	-	Not stated	Loret et al., 2008
	Sand filtration	0-3.4		
	GAC filtration	1.4		
Spain	River	-	*Acanthamoeba, Echinamoeba, Naegleria, Vannella*	Corsaro et al., 2009
	Sand filtration	1.6-3.7	*Echinamoeba, *Vermamoeba*	
	GAC filtration	1-1.7	*Vannella*	
Germany	Reservoir, source water	-	*Acanthamoeba, Naegleria*	Hoffmann & Michel, 2001
	Filtration	0-3.5	*Naegleria, Acanthamoeba*	
Korea	Source water	-	*Acanthamoeba*	Jeong and Yu, 2005
	Sand filtration	-	*Acanthamoeba*	
	Carbon filtration	-	*Acanthamoeba*	

**Vermamoeba* referred to as *Hartmanella* in the references cited

2.4.3 Occurrence of FLA in drinking water distribution systems

Even if absent at exit from the treatment plant, FLA may be introduced at each of the steps during distribution. It is therefore important to investigate their prevalence and biodiversity throughout the system to control possible re-introduction after biocide treatment (Loret and Greub, 2010). FLA multiply in engineered like water distribution systems (pipes and storage reservoirs for the treated water) despite disinfection with ozone and chlorine in water production plant (Hoffmann and Michel, 2001; Thomas et al., 2008; Corsaro et al., 2009). The persistent nature of amoebal cysts has made them colonizers of virtually any kind of water distribution system. Those cysts are resistant to extreme conditions of chemical (e.g. biocide exposure and pH changes) and physical (e.g. temperature and UV light) nature Greub and Raoult, 2004). Furthermore, low disinfectant levels in distribution networks have limited activity on FLA (Thomas et al., 2004), allowing occurrence of higher densities of 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 (Sriram et al., 2008).

Accumulation of sediment and biofilm are important factors that influence the occurrence and diversity of FLA in water distribution systems. Corsaro et al. (2009) compared the prevalence of FLA in water, biofilm and sediment samples and reported that the frequency and diversity of FLA were highest in sediment, followed by biofilm and least in water samples collected at the reservoir, indicating a high risk of re-contamination during distribution especially in areas where biofilm and sediment accumulate. This confirmed earlier findings by Barbeau and Buhler (2001) and Pickup et al. (2007). Hoffmann and Michel (2001) also detected higher levels of FLA in reservoirs further away from the treatment plant.

Thomas and Ashbolt (2011) proposed a theoretical model for FLA interactions with possible intracellular microorganisms like ARB from the end of drinking water plant to the endpoint in-premise taps of water distribution systems. The first stage of the model is at the start of the distribution where low FLA density and diversity are expected as most genera are eliminated by disinfection campaigns in the water treatment process. The second stage is the middle of the water distribution characterized by pipelines further downstream of the distribution system. FLA density and diversity start to increase as disinfectants concentrations are reduced and amoebae feed on other microorganisms in the biofilms. The final stage is downstream the distribution system at the endpoint in-premise where most of the FLA are infected with pathogenic microorganisms and FLA density and diversity are expected to be highest. At this stage, water retention time in the system is high offering better conditions for FLA re-growth as biofims are formed. This theory has been supported by various studies which reported the recovery of FLA from swimming pools (Vesaluoma et al., 1995), dental unit waterlines (Singh and Coogan, 2005), cooling towers (Berk et al., 2006; Declerck et al., 2007), domestic tap water (Shoff et al., 2008; Marciano-Cabral et al., 2010) and hospital water networks (Rohr et al., 1998; Thomas et al., 2006). Focus for this study will be on domestic and healthcare water distribution systems in respect to the occurrence of FLA and ARB.

2.4.4 Occurrence of FLA at the point of use

FLA are regularly detected at the point of use worldwide but the frequency of detection, the levels of contamination and the diversity of FLA in these drinking water distribution systems vary among different reports (Table 2.4). For example, Kahane et al. (2004) isolated FLA from 92% of their samples compared to the 8% reported by Houang et al. (2001) (Table 2.4). This is understandable given the vast number of factors which may affect the quality of drinking water reaching the end user. Another important consideration is the vast difference in sample size reported in the literature. A study done in Bulgaria reported a prevalence of FLA in 18% of 60 tap water samples (Tsvetkova et al., 2004), whereas a study in Germany reported a prevalence of 66 % for 3 tap water samples (Hoffmann and Michel, 2001) (Table 2.5).

Table 2.5: Detection of FLA at endpoint in-premise taps

Country	N	FLA present (%)	Main FLA genera/ species identified	References
Mexico	27	13 (49) (Cistern); 6 (22) (Tank)	*Acanthamoeba*	Bonilla-Lemus et al., 2010
Bulgaria	60	11(18)	**Hartmannella, Acanthamoeba*	Tsvetkova et al., 2004
Germany	3	2 (66)	*Acanthamoeba, *Hartmannella*	Hoffmann and Michel, 2001
Israel	26	24 (92)	*Acanthamoeba polyphaga*	Kahane et al., 2004
Canada	18	18 (100)	*Vannella, Vahlkampfia, Acanthamoeba*	Barbeau and Buhler, 2001
Nicaragua	74	17 (23)	*Acanthamoeba, Naegleria*	Leiva et al., 2008
West Indies	180	65 (36)	*Acanthamoeba*	Lorenzo-Morales et al., 2005
Hong Kong	100	10 (10)	*Acanthamoeba*	Boost et al.,2008
China	90	7 (8)	*Acanthamoeba*	Houang et al., 2001
Korea	207	97 (47)	*Acanthamoeba*	Jeong and Yu, 2005
United Kingdom	27	24 (89)	*Acanthamoeba, *Hartmannella, Naegleria, Vahlkampfia, Vannella*	Kilvington et al., 2004
	50	24 (48) Tank 13 (26) Mains	*Acanthamoeba, *Hartmannella, Vahlkamfia, Vannella, Platyamoeba, Filamoeba Nucleari*a	
Spain	148	89 (60)	*Acanthamoeba*	Lorenzo-Morales et al., 2005
Poland	31	18 (58)	*Acanthamoeba*	Lanocha et al., 2009
U.S.A	2424	959 (39)	*Vahlkampfia, Acanthamoeba, *Hartmanella*	Stockman *et al*., 2011
	283	54 (19)	*Vexillifera, *Hartmannella, Acanthamoeba, Vahlkampfia, Vannella, Cochliopodium, Limax, Platyamoeba, Mayorella, Echinamoeba, Parvamoeba, Saccamoeba*	Shoff et al., 2008
	207	145 (70)	**Hartmannella, Acanthamoeba, Vahlkampfia, Rosculus*	Sanden et al., 1992
Korea	207	97 (47)	*Acanthamoeba*	Jeong and Yu, 2005
Pakistan	52	19 (37)	*Acanthamoeba, Naegleria fowleri*	Yousuf et al., 2013

**Hartmanella vermiformis* now known as *Vermamoeba vermiformis*

Despite these differences, it is clear from the literature that drinking water distribution systems offer ideal conditions for amoebal growth as disinfectant levels and water pressure decrease during distribution, resulting in the formation of biofilms (Barbeau and Buhler, 2001; Thomas and Ashbolt, 2011). FLA that have been isolated from in-premise point of use taps are similar to those recovered from environmental water, supporting the theory that FLA are spread from sources like rivers and lakes to man-made systems and include members of the genera *Vermamoeba, Acanthamoeba, Naegleria, Vahlkampfia, Vannella, Platyamoeba, Echinamoeba, Parvamoeba, Saccamoeba and Vexillifera* (Bonilla-Lemus et al., 2010; Hoffmann and Michel, 2001; Kilvington et al., 2004; Shoff et al., 2008). *Acanthamoeba* has previously been the most frequently isolated genera in tap water as they are relatively easy to distinguish from the other genera due to their cyst morphology. However as molecular methods become more readily available this scenario is changing and more diverse FLA are isolated from drinking water sources (Bonilla-Lemus et al., 2010; Boost et al*.,* 2008; Houang et al., 2001).

Seasonal temperature fluctuations has been shown to also affect detection frequency and diversity of FLA at endpoint in-premise taps (Hoffmann and Michel, 2001; Marciano-Cabral et al., 2010), where increases have been observed in summer months (Carlesso et al., 2010). A study by Hoffmann and Michel, 2001 reported *Acanthamoeba* spp. isolation from tap water throughout the year, even at temperature as low as 0.5 °C while thermophilic *Naegleria* spp. were only isolated during the warmer summer months. Several studies have also reported a higher prevalence of FLA in cold water compared to hot water with prevalence of FLA significantly lower for temperatures above 60°C (Kilvington et al., 2004; Ménard-Szczebara et al., 2008; Thomas et al., 2006). Storing treated drinking water in tanks prior to distribution at the tap water has been identified as another contributing factor to FLA occurrence and diversity (Bonilla-Lemus et al., 2010; Shoff et al., 2008).

A Study by Kilvington et al., 2004 in the United Kingdom (UK) of 27 households where water is stored in a tank in the roof prior to use, isolated FLA from 89% of the samples. This study suggested that the high rates of amoebic keratitis in the UK compared to the USA (15 times higher) could be attributed to the use of roof tanks in the UK for storage of mains water. An earlier study in the UK had also proposed FLA growth being enhanced by water tanks in the roof supplying the mains as FLA were isolated from 48 % (n = 50) and 26 % (n = 50) of taps in the bathroom and kitchen respectively (Seal et al., 1992). Studies in Mexico (Bonilla-Lemus et al., 2010 and Hong Kong (Boost et al., 2008) also support this trend as storage tanks had higher rates of FLA detection compared to the mains water which they supply (Table 2.4).

2.4.5 Treatment processes for FLA removal in water

Despite the increasing health concerns caused by FLA, few studies have looked at their inactivation by various physical and chemical biocides. The studies that have reported treatment of FLA is mainly lab based and have not been applied in distribution systems.

2.4.5.1 Treatment with oxidising biocides

Chlorine, an oxidising agent, is a commonly used disinfectant for water treatment. The effects of chlorine on the viability of amoebae have been studied under laboratory conditions but there is no consensus about the efficacy of biocides in general, either under laboratory conditions or in water distribution systems. *Acanthamoeba* have been shown to resist exposure to 100 mg/ L free and combined chlorine for 10 min and 50 mg/L for 18 h (Kilvington and Price, 1990; Storey et al*.,* 2004). Siddiqui et al. (2008) studied the effects of biocide treatment on *B. mandrillaris* trophozoites and cysts. They concluded exposure to 25 mg/L chlorine had no effect on *B. mandrillaris* cysts or trophozoites. However treatment with other oxidising agents might be more successful. For example, Cursons and coworkers (1980) reported that trophozoites of *Acanthamoeba* and *Naegleria* species were inactivated by chlorine dioxide at concentrations of 3mg/L and 2mg/L for 30 minutes respectively. This was confirmed by Ercken et al. (2003) who reported the inactivation of *Naegleria lovaniensis* cysts by exposure to 3.9 mg/L chlorine dioxide. However, a study by Thomas and coworkers (2004) showed that continuous injection in water pipes of 0.5 mg/L chlorine dioxide could not eliminate amoebae. More research into the efficiency of biocide treatment under laboratory as well as field conditions is necessary to determine the efficiency of these disinfectants against a wide range of free living amoebae.

2.4.5.2 Ozone

Another oxidizing agent, ozone, has been used successfully in reducing amoebae, particularly *Acanthamoeba* populations in drinking water plants (Loret et al., 2008; Thomas et al., 2008). However, it has not been used in water distribution systems to control amoebae maybe due to the fact that it is limited to the treatment of a limited volume of circulating water and it has no residual activity against FLA. Treatment with heat, physical agents and ultraviolet radiation

2.4.5.3 Heat treatment

Moist heat is successful in eliminating amoebal trophozoites and to a lesser extent cysts, which require higher temperatures than trophozoites for inactivation (Thomas et al*.,* 2010). Turner and coworkers (2000) successfully inactivated *A. castellanii* trophozoites at 46°C after exposure for 30 minutes. *Balamuthia mandrillaris* cysts may remain active after exposure at 60°C for one hour (Siddiqui et al*.,* 2008). Several studies reported effective physical removal (log 1-3) of FLA by clarification and filtration processes (Hoffmann and Michel, 2001; Jeong and Yu, 2005; Thomas et al., 2008; Loret et al., 2008). Ultra-violet (UV) radiation has been widely used for water disinfection in water production plants. Treatment with ultraviolet light is relatively effective for the inactivation of amoebal trophozoites but the cysts or some species, particularly *Acanthamoeba* and *Balamuthia* cysts, are more resistant (Maya et al*.,* 2003; Hijnen et al*.,* 2006; Siddiqui et al*.,* 2008).

2.4.5.4 Point of use filtration

Exner and coworkers (2005) recommended the use of terminal point-of-use filters as means of prevention and control of health care-associated waterborne infections in health care facilities. However, Thomas et al. (2010) recommended that more studies be done on the use of these filters as they could encourage biofim formation and amoebal growth.

2.5 IMPLICATIONS OF FLA AND ARB RESISTANCE TO WATER TREATMENT

Free living amoebae are highly resistant to water treatment products. Their resistance to biocides, chlorine in particular, is of critical importance in drinking water treatment especially in hospitals where immunocompromised patients may come into contact with contaminated tap water (McDonnell, 2007: Loret et al., 2008). A study done by Storey et al. (2004) showed that amoebal cysts can survive chlorine concentrations as high as 100 mg/L for 10 minutes. Thomas et al. (2004) demonstrated that FLA, including *Acanthamoeba*, *Vermamoeba* and *Vahlkampfia* species resisted treatment with ozone, chlorine dioxide, monochloramine, copper-silver and chlorine This enhanced resistance of FLA and their endosymbionts (ARB) to water treatment processes has major implications for disease transmission and water treatment measures. There is therefore a need for more studies on the occurrence of FLA and ARB, as well astheir resistance to biocides in water treatment and distribution systems.

The development of biocide and antibiotic resistance among microbial populations has received increasing attention in the last few years. Bacterial resistance is a problem with serious economic and health consequences. Understanding the basis of resistance may assist to develop to strategies to improve the efficiency of water treatment programs. The efficiency and/or killing rate of disinfectants are influenced by (Gerba, 2002):

- *Temperature*: Temperature controls rate of chemical reactions. As the water temperature increases, the killing rate with chemical disinfectants increases.
- *pH value*: The pH of the water affects the ionization of disinfectants and microorganism viability. Most waterborne organisms are adversely affected by pH values < 3 and > 10. In the case of halogens such as chlorine, pH controls the amount of HOCl (hypochlorous acid) and –OCl (hypochlorite) in solutions. With chlorine, the C x t increases with pH (where C is the concentration of the disinfectant and t is the time required to inactivate a certain percentage of organisms of under a specific set of conditions).
- *Planktonic or sessile state*: Sessile microorganisms are generally more resistant to water treatment than planktonic organisms. Particulate matter may interfere by either acting chemically to react with the disinfectant, thus neutralizing the action of the disinfectant, or by physically shielding the organism from the disinfectant.
- *Size*: The sizes of the targeted microorganisms also affect disinfectant efficiency. Smaller organisms are more difficult to reach than bigger particles. Removing particulates or turbidity in drinking water prior to treatment is believed to improve the effectiveness of disinfection.

- *Chemical substances*: The presence of chemical substances like organic compounds, inorganic and organic nitrogenous compounds, iron, manganese, hydrogen sulphide may decrease the efficiency of biocide action.
- *Presence of protozoa*: Amoebal cyst walls contain cellulose that forms a physical barrier which protects the ARB from physical extremes of temperatures (Storey et al., 2004; Winiecka-Krusnell and Linder, 2001), ultraviolet radiation (Aksozek et al., 2002) and disinfectants used in water treatment (Coulon et al*.,* 2010; Storey et al., 2004).

2.5.1 Clinical implications of FLA and ARB presence in water distribution systems

The clinical implications of FLA and ARB in water distribution systems are twofold: (i) the presence of pathogenic FLA are an important and often neglected risk of waterborne infection; (ii) FLA may act as carriers for ARB increasing the risk of waterborne infection indirectly. Not only do they carry the bacterial pathogens, but studies have shown an increase in virulence and antibiotic resistance in the majority of ARB after passage through amoebae, as well as an increased ability to infect human macrophages (Greub and Raoult, 2004; Salah et al*.,* 2009).

2.5.1.1 Increased virulence

ARB released when FLA lyse are more virulent to humans than there were prior to FLA infection (Cirillo et al., 1994; Cirillo et al., 1997; Neumeister et al., 2000) as their virulent genes are up-regulated (Schmitz-Esser et al., 2010).

2.5.1.2 Increased resistance to antibiotics

The widespread use of antibiotics for clinical purposes has caused a growing problem worldwide as different microorganisms have developed resistance to these antibiotics over the years. It is interesting to note that the interaction of amoebae with pathogenic ARB has decreased the susceptibility of these pathogens to antibiotics (Loret and Greub, 2010). Although the mechanism used by ARB to acquire drug resistance is not known, some studies have shown that amoebae harboring *L*. *pneumophila* and *M*. *avium* were found to enhance decreased antibiotic susceptibility to these bacteria (Barker et al., 1995; Miltner et al., 2000).

2.5.1.3 Increased resistance to macrophages

The adaptation of *Legionella* sp. to FLA and macrophages has been studied extensively at both cellular and molecular levels. At the cellular level, the way in which *Legionella* sp. are adapted to amoebae and macrophages is similar. *Legionella* sp. enter both amoebae and macrophages by coiling phagocytosis; phagosome-lysosome fusion is absent and is characteristic of the survival of *Legionella* spp. in both macrophages and amoebae (Bozue and Johnson, 1996). *Legionella* spp. also induces the lysis of both macrophages and amoebae (Horwitz and Silverstein, 1980; Rowbotham, 1980). Furthermore, studies by

Cirillo et al. (1994) and Neumeister et al. (2000) showed that *Legionella* species grown in *A. castellanii* are more invasive for human cells lines than their extracellular counterparts.

2.5.2 Human health implications of FLA and ARB presence in hospital water distribution systems

Drinking water systems in health care institutions can be a source of contamination to patients and healthcare personnel. Hospitals, health centres, hospices, residential care facilities, dental offices, and dialysis centres have been described by WHO Guidelines for Drinking Water Quality as part of healthcare facilities where drinking water systems should be monitored for chemical and bacteriological contamination (WHO, 2011). Once water enters the building, the health care institution is responsible for making sure that the drinking water is suitable for human consumption. However, water from the public network may not be useful for domestic use or even personal hygiene for some groups of patients. In this case additional treatment or processing such as microfiltration or sterilization is required to prevent exposure a patients to contaminants, especially for immune-suppressed persons. For example, the presence of endotoxins, microorganisms, toxins and chemical contaminants like chloramines in municipal water must be minimized by further special processing before it can be used in dialysis patients (WHO, 2011). This review will focus on bacteriological contamination, particularly ARB, in health care facilities, population at risk and their association with FLA.

2.5.2.1 Waterborne pathogens in hospital water system

Hospital water systems can be a significant source of contamination, and poor management of these systems can contribute to outbreaks of disease and illness in patients and staff. Water used in hospitals is generally supplied by municipal water. As water travels from the treatment through biofilm-laden pipes, water quality is altered before it enters the hospital. In the hospital water distribution, biofilm formation caused by water stagnation in receiving tanks and water flowing through smaller diameter can further compromise the quality of water (Ortolano et al., 2004). This biofilm of plumbing systems is mainly responsible for production of waterborne pathogens that are sources of hospital acquired (nosocomial) infections. Rates of nosocomial infections in developed countries have been estimated to be between 5 and 10% and can be even higher in developing countries. Although contamination of hospital water distribution systems with potentially pathogenic organisms is common, hospital water has been overlooked as a possible source of nosocomial infections (Anaissie et al., 2002). Hospitals have a large population of patients with weakened immune systems who can be exposed to many opportunistic waterborne pathogens. Patient exposure can occur during drinking (ingestion), showering (inhalation or aspiration aerosols) or bathing (contact) (Figure 2.8). Health care personnel can also expose patients to waterborne pathogens when they wash their hands with tap water (Emmerson et al., 2001; Stout et al., 1994; Anaissie et al., 2002). Potable water distribution system harbours a variety of waterborne pathogens which include bacteria, viruses, protozoa and helminths (Figure 2.8). Among these, this review will focus on ARB that has been implicated in nosocomial infections.

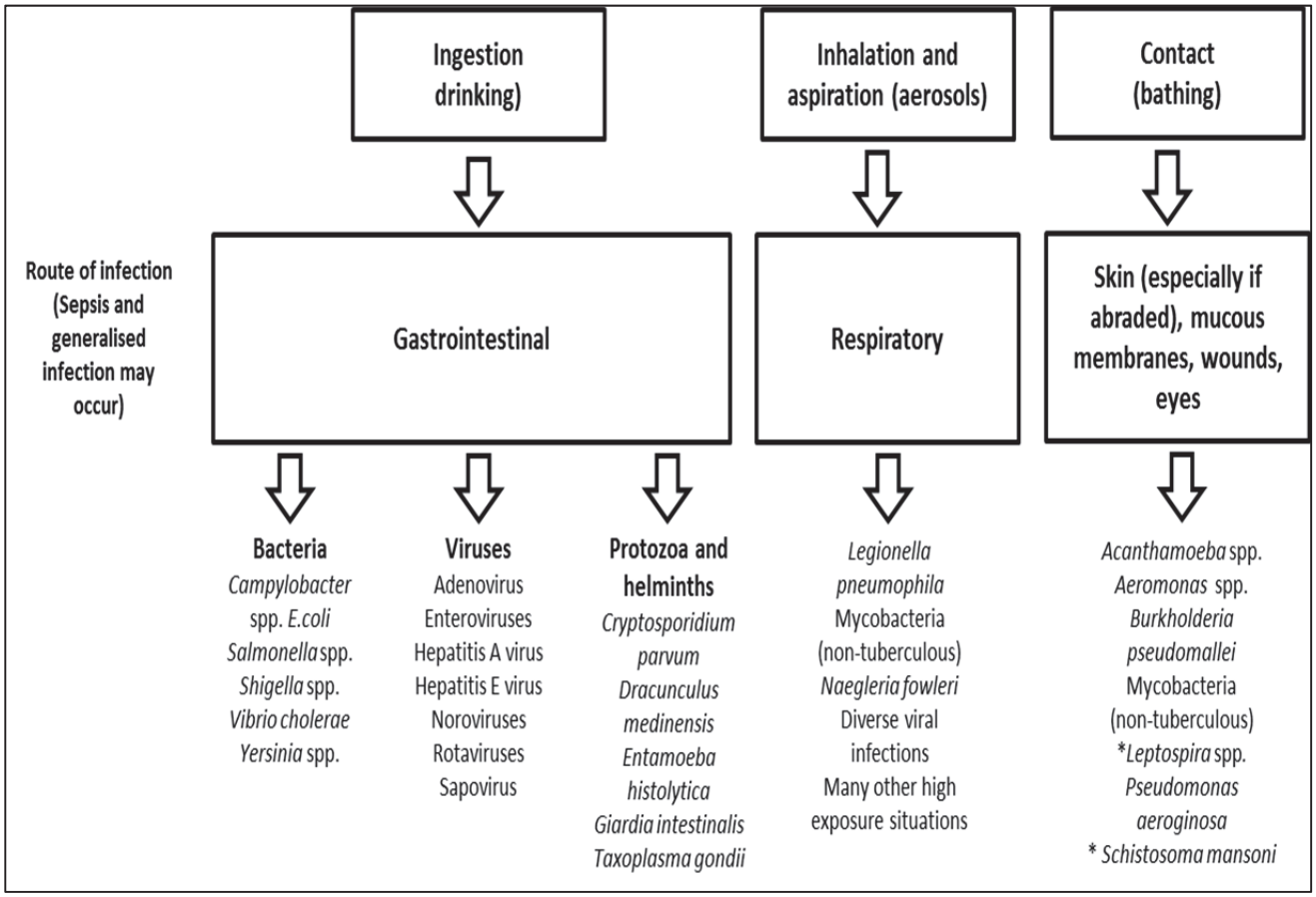

Figure 2.8: Transmission pathways of the most relevant waterborne pathogens (Anaissie et al*.*, 2002)

2.5.2.2 Patients population at risk and sources of contamination

Prevalence of waterborne pathogens in hospital settings presence a huge risk to the large population of patients with weakened immune systems. High risk groups include: neonates with immature immune systems (Vochem and Doring, 2001), intensive care ward patients (Kusnetsov et al., 2003), organ transplants patients (Stelzmueller et al., 2005; Zhou et al., 2013), hematologic procedures Orenn et al., 2002, HIV and AIDS patients (Hillebrand-Haverkort et al., 1999), patients undergoing renal dialysis (Chertow, 2000) and the elderly patients with waning immunity (Kay et al., 2007) (Table 2.6). Risks of exposure to waterborne pathogens should be minimized in these groups of patients. A review by Glasmacher et al., 2003 and a study by Engelhart et al., 2001 reported risk reduction in vulnerable patients. Table highlights some of the populations at risk of infection from waterborne pathogens and actions that can reduce risk in health care settings. In these settings, sources that include faucets and sinks (Su et al*.*, 2001), showers and shower heads Campins et al., 2000, humidifiers (Moiragh et al.,1987) , hemodialysis fluid (Engelhart et al., 2001), and tap water aerators (Kappstein et al., 2000) have been reported to be the sources of contaminated water that may reach the patient.

Table 2.6: Patient populations at risk for infection from waterborne pathogens and measures to reduce risk (Adapted from Ortolano et al., 2005)

Patients population at risk	Measures to reduce risk	Immunosuppression level
Stem cell transplantation	Use sterile water only to wash, drink and mouth	Extreme
Neonates		Severe
AIDS with a count of CD4+ cells less than 200 µL		
Solid organ transplant under intensive treatment phase		
Acute or chronic leukemia, malignant		
Second, third and fourth degree burns		
Long-term corticosteroid therapy	Bathroom installations should be controlled for bacterial reservoirs and avoid drinking water direct from taps	Moderate
AIDS with a count of CD4+ cells less than 200 µL		
Second degree burns	Avoid drinking water from uncontrolled sources like taps	Mild
Cystic fibrosis Surgical patients		
Acute or chronic leukemia		
Renal dialysis		
Blood component transfusion recipient		
Endoscopy, laparoscopy and colonoscopy procedures	Avoid exposure of medical devices to tap water	None

2.5.2.3 ARB as etiologic agents for nosocomial infections

In South Africa, the increase in mortality and morbidity in patients receiving health care has been attributed to nosocomial infections. These infections also have direct or indirect financial implications to the health delivery system (Brink et al., 2006). In the United States, nosocomial infections as being part of preventable events are responsible for 44,000 to 98,000 deaths annually, representing a cost of $17 to $29 billion as reported by the Institute of Medicine in Washington DC. In developing countries like South Africa, there is a need for more studies to prevent infections, starting with prevalence of pathogens in healthcare settings. This will help to manage and control nosocomial infections considering the limited financial resources available to treat the infections.

Legionella species

Legionella species have been isolated from fresh water habitats, such as lakes and rivers and engineered water systems, such as cooling towers and hospital water systems (Fields, 2002; Leoni *et al*., 2005; Newton et al., 2010; Bargellini et al., 2011). They can survive in man-made water systems at temperatures ranging from 0 to 60ºC, with an optimum growth of between 25 to 42ºC (Fields, 2002; Carvalho et al., 2008). This ability to survive at relatively high temperatures has made *Legionella* species successful colonizers of hot water distribution systems of hospitals (Patterson et al., 1997; Yu et al., 2000; Lasheras et al., 2006). *Legionella* species are known to naturally infect and multiply within amoebae under adverse conditions like the presence of biocides, allowing them to further proliferate in man-made water system like hospital water

(Steinert et al., 1997; Bichai et al., 2008). This ability of *Legionella* species to resist phagocytosis and multiply inside amoebaei in aquatic environments can has been supported by studies done by Barker et al. (1992) and Ohno et al. (2008). Furthermore, *Legionella* species interactions with FLA in engineered water systems thrive in biofilms protecting them from disinfectants and biocides used in water systems. Planktonic Legionella can then be release from biofilm when conditions are conducive resulting in human infection (Declerck, 2010).

Infections caused by *Legionella pneumophila* account for 90% of legionellosis in the world. Legionellosis can take the form of two types of disease: Legionnaires disease and Pontiac fever (Fields, 2001). Legionnaires' disease is a severe form of infection which may cause pneumonia. Pontiac fever on the other hand is a milder, self-limiting flu-like illness (Fields, 2002). Hospital acquired pneumonia caused by Legionnaires disease has been known to be part of outbreaks Kool et al., 1998; Yu, 2000). In a hospital water setting, patients are often exposed by inhaling contaminated aerosol and/or aspiration or ingesting contaminated water (Venezia et al., 1994; Woo et al., 1992; Kool et al., 1999). Patient most at risk of infection include those with chronic lung disease, underlying disease conditions such as pulmonary or liver dysfunction and following surgery (Johnson et al., 1985; Kool et al., 1998; Sabria and Campins, 2003). This has important implications in the health care industry within South Africa due to increasing numbers of immuno-compromised individuals typically suffering from HIV/AIDS. This raises concerns since South Africa has the largest epidemic in the world of HIV/AIDS with an estimated 5.6 million people that were living with HIV and AIDS in 2009 (UNAIDS 2010).

Since the first isolation of *Legionella pneumophila* in 1976, numerous outbreaks of *Legionella* infections have been documented worldwide (http://hcinfo.com/outbreaks-news.htm). Most outbreaks, however, have been reported in developed countries. For example, an outbreak of Legionnaires' disease affected visitors at a flower show in Netherlands, resulting in 133 confirmed cases and 55 probable cases (Den Boer et al., 2000). In Europe, another outbreak was reported in Spain and resulted in 449 confirmed cases of Legionnaires disease from the over 800 suspected cases (García-Fulgueiras et al., 2003). Another very large outbreak occurred in Australia where there were 125 Legionnaires disease cases caused by *L. pneumophila* that were associated with the Melbourne Aquarium (Greig et al., 2004). In South Africa, between 1985 and 1986, 12 cases of Legionnaires disease were identified at a Johannesburg teaching hospital (Strebel et al., 1988). Although legionellosis cases are often diagnosed clinically, very few of these cases are reported although legionellosis became a notifiable disease in South Africa in 1990.

Non-tuberculous Mycobacteria (NTM)

Members of NTM share same aquatic habitats as FLA and have been isolated from fresh water , marine environments and hospital water systems in which FLA are frequently isolated (Ettinger et al., 2003; Liu et al., 2006; Thomas and McDonnell**,** 2007; Baird et al., 2011). The ability of NTM to survive and grow in FLA has been demonstrated in several experimental studies. For example, a study by Ben Salah and Drancourt, 2010 has shown that MAC organisms can survive *Acanthamoeba polyphaga* cysts. Another earlier study by Danelishvili et al. (2007) demonstrated that *M. avium* subsp. *avium* can survive in *A. castellanii.* A much recent study by Ovrutsky et al., 2013 has also shown the co-occurrence of NTM species with FLA

Acanthamoeba spp. and *H. vermiformis* in hospital water networks. Survival of NTM within FLA offers protection to NTM species from disinfectants, like chlorine and glutaraldehyde, used in water treatment systems and hospitals (Cirillo et al., 1997; Whan et al., 2006; Coulon et al*.,* 2010). NTM species can therefore be transmitted in water distribution systems of hospital, posing a great risk to public health. Furthermore, the ability of NTM species to survive within FLA also reduces the effectiveness of antimicrobials used to treat AIDS patients exposed to *M. avium* (Miltner and Bermudez, 2000).

NTM species are responsible for infections and disease called pneumonitis. This disease, which occurs in especially in immune-compromised patients causes an inflammation of the lung tissue and is commonly referred to as 'hot tub lung' (Lumb et al., 2004; Hanak et al., 2005; Marras et al., 2005). The most common symptoms of MAC lung disease include dyspnea, cough, hypoxia and fever (Field et al., 2004). Clinical presentation of then infection depends on the NTM species involved and the medical conditions of the patient. Inhalation of contaminated aerosols exposes patients in hospital settings (American Thoracic Society, 1997). Most clinical NTM infections are however characterized by septic emboli, bacteraemia, lung colonization and soft tissue and bone infections (Baird et al. 2011).

NTM species as opportunistic pathogens cause infections in elderly women, cervical nodes of young children with erupting teeth and in immuno-compromised individuals, especially AIDS patients (Horsburgh et al., 2001; Aksamit et al., 2002). In hospital settings, the presence of NTM species in water systems has also caused nosocomial infections in patients undergoing dialysis (Vera, 1999) patients with chronic lung disease (Costrini et al., 1981). Studies in some African countries such as Kenya, Uganda and Tanzania reported MAC infections to be absent or uncommon (Gilks et al., 1995; Okello et al., 1990; Archibald et al., 1998). However, a study in South Africa by Pettipher et al., 2001 reported the point prevalence of MAC infections for a sample of 100 AIDS patients to be 54%. *Mycobacterium avium* complex infections have also been reported in immunocompetent persons who use hot tubs regularly.

Methicillin-resistant *Staphylococcus aureus* (MRSA)

Methicillin-resistant *S. aureus* has been isolated from aquatic environments, mainly in hospital and public water distribution systems. Free-living amoebae also commonly inhabit the same areas within healthcare environments and water distribution systems (Rohr et al., 1998; Thomas et al., 2006). Some recent studies have shown that MRSA survive and replicate inside FLA, particularly *Acanthamoeba* spp., and may thus be spread through water distribution systems. Huws et al. (2006) investigated interactions of *A. polyphaga* with epidemic isolates of MRSA. This study showed that MRSA can survive and multiply within amoebae. Another study by Lone et al., 2009, also documented the infection and survival of MRSA strain inside *Acanthamoeba castellanii* cytoplasm (Lone et al., 2009). FLA other than *Acanthamoeba* like *Enthamoeba histolytica* (Bracha et al., 1982) and *Hartmannella vermiformis* (Pickup et al*.,* 2007) have also been shown been shown to support intracellular survival and growth of MRSA.

The control of healthcare-associated MRSA infections has been of concern worldwide since the first outbreak was reported in 1968 (Barrett et al., 1968). Since that time, MRSA has been shown to be a major problem in nosocomial infections, prompting infection control programmes to control its dissemination.

Hospital-acquired MRSA (HA-MRSA) infections occur throughout the world where MRSA strains have been isolated from clinical samples (Kluytmans et al., 1997; Shopsin et al., 2008). Such HA-MRSA infections are often associated with pneumonia, urinary tract infections, bacteremia and surgical wound infections (Cosgrove and Fowler, 2008; Decker, 2008). The multiple resistance nature of MRSA to antibiotics and the ability of infections to spread quickly, especially in immune-compromised patients make them successful colonizers of hospital settings (Cunha, 2005). Established risk factors for HA-MRSA infections include dialysis, recent hospitalization or surgery, surgical wounds patients and indwelling catheters (Graffunder and Venezia, 2002; Chavez and Decker, 2008). The presence of these MRSA infections in health care environments has made them difficult to manage and costly to treat in developed countries where they have been mostly reported (Barnes and Jinks, 2008; Scudeller et al., 2000).

Salmonella species

Salmonella and amoebae are widely distributed in the environment where they have been recovered from soil and water. Studies have shown that *Salmonella* and amoebae interact in these habitats. Tezcan-Merdol et al*.,* 2004 provided data on the interaction between *Salmonellae* and free-living amoebae. They studied the establishment of conditions for the growth and replication of salmonellae in an amoebic intracellular environment and found that bacterial growth conditions affect the survival, replication, and cytotoxicity of *Salmonellae* within *Acanthamoeba rhysodes*. A more recent study by Douesnard-Malo and Daigle, 2011 has shown that *S.* Typhi can survive for a long time (three weeks) when grown in A. *castellanii* compared to less than 10 days when *S. typhi* is grown as singly cultured bacteria under same conditions.These studies provide evidence that FLA function as hosts of *Salmonella* species contributing to their persistence and transmission in the environment.

Salmonellosis is a collective description of infectious diseases caused by members of the genus *Salmonella* (Threlfall et al., 1992; McConkey and McConkey, 2002). Clinical symptoms of salmonellosis include nausea, vomiting, abdominal cramps, minor diarrhoea, fever, and headache. Chronic symptoms such as arthritis may follow 3-4 weeks after onset of acute symptoms (Jones et al., 1994; Jensen et al., 1998; Chin, 2000). Transmission occurs mainly by ingestion of faecally contaminated food and water (Abdullahi, 2010). The infecting dose of *Salmonella* in immune-compromised people has been reported to be lower than that in immune-competent people (Crum-Cianflone, 2008). A review by Lee and Greig, 2013 to identify *Salmonella* outbreak reports in hospital settings published between 1995 and 2011 showed outbreaks from neonatal units, paediatric wards, tertiary care hospitals, the elderly hospitals psychogeriatric hospital and a renal transplant unit. In South Africa, Wadula et al. (2006) reported a *Salmonella* outbreak in which nine paediatric patients died with over three-quarters were co-infected with human immunodeficiency virus.

Parachlamydia acanthamoebae

Parachlamydia acanthamoebae was first described by Birtles et al. (1997), when the *P. acanthamoebae* strain, Hall's coccus present intracellularly within amoebae, was isolated from the source of an outbreak of humidifier-associated fever. This association provided the first evidence supporting the medical importance of *P. acanthamoebae.* Since then it has been isolated from soil and aquatic environments where it naturally infects free-living amoebae (Greub and Raoult, 2002). In these environments, *P. acanthamoebae* poses a

health concern as a potential agent of respiratory tract infections in humans as it is associated with bronchitis (Corsaro et al., 2002), bronchiolitis (Kahane et al.,1998), community acquired pneumonia (Greub et al., 2003) and aspiration pneumonia (Greub et al., 2003). The role of *P. acanthamoebae* as a common agent of inhalation pneumonia and community acquired pneumonia in AIDS patients and in organ transplant recipients receiving immunosuppressive therapy has also been reported (Greub et al., 2003; Casson et al., 2008).

2.6 METHOD FOR ISOLATION OF AMOEBA RESISTANT BACTERIA FROM WATER DISTRIBUTION SYSTEMS

Amoebal coculture and amoebal enrichment techniques used in conjunction with traditional and molecular microbiological methods are very successful in identifying a large biodiversity of pathogens from clinical and environmental samples. Amoebal enrichment and co-culture techniques are reasonably simple and cost effective laboratory methods used globally to improve the culturability of environmental bacteria. The principle of amoebal coculture and -enrichment is illustrated in Figure 2.9.

2.6.1 Amoebal coculture

Amoebal co-culture involves the inoculation of a cell culture system in which axenically grown free living amoebae are used as the cellular base (Greub and Raoult, 2004). Clinical or environmental samples that potentially contain ARB are seeded onto axenic amoebae, incubated and viewed microscopically for intracellular bacteria and / or amoebal disruption. Amoebal co-culture has the advantage of reducing interference from rapidly growing species present in the sample that may overwhelm slower growing species on traditional laboratory media, allowing fastidious and slow growing species to recovery and multiply intracellularly (Thomas et al., 2006). Amoebal co-culture is successfully used worldwide to recover amoeba resistant bacteria from clinical and environmental samples.

2.6.2 Amoebal enrichment

Amoebal enrichment involves two steps. The sample is inoculated onto a non-nutrient agar medium flooded with gram negative bacteria which will act as a source of nutrients for amoebae that may be present in the sample. These amoebae are allowed to grow out onto the medium and are purified by subculturing onto fresh non-nutrient agar containing gram negative bacteria. The amoebae are then washed from the agar plate and placed into tissue culture plates with a saline solution and viewed microscopically for the presence of intracellular bacteria. If a sample contains intracellular bacteria, the amoebae are broken to release the bacteria which are then identified using normal staining, culture and molecular techniques. The amoebae from the sample can also be inoculated onto a layer of an axenic type strain of amoebae in tissue culture wells, incubated to allow the intracellular bacteria to adhere to and proliferate inside their amoebal host before final identification (Corsaro et al*.,* 2006; Greub and Raoult, 2004; Thomas et al*.,* 2006).

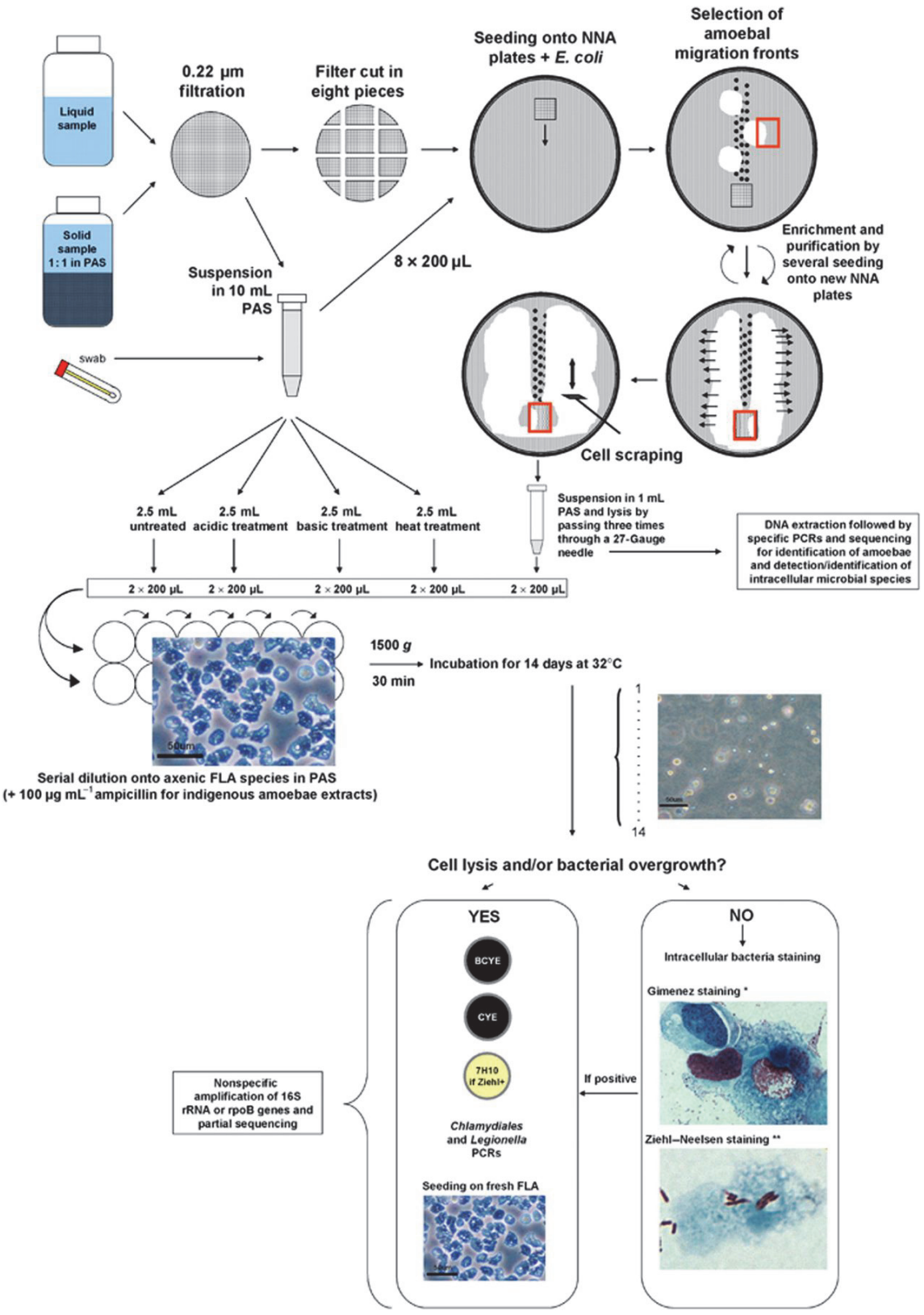

Figure 2.9: Schematic representation of amoebal coculture and amoebal enrichment (Thomas et al., 2006)

CHAPTER 3: METHODOLOGY

3.1 INTRODUCTION

Written permission to conduct the study was obtained from the management of three public health care institutions in Johannesburg, Gauteng. This was an observational, descriptive study using a systematic random sampling technique. The sampling strategy was developed with the assistance of a statistician/ epidemiologist. Meetings and walkthrough assessments were conducted, in collaboration with infection control staff, facility managers and medical practitioners, to determine the most appropriate, high risk sampling areas at each facility. The amoebal enrichment method used to detect amoebae and amoebae resistant bacteria was optimized and validated prior to sampling and testing.

3.2 STUDY SITES

The study was conducted in three public hospitals serving communities around Johannesburg, Hospital A, Hospital B and Hospital C.

3.2.1 Hospital A

Hospital A has approximately 700 beds and consists of medical, surgical, orthopaedic and psychiatric wards which provide in-patient facilities for around 350 patients and admits approximately 45 patients per day. The hospital consists of nephrology, pulmonology, cardiology and neurology departments and a tuberculosis isolation ward.

3.2.2 Hospital B

Hospital B consists of four in-patient wards and a large out-patient unit where patients attend weekly clinics. The in-patient facilities include a neonatal unit with a six-bed neonatal intensive care unit, a premature unit and a mother care program.

3.2.3 Hospital C

Hospital C consists has facilities for >1000 in-patients and large out-patient units focusing on adult, mother and child health.

3.3 WALKTHROUGH ASSESSMENTS

Walkthrough assessments were conducted for each study site. The aim of the assessments was to determine areas of high risk for infection by waterborne pathogens to patients, health care professionals and water treatment and maintenance personnel. Therefore the objectives of the assessments were to:

- Identify and review treatment care areas that pose the greatest risk of contracting waterborne pathogens taking into consideration the three most likely exposure routes: inhalation, ingestion and contact
- Reviewing cases and history of waterborne infections associated with exposure of water at the hospital
- Identify areas in the water distribution system where water may stagnate such as storage tanks or infrequently used taps and showers
- Note the number of water sources i.e. tap and showers and the population they serve in each high risk area

3.4 SAMPLING STRATEGY

The sampling strategy was designed to include collected from all the identified basin taps since there were few sampling areas identified during the walk-through surveys for bath taps and showerheads. However, using a systematic random sampling strategy for basin taps, a proposed 350 samples were determined using a four step process (Figure 3.1).

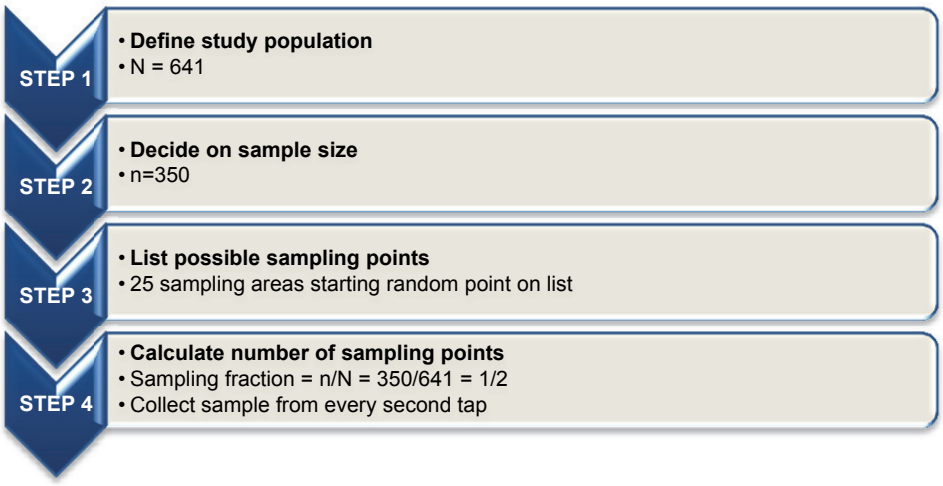

Figure 3.1: Sampling strategy development process

A small pilot study consisting of 15 samples collected at the municipal water inlet of Hospital A indicated an expected frequency of 6.7% for the presence of FLA. The Epi Info (CDC, Atlanta, USA) Population Survey tool was used to determine the number of samples to collect from each facility (CI 80%, variance 2.3). Seventy (23.3%), 13 (4.2%) and 217 (72.5%) samples were collected from Hospitals A, B and C respectively as summarised in Figure 3.1.

3.4.1 Sampling sites

A total of 677 possible sampling points were identified during the walkthrough assessments, 159 from Hospital A, 29 from Hospital B and 489 from Hospital C (Table 3.1). These sampling sites included 641 basin taps, 33 bath taps and showerheads and 3 municipal water inlet points (Tables 3.1-3.5). The sampling areas:

Table 3.1: Summary of sampling areas within the three hospitals

SAMPLING AREA	HOSPITAL A	HOSPITAL B	HOSPITAL C	TOTAL
Water inlet	1	1	1	3
Renal unit	39	-	50	89
ICU	8	-	72	80
Theatre complex	76	-	195	271
TSSU	6	-	6	12
CSSU	11	-	10	21
Endoscopy / Bronchoscopy	18	-	2	20
Neonatal ward	-	17	-	17
Neonatal ICU	-	6	-	6
Diarrhoea ward	-	3	-	3
Milk kitchen	-	2	5	7
Radiation / Oncology	-	-	40	40
Trauma high care	-	-	30	30
Oncology / Haematology	-	-	39	39
Pediatric ICU	-	-	14	14
Pediatric oncology	-	-	25	25
Total	159	29	489	677

Table 3.2: Sampling areas for Hospital A

SAMPLING AREA	BASIN TAPS	BATH TAPS	SHOWERHEADS	TOTAL
Renal unit	39	0	0	39
Intensive care unit	8	0	0	8
Theatre	70	0	6	76
TSSU	6	0	0	6
CSSU	11	0	0	11
Endoscopy/bronchoscopy	15	2	1	18
Water inlet	1	0	0	1
Total	150	2	7	159

Table 3.3: Sampling areas for Hospital B

SAMPLING AREA	BASIN TAPS	BATH TAPS	SHOWERHEADS	TOTAL
Neonatal ward	17	0	0	17
Neonatal ICU	6	0	0	6
Diarrhea ward	2	1	0	3
Milk kitchen	2	0	0	2
Water inlet	1	0	0	1
Total	28	1	0	29

Table 3.4: Sampling areas for Hospital C

SAMPLING SITE	BASIN TAPS	BATH TAPS	SHOWERHEADS	TOTAL
Renal Unit	50	0	0	50
Intensive care unit	70	0	2	72
Theatre	149	0	10	159
TSSU	6	0	0	6
CSSU	10	0	0	10
Endoscopy/bronchoscopy	2	0	0	2
Radiation oncology unit	40	0	0	40
Trauma high care unit	22	6	2	30
Oncology-haematology	38	0	1	39
Milk kitchen	5	0	0	5
Pediatric ICU	14	0	0	14
Pediatric oncology ward	22	2	1	25
Water inlet	1	0	0	1
Total	429	8	16	453

Table 3.5: Breakdown of sampling areas (total of all three sites)

AREA		A	B	C	TOTAL
ADULTS					
High care and ICU	Trauma high care	-	-	30	30
	ICU	8	-	72	80
Renal units		39	-	50	89
Oncology	Radiation	-	-	40	40
	Haematology	-	-	39	39
Theatre complexes	General	76	-	195	271
	Bronchoscopy / Endoscopy	18	-	2	20
CHILDREN					
High care and ICU	Neonatal ICU	-	6	-	6
	Pediatric ICU	-	-	14	14
Oncology		-	-	25	25
Diarrhoea ward		-	3	-	3
Neonatal ward		-	17	-	17
Milk kitchen		-	2	5	7
GENERAL AND MUNICIPAL WATER					
Sterilization units	Central	11	-	10	21
	Theatre	6	-	6	12
Municipal water inlet		1	1	1	3
TOTAL		159	29	489	677

A-Hospital A; B-Hospital B; C-Hospital C

3.4.2 Sample collection

Radiation oncology unit, Oncology / Haematology and Pediatric oncology were not considered for sample collection as shown in Figure 3.2. A total of 146 water and 129 biofilm samples (n=275) were collected from the three hospitals. Samples were collected randomly using sterile bottles and swabs. Water samples were collected after running the taps/shower for 1-2 minutes in 1 L sampling bottles containing 5 mg/L sodium thiosulfate (Merk, SA) to neutralize the chlorine disinfectant. Biofilm samples were collected by swabbing 20 cm^2 of surfaces prior to opening taps.

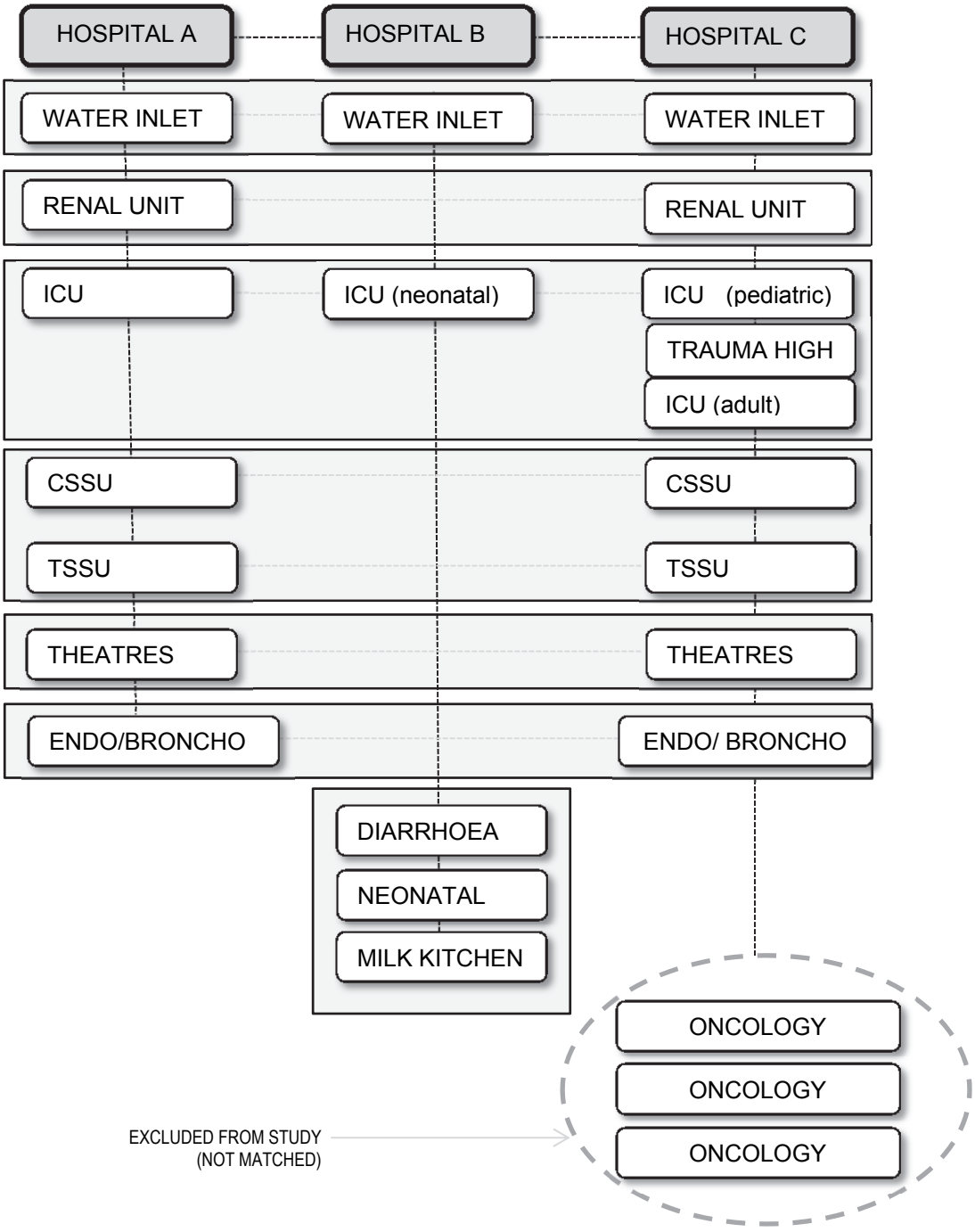

Figure 3.2: Detailed sampling strategy

3.5 EXPERIMENTS USING SEEDED SAMPLES

3.5.1 Growth conditions

The type strains *Acanthamoeba castellanii* ATCC 30010, *Legionella pneumophila* ATCC 33152, *Mycobacterium avium* ATCC 49601, *Escherichia coli* ATCC 25922 were obtained from the American Type Culture Collection. The freeze-dried amoebal and bacterial type strains were reconstituted, stored and sub-cultured according to the supplier's instructions. The growth and maintenance conditions of axenic *A. castellanii*, *L. pneumophila* and *M. avium* were optimised by our previous study (Muchesa et al., 2014). The optimized conditions (sample concentration methods, incubation temperatures and food sources) were then applied to test water samples seeded with type strains of the bacteria and amoebae as well as non-seeded wastewater samples. The validation of these methods is an ongoing procedure. The techniques have so far been successfully applied to isolate FLA and ARB from > 800 water and biofilm samples from various sources (wastewater, borehole water and drinking water) (results not part of this deliverable, not shown). Briefly, this report will show how the amoebae enrichment technique was validated using seeded samples and then applied to hospital samples.

3.5.2 Experimental procedures

Nine sterile and non-sterile tap water samples were seeded with the type strains of *A. castellanii, L. pneumophila* and *M. avium* in different combinations (Table 3.6). Each 500 ml sample was inoculated with 1.5 ml of the bacterial suspension at an optical density of 0.1 at 595 nm (Powerwave-X spectrophotometer, USA) to standardize bacterial numbers. Amoebal trophozoites for the seeding experiment were harvested from axenic growth in tissue culture flasks, washed three times by centrifugation, resuspended in 1 ml Page's amoebal saline (PAS) and added to the water sample. Each seeded water sample was then split into 10 equal portion (50 ml) and analysed by amoebae enrichment technique.

Table 3.6: Combinations of seeded samples analysed

Sample type	Type strains	Samples processed
Non-sterile tap water	Ac	10
	Ac + Lp	10
	Ac + Ma	10
	Ac + Lp + Ma	20
Sterile tap water	Ac	20
	Ac + Lp	20

Ac: *Acanthamoeba castellanii*; Lp: *Legionella pneumophila*; Ma: *Mycobacterium avium*

3.5.3 Amoebal enrichment

Amoebal enrichment technique was adapted from Thomas et al. (2006) and Lamoth and Greub (2010). The 90 split seeded samples were concentrated by filtration using a 0.45 µm pore size cellulose nitrate membrane (Millipore, SA). The membrane was placed upside down onto a non-nutrient agar heat-killed *E.coli* (NNA-HKEC) plate with a few drops sterile PAS, incubated aerobically at 32°C and checked daily under a light or inverted microscope for the appearance of amoebal trophozoites and cysts. The density of amoebal growth on the plates was recorded as (the average in 10 fields): <10 per field (+), 10-100 per field (++) or > 100 per field (+++). 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 removed from the agar by gentle scraping, re-suspended in sterile PAS and washed at least three times at 1000 x g for 20 minutes to remove extracellular bacteria and debris. The concentrate was then resuspended in sterile PAS, inoculated into a sterile 24 well flat-bottomed microtiter plate (Nunc, USA) and again incubated at 32°C. The plates were checked for the morphological appearance of trophozoites and/or cysts under an inverted microscope (Leica, Germany), equipped with a 40 X objective, at regular intervals. Fifty microlitres of the amoebae suspension was harvested from the microtitre plate, heat-fixed on microscope slides and Giemsa stained to screen for the presence of amoebal trophozoites and/or cysts.

3.5.4 Cultivation of amoebae and infection with *Legionella pneumophila*

The *A. castellanii* type strain was inoculated into 25 cm^2 tissue culture flasks with 10 ml plate count broth (PCB) and incubated at 32ºC until they formed a homogenous layer. Amoebal trophozoites from the surface of the tissue culture flasks were loosened by 'tapping'. Using a sterile micropipette, 1ml of the amoebae suspension was transferred to a sterile Eppendorf tube containing 1ml PAS and mixed by vortexing at maximum speed for 30 s. The amoebae cells were then washed three times by centrifugation at 1500 x g for 10 minutes. The supernatant was removed, leaving approximately 1 ml covering the pellet. The pellet was suspended by vortexing at maximum speed for 30 s. After the last centrifugation, each of the 1 ml of the re-suspended amoebal cells was transferred into a 25 cm^2 tissue culture flask; the concentration of cells was adjusted to 5 X 10^5 cells/ml. One ml of this suspension was transferred into each of the wells of a 24 flat-bottom microtitre plate and incubated for one hour at room temperature to allow attachment of amoebae to the bottom of the wells. Each well was inoculated with bacterial suspension at an optical density of 0.1 at 595 nm (Powerwave-X spectrophotometer, USA) and co-culture suspension screened by transmission electron microscopy.

Amoebal trophozoites and cysts were recovered from all the seeded sample portions after primary culture on NNA plates seeded with heat killed *E. coli*. After enrichment and purification, trophozoites were observed containing live (moving) bacteria in 74 / 90 (82.2%) of the samples by direct microscopy. In 86 (95.6%) samples, amoebae with intracellular bacteria were observed after Giemsa staining (Figure 3.3). Of the 30 samples subjected to Ziehl Neelsen staining, 26 (86.7%) contained acid fast bacteria indicating the presence of *M. avium*. Using transmission electron microscopy, *A. Castellani* was shown to habour *L. pneumophila* after setting up of co-cultures (Figure 3.2) *L. pneumophila* and *M. avium* were confirmed by culture from 48 /

50 (96.0%) and 29 / 30 (96.7%) of the seeded samples respectively. The three samples from which the bacteria were not recovered by culture were those originally seeded with all three type strains. This may have been due to the bacteria being present in concentrations too low to be detected by culture, the VBNC state or the use of an inappropriate multiplicity of infection (MOI) for the seeding experiments. Nevertheless, we showed that amoebal enrichment can be used under our laboratory conditions to recover FLA and ARB from seeded samples.

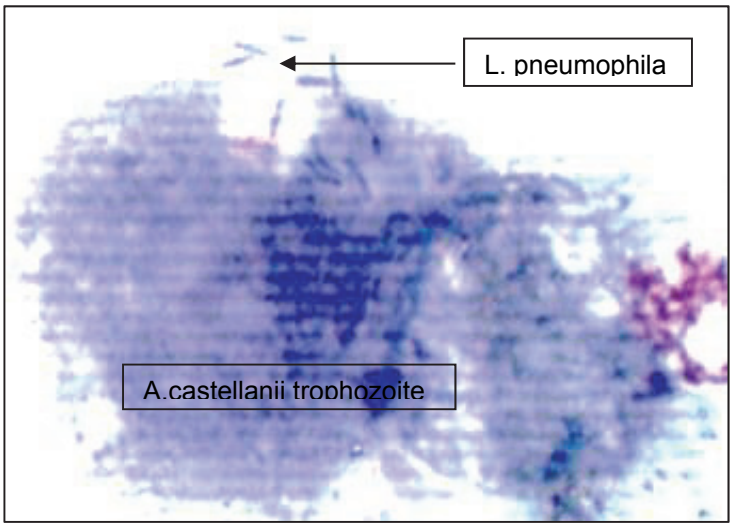

Figure 3.3: *L. pneumophila* phagocytosis by *A. castellanii* (Giemsa, 400x)

3.6 PHYSICO-CHEMICAL ANALYSIS

At each sampling site, water temperature, pH, turbidity, conductivity and total organic carbon (TOC) were measured with a portable COMBO TESTER® (Hanna, SA) according to the manufacturer's instructions. Free and total chlorine were measured using were also measured with a chlorine photometer (Hanna, SA) according to the manufacturer's instructions. The samples were then collected and transported in cooler boxes to the laboratory and processed on the day of receipt. The samples were transported to the lab at ambient temperature and processed within 24 hours.

3.7 MICROBIOLOGICAL ANALYSIS

The amoebal enrichment technique applied in the isolation and identification of FLA and ARB for seeded and wastewater samples were also used for hospital water and biofilm samples. The technique was used together with molecular based methods (PCR and sequencing), staining methods (TEM and Giemsa staining) and biochemical methods (VITEK analysis) to detect FLA and possible ARB (Figure 3.4)

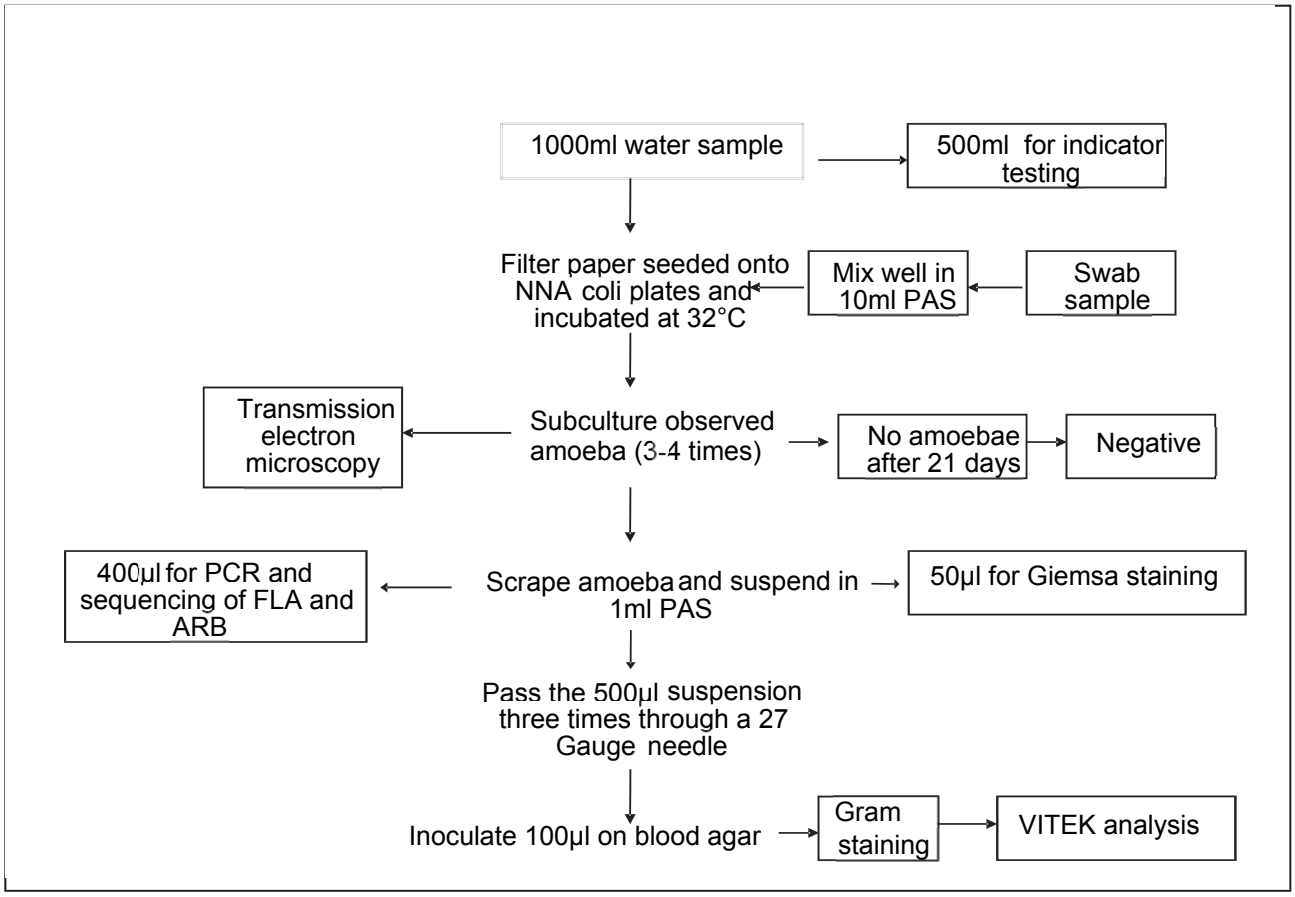

Figure 3.4: Protocol used to isolate and identify FLA and ARB in hospital water and biofilm samples

3.7.1 Testing for microbial indicator organisms

One portion of 100 ml (from the 500 ml) of each water sample was mixed with Colilert® media (IDEXX Laboratory) in sterile 100 mL glass funnels with lids and sealed in Quanti-Tray®/2000 containers. The trays were incubated for 24 h at 35ºC. After incubation, the trays were examined under long wave (366 nm) ultraviolet light, and wells that both turned yellow and fluoresced were counted as *E. coli* positive. *E. coli was used as a positive control and K. pneumoniae* and *P. aeruginosa* as negative controls.

3.7.2 Isolation of free living amoebae

Five hundred millilitres of water sample was concentrated by filtration using a cellulose nitrate membrane (Millipore, SA) with a pore size of 0.45 µm to allow growth of indigenous amoebae. The swabs containing biofilm were vortexed at maximum speed for 30 seconds in 10 ml Page's amoebal saline buffer (PAS) in individual sterile tubes to release the biofilm and the suspension was then concentrated by membrane filtration as described for water samples. The filter membrane was placed upside down onto non-nutrient agar (NNA) overlaid with a suspension of heat-killed *E. 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 X 10 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 cells as much as possible. Once purified, amoebae cells were harvested by gently scraping the agar surface and re-suspending in 1 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 re-suspended in 1 ml PAS. Nine hundred and fifty microlitres was inoculated in 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 morphological appearance of FLA. Transmission electron microscopy (TEM) was used to determine the presence of intracellular bacteria.

3.7.3 Detection of free-living amoebae using molecular methods

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).

3.7.4 Screening for potential ARB

To screen for ARB, the 24 well flat-bottom microtitre plates were first checked for the morphological appearance of trophozoites and/or cysts with intracellular bacteria under an inverted microscope (Leica, Germany) equipped with a X 40 objective for up to three days. Photographs were taken from day 1 to day 3 at various stages of the process. Staining, molecular and biochemical analysis were then performed to identify the possible intracellular bacteria. Amoebae potentially containing intra-amoebal bacteria were harvested by scraping the bottom of the microtitre well to suspend amoeba cells and heat-fixing 50 µl portions on microscope slides. Gimenez staining, to screen for the presence of bacterial proliferation, amoebal trophozoites and cysts potentially containing ARB, was performed using standard methodology.

3.7.5 Characterization of bacterial isolates using the VITEK® 2 Compact System

Bacteria isolates were characterized using the VITEK® 2 Compact System (BioMérieux, Inc.) which identifies organisms using biochemical reactions and newly developed substrates measuring carbon source utilization, enzymatic activities, and resistance. Gram stains were performed on the colonies to group them a Gram positive or negative (Silhavy et al., 2010). Pure colonies were placed in a tube containing 3ml saline (BioMérieux, Inc.) and the density was monitored with the VITEK® 2 DensiCHEK™. Bacterial suspensions not within the appropriate zone were adjusted to 0.5-0.63 [McFarland No. (McF)]. Once the correct density was achieved, the appropriate VITEK® 2 Identification Card was selected and inserted into the tube containing the suspension. Thus the VITEK® 2 Identification Card for Gram positive organisms were placed in the tube containing the Gram positive bacterial suspension and the VITEK® 2 Identification Card for Gram negative card were placed in the tube containing Gram negative bacterial suspension. Thereafter, the instructions on data entry and how to load the cassette into the instrument was followed as directed by the instrument. After insertion of the cassettes into the instrument, the cassettes were incubated for 18-24 h, after which the organisms were identified using the VITEK® 2 Compact System software.

3.7.6 MOLECULAR DETECTION OF *LEGIONELLA* SPECIES

A two-hundred microlitre suspension of lysed amoebae (of 150 positive samples) was used to extract whole genome using QIAmp DNA Blood Mini Kit (Qiagen, Hilden, Germany) following the protocol provided by the manufacturer. Following the extraction process, quantitative and qualitative polymerase chain reaction in accordance to the ISO/TS 12869 (first edition, 2012-11-01) using Quantitec Probe PCR Kit (Qiagen, Hilden, Germany) in a Rotor-Gene 6000 Cycler (Corbett Life Science, Mortlake, Australia) was conducted.

Polymerase chain reactions detected DNA of *Legionella* spp. by using the (LEG-225 5'-AAGATTAGCCTGCGTCCGAT-3)' and the LEG-858 (5'-GTCAACTTATCGCGTTTGCT-3'), which amplifies

approximately 654 bp of the 16 rRNA gene (Miyamoto, 1997). The primers at a concentration of 10 µM each were transferred into a reaction tube containing 10 µl template DNA, 2 mM MgCl2 , 2.5 U of taq DNA polymerase (Life Technologies, SA), 100 µM each deoxynucleoside triphosphate and 8 µl Ultra pure water (Fermentas, Canada) and 0.2 µl. hotStar Taq Polymerase. A positive control was used in each experiment which comprised of all the reagents mentioned above other than template DNA which was replaced with 10 µl of genomic DNA extracted from the reference strains of *Legionella* spp*.* A negative control was also used in each experiment which comprised of all the reagents mentioned above other than template DNA which was replaced with 10 µl of PCR water. To detect *Legionella* spp., the reaction tubes were initially activated at 95°C for 90 s, followed by 45 cycles of amplification using denauration 94°C for 1 minute. Annealing was done at 55°C for 30 s and extension at 72°C for 1 minute followed with a final extension cycle at 72°C for 2 minutes. DNA was analyzed in a horizontal 2% (w/v) agarose slab gel (FP Agarose from Promega) with ethidium bromide (0.5 µg/ml) in a TAE (40 mM Tris acetate; 2 mM EDTA, pH 8.3) buffered system. 5 µl of 100 bp DNA marker (Fermentas O‟GeneRuler DNA ladder; Canda) was loaded into the first well of the gel, into the remaining wells 10 µl each sample (including positive and negative controls) mixed with 3 µl of loading dye (Fermentas Orange x 6 Loading Dye; Canda).

L.pneumophila was detected using quantitative PCR (qPCR) with primers LpneuF (5'-CC GATGCCACATCATTAGC-3') and LpneuR (5_-CCAATTGAGCGCCACTCATAG-3_) and a TaqMan probe, LpneuP (5_- 6-carboxyfluorescein [FAM]-TGCCTTTAGCCATTGCTTCCG-BHQ1-3_), which target the *mip* gene. The amplification mixture for the detection of *L. pneumophila* using primers LpneuF and LpneuR and the probe LpneuP consisted of 25 µl of iQ supermix (Bio-Rad Laboratories B.V., Veenendaal, Netherlands), 0.4 mg/mL of bovine serum albumin (BSA; PCR grade; Roche Diagnostics, Almere, Netherlands), 0.2µM of each primer and probe, 0.2 µM of each primer, and 10 µl of DNA template in a total reaction volume of 50 µl. To detect *Legionella* spp., the reaction tubes were initially activated at 95°C for 90 s, followed by 43 cycles of amplification using denauration 95°C for 3 minute. Annealing was done at 95°C for 20 s and extension at 60°C for 1 minute. Detection by Q-PCR and data analysis was performed with a real-time PCR Rotor-Gene 6000 Cycler (Corbett Life Science, Mortlake, Australia).

Table 3.7: Primers used for amplification.

ORGANISM	TARGET	PRIMER	SEQUENCE	BP	REFERENCE
FLA	18S rRNA	Ami6F1 Ami6F2 Ami9R	5′CCAGCTCCAATAGCGTATATT3′ 5'CCAGCTCCA AGAGTGTATATT3' 5′GTTGAGTCGAATTAAGCCGC3′	700	Thomas et al., 2006
Acanthamoeba spp.	18S rRNA	JDP1 JDP2	5'GGCCCAGATCGT TTACCGTGAA3' 5'TCTCACAAGCTGTAGGGGAGTCA3'	1000	Schroeder et al., 2001
Legionella spp.	16S rRNA	Leg225 Leg858 LpneuF LpneuR	5′AAGATTAGCCTGCGTCCGAT3′ 5′GTCAACTTATCGCGTTTGCT3′ 5'CCGATGCCACATCATTAGC3' 5'CCAATTGAGCGCCACTCATAG3'	660	Miyamoto et al., 1997 Omiccioli et al., 2015

bp=base pairs

CHAPTER 4: RESULTS AND DISCUSSION

4.1 INTRODUCTION

The occurrence of free-living amoebae (FLA) and its associated bacteria in healthcare water systems has been described worldwide. However contamination of water systems with these potentially pathogenic microorganisms has not been reported in water distribution systems of health care settings in South Africa. Colonisation of hospital water distribution systems can be a source of nosocomial infections. There is therefore a need to determine the occurrence and diversity of free living amoebae and amoeba resistant bacteria in these systems and monitor possible exposure of patients and hospital personnel.

4.2 WALKTHROUGH ASSESSMENTS AND SAMPLING SITES

4.2.1 Hospital A

4.2.1.1 Municipal water supply and inlet

The hospital is over 50 years old; there is thus no official record of the layout of the original water distribution system. The hospital receives water from the Johannesburg municipality which is stored in four reservoir tanks on the roof of the building. From here it is distributed to the different wards and sections of the hospital. The water system also contains three hot water tanks which provide steam for heating. The steam is transferred to calorifiers which then transfer the heat (from the steam) to the hot water distribution system and from there to the different sections of the hospital.

4.2.1.2 Sampling sites

A walkthrough assessment of the water system was conducted in collaboration with infection control practitioners and the facility manager. Through recommendations by infection control practitioners, seven areas were identified as high risk of exposure to waterborne pathogens. These areas are: (i) municipal water inlet, (ii) renal unit, (iii) intensive care unit (ICU), (iv) theatre complex, (v) endoscopy/bronchoscopy unit, (vi) central sterilization service unit (CSSU) and (vii) theatre sterilization service unit (TSSU) (Table 4.1). The infection control staff did not have records of any previous outbreaks of waterborne pathogens in the areas included in the walkthrough assessment. Water quality testing is only carried out when there are renovations or when new water pipes are being installed, and then only for the traditional indicator organisms (coliforms, faecal coliforms and *E. coli*).

Table 4.1: Sampling sites for Hospital A

SAMPLING SITE	BASIN TAPS	BATH TAPS	SHOWERHEADS	TOTAL
Renal unit	39	-	-	39
Intensive care unit	8	-	-	8
Theatre	70	-	6	76
TSSU	6	-	-	6
CSSU	11	-	-	11
Endoscopy/bronchoscopy	15	2	1	18
Water inlet	1	-	-	1
TOTAL	150	2	7	159

CSSU-Central sterilization service unit; TSSU-theatre sterilization service unit

- Renal Unit: The renal unit is a 19 bed ward with a total of 39 basin taps. A hydroboil unit for hot water supply is installed at one of the basin taps. There are no showers in the renal unit. Water for renal dialysis is treated by reverse osmosis (RO) before being transferred to the dialysis units. The RO water treatment is outsourced to a contractor who manages the treatment system. It is unclear whether the contractor tests the water quality at any point during the treatment process or before entering the dialysis machines.
- Adult intensive care unit: The adult intensive care unit (ICU) contains ten beds of which eight were occupied at the time of the assessment. The ward has a total of eight functional taps and no showers. The staff reported that the water from the taps has a dark, reddish colour from time to time.
- Theatre complex: The theatre complex consists of twelve theatre rooms with a total of 70 basin taps and 6 showers. A hydroboil unit for hot water supply is installed at one of the basin taps. Three of the six showers are not frequently used.
- Endoscopy/Bronchoscopy unit: The endoscopy / bronchoscopy (EB) unit has a total of 15 basin taps, two bath taps and one shower.
- Central sterilization service unit and theatre sterilization service unit: There are eleven basin taps in the central sterilization service unit (CSSU) and six in the theatre sterilization service unit (TSSU).

4.2.2 Hospital B

4.2.2.1 Municipal water supply and Inlet

The hospital receives water from the Johannesburg municipality; this water is stored in two reservoir tanks situated on the roof of the building. For the hot water system, the municipal water is salted and heated in boilers to provide steam for heating. The steam is transferred to calorifiers which then transfer the heat (from the steam) to the hot water distribution system and from there to the different sections of the hospital.

4.2.2.2 Sampling sites

A walkthrough assessment was conducted at Hospital B in close collaboration with the infection control practitioners and the facility manager. Five potentially high risk areas were identified: (i) municipal water inlet, (ii) neonatal ward (NW), (iii) neonatal intensive care unit (NICU), (iv) diarrhoea ward and (v) milk kitchen (Table 4.2). There is no known history of outbreaks related to waterborne pathogens in any of these wards. Water quality testing is only carried out when renovations are done or when new water pipes are being installed and then only for the traditional indicator organisms (coliforms, faecal coliforms and *E. coli*). The hospital is more than 70 years old; there is thus no official record of the layout of the original water distribution system.

Table 4.2: Sampling sites for Hospital B

SAMPLING SITE	BASIN TAPS	BATH TAPS	SHOWERHEADS	TOTAL
Neonatal ward	17	-	-	17
Neonatal ICU	6	-	-	6
Diarrhoea ward	2	1	-	3
Milk kitchen	2	-	-	2
Water inlet	1	-	-	1
TOTAL	28	1	0	29

- Neonatal ward (NW) and neonatal intensive care unit (NICU): The neonatal ward is a 35 bed unit with a total of 17 basin taps. This ward houses ill or premature newborn infants. The NICU is an intensive care unit specializing in the care of critically ill or prematurely born infants. The NICU consists of six beds and has a total of six functional water taps: one in the isolation room, two in the cleaning area, two in the blood washing area and one in the staff tea room.
- Diarrhoea ward: The diarrhoea ward for children contains eight beds in the general ward and two beds in the isolation room. The ward has a total of six functional taps and no showers.
- Milk kitchen: The milk room is divided into mixing and washing areas with two basin taps.

4.2.3 HOSPITAL C

4.2.3.1 Municipal water supply and inlet

Water from the municipality is received in reservoir tanks situated in the basement of the hospital building. The water is then pumped to the top of building before being distributed by gravity to different sections of the hospital. For the hot water distribution system, the municipal water is salted and heated in boilers to provide steam for heating. The steam is transferred to calorifiers which then transfer the heat (from the steam) to the hot water distribution system and from there to the different sections of the hospital.

4.2.3.2 Sampling sites

A walkthrough assessment was conducted at Hospital C in collaboration with the infection control practitioners, facility manager and facility engineer. Eight units with specialized wards were identified as high risk areas for exposure to waterborne pathogens during the assessment. These areas are: (i) intensive care unit (ICU), (ii) theatre complex, (iii) endoscopy unit, (iv) renal unit, (v) milk room, (vi) central sterilization service unit (CSSU) and (vii) theatre sterilization service unit (TSSU) (Table 4.3). There is no known history of outbreaks related to waterborne pathogens in any of the areas included in the walkthrough assessment. Water quality testing is only carried out when renovations are done or when new water pipes are being installed and then only for the traditional indicator organisms (coliforms, faecal coliforms and *E. coli*).

Table 4.3: Sampling sites for Hospital C

SAMPLING SITE	BASIN TAPS	BATH TAPS	SHOWERHEADS	TOTAL
Renal Unit	50	-	-	50
Intensive care units	70	-	2	72
Theatre	149	-	10	159
TSSU	6	-	-	6
CSSU	10	-	-	10
Endoscopy/bronchoscopy	2	-	-	2
Milk kitchen	5	-	-	5
Renal unit	8	-	-	8
Water inlet	1	-	-	1
TOTAL	301	0	12	313

CSSU-Central sterilization service unit; TSSU-theatre sterilization service unit

- Intensive care unit (ICU): The ICU is divided into four specialised units: cardio-thoracic ICU, neurosurgical ICU, cardiac ICU and trauma ICU. The cardio-thoracic ICU has three adult cubicles, a pediatric cubicle and an equipment room with a total of 13 basin taps. The neurosurgical ICU has eight cubicles, a kitchen, toilets and a corpse dressing room with a total of 26 basin taps. The cardiac ICU consists of a general ward with six beds, an isolation cubicle and a kitchen with a total of 9 basin taps. Trauma ICU has nine cubicles and a kitchen with a total of 22 basin taps.
- Theatre complex: The theatre complex is divided into nine specialised sections: emergency, general surgery, neurosurgery, pediatrics, orthopedics, urology, cardiac, eye and vascular sections. There are a total of 149 basin taps and 10 showers.
- Endoscopy unit: Endoscopy unit consists of a scope room, a procedure room and a kitchen with a total of four basin taps.
- Renal unit: This ward has its own reverse osmosis treatment used to supply dialysis points. Eight dialysis points were identified in this ward.

- Milk room: The milk room is divided into the dirty, mixing and washing areas with a total of five basin taps.
- Central sterilization service unit (CSSU) and theatre sterilization service unit (TSSU): The two sterilization service units contain a total of eight basin taps in the areas where instruments are washed and in the toilets.

4.3 PHYSICOCHEMICAL PARAMETERS

The physicochemical parameters of the water at the three hospitals were measured at the time of sampling and are summarised in Table 4.4.

Table 4.4: Physicochemical parameters of the three hospitals

Hospital	Temperature (°C)			pH			TDS (mg/L)			Residual chlorine (mg/L)		
	Range	M	Me	Range	M	Me	Range	M	Me	Range	M	Me
A	14.8-27.3	23.1	22.30	1.7-8.8	7.77	7.89	10-187	126	124	0.01-0.35	0.12	0.1
B	18.3-30.8	22.6	22.55	7.6-8.5	7.97	7.97	114-148	120	118	0.06-0.33	0.23	0.245
C	20.0-25.7	22.6	22.20	1.8-8.1	7.80	7.45	109-143	124	123	0.01-0.34	0.20	0.19
All	14.8-30.8	22.8	22.30	1.7-8.8	7.80	7.91	10-187	123	123	0.01-0.35	0.28	0.18
South African Standard (SANS 241:2015)				6.0-9.0			≤1200 mg/L			≤ 5 mg/L		

TDS – total dissolved solids; M – mean; Me – median

4.3.1 Water temperature

The water temperature in the three hospitals at the time of sampling ranged between 14.8°C and 30.8°C with no significant differences among the hospitals. The temperatures ranged between 14.8 °C and 27.3°C (mean 23.1°C) at Hospital A, between 18.3 °C and 30.8°C (mean 22.6°C) at Hospital B and between 20.0°C and 25.7°C (mean 22.6°C) at Hospital C.

4.3.2 pH levels

The pH of the water collected at the three hospitals ranged between 1.74 and 8.77 (mean 7.77). The lowest pH recorded (1.74) was at the reverse osmosis treatment plant which feeds the renal unit of Hospital A. The pH levels recorded ranged between 7.56 and 8.47 (mean 7.97) at Hospital B and between 7.56 and 8.06 (mean 7.89) at Hospital C.

4.3.3 Total dissolved solids

The TDS levels were all within acceptable limits and ranged between 10-187 mg/L (mean 126 mg/L); 10-187 mg/L (mean 126 mg/L) at Hospital A, 114-148 mg/L (mean 120 mg/L) at Hospital B and 119-143 mg/L (mean 126 mg/L) at Hospital C.

4.3.4 Residual chlorine

The residual chlorine levels of all three hospitals ranged between 0.01 and 0.34 mg/L (mean 0.28 mg/L) at the time of sampling. The chlorine levels of the individual hospitals were 0.01-0.35 mg/L (mean 0.12 mg/L) for Hospital A, 0.06-0.33 mg/L (mean 0.23 mg/L) for Hospital B, and 0.01-0.34 mg/L (mean 0.20 mg/L) for Hospital C.

4.4 DETECTION OF FREE-LIVING AMOEBAE BASED ON MORPHOLOGY

4.4.1 Isolation of amoebae in Hospital A

A total of 51 water and 46 biofilm samples (n=97) were collected from the municipal water inlet of the hospital, theatre complex, TSSU, CSSU, endoscopy/bronchoscopy unit, intensive care unit (ICU) and the renal unit. Based on morphological characteristics, free-living amoebae were observed using light and inverted microscopy in 77 (79.4%) of the 97 samples (40 water and 37 biofilm) after amoebal enrichment. Amoebae were present in both the water and biofilm samples from all sampling sites, except for one water sample from the intensive care unit. Of the positive samples, 6 (7.80%) and 26 (33.8%) resembled *Acanthamoeba* and *Vermamoeba* species (formerly *Hartmanella* spp.) respectively. The FLA observed in the rest of the samples could not be clearly distinguished morphologically and were categorised as “other FLA” (Figure 4.1).

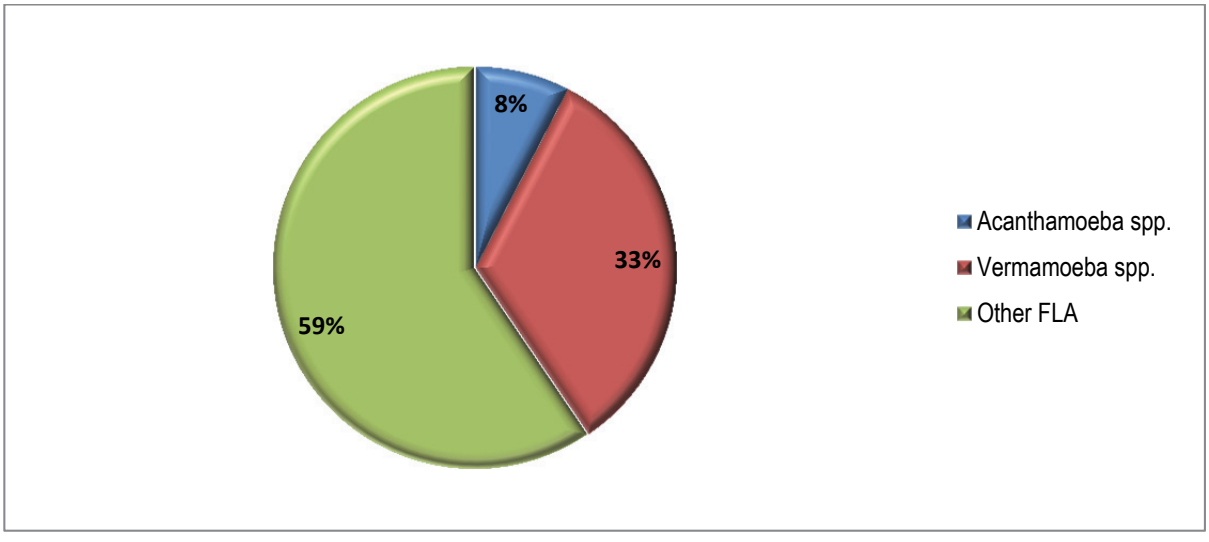

Figure 4.1: Percentage distribution of amoebae isolated in Hospital A

Vermamoeba and *Acanthamoeba* species were isolated from all the sampling areas except the endoscopy/bronchoscopy unit and the ICU which did not contain *Acanthamoebae* (Figure 4.2). One sample from the CSSU and two from the theatre complex contained both *Acanthamoeba* and *Vermamoeba* species. The typical morphology of *Acanthamoeba* cysts and *Vermamoeba* trophozoites is shown in Figure 4.3.

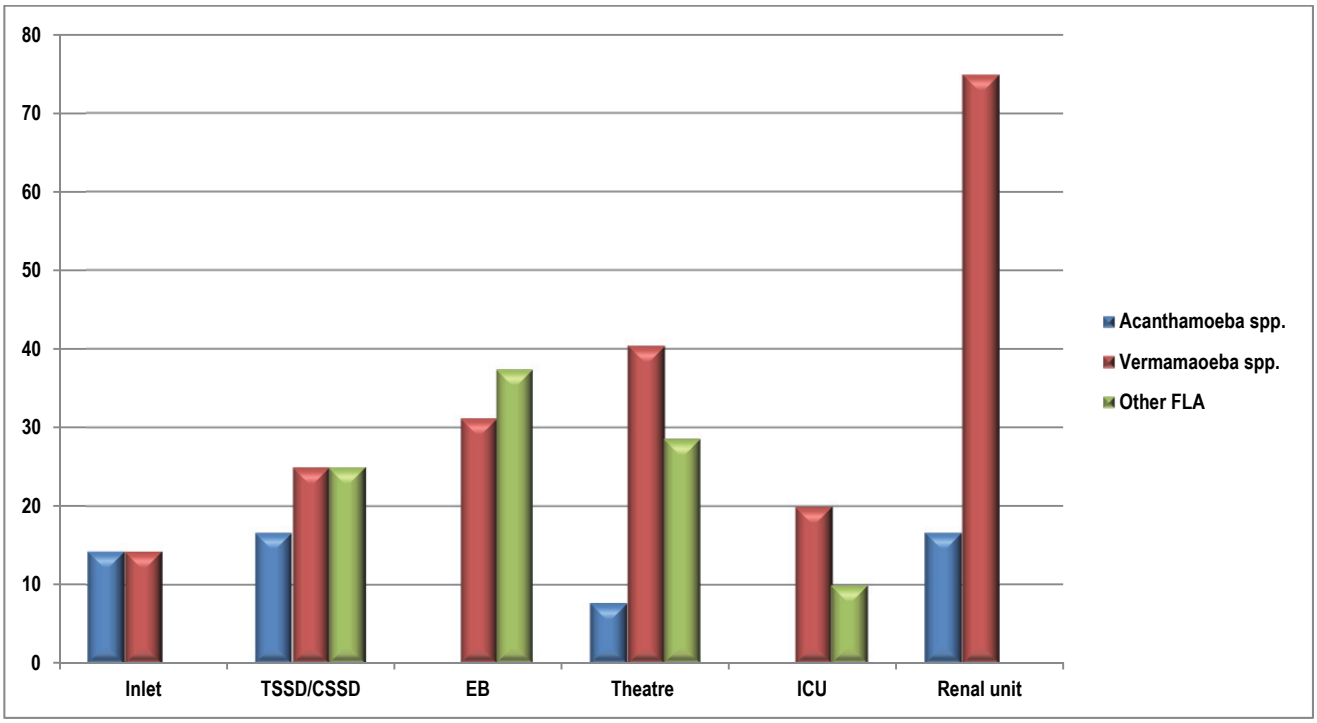

Figure 4.2: FLA morphology in Hospital A

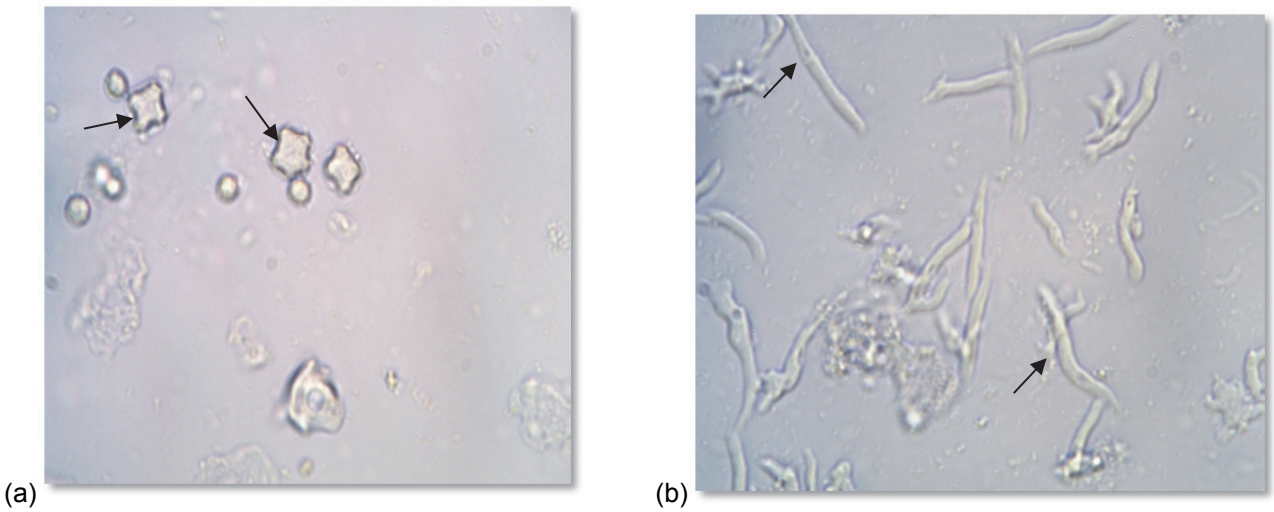

Figure 4.3: Typical morphology of *Acanthamoeba* cysts and *Vermamoeba* trophozoites (a) *Acanthamoeba* cysts and (b) *Vermamoeba* trophozoites (arrows), scale bar 100µm

4.4.2 Isolation of amoebae in Hospital B

Eighteen (18) water and twelve (12) biofilm (n=30) samples were collected from the municipal water inlet, neonatal ICU, neonatal ward, milk room and the diarrhoea ward. Free-living amoebae were observed in seven (46.7%) water and eight (53.3%) biofilm samples after amoebal enrichment. Based on morphology, three of the positive samples contained clearly distinguishable *Vermamoeba* trophozoites. The FLA present in the other twelve samples could not be distinguished morphologically and were placed in the category "other FLA" (Figure 4.4). None of the samples contained clearly distinguishable *Acanthamoeba* cysts. *Vermamoeba* species were identified from the municipal water inlet, neonatal ICU and the diarrhoea ward. No FLA were isolated from the milk room (Figure 4.4).

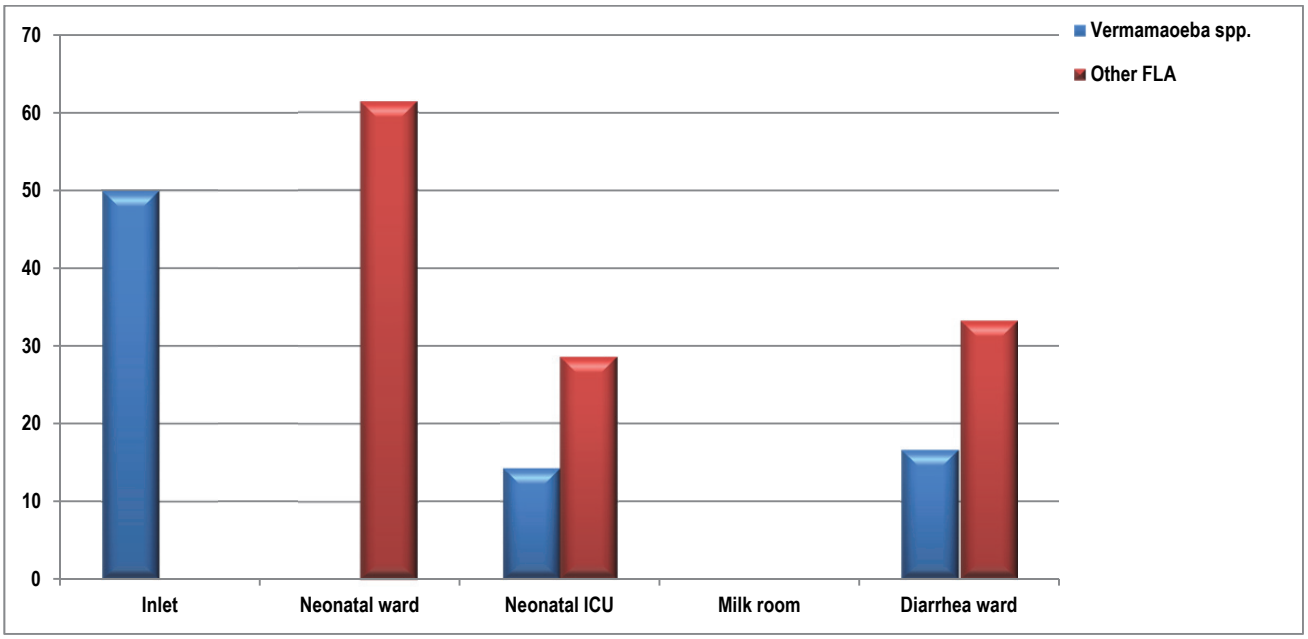

Figure 4.4: FLA identified based on morphology examination from different sampling sites of Hospital B

4.4.3 Isolation of amoebae in Hospital C

A total of 148 samples (77 water and 71 biofilm) were collected from the municipal water inlet, cardio-thoracic intensive care unit, neurosurgical intensive care unit, cardiac intensive care unit, trauma intensive care unit, milk room, endoscopy unit, renal unit and CSSU/TSSU of Hospital C. Free-living amoebae were isolated from 62 (41.2%) of the samples, 24 (38.7%) water and 38 (64.0%) biofilm, after amoebal enrichment. *Vermamoeba* species were present in most (56.0%) of the samples, while *Acanthamoeba* species were observed in only 15.0% of the samples. FLA were isolated from all the sampling areas except the municipal water inlet. Fifty percent of the samples collected at the cardiac ICU and sterilization units contained amoebae (Figure 4.5). Three samples (one each) from the neurosurgical ICU, trauma ICU and CSSU units contained both *Acanthamoeba* and *Vermamoeba* species.

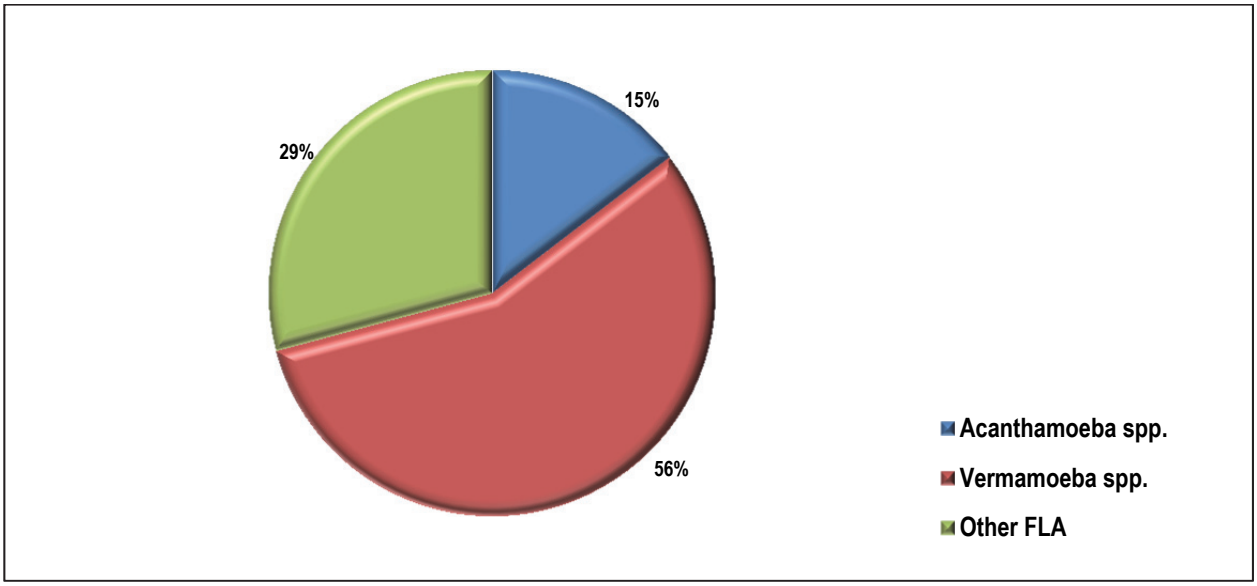

Figure 4.5: Percentage distribution of amoebae isolated from Hospital C

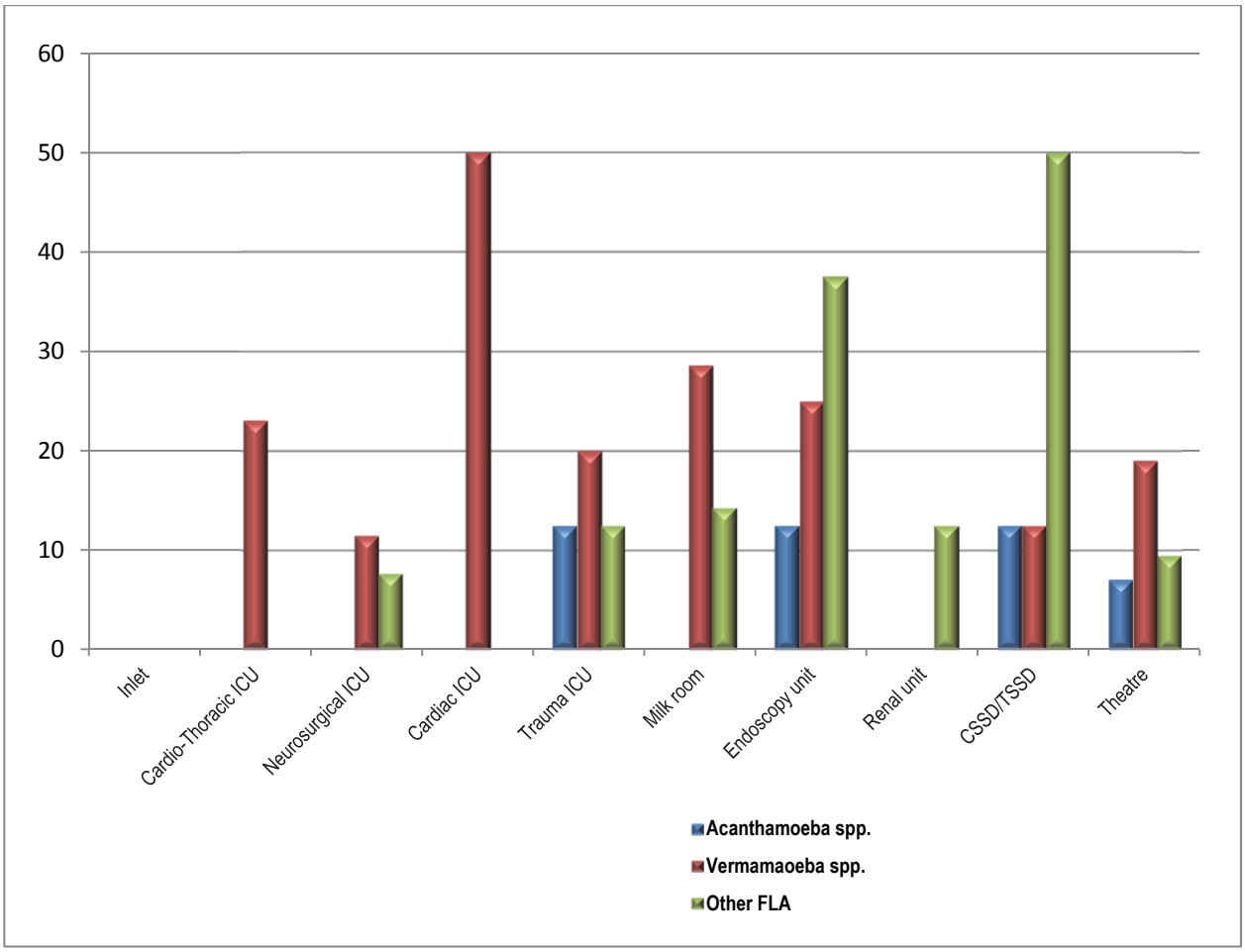

Figure 4.6: Free-living amoebae isolated at different sampling sites of Hospital C

4.5 IDENTIFICATION OF FLA BY PCR AND DNA SEQUENCING

4.5.1 Biodiversity of FLA in Hospital A

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. From these 53 amoebae, *Vermamoeba vermiformis* (87.0%) were the most abundant amoebae identified from all the sampling areas, followed by *Acanthamoeba* spp. (9.0%), *Naegleria* sp. (2.0%) and *Amoebozoa* (2.0%) (Figure 4.7).

A total of 23 *Vermamoeba vermiformis* isolates (GenBank accession numbers shown in Table 4.5), five *Acanthamoeba* spp. isolates (GenBank accession numbers: KT183616, KT183582, KT183604, KT183575, KT183576), one isolate each for *Amoebozoa* sp. (GenBank accession number KT183581) and *Naegleria* sp. (GenBank accession number KT183611) were identified based on 18 rRNA gene regions from different sampling areas of Hospital A. Furthermore, other protozoa *Pseudoparamoeba pagei* (GenBank accession number KT183621) and *Schizoplasmodiopsis amoeboidea* (GenBank accession number KT183622) were also detected in four samples (Table 4.5).

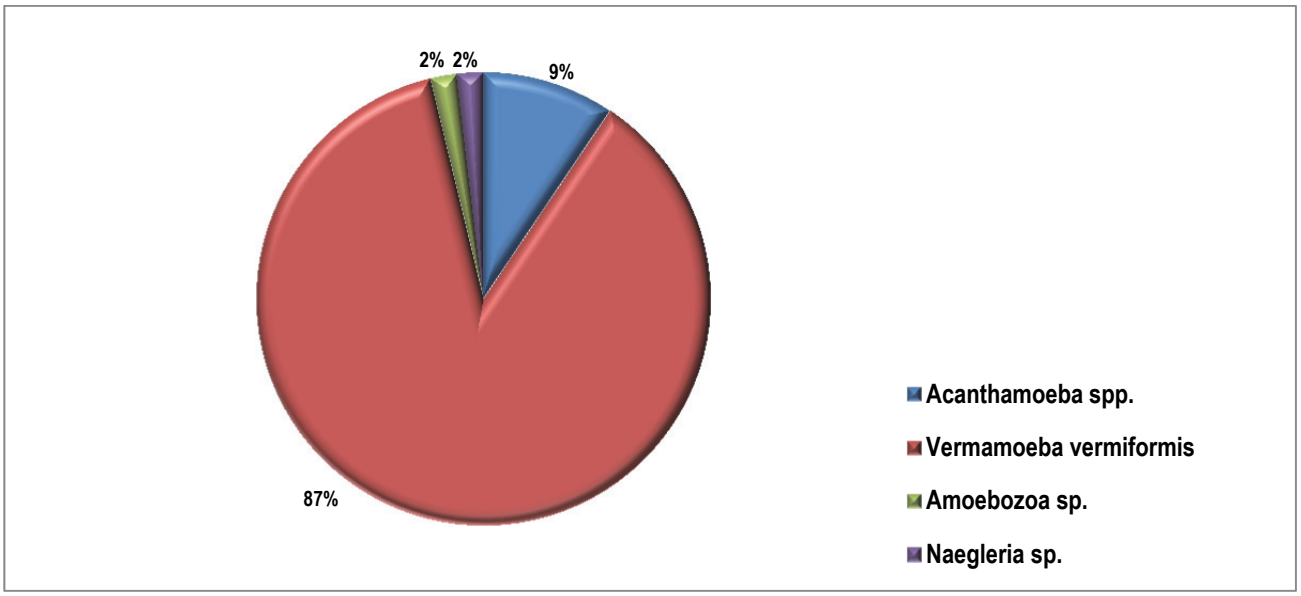

Figure 4.7: Percentage distribution of amoebae detected in Hospital A using PCR and DNA sequencing

Table 4.5: Biodiversity of FLA isolated from Hospital A

Origin	GenBank Accession No.	Published closest described species
Inlet	KT183594	*Vermamoeba vermiformis*
TSSU/CSSU	KT183577	*Vermamoeba vermiformis*
	KT183616	*Acanthamoeba* spp.
Endoscopy/Bronchoscopy unit	KT183578	*Vermamoeba vermiformis*
	KT183608	*Vermamoeba vermiformis*
	KT183579	*Vermamoeba vermiformis*
	KT183580	*Vermamoeba vermiformis*
	KT183588	*Vermamoeba vermiformis*
	KT183581	*Amoebozoa* species
	KT183582	*Acanthamoeba* species
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 species*
	KT183575	*Acanthamoeba species*
	KT183621	*Pseudoparamoeba pagei*
	KT183611	*Naegleria* species
Intensive care unit	KT183618	*Vermamoeba vermiformis*
	KT183574	*Vermamoeba vermiformis*
	KT183593	*Vermamoeba vermiformis*
	KT183591	*Vermamoeba vermiformis*
Renal unit	KT183576	*Acanthamoeba* species
	KT183612	*Vermamoeba vermiformis*
	KT183600	*Vermamoeba vermiformis*
	KT183625	*Vermamoeba vermiformis*
	KT183620	*Vermamoeba vermiformis*
	KT183619	*Vermamoeba vermiformis*
	KT183622	*Schizoplasmodiopsis amoeboidea*
	KT183584	*Pseudoparamoeba pagei*

4.5.2 Biodiversity of FLA in Hospital B

Fifty amoebae positive samples were analyzed using 18S rRNA PCR and DNA sequencing. Two samples were overgrown with fungi and were discarded. Nine samples (69.2%) contained *Vermamoeba vermiformis* and four (30.8%) from the 13 amoebae positive samples. A total of eight *Vermamoeba vermiformis* isolates (GenBank accession numbers shown in Table 4.6) and four *Acanthamoeba* spp. isolates (GenBank accession numbers: KT385816, KT385819, KT385809 and KT385814.

Table 4.6: Biodiversity of FLA isolated from Hospital B

Origin	GenBank Accession No.	Published closest described species
Neonatal ward	KT385808	*Vermamoeba vermiformis*
	KT385810	*Vermamoeba vermiformis*
	KT385815	*Vermamoeba vermiformis*
	KT385816	*Acanthamoeba* species
	KT385819	*Acanthamoeba* species
	KT385821	*Vermamoeba vermiformis*
Neonatal ICU	KT385807	*Vermamoeba vermiformis*
	KT385809	*Vermamoeba vermiformis*
	KT385817	*Acanthamoeba* species
Gastrointestinal ward	KT385811	*Vermamoeba vermiformis*
	KT385814	*Acanthamoeba* species
	KT385816	*Vermamoeba vermiformis*

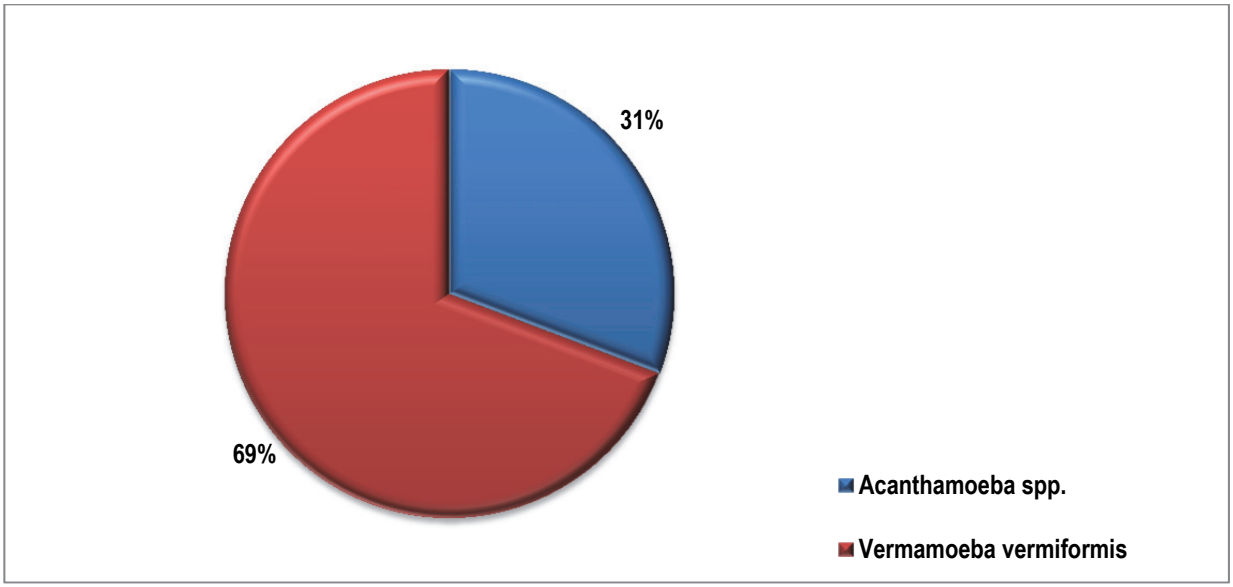

Figure 4.8: Percentage distribution of amoebae detected in Hospital B using PCR and DNA sequencing

4.5.3 Biodiversity of FLA in Hospital C

A total of 62 amoebae positive samples were analysed using 18S rRNA PCR and DNA sequencing. Of these, three showed no bands on agarose gel while ten were overgrown with fungi and were discarded. Therefore 49/62 (79.0%) of the samples were positive for amoebae and of these, 44 (89.8%) were confirmed as *Vermamoeba vermiformis,* four (8.2%) as *Acanthamoeba* species and one (2.0%) as *Naegleria* species (Figure 4.9).

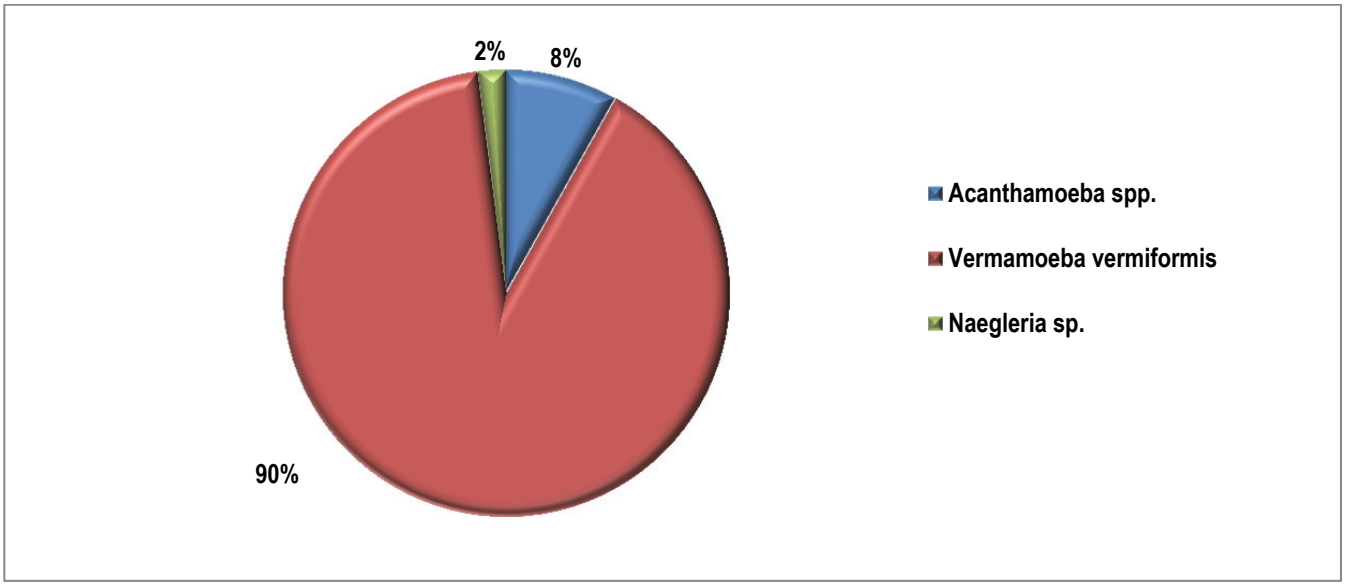

Figure 4.9: Percentage distribution of amoebae detected in hospital C using PCR and DNA sequencing

A total of 39 *Vermamoeba vermiformis* isolates (GenBank accession numbers shown in Table 4.7), 4 *Acanthamoeba* species isolates (GenBank accession numbers: KT385852, KT385836, KT385864, KT385844), one isolate of *Naegleria* species (GenBank accession number KT385863) were detected based on 18 rRNA gene regions from different sampling areas of Hospital C. Five *Trichia scabra* isolates (GenBank accession numbers KT385825, KT385828, KT385830, KT385837, KT385841) and four *Diderma testaceum* isolates (GenBank accession numbers KT385839, KT385870, KT385870, KT385864) were also detected.

Table 4.7: Biodiversity of FLA species isolated from water distribution system of Hospital B

Origin	GenBank Accession No.	Published closest described species
Cardio-Thoracic ICU	KT385822	*Vermamoeba vermiformis*
	KT385824	*Vermamoeba vermiformis*
	KT385831	*Vermamoeba vermiformis*
	KT385835	*Vermamoeba vermiformis*
	KT385832	*Vermamoeba vermiformis*
Neurosurgical ICU	KT385827	*Vermamoeba vermiformis*
	KT385829	*Vermamoeba vermiformis*
	KT385834	*Vermamoeba vermiformis*
	KT385838	*Vermamoeba vermiformis*
	KT385840	*Vermamoeba vermiformis*
	KT385842	*Vermamoeba vermiformis*
	KT385843	*Vermamoeba vermiformis*
	KT385846	*Vermamoeba vermiformis*
	KT385849	*Vermamoeba vermiformis*
	KT385852	*Acanthamoeba* species
	KT385856	*Vermamoeba vermiformis*
	KT385858	*Vermamoeba vermiformis*
	KT385859	*Vermamoeba vermiformis*
	KT385860	*Vermamoeba vermiformis*
Milk room	KT385854	*Vermamoeba vermiformis*
	KT385855	*Vermamoeba vermiformis*
Endoscopy	KT385836	*Acanthamoeba species*
	KT385844	*Vermamoeba vermiformis*
	KT385845	*Vermamoeba vermiformis*
	KT385850	*Vermamoeba vermiformis*
	KT385857	*Vermamoeba vermiformis*
	KT385871	*Vermamoeba vermiformis*
Theatre complex	KT385862	*Vermamoeba vermiformis*
	KT385863	*Naegleria* species
	KT385865	*Vermamoeba vermiformis*
	KT385866	*Vermamoeba vermiformis*
	KT385868	*Vermamoeba vermiformis*
	KT385869	*Vermamoeba vermiformis*
	KT385871	*Vermamoeba vermiformis*
	KT385872	*Vermamoeba vermiformis*
	KT385873	*Vermamoeba vermiformis*
	KT385874	*Vermamoeba vermiformis*
	KT385875	*Vermamoeba vermiformis*
	KT385876	*Vermamoeba vermiformis*
	KT385877	*Vermamoeba vermiformis*
	KT385878	*Vermamoeba vermiformis*
	KT385816	*Vermamoeba vermiformis*
	KT385864	*Acanthamoeba* species

4.6 PHYLOGENETIC ANALYSIS OF *ACANTHAMOEBA* SPECIES

Neighbour joining (NJ) analysis was performed using the genus specific primer set JDP-1 and JDP-2 to show relationships between the *Acanthamoeba* positive isolates in this study, and reference strains from NCBI GenBank for the genotypes T1-T20. A total of 13 *Acanthamoeba* spp. isolates (GenBank accession numbers: KT183616, KT183582, KT183604, KT183575, KT183576, KT385816, KT385819, KT385817, KT385814, KT385852, KT385836, KT385864, KT385867) from both Hospital A and B were used to construct two NJ trees. Five *Acanthamoeba* isolates from Hospital A (KT183616, KT183582, KT183604, KT183575, KT183576) in the first analysis were 91-95% (mean 93%) similar to the T20 genotype sequence (GenBank accession number DQ451162) as illustrated in Figure 4.10. For the second analysis, the neighbour joining method constructed the phylogenetic tree with bootstrap test using 1,500 replicates. Seven *Acanthamoeba* isolates (GenBank accession numbers: KT385816, KT385819, KT385817, KT385814, KT385852, KT385836, KT385864) closely resembled T1 and T3 genotypes, while one isolate (GenBank accession number: KT385867) closely resembled the genotype T20 (Figure 4.11).

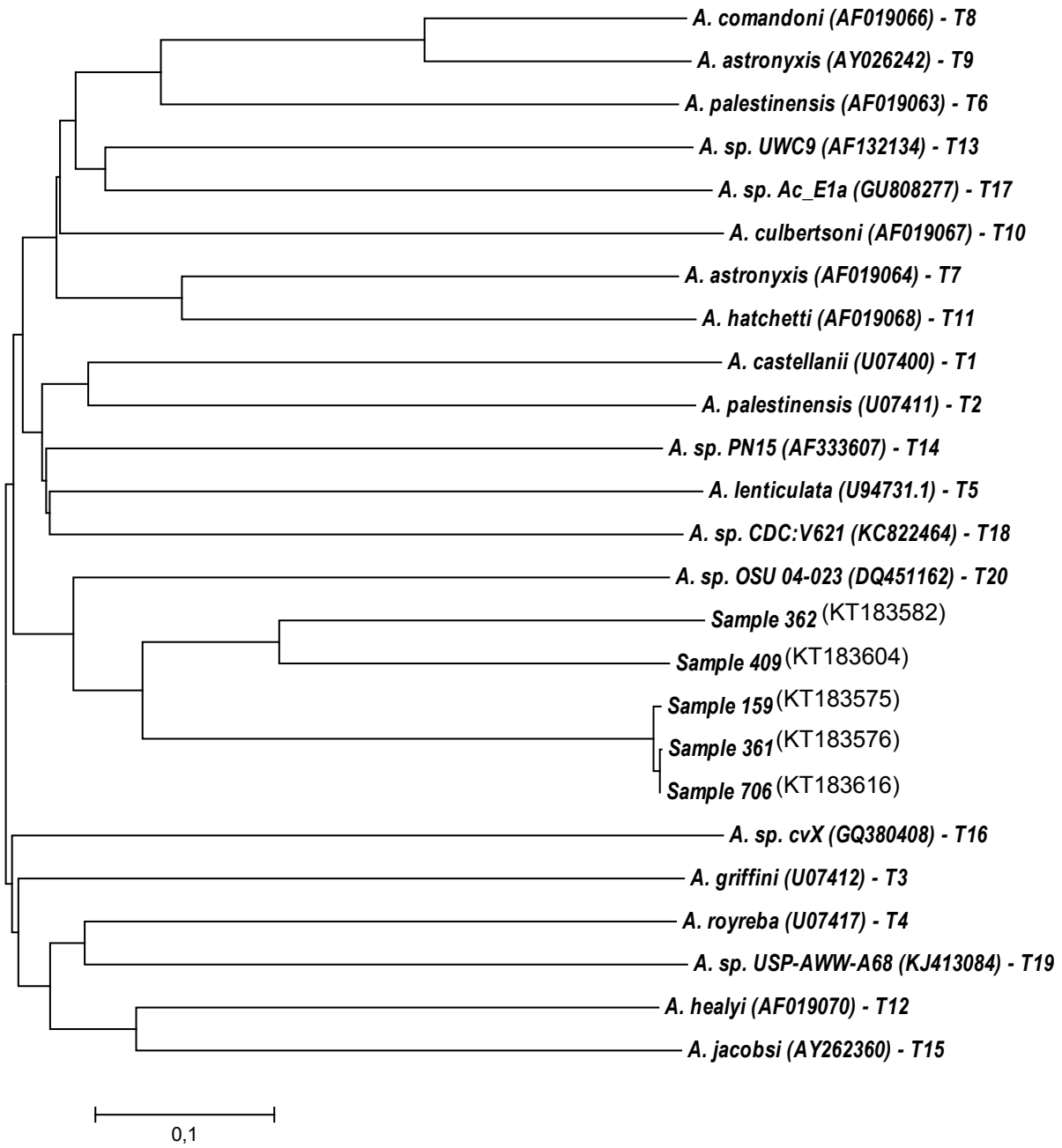

Figure 4.10: Phylogenetic relationships of the PCR products of *Acanthamoeba* species obtained from hospital A and reference strains for *Acanthamoeba* subtype T1-T20 from the NCBI GenBank inferred by neighbour-joining analysis using MEGA 6.06. Sample labeling consists of the Taxa, the accession number (in brackets) and the corresponding subtype, scale 1nm

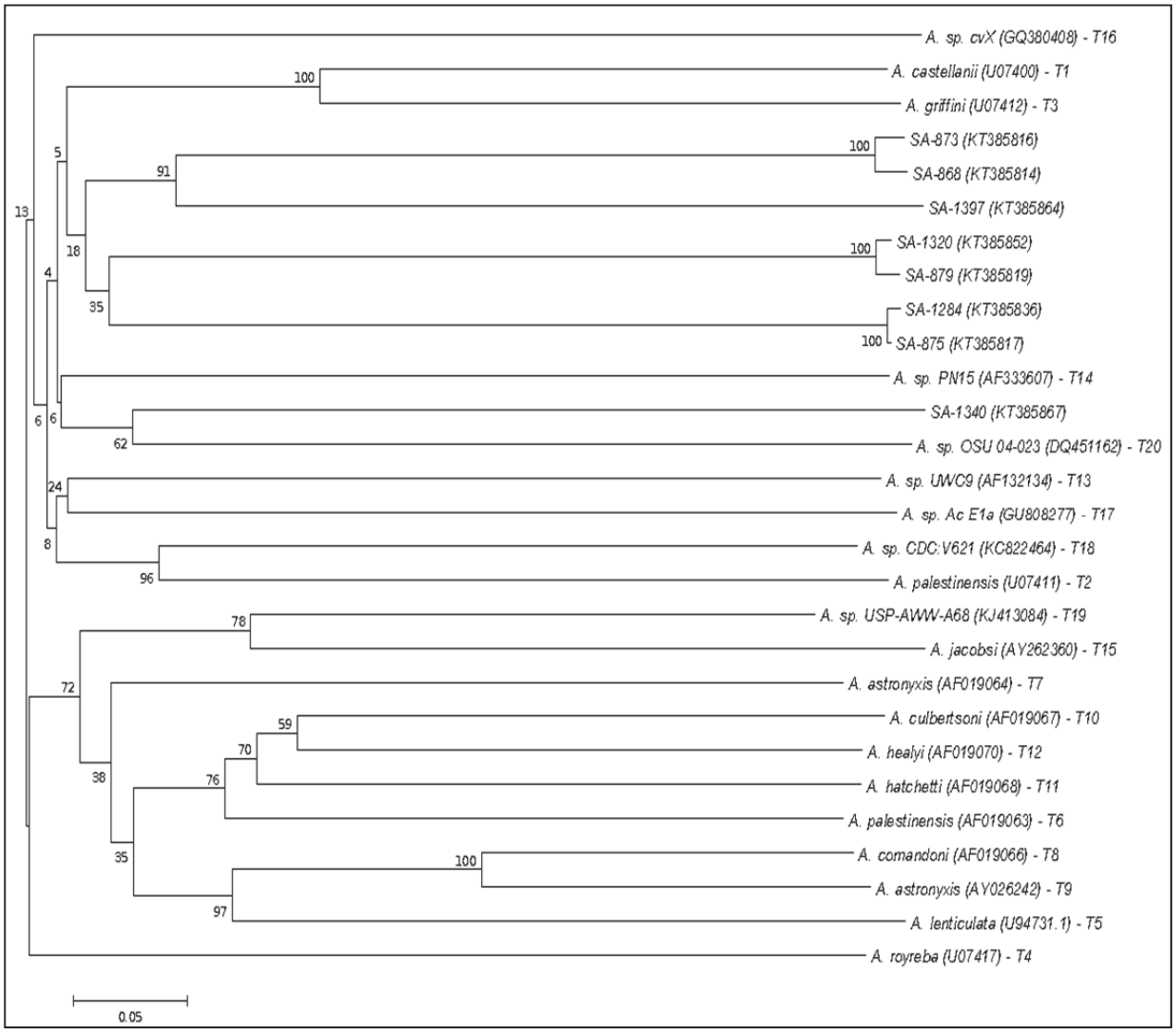

Figure 4.11: Phylogenetic relationships of the PCR products of *Acanthamoeba* species from Hospital A and reference strains for *Acanthamoeba* subtype T1-T20 from the NCBI GenBank inferred by neighbour-joining analysis using MEGA 6.06. Sample labeling consists of the Taxa, the accession number (in brackets) and the corresponding subtype, scale 0.05nm

4.7 BACTERIAL SPECIES DETECTED USING VITEK® ANALYSIS

4.7.1 Bacterial diversity in Hospital A

Thirty-eight bacterial species belonging to 29 genera of were identified using the VITEK® analysis from samples that were positive for amoebae that is all the sampling sites except the milk room. The genera *Pseudomonas, Staphylococcus, Moraxella and Brevundimonas* were the most prevalent with at least two species being detected at different sampling areas (Table 4.8).

Table 4.8: Distribution of bacterial species among sampling points of Hospital A

Bacterial species	Inlet (n=2)	TSSU/CSSU (n=8)	EB (n=11)	Theatre (n=32)	ICU (n=3)	Renal unit (n=11)
Budvicia aquatica	0	1	0	0	0	0
Sphingomonas paucimobilis	0	3	2	7	0	1
Serratia marcescens	1	1	0	1	0	0
Aeromonas salmonicida	0	1	6	4	1	3
Granulicatella elegans	0	1	0	0	0	0
Stenotrophomonas maltophilia	0	1	2	2	0	0
Pantoea spp	0	1	0	2	0	0
Moraxella Lucunta	0	0	2	3	1	1
Moraxella nonliquefaciens	0	0	1	2	1	1
Moraxella osloensis	0	0	1	2	1	1
Kocuria kristinae	0	0	2	2	0	0
Pseudomonas putida	0	0	3	2	0	0
Pseudomonas aeruginosa	0	1	0	4	0	0
Pseudomonas luteola	0	0	1	0	0	0
Pseudomonas fluorescens	0	0	0	3	0	2
Enterobacter cloacae complex	0	0	1	2	0	0
Escherichia coli	0	0	1	0	0	0
Aerococcus viridians	0	0	1	0	0	0
Leuconostoc mesenteroides ssp	0	0	1	1	0	0
Staphylococcus hominis ssp	0	0	1	0	0	0
Staphylococcus epidermidis	0	0	0	1	0	0
Staphylococcus lentus	0	0	0	0	0	1
Granulicatella adiacens	0	0	1	0	0	0
Acinetobacter lwoffii	0	0	0	2	0	0
Dermacoccus nishinomiyaensis	0	0	0	1	0	0
Kytococcus sedentarius	0	0	0	1	0	0
Comamonas testosterone	0	0	0	9	0	0
Delftia acidovorans	0	0	0	10	0	0
Brevundimonas diminuta	0	0	0	2	0	0
Brevundimonas vesicularis	0	0	0	2	0	0
Methylobacterium spp	0	0	0	1	0	0
Pasteurella pneumotripica	0	0	0	1	0	0
Myroides spp	0	0	0	1	0	0
Rhizobium radiobacter	0	0	0	1	0	1
Burkholderia mallei	0	0	0	1	0	0
Brucella melitensis	0	0	0	1	0	0
Achromobacter denitrificans	0	0	0	0	0	1
Micrococcus luteus	0	0	0	0	0	2
Total	1	10	26	70	4	14

CT/ICU – Cardio-Thoracic ICU; N/ICU – Neurosurgical ICU; C/ICU – Cardiac ICU; T/ICU – Trauma ICU; EU – Endoscopy unit; CSSU –Central sterilization service unit; TSSU-theatre sterilization service unit

The most representative species were *Aeromonas salmonicida* (n=14), *Sphingomonas paucimobilis* (n=13), *Delftia acidovorans* (n=10), *Comamonas testosteroni* (n=9) (Figure 4.12).

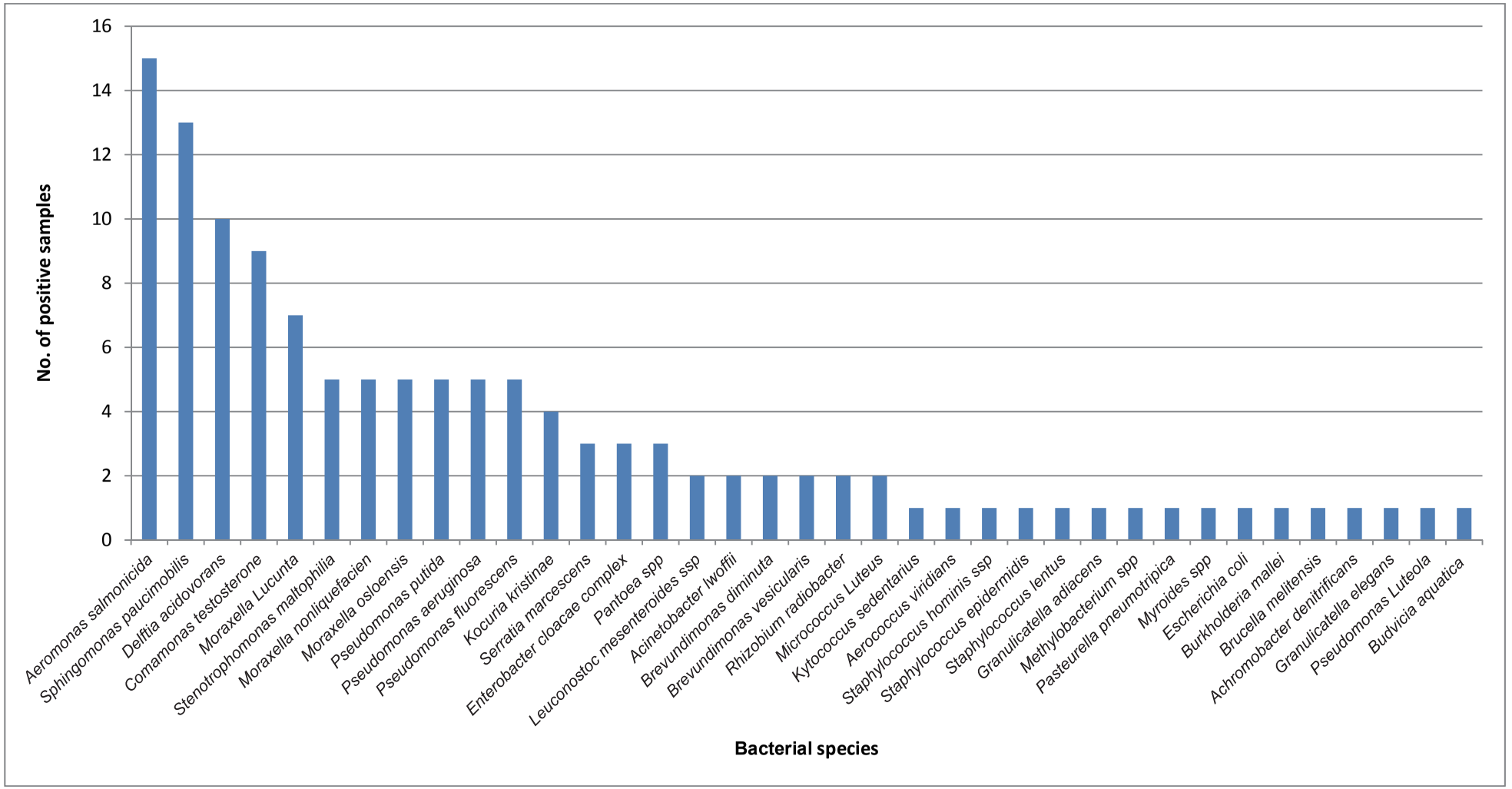

Figure 4.12: Bacterial species isolated from Hospital A

4.7.2 Bacterial diversity in Hospital B

Six bacterial species were identified using the VITEK® analysis from samples that were positive for amoebae that is all the sampling sites except the milk room. The most representative species were *Serratia marcescens* (n=5), *Stenotrophomonas maltophilia* (n=4), (Figure 4.13).

Table 4.9: Distribution of bacterial species among sampling points in Hospital B

Bacterial species	Inlet (n=4)	NW (n=8)	N/ICU (n=5)	DR (n=3)
Serratia marcescens	1	2	1	1
Stenotrophomonas maltophilia	0	3	1	0
Pseudomonas luteola	0	1	0	0
Rhizobium radiobacter	0	1	0	0
Achromobacter denitrificans	0	1	0	0
Sphingomonas paucimobilis	0	0	0	2
Total	1	7	2	3

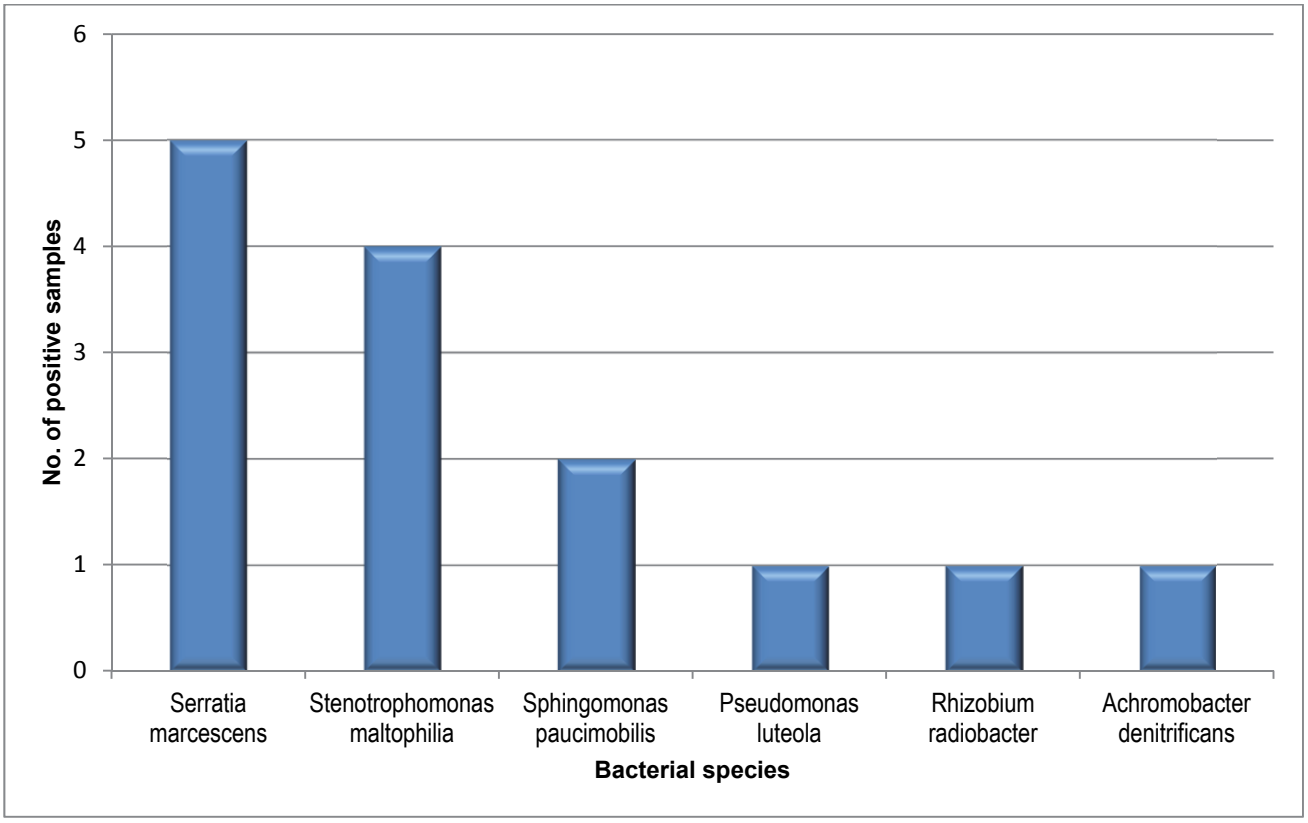

Figure 4.13: Number of bacterial species detected from Hospital B

4.7.3 Bacterial diversity in Hospital C

Twenty-one bacterial species belonging to 18 genera of were detected using the VITEK® analysis from amoebae positive samples of Hospital C. These bacteria were isolated from all sampling areas except the renal unit. The genera *Pseudomonas* and *Staphylococcus* were most abundant with at least two species detected at different sampling areas (Table 4.10). The most representative species were *Serratia marcescens* (n=25), *Stenotrophomonas maltophilia* (n=23)*, Delftia acidovorans* (n=9) (Figure 4.14).

Table 4.10: Distribution of bacterial species among sampling points in Hospital C

Bacterial species	CT/ICU (n=3)	N/ICU (n=8)	C/ICU (n=4)	T/ICU (n=14)	Milk oom (n=3)	EU (n=5)	CSSU/TSSU (n=6)	Theatre (n=14)
Sphingomonas paucimobilis	0	1	2	0	0	1	0	3
Serratia marcescens	0	1	0	4	2	3	2	13
Stenotrophomonas maltophilia	0	3	2	5	1	1	2	9
Pseudomonas luteola	0	0	1	1	0	0	0	0
Pseudomonas fluorescens	0	0	0	1	0	0	0	1
Escherichia coli	0	0	0	1	0	0	0	0
Achromobacter xylosoxiadans	0	0	0	0	0	0	1	0
Staphylococcus hominis ssp	0	0	0	1	0	0	0	0
Staphylococcus epidermidis	0	0	0	1	0	0	0	0
Enterococcus faecium	0	1	0	0	0	0	0	0
Staphylococcus hominis ssp	0	0	0	1	0	0	0	0
Aerococcus viridans	0	0	0	1	0	0	0	0
Dermacoccus nishinomiyaensis	0	1	1	1	0	0	0	1
Acinetobacter lwoffii	0	0	0	1	0	0	0	0
Aeromonas salmonicida	0	0	0	1	0	0	0	0
Comamonas testosteroni	0	0	0	2	0	0	0	4
Defiltia acidovorans	0	0	0	3	0	0	0	6
Rhizobium radiobacter	0	0	0	1	0	1	0	2
Kocuria varians	0	0	0	1	0	0	0	1
Kocuria kristinae	1	0	0	1	0	0	0	0
Ochrobactrum anthropi	0	0	0	1	0	0	0	0
Total	1	7	6	28	3	6	5	40

CT/ICU – Cardio-Thoracic ICU; N/ICU – Neurosurgical ICU; C/ICU – Cardiac ICU; T/ICU – Trauma ICU; EU – Endoscopy unit; CSSU-Central sterilization service unit; TSSU-theatre sterilization service unit

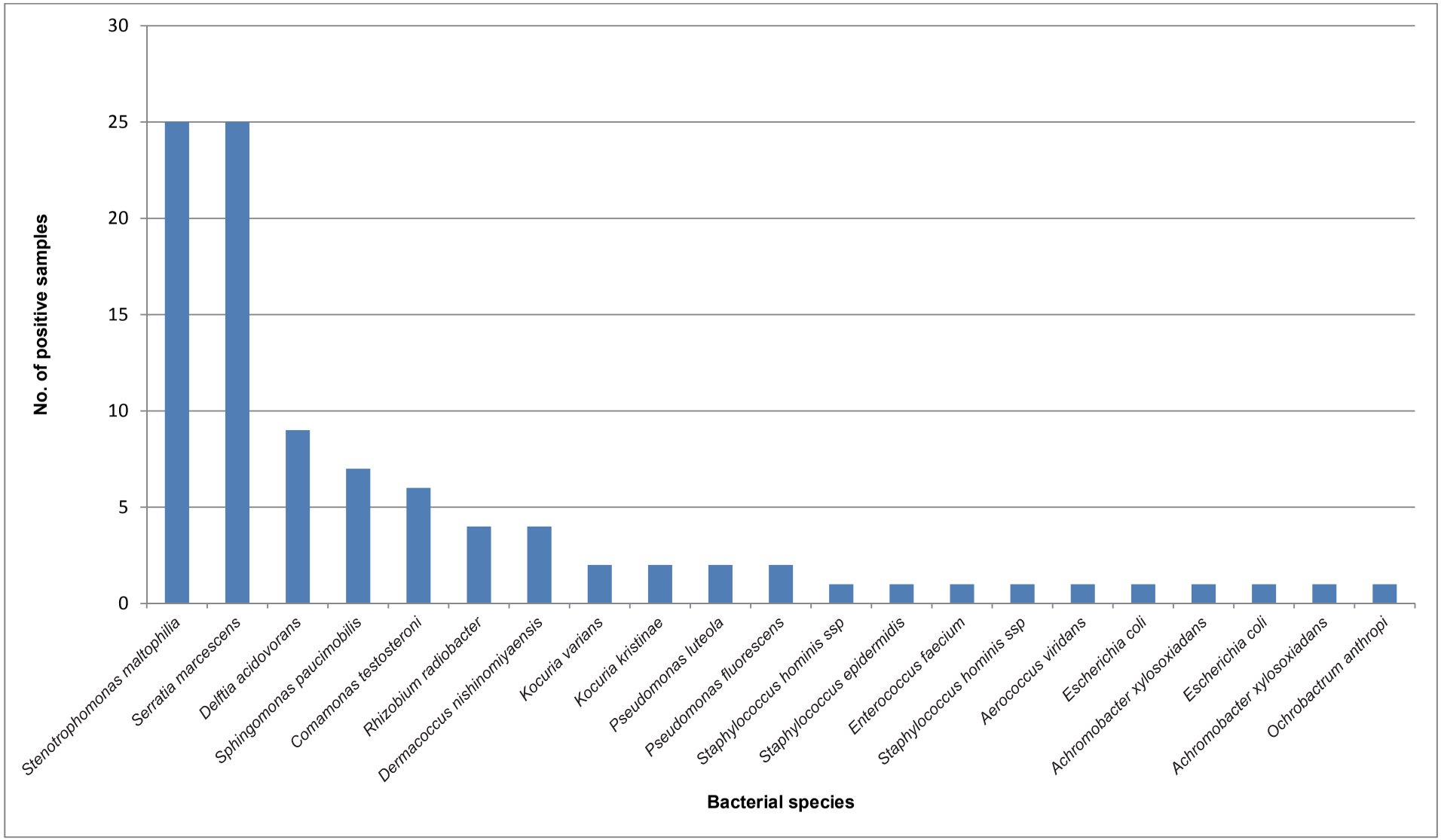

Figure 4.14: Number of bacterial species detected from Hospital C

4.8 DETECTION OF *LEGIONELLA* SPECIES BY PCR

A total of 7(5 water and 2 swab) out of 154 amoebae positive samples showed a strong positive for *Legionella spp*. on 2% gel wit (w/v) agarose slab gel with ethidium bromide (0.5 µg/ml) in a TAE (40 mM Tris acetate; 2 mM EDTA, pH 8.3) buffered system. The positive samples were isolated from the central sterilization service department (CSSU) (167) and theatre tap (402); neonatal ward cubicle tap (870); and from the cardiothoracic ICU (1109) and trauma ICU (1130; 1302; 1286) (Figure 4.15). Using qPCR, the positive samples was quantified to determine the genomic units per litre (GU/L). Sample 1302 had the highest concentration of 3.8x10^2 GU/L, corresponding to the strongest band signal on the agarose gel. Sample 870 had the lowest concentration of 2.7x10^0 GU/L, corresponding to the weakest band signal on the agarose gel (Table 4.11).

Table 4.11: *Legionella* spp. and/or *Legionella* pneumophila isolated from hospital water

Hospital	Sample number (type)	Sampling area	L.pneumophila in GU/L
Hospital A	167 (S)	CSSU	2.7x10^0
	402 (W)	Theatre	1.6x10^1
Hospital B	870 (W)	Neonatal ward	2.9x10^0
Hospital C	1109 (W)	Cardiothoracic ICU	4.7x10^1
	1130 (W)	Trauma ICU	1.4x10^1
	1302 (W)	Trauma ICU	1.6x10^1
	1286 (W)	Trauma ICU	3.8x10^2

S-swab; W-Water; GU/L – genetic units per litre

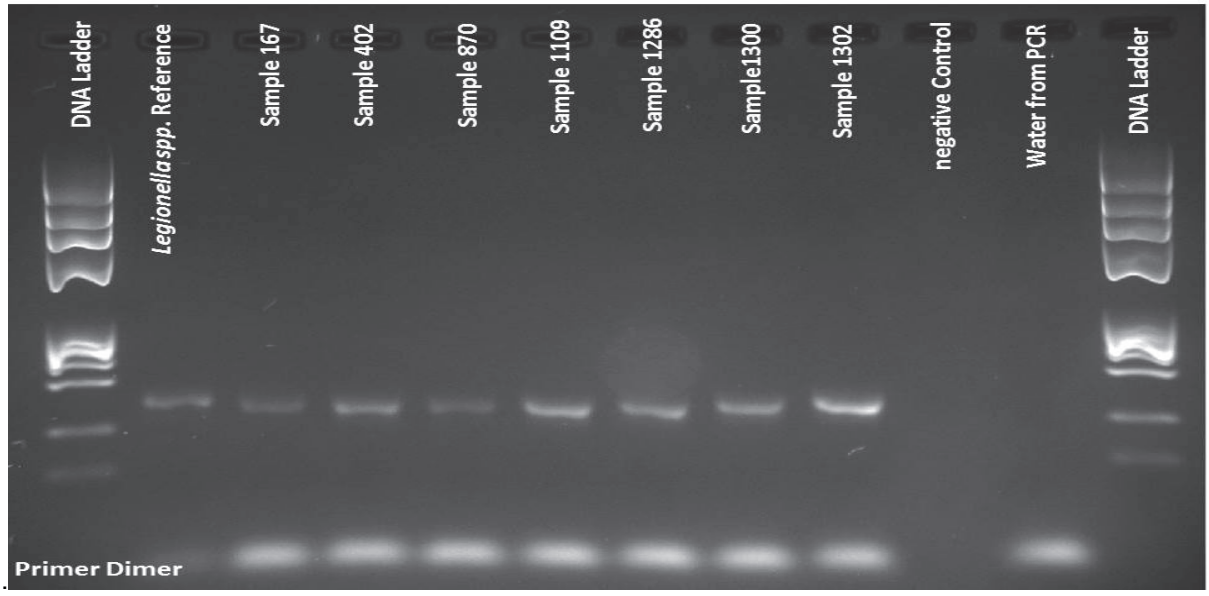

Figure 4.15: Agarose gel (2%) electrophoresis of the first-step PCR products

4.9 GENERAL DISCUSSION

Contamination of hospital water with potentially pathogenic microorganisms is very common. A wide range of bacteria, viruses, fungi and protozoa may be present in the water supply and may cause a substantial increase in the risk of nosocomial infections among patients and medical personnel. The drinking water quality supplied to and distributed in public health care facilities, with special reference to free living amoebae (FLA) and amoeba resistant bacteria (ARB), has not been studied previously in South Africa.

This study thus aimed at providing an overview of the presence of FLA and ARB in the municipal water supplied to and during distribution through three public health care institutions in Johannesburg, South Africa. Potential high-risk areas for waterborne nosocomial infections were determined during walkthrough assessments conducted by members of the study team and key staff members at each institution. This is, to our knowledge, the first report on the presence of FLA and ARB in South African public health care institutions.

Public health care institutions (PHIs) in South Africa house large numbers of immunocompromised patients under conditions that are often far from ideal. The majority of the facilities are old, poorly maintained and were not designed with infection control in mind. Overcrowding, shortages of well-trained medical personnel, constant equipment breakdowns, water and electricity shortages and funding restrictions are experienced at the majority of PHCIs throughout the country. The three public hospitals included in the study were built more than 50 years ago. They are located within a radius of 10 kilometres from inner Johannesburg and cater for the majority of its citizens. Hospital A has approximately 700 beds and consists of medical, surgical, orthopaedic and psychiatric wards which provide in-patient facilities for around 350 patients. The hospital consists of nephrology, pulmonology, cardiology and neurology departments and a tuberculosis isolation ward. Hospital B consists of four in-patient wards and a large out-patient unit where patients attend weekly clinics. The in-patient facilities include a neonatal unit with a six-bed neonatal intensive care unit, a premature unit and a mother care program. Hospital C has facilities for over 1000 in-patients as well as large out-patient units focusing on adult, mother and child health. The study team could not obtain official plans of any of the buildings or their water distribution systems but the infection control staff and facility management of each hospital provided information about the water distribution systems and the areas where patients and staff are most at risk of nosocomial infections.

All three hospitals use municipal drinking water supplied from treatment plants and reservoirs situated around the city. The water enters the facility at a central point from where it is distributed through the building. Municipal water is tested for indicator organisms and disinfected at the treatment plant, but not again upon entry or during distribution through the facility. The municipal water inlet at each hospital was tested to determine the quality of drinking water entering the facility. The water from all three hospitals conformed to the South African drinking water quality standards as specified in SANS 241:2015 as determined by routine water testing methods.

4.9.1 Physico-chemical parameters

The water temperature, pH, total dissolved solids (TDS) and residual chlorine content were measured at the time of sampling. The measurements were within normal limits except at the reverse osmosis (RO) plants feeding the renal units of Hospital A and Hospital C, where the pH ranged from 1.7 to 8.8 and 1.8 to 8.1 respectively. The TDS ranged from 10 to 187 mg/L at the RO plant in Hospital A.

4.9.1.1 Renal dialysis

During dialysis each patient is exposed indirectly to 15,000-30,000 L of water per year (Vorbeck-Meister et al*.,* 1999), therefore the chemical and microbiological quality of water used in dialysis centres plays a critical role in the quality of patient care. Infectious disease is a common cause of mortality in dialysis patients. These infections can be due to contamination of the shunts used during dialysis, the dialysate and the water used to prepare the dialysate. Gram negative bacteria also produce endotoxins which may penetrate dialyser membranes and cause pyrogenic reactions in dialysis patients. In addition, the chemical composition of dialysis water may cause acute and chronic complications (Vorbeck-Meister et al*.,* 1999).Tap water is clearly not suitable for this purpose and additional treatment, usually through a process of ion exchange and reverse osmosis (RO), followed by UV disinfection, is used at most centres to improve the quality of the water used in dialysis machines and for the dilution of dialysate.

4.9.1.2 Water treatment in renal dialysis centres

The South African Renal Society recommends the use of pure water which complies with the Association for the Advancement of Medical Instrumentation (AAMI) Standard in dialysis centres. The minimum requirements are: (i) Bacteria: < 200 cfu/mL, (ii) Inorganic contaminants: Total solutes: <20 mg/mL; Aluminium: < 10 ppm, (iii) Endotoxins: <10 EU/mL, (iv) Conductivity: < 5µS/cm. The microbiology of water feeding dialysis machines must be monitored weekly for new system validation and monthly for surveillance and/or maintenance of existing systems.

Reverse osmosis is based on a filtration process in which water is pushed by high pressure through semipermeable membranes that can reject the majority of contaminants: 95-98% of dissolved salts and up to 99% of bacteria, endotoxins and substances of > 200 Da molecular weight (Pantea et al*.,* 2011). RO membrane performance is measured by percent rejection; the final product water quality can be determined by measuring either conductivity in µs/cm or total dissolved solids (TDS) in mg/L or parts per million (ppm). Conductivity is the ability of a solution to pass an electric current between two electrodes. The current is carried by ions, therefore the conductivity increases with the number of ions present in solution and their mobility. The conductivity of water provides information on its chemical composition (Pantea et al*.,* 2011). In an RO system, the conductivity is usually measured before and after the water passes through the membrane.

The Guidelines for the Optimal Care of Patients on Chronic Dialysis in South Africa (South African Renal Society, 2006) states that “haemodialysis requires the use of pure water complying at a minimum with the Association for the Advancement of Medical Instrumentation (AAMI) standards that takes into consideration both chemical and bacteriological purity”. The guidelines require routine monitoring of the water feeding dialysis machines; weekly during the validation phase of new systems and monthly during the surveillance and monitoring phase of systems. The water treatment system should include pre-treatment (softener, activated carbon, downsizing microfilters) and a RO unit which should be implemented in series. Disinfection should be performed at least once a month (South African Renal Society Guidelines, 2001). The Australian Guidelines for the Pre-treatment of Dialysis Water (2008) also stresses the importance of temperature, pH, chlorine/chloramine levels and the cleanliness of RO membrane surfaces for optimal performance. Water temperature influences its ability to cross the RO membrane. The colder the water, the more resistant it is to crossing the membrane.

Although RO membranes generally have a wide pH tolerance (2-11), the optimum pH range for membranes to perform optimally is > 5.0 and < 8.5 (Water Guidelines NSW, 2008). In some instances the pH of municipal water is deliberately increased to minimize leaching of metals from pipes. Because carbon filtration and osmosis devices do not work effectively at pH > 8.5 (Northwest Renal Network, 2005), the pH is decreased to improve the functioning of the treatment system. This may increase the risk of bacterial contamination when organic acids rather than inorganic acids are added to the system. The acid must also be fed at the appropriate rate; therefore the pH must be monitored daily from a sample port just downstream of the acid feed pump (Northwest Renal Network, 2005). The ideal range of pH at this point is between 7.0 and 8.0. The chlorine/chloramine levels should be recorded at least once per dialysis session. The Guidelines require chlorine/chloramines levels to be tested when there is maximum water flow through the carbon tanks, with special requirements for direct- and indirect feed systems. Similarly, the requirements for the construction and disinfection of distribution piping systems in renal units are clearly stated in the Guidelines which strongly advise against the use of copper, brass, aluminium or other toxic substances in the piping. Disinfection should be carried out regularly.

Re-growth on the surfaces of the tubing, filters and ion exchangers of the dialysis system is an important cause of bacterial recontamination even after RO. It is therefore important to monitor the bacterial levels in water as well as biofilm at different temperatures and at different areas of the dialysis centre, including the outlet of the dialysis machines. Previous studies showed increased bacterial counts in samples collected at the outlets of dialysis machines, where stagnancy and elevated temperatures increase the risk of contamination and ultimately the risk to the patient. Vorbeck-Meister et al. (1999) recommended testing the microbiological quality of the water and biofilm at 22°C as well as at 37°C (the temperature to which the water in dialysis machines is heated) at various points during the treatment and dialysis process to ensure recovery of a wide range of possible contaminants. Testing water only for indicator organisms is not sufficient.

4.9.2 Microbiological analysis of water and biofilm samples

Culture-based methods for indicator microorganisms have traditionally been key in determining the quality of drinking water in South Africa (Department of Water Affairs and Forestry, 1996). In this study no culturable indicator organisms were detected in all water and biofilm samples from the three hospitals using the micro-most probable number (MPN) method. Concentrations of indicator organisms in the water samples might have been too low to be detected by the MPN method used in this study. In addition some studies have reported false negative results due to the presence other interfering bacteria and because of viable but non-culturable bacteria in the samples (Chao et al., 2004; Lamoth and Greub, 2010). In addition, some pathogens (FLA included) can persist in treated water longer than indicators used to test water quality (Codony et al., 2012). Therefore indicator testing is not reliable for monitoring quality of drinking water especially in health care institutions where immunocompromised patients are exposed daily to potentially contaminated water through contact, inhalation or ingestion. Several recent studies stressed the importance of testing for FLA and ARB in addition to indicator organisms. For example, using amoebal co-culture, Thomas et al. (2006) showed a statistically significant association between the occurrence of FLA and *Legionella* species and a strong association with *Mycobacteria* species in a hospital water network. Using amoebal co-culture, they recovered at least one ARB (including *Mycobacteria* and *Legionella*) from 45% of the 200 samples they tested; in some samples more than one species were present. Another study by Yli-Pirilä et al. (2007) showed that amoebae can improve the cytotoxicity and pro-inflammatory properties of certain microbes associated with moisture-damaged buildings in Finland.

4.9.3 Free living amoebae in hospital water and biofilm samples

Free living amoebae were isolated from the water and biofilm samples collected at the municipal water inlet points of Hospitals A and B but not at Hospital C. *Acanthamoeba* species were present in 15% of the samples from Hospital A but not in those from Hospital B; neither of the other pathogenic FLA, *Naegleria* or *Balamuthia* species, were detected. *Vermamoeba vermiformis* was present in 15% and 50% of the samples collected at Hospital A and Hospital B respectively, indicated a potential risk for the presence of ARB at the point of entry into the hospital water distribution systems.

A total of 275 water and biofilm samples were collected from hospital A (n=127) and B (n=148). Using the amoebal enrichment technique, a total of 154 (56.0%) were isolated in the two hospital water systems. This supports the ubiquitous nature of FLA as they have also been isolated other man-made water systems such as swimming pools, cooling towers, wastewater and drinking water plants (Greub and Raoult, 2004; Pagnier et al., 2009: Magnet et al., 2013). From the amoebae positive samples, 92 (74.4%) were isolated from Hospital A and 62 (41.2%) from Hospital B. The prevalence of FLA in this study is higher than in studies done by Ovrutsky et al. (2013) in the USA and Khurana et al. (2015) in India where amoebae were detected in 13 of 88 (52.0%) and in 14 of 100 (14.0%) hospital water samples analysed respectively. Rohr et al. (1998) from Germany and Lasheras et al. (2006) from France recovered amoebae from 52% and 68.9% or their samples respectively. The latter two studies, however 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 23.1ºC (range, 14.4-27.3ºC). Thomas et al. (2006) reported a higher prevalence of FLA in cold water compared to hot water, with a prevalence of FLA significantly lower for temperatures above 60ºC. This might explain the higher prevalence of FLA in our study compared to those other studies. Differences in the number of water samples analyzed can also influence the recovery rate of FLA. For example, a study done in the USA had a prevalence of 39% for 2424 tap water samples analyzed (Stockman et al., 2011), whereas a study in Germany reported a prevalence of 66 % for 3 tap water samples (Hoffmann and Michel, 2001

Furthermore, the variability in the prevalence values of FLA in Hospital A (74.4%), Hospital B (41.2%) and other studies referred to above can also be explained by the difference in water source, geographical areas and the different methods used in the identification of FLA. Prevalence of FLA can also be influenced by accumulation of biofilm in a water distribution system. Several studies have demonstrated that bacteria in biofilm provides favourable conditions for FLA proliferation compared to compared to bacteria suspended in water (Barbeau and Buhler, 2001; Pickup et al., 2007; Corsaro et al., 2009). Some factors that influence biofilm formation in water distribution systems include: water stagnation in dead-legs and holding tanks and pipe corrosion which lowers efficiency of chlorine on biofims (Bagh et al., 2002; Sehulster et al., 2003). However, few studies have directly compared the occurrence of FLA in water and swab (biofilm) samples. 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 three (20.0%) were water samples. This was in agreement with our study as there was a high detection rate in biofilm samples, that is, 38.7% for water samples 64.0% and for swab samples in hospital A and 69.6% for water samples and 75.9% swab samples for hospital B. This indicates that the accumulation of biofilm in health care water distribution systems could facilitate the re-growth of FLA.

A wide diversity of FLA that includes *Hartmannella, Acanthamoeba, Naegleria, Vahlkampfia, Vannella, Platyamoeba, Echinamoeba, Parvamoeba, Saccamoeba* and *Vexillifera* have been isolated in end-point in-premise taps (Kilvington et al., 2004; Shoff et al., 2008; Bonilla-Lemus et al., 2010; Yousuf et al., 2013). Using both culture-based and molecular based methods, genera belonging to *Acanthamoeba* and *Vermamoeba* (formerly *Hartmanella*) and *Naegleria* were detected in Hospital A and Hospital B. The universal primers, Ami6F1, Ami6F2 and Ami9R, used in this study did not specify if the two *Naegleria* spp detected from hospital A (GenBank accession number KT183611) and hospital B (GenBank accession no. KT385863) 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 (32ºC) used in this study may not have been conducive to allow the growth of other FLA, particularly *Naegleria* spp. which grow better at higher temperatures. Other studies have however reported a higher diversity of amoebae in hospital environments. A study of six hospitals in Germany of 56 hot water taps and 49 swabs detected genera *Acanthamoeba*, *Vermamoeba, Naegleria, Vanella* and *Vahlkampfia* (Rohr et al.,1998). Another study in a new hospital building, also in Germany by Michel et al. (1995), isolated *Acanthamoeba, Vermamoeba, Naegleria, Echinamoeba* species. The other FLA could not be morphologically identified (43% for Hospital A and 30% for Hospital B) and further molecular methods are needed to identify them. Despite the requirement of using

culture-based methods like amoebal enrichment to detect FLA, most studies have applied both the culture-based and molecular (Lamoth and Greub, 2010). This is because culture-based methods are relatively simple and cost effective in addition to making the organism available for further classification and for testing its infectivity for human macrophages (Dowell et al., 2001; Greub and Raoult, 2004).

The most abundant amoebae species in both Hospital A and Hospital B was *Vermamoeba vermiformis*, both with culture and molecular based methods. 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 (Coşkun et al., 2013). Similar results were described by Thomas et al. (2006) in a study carried out in Switzerland to determine the biodiversity of FLA in a hospital water network where V. vermiformis had a prevalence of 86.7%. Our results also correlated well with those published by Pagnier et al. (2015) who studied the prevalence of FLA in hospital water in France and concluded that *V*. vermiformis is more commonly associated with amoeba resistant microorganisms than *Acanthamoeba* species. They proposed the use of *V. vermiformis* in conjunction with Acanthamoeba species in co-culture experiments when testing water for the presence of potential ARB. 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). *V. vermiformis* is also a well-known carrier of a number of amoeba resistant pathogens including *Legionella* species.

The FLA detected in the selected sampling areas were: *Acanthamoeba* spp., where 13% of the samples were from Hospital A. In contrast, other studies have reported relatively higher prevalence of *Acanthamoeba* species. Bagheri et al*.,* 2010 demonstrated 48% the samples collected in different wards of hospitals in Iran were *Acathamoeba* species. Two separate studies by the same research group in hospital environments have also reported the presence of *Acathamoeba* species in 34% (Carlesso et al*.,* 2010) and 23% of the samples (Carlesso et al., 2007). Studies in Tunisia and Egypt have also shown a high detection rate of 32.6-42.9% of *Acathamoeba* species in haemodialysis and dental waters (Dendana et al., 2008; Trabelsi et al., 2010 Hassan et al., 2012). Generally, *Acanthamoeba* has been the most frequently isolated genera in tap water as they are easily identified morphologically compared to the other genera. This could have caused bias in the other results from other studies which based their testing on culture based methods which uses morphology as a basis for identification, rather than molecular methods.

The first phylogenetic analysis of the five samples positive for *Acanthamoeba* spp. showed isolates closely resembling the T20 genotype. The T20 genotype was recently described by Fuerst et al. (2015) and contains several *Acanthamoeba* strains previously miss-assigned to genotypes T16 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). This is, to our knowledge, the first report of T20 in Africa. Other *Acanthamoeba* species isolated during this study closely resembled T1 and T3 genotypes. The T1 genotype has not been associated with human disease; the T3 genotype 9 which

includes *A. griffin, A. pearcei* and *A. hatchetti* has been associated with *Acanthamoeba* keratitis (Gonzalez-Robles et al., 2014). Although genotype T3 is not commonly isolated from environmental samples, it is frequently isolated from clinical samples worldwide (Booton et al., 2005; Mahsood et al., 2005; Lorenzo-Morales et al., 2013).

The presence of the genera *Vermamoeba* and *Acanthamoeba* in our study is a possible risk factor to a large population of immunocompromised individuals and medical personnel who might be exposed to these opportunistic FLA through ingestion, inhalation or aspiration of aerosols or 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).

4.9.4 DETECTION OF AMOEBA RESISTANT BACTERIA IN WATER AND BIOFILM SAMPLES

Bacteria and amoebae live together both in nature and man-made aquatic systems were they constantly interact with each other in different ways. Some bacteria are digested and used as food by amoebae while others can survive, multiply and lyse amoebae resulting in spread to the environment in large numbers. Amoebal cyst walls contain cellulose that forms a physical barrier, offering bacteria against hostile conditions such as extremes of temperature, UV light ozone, chlorine dioxide, monochloramine, copper-silver (Greub and Raoult, 2004; Thomas et al., 2004; Bagheri et al., 2010). Studies have also shown that passage through amoebae is likely to increase the virulence and antibiotic resistance of bacteria substantially enabling them to prepare for subsequent survival in macrophages (Miltner et al., 2000; Schmitz-Esser et al., 2010). Therefore association of amoebae with ARB can be a potential reservoir of bacteria that have implications in public and environmental health.

Intra-amoebal growth increases the virulence of some amoeba resistant bacteria, prompting the concern that other intracellular bacteria recovered from amoebae, even those that are not necessarily pathogenic to healthy individuals, may pose a health risk to patients and staff.

4.9.5 Presence/absence of indicator organisms

All the three hospitals in this study do not carry out routine testing of water to check for bacterial contaminants, water testing is usually done by checking for indicator organisms when there renovations as indicated in the walk-through assessments. Conventional indicator testing has traditionally been key in determining the quality of drinking water systems in South Africa (Department of Water Affairs and Forestry, 1996). In this study no culturable indicator organisms was detected in all water samples from the three hospitals using the micro-most probable number (MPN) method. However, indicator testing has limitations which make it not suitable for testing water quality in water systems, especially in hospital water systems

where immunocompromised individuals may be exposed. Concentrations of indicator organisms in the water samples might have been too low to be detected by the MPN method used in this study as small volume of 100ml water was analyzed. Furthermore, some studies have reported false negative results due to the presence other interfering bacteria and because of viable but non-culturable bacteria in the samples (Chao et al., 2004; Lamoth and Greub, 2010). Results of important water-related pathogens in hospital settings, such as, environmental *Mycobacteria, Legionella* and *Pseudomonas* species that can survive inside amoebae, shows that there is no correlation with conventional indicator organism counts. Some studies have stressed the importance of testing for ARB in addition to indicator organisms. For example, using amoebal co-culture, Thomas et al. (2006) showed a statistically significant association between the occurrence of FLA and *Legionella* species and a strong association with *Mycobacteria* species in a hospital water network. Using amoebal co-culture, they recovered at least one ARB (including *Mycobacteria* and *Legionella*) from 45% of the 200 samples tested; in some samples more than one species were present

4.9.6 Biodiversity of bacteria in the hospital water systems

In this study, we investigated the biodiversity of bacteria in the water networks of three hospitals with no recent history of epidemics involving such microorganisms. Culture and biochemical approaches using the VITEK® were using to identify bacteria isolated with amoebae using amoebal enrichment method. Bacterial analysis was done on all the 174 amoebae positive samples from the three hospitals to ascertain their association. Rowbotham (1980) conducted the first study to prove the association between Legionella and the free-living amoebae (FLA), *Acanthamoeba*. Numerous other studies have also shown that amoebae in man-made water systems such as drinking water, tap water, swimming pools, and cooling towers can be infected by many other relevant ARB which could use amoebae as a platform for multiplication (Michel et al., 1998; Greub and Raoult, 2004; Molmeret et al., 2005; Thomas et al., 2006; Pagnier et al., 2008).This study is the first in South Africa where any association between amoebae and bacteria is reported in hospital water systems.

In this study, the most representative species for hospital A: were *Aeromonas salmonicida, Sphingomonas paucimobilis, Delftia acidovorans* and *Comamonas testosteroni*; Hospital B: *Serratia marcescens* and *Stenotrophomonas maltophilia*; Hospital C: *Serratia marcescens, Stenotrophomonas maltophilia* and *Delftia acidovorans*. All these species are waterborne nosocomial pathogens that have been reported by several studies (See Section 2.2.1), with the exception of *Aeromonas salmonicida* which has been reported in fish associated infection but has recently been isolated from a human blood sample (Tewari et al., 2014).

The non-fermenting bacteria: *Sphingomonas paucimobilis, Delftia acidovorans, Stenotrophomonas maltophilia* and *Comamonas testosterone*, cause rare but clinically important opportunistic infections. Due to their minimal nutritional requirements and tolerance to a variety of physical conditions, these non-fermenters have all been linked with outbreaks and nosocomial infections associated with tap water and distilled water used in healthcare settings. Outbreaks caused by this group of bacteria have also been reported in dialysis machines, ventilators and nebulisers, mainly in intensive care units (Schulster and Chinn, 2003). *Serretia marcescens*, a member of *Enterobacteriaceae* has been reported in bloodstream infections, urinary tract

infections, wound infections and pneumonia (Mahlen , 2011) *Serratia marcescens* and *S. maltophilia* can survive in amoebic vacuoles without multiplication resulting in complete lysis of amoebae while *Delftia acidovorans* result in partial lysis of amoebae (Thomas et al., 2010). A study by Laganà et al. (2014), on the occurrence of FLA and Gram-negative bacteria in an Italian hospital water system, showed that *Vermamoeba* spp. were associated with the occurrence of *Pseudomonas fluorescens* and *Pseudomonas. aeruginosa, Acinetobacter lwoffi, S. maltophilia, C. testosteroni* and *Sphingomonas paucimobilis.* In the present study these bacterial species were detected together with *Vermamoeba vermiformis.*

4.9.7 Detection of *Legionella*

The growth and survival of *Legionella* in the environment is enhanced by their ability to form symbiotic relationships with other larger microorganisms. *Legionella* have been found to infect and incorporate themselves into at least 13 species of amoebae including *Acanthamoeba, Hartmanella, Valkampfia* and *Naegleria*, and two strains of ciliates, *Tetrahymena and Cyclidium* (Lee and West, 1991; Paszko-Kolva et al., 1993; States et al., 1989; Kramer and Ford, 1994; Henke and Seidel, 1986; Fields, 1996; Vandenesch et al., 1990). This study managed to isolate *Legionella* water and swab samples from different ares of the three hospitals where FLA had been detected. Although relatively few samples were positive (7/150), it's a still a cause for concern for inhabitants of these settings as *Legionella* replicate rapidly intracellularly within protozoan hosts for prolonged periods of time, amoebic vesicles can contain hundreds of *Legionella* cells (Berk et al. 1998). In addition, this replication within protozoa may contribute to enhanced virulence of Legionella (Kramer and Ford 1994). The ability of *Legionella* to thrive within protozoa also allows them to survive over a wider range of environmental conditions and to resist the effects of chlorine, biocides, and other disinfectants (Fields, 1996; Kramer and Ford, 1994; Paszko-Kolva et al., 1993; States et al., 1989).

Legionella pneumophila quantified in this study with the highest concentration of 3,8 x10^2 GU/L is responsible for 90% of Legionnaires disease and Pontiac fever in the world (Fields, 2001). This raises public health concerns in South Africa where outbreaks have not been recorded. Studies by Kool et al. (1998) and Yu (2000) have reported however Legionnaires disease outbreaks connected to hospital water distribution. In this study *Legionella* was isolated from ICU, theatre and central sterilization service unit indicating that patients and health care personnel are at risk of Legionnaires ' disease. Several other patient populations (e.g., renal transplant patients, especially those requiring hemodialysis) are at an extremely high risk for legionnaires' disease, as they have both an increased risk of exposure (via their surgery and other ventilation needs), and an increased susceptibility (due to corticosteroid therapy and dialysis) (Woo et al. 1986, LeSaux et al. 1989). In addition, the increased rate of HIV infections in South Africa also increases risk of developing more severe Legionnaires' disease.

Angenent et al. (2005) reported on the presence of potential pathogens in the water and air samples of a hospital hydrotherapy pool after workers were diagnosed with hypersensitivity pneumonitis and M avium infections. Their results showed that, despite using a state-of-the-art UV disinfection system, the numbers of bacteria in the therapy pool water was relatively high compared with the potable water used to fill the pool.

Direct microscopic counts were usually about 1000 times higher than conventional plate counts. A survey of >1,300 rRNA genes yielded a total of 628 unique sequences; the majority of these were nearly identical to *M. avium* strains. This suggested that these organisms comprised a significant portion of the microbes in the pool water (>30%). This study also showed that culture methods currently used by public health facilities are seriously inadequate for the detection and enumeration of potential pathogens

This study showed that FLA could survive even in residual chlorine levels as high as 0.35 mg/L. Storey et al. (2004) showed that amoebal cysts can survive chlorine concentrations as high as 100 mg/L for 10 minutes. This has major environmental implications as the three hospitals in this study all rely on chlorine as a biocide, with a residual chlorine range of 0.01-0.35 mg/L for samples analyzed. Other studies have also shown amoebal cysts to be tolerant to extremes of temperature, ultraviolet radiation, ozone, chlorine dioxide, monochloramine, copper-silver (Greub and Raoult, 2004; Thomas et al., 2004; Bagheri et al., 2010). This enhanced resistance of amoebal cysts allows the transmission of amoebae resistant bacteria (ARB) such as *Legionella*, *Mycobacteria* and *Salmonella* as amoebal cyst walls contain cellulose that forms a physical barrier, which protects the ARB (Winiecka-Krusnell and Linder, 2001; Coulon et al*.,* 2010). Studies have shown that passage through amoebae is likely to increase the virulence and antibiotic resistance of bacteria substantially (Miltner et al., 2000; Schmitz-Esser et al., 2010). Potential ARB were detected using Gimenez staining and transmission electron microscopy showing amoebae filled with intracellular bacteria.

The bacteria isolated in this study are all known amoeba resistant bacteria; the majority are known causative agents for nosocomial pneumonia. According to Greatorex and Oosthuizen, (2015) mortality from severe sepsis in intensive care units worldwide ranges from 28-50%. These cases are mostly caused by the so-called ESKAPE pathogens: *Enterococcus faecium, S. aureus, K. pneumonia, Acenitobacter baumannii, P. aeruginosa* and *Enterobacter* species. A recently published audit from an intensive care unit at a public hospital in Durban, South Africa, reported the presence of gram negative bacteria in 62% of samples from patients on ventilators. *A. baumannii*, *K. pneumoniae* and *E. coli* were the most prevalent gram negative bacteria. *S. aureus* was isolated from 10% of patients (Brink et al., 2008; Greatorex and Oosthuizen, 2015).

Acenitobacter baumannii is a common cause of nosocomial infections. Current studies indicate that the resistance and virulence of *A. baumannii* are increasing. A study recently conducted in Turkey reported that *A. baumannii* was responsible for 21.8% of ICU-associated infections (Ulu-Kilic et al*.,* 2013). *Acenitobacter* species are opportunistic pathogens in hospitalised and critically ill patients. The bacterium is strongly associated with environmental contamination. It has adapted to survive in the hospital environment and is often implicated in outbreaks of nosocomial infectious disease. *A. baumannii* is among the most common causes of late-onset VAP and the second most common pathogen to cause nosocomial bloodstream infections (Cateau et al*.,* 2014; Ulu-Kilic et al*.,* 2013). *Acenitobacter* species are common inhabitants of hospital water systems from where it is often isolated via amoebal enrichment and -coculture (Cateau et al*.,* 2014).

CHAPTER 5: CONCLUSIONS AND RECOMMENDATIONS

This was the first study of the presence and potential impact of free living amoebae and amoeba resistant bacteria in South African public health care institutions. Our results indicated a high level of contamination of the water supplied to the three hospitals, even at the inlet points, of potentially pathogenic free living amoebae. Even if not pathogenic, the presence of these amoebae indicate an increased risk of waterborne, amoebae resistant pathogens entering and being distributed through the water distribution systems in these facilities. The water quality, as determined by traditional methods, conformed to the South African Standards for Drinking Water Quality (SANS 241: 2015) at all the sampling points. However, not only did the vast majority of samples contain FLA, but a wide variety of known amoeba resistant bacteria were present in the water and especially in the biofilms collected from most sampling areas. The physico-chemical characteristics and presence of FLA and ARB in water collected, after the reverse osmosis process, in the two renal units are of particular concern. It is not clear how and how often the RO plants are maintained but this raised the concern that renal dialysis patients may be at risk of infections and other complications resulting from the water used in the dialysis process.

Our recommendations are therefore:

- To screen hospital water at the municipal water inlet and various points in the distribution system routinely for the presence of indicator organisms as well as free living amoebae as this may indicate a possible increased risk of the presence of amoeba resistant organisms, which may increase the risk of nosocomial and occupational infections, in the distribution system
- To raise awareness and provide training to hospital personnel and health care professionals on the risks of using tap water for patient care, especially for critically ill, young or elderly patients receiving specialised treatment and the importance of considering water contamination as a possible cause of nosocomial infections
- To ensure that the water used in the renal units are free from potentially harmful substances and organisms. Frequent testing would provide useful information in this regard. The water circulating through the units should also be tested and treated regularly.

There are a number of simple, low cost interventions that may be useful to decrease the risk of nosocomial infection caused by the amoeba resistant bacteria isolated in this study (Ulu-Kilic et al., 2013). These include

- The provision of educational programs and evaluating the effectiveness of these programs
- Surveillance activities
- Hand hygiene training and promotion
- Rational use of antibiotics
- Upgrading of infection control measures

In the presented study, presence/absence was performed on samples analyzed by amoebal enrichment technique to isolate amoebae. Therefore was no statistical correlation between the isolated amoebae and possible intracellular bacteria. Nevertheless, the detection of nosocomial bacteria in samples positive for amoebae suggests that these amoebae may harbour and act vectors for these opportunistic pathogens. The association of bacteria and amoebae in examined water samples of the three hospitals in this study underlines the importance of hospital water surveillance through the application of monitoring and prevention measures to ensure safe potable water for patients and workers. Therefore a comprehensive water safety plan is recommended, which should include:

- The microbiological examination of water from the healthcare facility environment is necessary both in the routine monitoring of decontamination procedures
- Sampling should be undertaken by staff trained in the appropriate technique for taking water samples including the use of aseptic technique to minimise extraneous contamination.
- Correct temperatures must be maintained throughout the water distribution system.
  - Keep hot water hot and cold water cold
  - Hot water should leave the calorifier above 60°C
  - Circulate hot water in the system flow at 55°C
  - Cold water should be stored and distributed below 20°C
- Cleaning regimens must be maintained to reduce biofilm and scale build-up
- Appropriate maintenance of hospital water distribution systems, by mechanic cleaning-out of the tanks of possible organic matter, followed by their washing-out with disinfectants (i.e. sodium hypochlorite
- The Infection prevention and control team should have an active surveillance programme in place in each healthcare facility to detect alert organisms such as *Pseudomonas aeruginosa*
- The Infection prevention and control team should identify clinical areas where patients may be at high risk of waterborne
- The Infection prevention and control team should encourage a culture of adherence to hand hygiene to prevent potential. Regular hand hygiene audits are performed, reported and auctioned
- The Infection prevention and control team should ensure that clinical hand wash sinks should be dedicated for the purposes of hand washing only
- Cleaning staff must clean clinical hand wash sinks in a manner that minimises the risk of contamination of the tap from organisms in the basin

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