Acanthamoeba- associated *Pseudomonas* species at Kenyatta National Hospital Intensive Care Unit, Kenya

By

Zipporah Wanjiku Macharia

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A Thesis Submitted in Partial Fulfillment for the Requirements for the Award of the Degree of Master of Science in Medical Microbiology, University of Nairobi

2019

DECLARATION

I declare that this is my original work and to the best of my knowledge has not been presented by any other person for research purpose, degree or otherwise in any other university or institution.

Signed……Date……

Zipporah Wanjiku Macharia

H56/80959/2015

APPROVALS

This thesis has been submitted for review in partial fulfillment for the award of Master Degree in Medical Microbiology with our approval as supervisors:

Supervisors

Dr. Kariuki Njaanake

Department of Medical Microbiology

University of Nairobi

Signature…… Date……..

Dr. Moses Masika

Department of Medical Microbiology and

KAVI –institute of Clinical Research

University of Nairobi

Signature…… Date……..

Dr. Marianne Mureithi

Department of Medical Microbiology and

KAVI –institute of Clinical Research

University of Nairobi

Signature…… Date……..

DEDICATION

I dedicate this work to my dear family and to everyone who has made this work a success.

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I will forever be grateful to God for His grace, strength and guidance throughout my studies and research work.

My heartfelt appreciation is to my husband Mr. Samuel Njogu who has sacrificially supported me and my children Shawn, Glenys and Prince whose prayers and love kept me going.

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LIST OF ABBREVIATIONS

AAMs -Amoeba Associated Microorganisms

ATCC -American Type Culture Collection

AIBBC - Africa International Biotechnology and Biomedical Conference

AIDS -Acquired Immune Deficiency Syndrome

AK -Amoebic Keratitis

ARMs -Amoeba Resistant Microorganisms

BA -Blood Agar

^{0}C -Degrees Centigrade

CDC -Centers for Disease Control and Prevention

CL -Contact Lens

CM -Centimeters

CNS -Central Nervous System

CPE -Cytopathic Effect

DNA -Deoxyribonucleic Acid

ECG -Electrocardiography

EDTA -Ethylene Diamine Tetra Acetic acid

FLA -Free Living Amoeba

GAE -Granulomatous Amoebic Encephalitis

HCL -Hydrochloric Acid

HEK -Human Embryonic Kidney

HIV -Human Immunodeficiency Virus

ICU -Intensive Care Unit

KCl -Potassium Chloride

KNH -Kenyatta National Hospital

Ksh -Kenya Shillings

M -Molar

MAC -MacConkey

ML -Milliliters

MLB -Multi-Lamellar Body

MRI -Magnetic Resonance Imaging

MRSA -Methicillin Resistant Staphylococcus Areaus

MSc -Master of Science

NaCl -Sodium Chloride

NNA -Non nutrient Agar

PAS -Page's Amoebic Saline

PAM -Primary Amoebic Meningoencephalitis

PBS -Phosphate Buffered Saline

PCR -Polymerase Chain Reaction

PHMB -Polyhexamethylene

PYG -Proteose Peptone Yeast Extract Glucose

RPM -Revolutions Per Minute

SDS -Sodium Dodecyl Sulfate

Sp -Species

SPSS -Statistical Package for the Social Science

TBE -Tris Borate EDTA

TEM -Transmission Electron Microscopy

µL -Micro Liters

UoN -University of Nairobi

US -United States

V -Voltage

χ^2 -Chi-square

ABSTRACT

Background: Free-living amoeba (FLA) such as *Acanthamoeba* spp. are ubiquitous unicellular eukaryotes that graze on bacteria, viruses, algae and are common contaminants in virtually all environments. However, amoeba resistant microorganisms (ARMs) evade amoebic killing and survive and multiply within FLA. *Pseudomonas* spp. is an ARM with known high antibiotic resistance, increased virulence, resistance to disinfectants and are etiologic agents of nosocomial infections. Co-existence of FLA and ARMs may perpetuate spread of pathogens across hospital environments that would be responsible for nosocomial infections and the antimicrobial resistance global surge today. Sensitization on the existence of ARMs and their potential impact on infection control and patient health is paramount.

Aim: To culture and isolate both *Acanthamoeba* spp. and bacteria as well as to detect *Pseudomonas* sp. genomic DNA within *Acanthamoeba* spp. isolates obtained from swabs collected from selected surfaces and equipment at Kenyatta National Hospital Intensive Care Unit (KNH ICU).

Methodology: This was a descriptive cross-sectional study done at KNH ICU. One hundred and fifty-three swabs were collected in duplicate (306 swabs). *Acanthamoeba* spp. cultures were performed on the first batch of 153 swabs while bacterial cultures were performed on the second batch of 153 swabs. *Pseudomonas* sp. genomic DNA was detected from *Acanthamoeba* spp. isolates using polymerase chain reaction (PCR) technique.

Results: The proportion of swabs with *Acanthamoeba* spp. isolates was 93.5% (143/153). Subcultures were done on 62.7% (96/153) primary *Acanthamoeba* spp. isolates and only 22.9% (22/96) were positive. PCR was conducted on all the 22 positive *Acanthamoeba* spp. subcultures and *Pseudomonas* sp. genomic DNA was detected in almost half (45.5%) of the tested subcultures. Of the total bacterial isolates, the proportion of *Pseudomonas* spp. was 10.7% (18/168).

Conclusion: To the best our knowledge *Acanthamoeba* spp. was for the first time in Kenya isolated upon culture and *Pseudomonas* sp. endosymbiotic relationship with the isolated *Acanthamoeba* spp. confirmed as a potential contributor to nosocomial infections and bacterial antimicrobial resistance burden at KNH ICU.

CHAPTER ONE: INTRODUCTION

1.1 Background information

Free living amoeba (FLA) are ubiquitous protozoa with a global distribution (Saeed *et al.*, 2012; Chow and Glaser, 2014; Lakhundi, Siddiqui and Khan, 2015; Fabres *et al.*, 2016). They feed by phagocytosing bacteria, algae, fungi, protozoa and particles rich in energy for nutrition (da Rocha-Azevedo, Tanowitz and Marciano-Cabral, 2009; Gimenez *et al.*, 2011; Aç *et al.*, 2013; Ovrutsky *et al.*, 2013; Cervero-Aragó *et al.*, 2015; Fabres *et al.*, 2016). However, some microorganisms known as amoeba resistant microorganisms (ARMs) resist amoebic killing and are capable of invading and proliferating within FLA. These ARMs are released into the environment as free pathogens or in vesicles that propagate transmission into different environments (Greub and Raoult, 2004; Barnard, 2015).

The interaction between ARMs and FLA pose a risk of increased virulence, spread of drug resistance genes and may also act as an evolutional crib for intracellular microorganisms (da Rocha-Azevedo, Tanowitz and Marciano-Cabral, 2009; Lamrabet *et al.*, 2012; Fabres *et al.*, 2016). In addition, FLA may enhance the transfer of bacterial gene fragments from the phagocytosed prey to other bacteria (Fukumoto *et al.*, 2016). This can make FLA potential facilitators of antimicrobial resistance especially in the hospital set up. Moreover, amoeba associated microorganisms (AAMs) have been associated with community and nosocomial infections (Berger *et al.*, 2006). Berger and others investigated amoeba- associated microorganisms in the diagnosis of nosocomial pneumonia in ICU patients suffering from severe pneumonia. They reported 12.9% AAMs as etiologies of pneumonia of which 4.8% were *Pseudomonas aeruginosa,* 3.8% *Acanthamoeba polyphagamimivirus,* 1.9% *Escherichia coli* and 1.4% *Legionnella pneumophila* (Berger *et al.*,

2006). Similarly, different studies have associated ARMs such as *Pseudomonas* sp., *Legionella pneumophilia* and *Acinetobacter baumannii* with nosocomial infections (Cateau *et al.*, 2011; Barnard, 2015). Of note, *Pseudomonas* spp. comes second among the leading causative agents of nosocomial infections that present with treatment and infection control challenges (Lim and Webb, 2005; Moradali, Ghods and Rehm, 2017).

More than 1.4 million people globally contract nosocomial infections that are a leading cause of death among hospitalized patients (Bereket *et al.*, 2012; Bousbia *et al.*, 2013; Altayyar *et al.*, 2016). Moreover, according to the Centers for Disease Control and Prevention (CDC), about 1.7 million inpatients in the United States (US) acquire nosocomial infections annually with over 6% mortalities. Worse still, in advanced and emerging countries, for every 100 inpatients, 7% and 10% acquire nosocomial infections respectively (Haque *et al.*, 2018). Having noted the potential role of FLA and ARMs in the nosocomial infections burden, it's important to suggest that, in etiologic investigations of nosocomial infections, ARMs should be suspected in all negative results obtained from routine microbiological tests (Berger *et al.*, 2006).

Free living amoeba can also aid in the isolation of disease-causing intracellular microorganisms from specimens as well as in the resuscitation of viable non cultivable bacteria such as *Legionella pneumophila* hosted within FLA (Greub and Raoult, 2004; Thomas *et al.*, 2005; García *et al.*, 2007; Dickinson and Pont-de-claix, 2012). Common human pathogenic ARMs include members of *Enterobacteriaceae* family such as *Escherichia coli, Klebsiella* sp.*, Enterobacter* sp.; *Staphylococcus aureus, Streptococcus pneumonia, Cryptococcus neoformans, Bacillus cereus, Francicella tularensis, Mycobacteraceae, Legionella pneumophilia*, *Coxiella burnetti, Listeria monocytogens, Helicobacter pylori, Vibrionaceae, Herpes viruses, Echo* viruses, *Coxsackie*

viruses, Enteroviruses, *Adeno* viruses and Mimiviruses (Greub and Raoult, 2004; Berger *et al.*, 2006; Lamrabet *et al.*, 2012; Aç *et al.*, 2013; Ovrutsky *et al.*, 2013; Crosatti, 2014; Guimaraes *et al.*, 2016). Some researchers argue that the documented number of ARMs is an underestimation since FLA and ARM interaction studies have been limited to *Acanthamoeba* spp. and many pathogenic microorganisms have not been studied as yet (Balczun and Scheid, 2017). Of note, majority of the known ARMs have been isolated from intensive care unit patients at one time or the other as etiologic agents of primary infections and or nosocomial infections (Njoki, 2009).

Most infections are only linked to well-known free etiologic agents and hence the contribution of pathogenic ARMs in disease burden is mostly overlooked (Khan and Siddiqui, 2014). Recently, Khan and Siddiqui redirected infection control focus towards killing FLA that harbor pathogenic microbes in hospital environments as a new approach in controlling the spread of infectious diseases and reducing incidences of nosocomial infections (Khan and Siddiqui, 2014).

Studies across the globe have documented diverse prevalence's of *Acanthamoeba* spp. isolates. In America for example, a prevalence of 47.1% was reported from hospital water samples, 41.9% from tap water and 19% from dust samples in Brazil (Winck, Caumo and Rott, 2011; Ovrutsky *et al.*, 2013). In Europe, 99.1% from water matrices samples in Spain, 71.2% from cooling tower samples in Austria, 39.1% from water sources in Italy and 24.2% from tap water in Turkey (Teixeira *et al.*, 2009; Aç *et al.*, 2013; Izquierdo *et al.*, 2013; Walochnik, Scheikl and Haller-Schober, 2015). In Asia, 55.8% from different water samples and 49% from hospital drinking water in Iran, 37.5% from underground water in Saudi Arabia and 11.7% from seasonal river water in Taiwan (Bagheri *et al.*, 2010; Kao *et al.*, 2013; Shokri *et al.*, 2016; Vijayakumar, 2018). Regrettably, only a few African countries have documented literature on *Acanthamoeba* spp.

studies. A prevalence of 69% of *Acanthamoeba* spp. from dental unit water was isolated in Tunisia, 33% and 42.9% from environmental and tap water respectively in Uganda, 42.9% from hemodialysis water in Egypt, 12.8% from waste water and 11.3% from biofilm samples in South Africa (Trabelsi *et al.*, 2010; Hassan *et al.*, 2012; Muchesa . , Leifels, 2013; Muchesa *et al.*, 2014; Barnard, 2015; Sente *et al.*, 2016). Of concern, Kenya has no documented literature on *Acanthamoeba* spp. and to the best of our knowledge, the present study is the first to report on the same in the country. Having identified a research gap on FLA studies in the developing countries and more so in Kenya, a quest to research on FLA particularly *Acanthamoeba* spp. arose.

The present study was driven by the quest to investigate on the occurrence of *Acanthamoeba* spp. at KNH ICU and to confirm the co-existence of *Pseudomonas* spp. with *Acanthamoeba* spp. isolates. The study aimed at shedding light on FLA such as *Acanthamoeba* spp. and ARMs such as *Pseudomonas* sp. as possible contributors of the increased nosocomial infection incidences and antimicrobial resistance within hospital environments. Swabs from selected sites at KNH ICU were cultured for *Acanthamoeba* spp. isolation and subsequent molecular detection of *Pseudomonas* sp. genomic DNA was done from the *Acanthamoeba* spp. isolates. Bacterial cultures were also performed on swabs collected from selected sites at KNH ICU as part of infection control measures.

1.2 Problem Statement

Free living amoeba are ubiquitously distributed facultative pathogens that can cause serious and fatal human infections. FLA also harbor pathogenic ARMs such as *Pseudomonas* sp. which is a known etiologic agent of nosocomial infections with high antimicrobial resistance. According to World Health Organization (WHO) more than 1.4 million people globally contract nosocomial infections despite stringent infection control measures in hospital setups (Altayyar *et al.*, 2016). Moreover, nosocomial infections lead to high morbidity, mortality and financial losses due to prolonged hospital stay. The interaction between FLA and ARMs may lead to formation of dissemination points of pathogens to patients resulting in unsuspected nosocomial infections. However, due to the lack of adequate knowledge on the existence of FLA, their importance in health and their possible contribution to the burden of nosocomial infections, health personnel usually do not consider them during diagnosis and infection control strategies. This is further exacerbated by the fact that common disinfectants used in hospital infection control are not effective against FLA and their related ARMs. This may result in a continuous circulation of these pathogens within hospital environments regardless of infection control endeavors. Of note, *Acinetobacter baumannii*, *Pseudomonas* sp., *Klebsiella* sp., *Enterobacter* sp., *Escherichia coli*, *Proteus* sp. and *Staphylococcus areaus* are persistent pathogens that have been commonly isolated from KNH ICU patients' specimens and from infection control swabs according to both KNH Microbiology Laboratory and ICU Infection Control Departments regardless of intensive infection control measures put in place(Njoki, 2009; Inyama *et al.*, 2011). There is therefore an urgent need to better elucidate the prevalence of FLA, associated ARMs and their importance in health in Kenya.

1.3 Justification

The literature on *Acanthamoeba* spp. research in Kenya and other developing countries in general is limited with only a few African countries such as South Africa, Egypt, Tunisia, Sudan and Uganda having published literature on the protozoa (Shanan *et al.*, 2015). Nonobligatory FLA are extensively disseminated in the environment and cause severe, chronic and terminal diseases such as granulomatous amoebic encephalitis (GAE) and primary amoebic meningoencephalitis (PAM) and are known to harbor ARMs (Qvarnstrom *et al.*, 2006; Panda *et al.*, 2015). They could be linked to hospital milieu contamination with ARMs, such as *Pseudomonas* sp., that has known resistance to commonly used germicides and antibiotics (Berger *et al.*, 2006; Paulo, 2015; Fukumoto *et al.*, 2016). Moreover, the high burden of nosocomial infections continues to be a global health problem despite great advances in hospital infection control measures (Altayyar *et al.*, 2016). Its therefore imperative to create awareness of FLA and ARM-associated infections among health personnel and to develop rapid diagnostic tools, treatment and robust infection control measures (da Rocha-Azevedo, Tanowitz and Marciano-Cabral, 2009; Clarke *et al.*, 2012; Lorenzo-morales, Khan and Walochnik, 2015). To date, no information is available on the potential of infection with FLA and ARMs in Kenya although elsewhere there is evidence of hospital contamination that can perpetuate nosocomial infections (Berger *et al.*, 2006; Barnard, 2015; Altayyar *et al.*, 2016). In the light of the prevalence of ARMs, detailed investigations on the sources and points of transmission of these ARMs in the hospital set up in the country are necessary. The present study is aimed at elucidating the prevalence of FLA (*Acanthamoeba* sp.) and associated ARMs (*Pseudomonas* sp.) at KNH ICU and will form an important scaffolding for follow up investigations and associated infection control mechanisms.

1.4 Objectives

1.4.1 Broad objectives

To culture and isolate *Acanthamoeba* species and bacteria from swabs collected from selected surfaces and equipment at Kenyatta National Hospital Intensive Care Unit (KNH ICU), as well as to detect *Pseudomonas* species genomic DNA within isolated *Acanthamoeba* spp. using polymerase chain reaction (PCR) technique.

1.4.2 Specific Objectives

1. To culture and isolate *Acanthamoeba* species from swabs collected from selected surfaces and equipment at KNH ICU
2. To detect *Pseudomonas* sp. genomic DNA from the isolated *Acanthamoeba* species using PCR technique
3. To culture and isolate bacteria from selected surfaces and equipment at KNH ICU

CHAPTER TWO: LITERATURE REVIEW

2.1 FLA phylogeny and ecology

Free living amoeba were first described in a drop of water in 1674 by Antonie van Leewenhoek and were classified as *Chaos proteus* by Carl Linnaeus in 1767. Subsequent studies have led to the discovery of several free living and parasitic amoeba species (Horn, 2001). Castellani was the first to describe *Acanthamoeba* spp. in cultures of *Cryptococcus pararoseus* and in 1931 Volkonsky defined the genus *Acanthamoeba* (Marciano-Cabral and Cabral, 2003).

There are over 1500 species of distantly related protists that are widely spread in water and soil ecosystems (da Rocha-Azevedo, Tanowitz and Marciano-Cabral, 2009; Bertelli and Greub, 2012; Aç *et al.*, 2013). Isolates have been obtained from rivers, sea water, swimming pools, mineral water, tap water, fresh water lakes, air conditioning systems, springs, industrial cooling system, oceanic sediments, moist soil, desert soil, sewages, dialysis machines, eyewash stations, contact lenses, air dust and biofilms (Horn, 2001; Dendana *et al.*, 2008; Łanocha *et al.*, 2009; Abd *et al.*, 2010; Aç *et al.*, 2013; Ovrutsky *et al.*, 2013; Rudell *et al.*, 2013; Hsueh and Gibson, 2015; Gomes *et al.*, 2016; Szenasi *et al.*, 2017). Recently, FLA were isolated in anthropic milieu such as table towels, dental brushes and cooling systems (Rubenina *et al.*, 2017).

The completion of FLA lifecycle takes place externally in different environments with no human or animal hosts involvement but occasionally entry into humans or animal hosts takes place leading to pathology (La Scola *et al.*, 2003; da Rocha-Azevedo, Tanowitz and Marciano-Cabral, 2009; Chomicz *et al.*, 2015). *Acanthamoeba* spp., *Naegleria* spp., *Balamuthia mandrillaris* and *Sappinia* species are potential human and animal pathogens that cause opportunistic and non-opportunistic

infections (Trabelsi et al. 2010; Booton et al. 2010; Dendana et al. 2008; Aç et al. 2013). In humans *Acanthamoeba* spp., *Balamuthia mandrillaris* and *Sappinia* spp. are known to cause granulomatous amoebic encephalitis (GAE) whereas *Naegleria fowleri* and *Paravahlkampfia francia* cause highly lethal primary amoebic meningoencephalitis (PAM) (Szénási *et al.*, 1998; Horn, 2001; Marciano-Cabral and Cabral, 2003; da Rocha-Azevedo, Tanowitz and Marciano-Cabral, 2009; Wannasan *et al.*, 2013; Chow and Glaser, 2014; Siddiqui and Khan, 2014). *Acanthamoeba* spp. also cause acanthamoeba keratitis (AK) in immunocompetent individuals especially in those wearing contact lens, those with corneal injury and those living in places with inadequate water supply (Chappell *et al.*, 2001; Khan, 2003; Booton *et al.*, 2010; Clarke *et al.*, 2012; Muchesa *et al.*, 2014). Localized and systemic infections with *Acanthamoeba* spp. have also been reported in immunosuppressed patients (Szénási *et al.*, 1998; Booton *et al.*, 2010).

2.2 FLA interaction with microorganisms

Protozoa endosymbiotic relationship with microorganisms has been reported to exist either naturally or due to adaptation of phagocytosed intracellular microorganisms within the protozoa (Szenasi *et al.*, 2017). FLA feed by phagocytosing bacteria, algae, fungi, protozoa and particles rich in energy from the environment for nutrition (da Rocha-Azevedo, Tanowitz and Marciano-Cabral, 2009; Gimenez *et al.*, 2011; Aç *et al.*, 2013; Ovrutsky *et al.*, 2013; Cervero-Aragó *et al.*, 2015; Fabres *et al.*, 2016). Enzymatic digestion takes place within the lysosomes and waste is expelled in form of vesicles or pellets into the environment (Paquet and Charette, 2016). However, ARMs evade amoebic killing mechanisms with about 25% of both environmental and clinical *Acanthamoeba* spp. isolates being reported to foster ARMs (Kara *et al.*, 2015; Szenasi *et al.*, 2017). Amoeba resistant microorganisms control FLA innate gene regulation and expression systems in

favour of ARMs (Greub and Raoult, 2004; Siddiqui, 2012; Rubenina *et al.*, 2017). As such, there are Type III or Type IV secretory systems expressed by majority of Amoeba Resistant Bacteria (ARB) whose effector proteins enhance bacterial invasion and survival within FLA (Shanan *et al.*, 2015). This has been particularly observed in *Pseudomonas aeruginosa,* an amoeba-extracellular bacteria that express Type III secretory system whose released effector proteins lyse FLA (Shanan *et al.,* 2015).

Free living amoeba mediate packaging of ARMs into unique lysosomal storage or secretory structures called Multi-Lamellar Bodies (MLBs). Multi-lamellar bodies are made of numerous lipid membranes arranged in coaxial layers with an enclosed core that shelters ARMs (Paquet *et al.*, 2013). Human pathogens including *Pseudomonas sp.*, *Salmonella enterica, Escherichia coli* 0157: H7, *Legionella pneumophila* and *Listeria monocytogens* have been isolated from protozoal MLBs (Trigui and Charette, 2016). Amoeba resistant microorganisms packaged in MBLs can withstand adverse conditions and have been reported to be highly resistant to antimicrobial agents (Singer, 2010; Siddiqui, 2012; Denoncourt *et al.*, 2014). For example, ARMs that have interacted with *Acanthamoeba castellani* have been reported to be capable of forming biofilms and spores, and are resistant to substances such as gentamycin, chlorine and biocides which pose a challenge in treatment and infection control endeavors (Paquet *et al.*, 2013; Denoncourt *et al.,* 2014). Bacteria and FLA interactions have been reported to mediate bacterial physiological changes such as increased virulence and the ability to revive and grow non-cultivable bacteria (da Rocha-Azevedo, Tanowitz and Marciano-Cabral, 2009; Lamrabet *et al.,* 2012; Kara *et al.*, 2015; Fabres *et al.,* 2016). Additionally, predator FLA contribute to the transfer of bacterial gene fragments from the phagocytosed prey to other bacteria and may perpetuate transmission amongst hosts (Singer, 2010; Siddiqui, 2012; Fukumoto *et al.*, 2016). Free living amoeba interaction with ARMS

through a phagocytic process, bacteria multiplication, transmission of released ARMs and selection of virulence traits is shown in Figure 1.

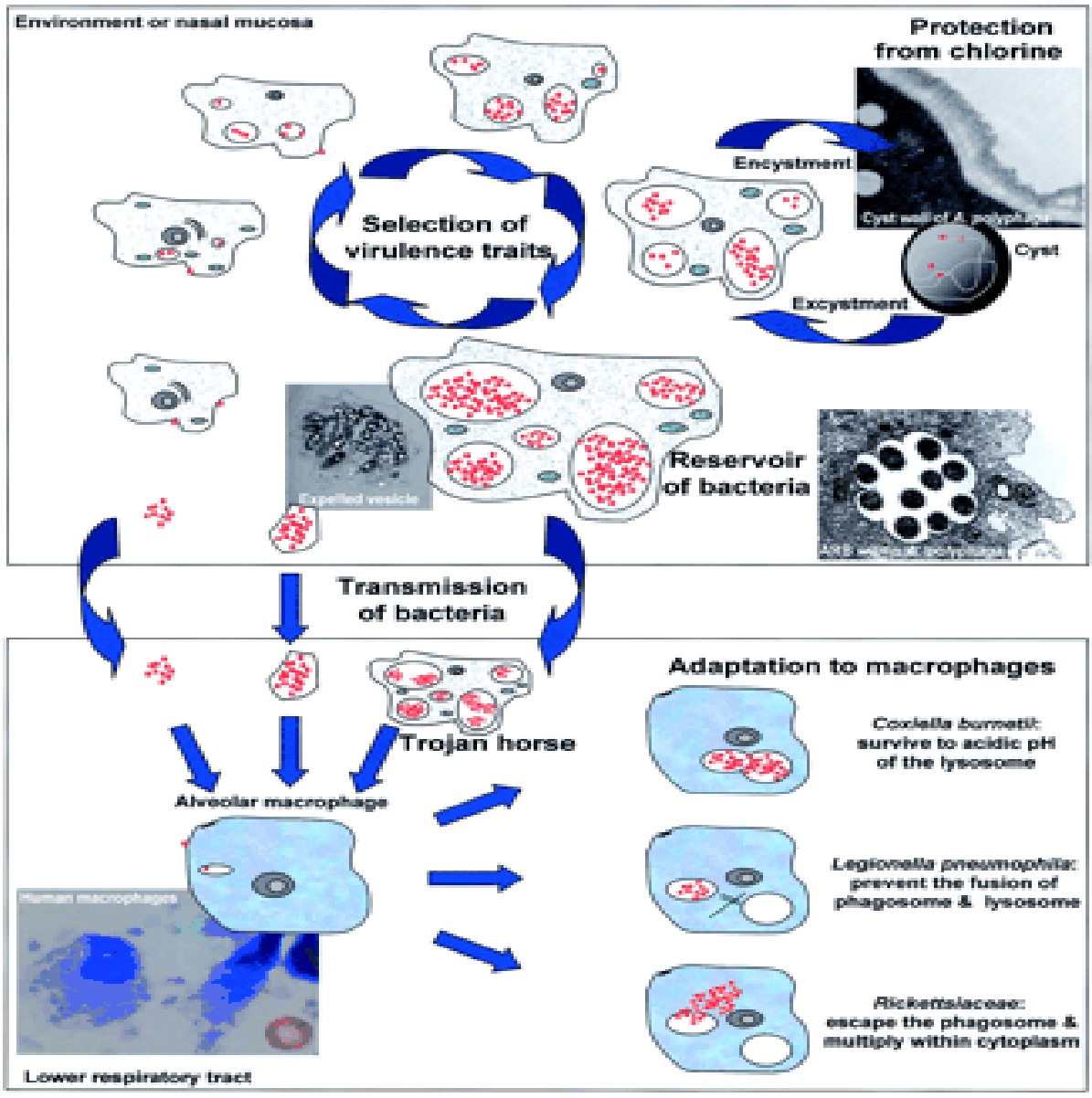

Figure 1: FLA phagocytosis, bacteria multiplication, transmission of released ARMs and selection of virulence traits

Obtained from: <http://cmr.asm.org/content/17/2/413/F2.expansion.html> or (Greub and Raoult, 2004).

2.3 Circulation of FLA in the natural and hospital environment

The ecology of FLA in the natural and hospital environment is complicated and poorly understood (Fukumoto *et al.,* 2016). There is no documented information on the existence of FLA in Kenya both in the natural and hospital environments. The fact that FLA are not fastidious in terms of habitat, supports a wide distribution of FLA in public places such as hospitals, schools, prisons and care centers commonly in air, peoples clothing, dust and soil (Costa *et al.,* 2010; Fukumoto *et al.,* 2016). As a result, FLA and the coexisting pathogenic microorganisms are in continuous circulation across the natural and hospital environments. For instance, visitors, staff and patients unknowingly bring in pathogens from the community to the hospital and carry other pathogens from the hospitals back to the community (Altayyar *et al.*, 2016). This circulation of ARMs across hospital environment predisposes especially the immunocompromised patients to FLA and ARM related infections (Barnard, 2015).

2.3.1 ARMs as potential agents of nosocomial infections

The current hospitalization rates have escalated due to the rise in various immune suppressing conditions such as cancer, diabetes, Human Immunodeficiency Virus (HIV) infections, organs failure and tuberculosis. Hospitalization predisposes to 5 - 15% patients to the development of nosocomial infections. Worse still, intensive care patients have a 5 to 10 times higher chance of developing nosocomial infections than patients in general wards (Lim and Webb, 2005; Weber, Raasch and Rutala, 2017). Therefore, to reduce the rate of nosocomial infections, information on predisposing factors, mode of transmission, source and reservoir of nosocomial pathogens is paramount (Rutala and Weber, 2017).

The level of hospital surface contamination with pathogenic microbes determines the rate of nosocomial infections (Altayyar *et al.,* 2016). For this reason therefore, continuous and proper infection control measures and hospital environment surveillance for pathogenic microorganisms and FLA contamination should be prioritized in an effort to lower the high incidence of nosocomial infections (Davane *et al.*, 2014).

Pseudomonas sp., a known ARM and an etiologic agent of nosocomial infections is ubiquitous in nature, commonly occurring as a colonizer in different environments such as soil, animals, plants, water bodies, hospital cut flowers, taps, toilets, sinks, mops, food, respiratory and dialysis equipment, moist environment, antiseptics, soap solutions, infusion fluids and disinfectants (Lim and Webb, 2005; Paulo, 2015; Moradali, Ghods and Rehm, 2017). The ubiquity nature of *Pseudomonas* spp. more so in hospital environments allows for vast spread to patients from many diverse sources including air, food, water, visitors, linen, contaminated medical personnel, contaminated surfaces and equipment such as catheters and ventilators (Davane *et al.*, 2014).

Immunocompetent individuals may harbor *Pseudomonas* spp. on the wet body parts such as the nose, gastrointestinal tract and the respiratory tract. However, colonization is exacerbated in hospitalized patients commonly in the alimentary canal, burnt skin, and in mechanically aerated respiratory tract predisposing patients to opportunistic and nosocomial infections (Molecolare and Microbiologia, 2005). Opportunistic human infections with *Pseudomonas* spp. mostly affect the eyes, skin, ears, bones, joints, circulatory system, surgical sites, urinary tract, gastrointestinal tract and respiratory tract infections commonly in the immunocompromised patients (Lim and Webb, 2005; Paulo, 2015; Altayyar *et al.*, 2016).

A low permeable outer membrane and large efflux pump systems are limitations in *Pseudomonas* sp. cell structure that allow for increased intake of drug resistant genes from the environment (Nasreen, Sarker and Malek, 2015). Recently, isolates of up to 10% multiple drug - resistant and pan - drug resistant strains of *P. aeruginosa* have been reported globally (Akyar, 2015; Nasreen, Sarker and Malek, 2015). This incidence unfortunately discredits the use of previously effective drug regime that presents a clinical crisis to medical personnel who get stranded and helpless in the face of patients (Yayan, Ghebremedhin and Rasche, 2015; Moradali, Ghods and Rehm, 2017).

2.4 FLA a new target for effective elimination of pathogenic ARMs

The ability of FLA to host and protect ARMs facilitates unsuspected transmission of pathogenic microorganisms within hospital environments (Khan and Siddiqui, 2014). Occasionally, the efficacy of hospital infection control disinfectants has been questioned in the face of the high burden of nosocomial infections and the persistent spread of highly resistant pathogens (Khan and Siddiqui, 2014; Biehl *et al.*, 2017). This has been of concern with the use of 5% Sodium Hypochlorite, a chlorine bleach commonly used in many hospitals, which though effective against majority of hospital pathogens is not effective against protozoa such as *Acanthamoeba* sp. that form cysts (Khan and Siddiqui, 2014). A new approach in infection prevention and control should thus direct efforts towards pathogens that are not susceptible to the commonly used disinfectants (Costa *et al.*, 2010; Khan and Siddiqui, 2014). However, research-based evidence has shown that ARMs within FLA cysts are highly resistant to antimicrobial agents and disinfectants than those within FLA trophozoites. Ultimately, use of cysticidal disinfectants targeted against FLA cysts may be an important additional strategy in the elimination of pathogenic ARMs (Khan and Siddiqui, 2014).

2.5 *Acanthamoeba* spp. classification and biology

Acanthamoeba spp. are amphizoic protozoa that exist naturally as free living amoeba in various environments including soil, air, water, the hospital environment and equipment such as dental units, used contact lenses, ventilators, dialysis units and ocular wash stations or as parasites in various host tissues (Moon et al. 2008; Marciano-Cabral & Cabral 2003;Jeong & Yu 2005; Lass et al. 2014; Teixeira et al. 2009). Isolates of *Acanthamoeba* spp. have been obtained from infected human specimens such as the lungs, brain tissues, corneal biopsies, cerebral spinal fluid and genitourinary tracts (Szénási *et al.*, 1998; Jeong and Yu, 2005; Dendana *et al.*, 2008; Siddiqui and Khan, 2012). Figure 1 depicts the phylogeny and classification of FLA.

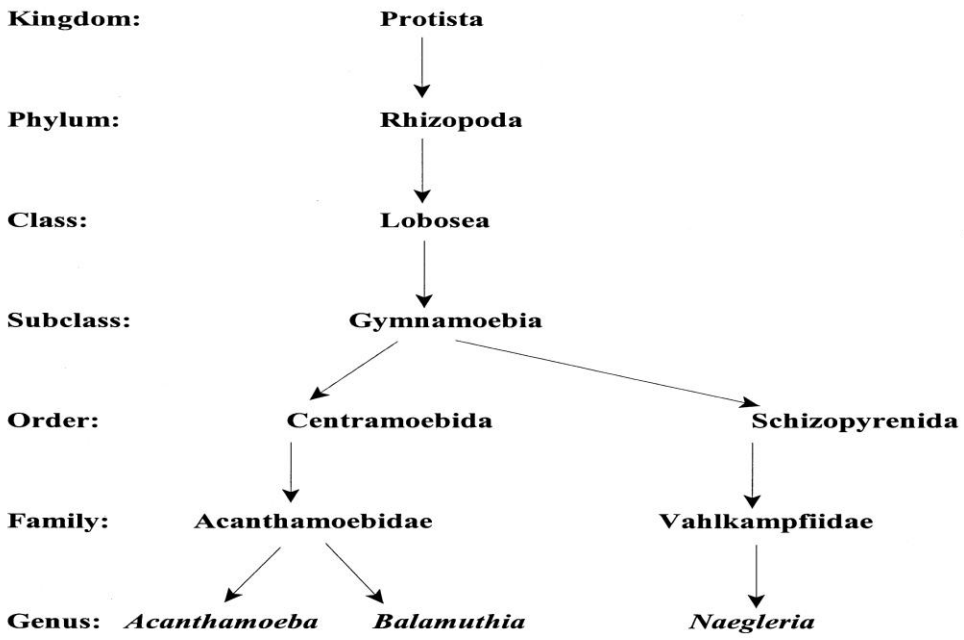

Figure 2: Phylogenetic scheme of genus Acanthamoeba, Balamuthia, and Naegleria
Adopted from (Marciano-Cabral and Cabral, 2003)

The genus *Acanthamoeba* has more than 21 species and 20 genotypes which have been described by 18s ribosomal RNA full gene sequencing (Gomes *et al.*, 2016). Of the more than 20 species, at

least eight are known to cause human infections, with *A. castellani* and *A. polyphaga* being the most frequently reported (Omaña-Molina *et al.*, 2013).The general life cycle of *Acanthamoeba* sp. is demonstrated in Figure 2.

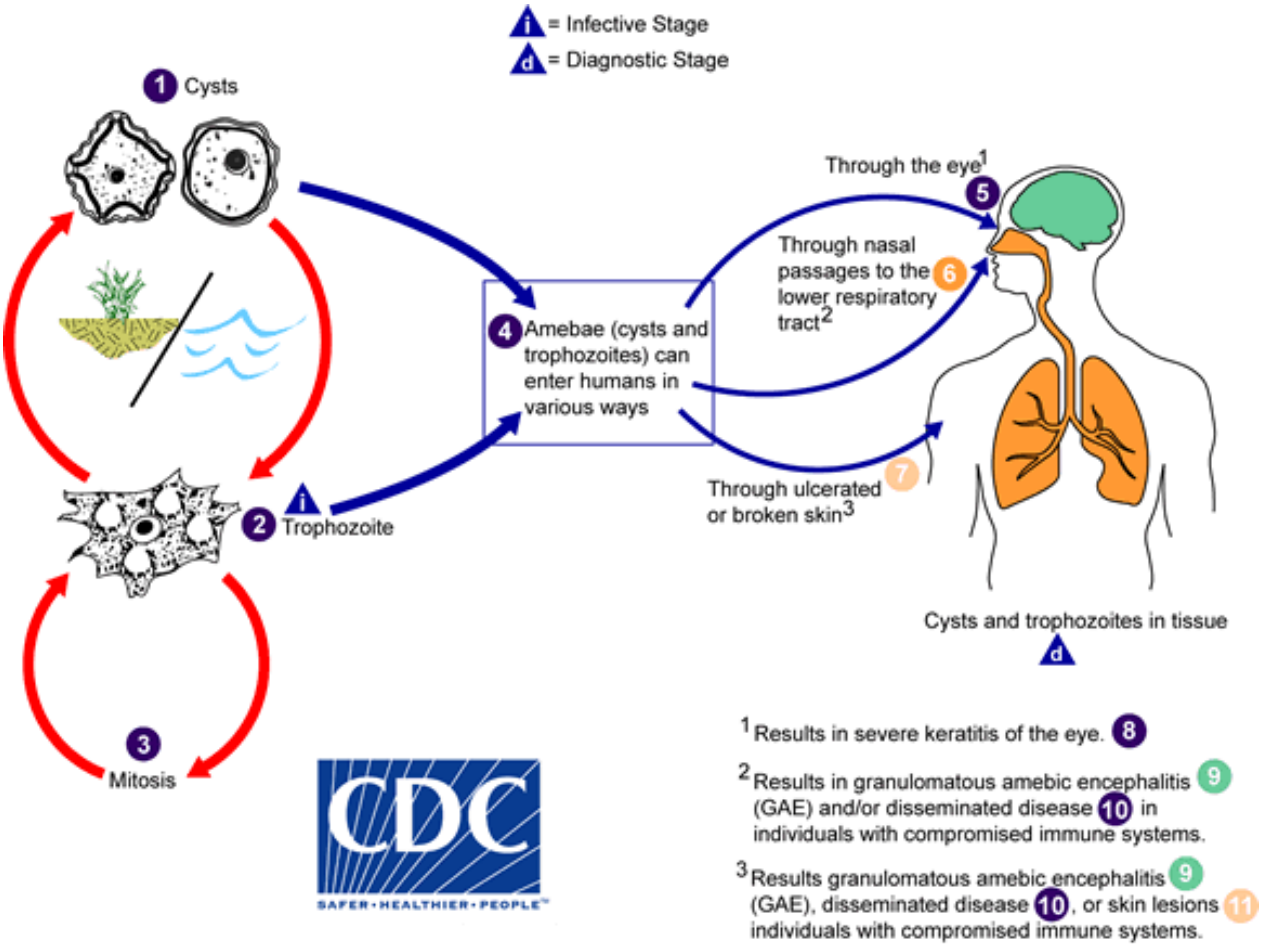

Figure 3: Life cycle of *Acanthamoeba* species
Obtained from <https://www.cdc.gov/dpdx/freelivingamebic/index.html> on 22/7/2019

Acanthamoeba sp. exists in two morphological forms, motile trophozoites and dormant cysts as shown in Figure 3 and Figure 4.

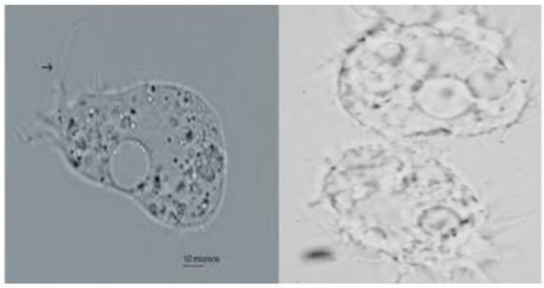

Figure 4: *Acanthamoeba* sp. trophozoites

(Auria *et al.*, 2012)

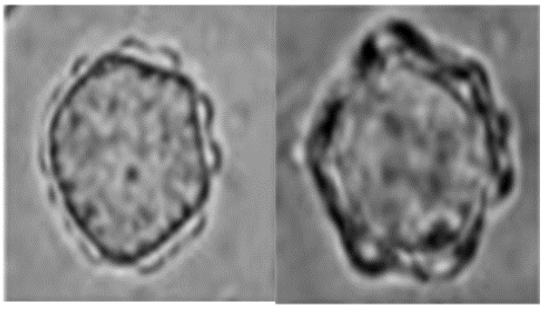

Figure 5: *Acanthamoeba* sp. cysts

(Duarte *et al.*, 2013)

The trophozoite is the pathogenic stage which is metabolically active, motile and in possession of unique spiny projections on the surface called acanthapodia which are easily seen under light microscopy. The cyst is the dormant stage and it is formed at the end of growth or under harsh environmental conditions (Marciano-Cabral and Cabral, 2003; da Rocha-Azevedo, Tanowitz and Marciano-Cabral, 2009; Booton *et al.*, 2010; Bertelli and Greub, 2012; Clarke *et al.*, 2012; Cervero-Aragó *et al.*, 2015; Chomicz *et al.*, 2015; Henst *et al.*, 2015). Cyst formation is also thought to be a survival strategy against host immune mechanisms (Moon et al. 2008; Omaña-

Molina et al. 2013; Marciano-Cabral & Cabral 2003). The highly resistant cyst is made up of a double wall that constitutes ectocysts and the endocysts (Marciano-Cabral and Cabral, 2003; Moon *et al.*, 2008; Henst *et al.*, 2015). Cysts survive desiccation, temperature changes, disinfectants, biocides, radiation, chlorination, pH changes, antibiotics, osmotic pressure variations and reduced nutrients (Marciano-Cabral and Cabral, 2003; Siripanth and Med, 2005; da Rocha-Azevedo, Tanowitz and Marciano-Cabral, 2009; Teixeira *et al.*, 2009; Trabelsi *et al.*, 2010; Clarke *et al.*, 2012; Zettler, 2012; Aç *et al.*, 2013; Hsueh and Gibson, 2015). They also act as reservoirs and sources of infections due to their ability to survive for several years in the environment (Marciano-Cabral and Cabral, 2003; Essa *et al.*, 2016). Both trophozoites and cysts have been isolated from the environment as well as infected human tissues (Khan, 2003; da Rocha-Azevedo, Tanowitz and Marciano-Cabral, 2009).

2.6 Pathogenesis of *Acanthamoeba* species

Free living amoeba were thought to be innocuous protozoa existing in water and soil for many years. However, pathogenic FLA have been isolated since the 1960s (Balczun and Scheid, 2017). Molecular sequencing using 18S nuclear small subunit ribosomal RNA (18S rRNA) genes has identified up to 20 genotypes (T1-20) of pathogenic *Acanthamoeba* spp. with T4 reported to be the most common human pathogen (Balczun and Scheid, 2017; Xuan *et al.*, 2017; Chan *et al.*, 2018; Ghaderifar *et al.*, 2018). *Acanthamoeba* spp. facultatively cause opportunistic and non-opportunistic infections (Trabelsi *et al.*, 2010; Aç *et al.*, 2013; Ghaderifar *et al.*, 2018). In humans, *Acanthamoeba* spp. cause GAE in immunocompromised individuals (Wannasan *et al.*, 2013; Chow and Glaser, 2014; Khan and Siddiqui, 2014; Mattana *et al.*, 2016; Szenasi *et al.*, 2017). *Acanthamoeba* spp. also cause AK in immune-competent individuals especially in those wearing contact lenses, those with corneal injury and also those individuals who live in places with

inadequate water supply (Chappell *et al.*, 2001; Khan, 2003; Booton *et al.*, 2010; Clarke *et al.*, 2012; Muchesa *et al.*, 2014). Localized and systemic infections by *Acanthamoeba* spp. have also been reported in immunosuppressed patients(Booton *et al.*, 2010; Al-Herrawy, Marouf and Gad, 2017; Szenasi *et al.*, 2017). Localized acanthamoeba infections involving the skin, ears, ocular organs, respiratory tract, genitourinary tract and the sinuses have been reported (Szénási *et al.*, 1998; Marciano-Cabral and Cabral, 2003; Paulo, 2015).

The most common transmission route of *Acanthamoeba* sp. is through water and air although establishment of infection depends on the inoculum size, the level of virulence, invasion potential of the amoeba strain involved and the host immunity level (Walochnik, Scheikl and Haller-Schober, 2015). Environmental exposure to *Acanthamoeba* sp. results in colonization of nasal mucosa in up to 24% of the population (Clarke *et al.*, 2012; Rahman *et al.*, 2013). Throat and nasal colonization may result in subclinical manifestations in healthy people as confirmed by the detection of between 50- 100% specific antibodies in immune-competent individuals (Teixeira *et al.*, 2009; Lass *et al.*, 2014).

Acanthamoeba spp. are able to host and transmit viruses, bacteria, fungi and protozoan parasites such as cryptosporidium oocysts to humans and other animals thus posing a risk to non-amoebic infections (Lass *et al.*, 2014). *Acanthamoeba* spp. have been associated with more than 100 species of pathogenic bacteria which may be transmitted to higher animals and even increase their virulence (Douesnard-Malo and Daigle, 2011; Gryseels *et al.*, 2012; Aç *et al.*, 2013; Fabres *et al.*, 2016).

2.6 Laboratory identification of *Acanthamoeba* species

Although culture is the gold standard for isolation of FLA and ARMs, routine cultures methods used for diagnostic isolation of free pathogens cannot be used for isolation of ARMs harbored

within FLA (Greub and Raoult, 2004; Bousbia *et al.*, 2013). FLA are therefore isolated from primary specimens on selective media such as Non Nutrient Agar (NNA), and upon growth, ARMs are released from FLA for identification using different methods such as PCR (Greub and Raoult, 2004; Bousbia *et al.*, 2013). Many pathogens can be detected from amoebic co-cultures (Evstigneeva *et al.*, 2017). *Acanthamoeba* spp. have been isolated from different environments including hospital water, domestic and environmental water, sewage, storage reservoirs and water puddles (Shin and Im, 2004; Ovrutsky *et al.*, 2013; Sente *et al.*, 2016). Clinical specimens from which the organism has been isolated include cerebral spinal fluid (CSF), brain tissues and autopsy material for GAE (da Rocha-Azevedo, Tanowitz and Marciano-Cabral, 2009; Siddiqui and Khan, 2012). Cornea scrapings, cornea biopsies, tissue sections and used contact lenses are considered the best for AK while skin scrapings are commonly used for cutaneous acanthamoebiasis (Marciano-Cabral and Cabral, 2003; Chomicz *et al.*, 2015; Lorenzo-morales, Khan and Walochnik, 2015). Urine and genital swabs can also be used for isolation of *Acanthamoeba* spp. from the genitourinary tract (Szénási *et al.*, 1998). Blood cultures, bronchoalveolar lavage, urine, water and swabs on hospital surfaces such as floors, sinks, drainages have been used for FLA and ARMs identification in diagnosis of ARM-associated nosocomial infections (Berger *et al.*, 2006; Bousbia *et al.*, 2013; Barnard, 2015; Fukumoto *et al.*, 2016)

The gold standard for isolation of *Acanthamoeba* spp. is culture technique which is relatively cheap and efficient (da Rocha-Azevedo, Tanowitz and Marciano-Cabral, 2009; Lorenzo-morales, Khan and Walochnik, 2015). Non-nutrient agar is routinely used for culture and isolation of *Acanthamoeba* spp. (Teixeira *et al.*, 2009). However, axenic cultures have also been performed on axenic media such as Proteose Peptone Yeast extract Glucose (PYG), Trypticase soy broth, Bacto Casitone Medium and Phosphate-biotriptase serum glucose medium (Bleasdale *et al.*, 2009;

Douesnard-Malo and Daigle, 2011; Omaña-Molina *et al.*, 2013; Lorenzo-morales, Khan and Walochnik, 2015). *Acanthamoeba* spp. can also be cultured in growth cells such as B103 rat neuroblastoma, Human Embryonic Kidney (HEK*),* l929 fibroblast and Human Embryonic Lung (HEL) (Marciano-Cabral and Cabral, 2003). The organisms graze on bacteria as a source of nutrition hence culture media are layered with live, inactivated or heat-killed non-mucoid bacteria such as *Escherichia coli, Enterobacter aerogenes* and *Aerobacter aerogenes* (Marciano-Cabral and Cabral, 2003; Dendana *et al.*, 2008; da Rocha-Azevedo, Tanowitz and Marciano-Cabral, 2009; Teixeira *et al.*, 2009; Clarke *et al.*, 2012; Ovrutsky *et al.*, 2013; Lass *et al.*, 2014). Growth temperature for *Acanthamoeba* spp. ranges from 4^0 C to 45^0 C with higher temperatures inducing early cyst formation (Greub and Raoult, 2004; Douesnard-Malo and Daigle, 2011; Chomicz *et al.*, 2015). The incubation period ranges from days to weeks depending on the *Acanthamoeba* spp. concentration in the specimen (da Rocha-Azevedo, Tanowitz and Marciano-Cabral, 2009). Moreover, animal inoculation has also been used for *Acanthamoeba* spp. isolation with mice being commonly used (Lorenzo-morales, Khan and Walochnik, 2015).

Light microscopy is commonly used for the morphological identification of *Acanthamoeba* spp. though different techniques such as electron microscopy, phase contrast microscopy, confocal microscopy and fluorescence microscopy have also been used (da Rocha-Azevedo, Tanowitz and Marciano-Cabral, 2009; Booton *et al.*, 2010; Siddiqui and Khan, 2012; Rusciano *et al.*, 2013; Chomicz *et al.*, 2015; Lorenzo-morales, Khan and Walochnik, 2015).

Different staining techniques are used for the detection and identification of *Acanthamoeba* spp. trophozoites and cysts. These stains include Gram stain, Periodic acid Schiff, Haematoxycillin and Eosin, Giemsa, Methylene blue, lactophenol cotton blue, silver stain, trichrome and immunostaining (Lorenzo-morales et al. 2015; Trabelsi et al. 2010; Marciano-Cabral & Cabral

2003; da Rocha-Azevedo et al. 2009). Special stains such as Acridine Orange and calcofluor white can also be used to aid identification (Rusciano *et al.*, 2013).

Molecular techniques are rapid, highly sensitive and specific but are expensive and require highly qualified personnel and they are not widely available (Chomicz *et al.*, 2015; Sifaoui *et al.*, 2015). They also do not differentiate between live and dead parasitic forms which makes it difficult for clinicians to decide on treatment (Rusciano *et al.*, 2013). Immunological techniques such as immune blotting and indirect immunoflourescence have been used in diagnosis of *Acanthamoeba* spp. and other FLA infections (Marciano-Cabral and Cabral, 2003; da Rocha-Azevedo, Tanowitz and Marciano-Cabral, 2009). One de-merit of immunological techniques is their lack of reliability as humans are usually exposed to *Acanthamoeba* spp. which triggers the production of *Acanthamoeba* specific immunoglobulins (Siddiqui and Khan, 2012; Lorenzo-morales, Khan and Walochnik, 2015).

According to the Centers for Disease Control and Prevention (CDC), about 1.7 million inpatients in the United States acquire nosocomial infections annually with over 6% mortalities. Moreover, in advanced and emerging countries, for every 100 inpatients, 7% and 10% acquire nosocomial infections respectively (Haque *et al.*, 2018). This trend has been observed despite great efforts being put in place in infection prevention and control systems in health care facilities with less noticeable achievement (Siddiqui and Khan, 2013). In this case therefore, the role of FLA and ARMs in the increasing rates of nosocomial infections needs to be keenly investigated. This study was aimed at confirming the possible co-existence of *Acanthamoeba* spp. and *Pseudomonas* sp., a known ARM, on selected surfaces and equipment at KNH ICU as a first step in the investigation of FLA and ARM contribution to nosocomial infections. This study will be a basis for more similar in-depth studies on this topic.

CHAPTER THREE: MATERIALS AND METHODS

3.1 Study Site

The study was conducted at Kenyatta National Hospital (KNH) main Intensive Care Unit (ICU). The hospital is the largest public, referral and teaching hospital in East and Central Africa and is ranked second largest in Africa (Gatero, 2010; Myers *et al.*, 2017). It is located approximately 3 kilometers from the Kenyan capital city Nairobi seated on about 304 acres of land (Inyama *et al.*, 2011). It receives a total of 2,500 patients daily, of whom 125 require intensive care unit beds although there are only 21 beds in the ICU. As a result, the ICU is always fully occupied and patients are frequently moved to general wards on slight improvement to create space for others. Both male and female medical patients, and surgical patients of all ages are admitted in the unit. There are approximately 1,200 admissions annually in the ICU (Ngumi, 2006; Inyama *et al.*, 2011). According to KNH's health information services, in 2016 the average monthly admissions were 58 patients, discharges 38 and deaths 21, with an average length of stay of 10.4 days/patient.

The main ICU is divided into wing A and wing B with two main entrances, a central area and a rear end. It contains a kitchen, a tea room, 4 toilets, a laboratory, an isolation room, a waiting room, a doctor's room, and a biomedical personnel room. There are 21 ventilators, 21 suction machines, 21 patients' lockers, 2 nurses' desks, 2 resuscitation trolleys and 1 defibrillator among others.

Varied incidences of nosocomial infections have been reported in the unit which include 18 % incidence of catheter-associated urinary tract infections (Inyama *et al.*, 2011), 28% incidence of ventilator-associated pneumonia in mechanically ventilated ICU patients (Njoki, 2009) and a current 42.9% incidence in ventilator associated pneumonia (Nduati, 2017 personal communication).

3.2 Study Design

The present study was a descriptive cross-sectional study aimed at culture and isolation of *Acanthamoeba* spp. and bacteria in swabs collected from selected surfaces and equipment at KNH ICU and subsequent molecular detection of *Pseudomonas* sp. genomic DNA within the *Acanthamoeba* spp. isolates.

3.3 Sampling Methods

Purposive sampling was used to select surfaces and equipment for swabbing at KNH ICU

3.4 Sample size determination

All available surfaces and equipment at KNH ICU that could potentially act as formites were identified for swab collection. As this is equivalent to a census no sample size calculation formula that was applicable. A total of 153 swabs were collected in duplicates (306 swabs) from these selected surfaces and equipment at KNH ICU following the hospital's infection control and prevention schedule (Nduati, 2017 personal communication).

The number of swabs per surface and equipment was carried out as follows: floor (6), sinks (21), door handles (9), telephone handles (3), drip stands (6), soap containers (6), walls (2), Electrocardiography (ECG) monitor screen (21), ventilator setting screens (16), suction machines (13), patient lockers (21), bed rails (21), resuscitation trolley (2), nurses desks (2) and defibrillator (2) as shown in details in Table 1.

Table 1: Selected surfaces and equipment swabbed at KNH ICU

Main swab collection site	Specific sites		Number of swabs
Floors	Main entrance	x2	6
	Central area	x2	
	Rear end	x2	
Sinks	Patients	x7	21
	Biomedical	x1	
	Laboratory	x1	
	Kitchen	x1	
	Tea room	x1	
	Toilet: physiotherapy, ladies, gents and doctors	x4	
	Decontamination	x1	
	Sluice room	x1	
	CPE sink	x1	
	Isolation sink 1	x1	
	Isolation sink 2	x1	
	Doctors room sink	x1	
Door handles	Main entrances	x2	9
	Laboratory	x1	
	Tea room	x1	
	Kitchen	x1	
	Doctors room	x1	
	Biomedical room	x1	
	Isolation room	x2	
Telephone handles	Nurses desk	x2	3
	Laboratory	x1	
Drip stands	Wing A	x3	6
	Wing B	x3	
Soap containers	Wing A sinks	x3	6
	Wing B sinks	x3	
Walls	Isolation area	x2	2
ECG monitor screens			21
Ventilator setting screen			16
Suction machines			13
Patients lockers			21
Bed rails			21
Resuscitation trolley			2
Nurses desks			2
Central monitor screen			2
Defibrillator			2
Total			153x2=306

A representation of the number of swabs that were collected in duplicates from selected surfaces and equipment at KNH ICU.

3.5 Specimen collection, culture, isolation and identification of bacteria, *Acanthamoeba* spp., molecular detection of *Pseudomonas* sp. genomic DNA and quality assurance

3.5.1. Specimen collection

Specimen collection was done randomly on a normal working day at KNH ICU for a few hours as the unit activities were still on course. The KNH ICU cleaning personnel were not informed before specimen collection and the assumption was that cleaning of both surfaces and equipment had been done on the unit's routine cleaning schedule. This was meant to depict the true level of infection control at the unit. A total of 153 selected surfaces and equipment from KNH ICU were aseptically swabbed in duplicates using sterile swabs to obtain 306 swabs. After collection, the swabs were carefully labeled per surface and equipment and delivered immediately to UoN Medical Microbiology laboratory for *Acanthamoeba* spp. and bacteria cultures.

3.5.2. Culture, isolation and identification of bacteria

In the laboratory, the first batch of 153 swabs were cultured on blood agar (BA) and MacConkey (MAC) agar following the streak plate procedure for isolation of bacteria. These were incubated aerobically at 37 ^{0} C for 18 to 24 hours as previously described by Sanders (2012). Negative controls were plain plates of BA and MAC, each incubated at the same conditions in parallel. *Pseudomonas* sp., ATCC 27853, was inoculated on a plate of BA and MAC and acted as a positive control. These were incubated at 37 ^{0} C for 18 to 24 hours to test whether BA and MAC agar were fit for their intended use. Bacteria identification was based on colonial morphology, type of haemolysis on BA, lactose fermentation, Gram stain and biochemical tests (Bisen et al. 2012) .

3.5.3. Culture, isolation and identification of *Acanthamoeba* spp.

Acanthamoeba spp. culture and detection of ARMs from the second batch of 153 swabs was carried out following the method described by Lagier and others (Lagier et al. 2015). Test for intended use of NNA media and confirmation of *Acanthamoeba* spp. morphological features was done by culturing *A. castellani* (ATCC 30010) trophozoites as a positive control (Kara *et al.*, 2015). The swabs for *Acanthamoeba* spp. culture were suspended in 2 milliliters (ml) sterile PAS saline in tubes and centrifuged at 1000 rpm for 10 minutes and the supernatant discarded (Fukumoto *et al.*, 2016). Sediments were inoculated on NNA culture plates overlaid with heat-killed *Escherichia coli* (Jeong and Yu, 2005; Trabelsi *et al.*, 2010; Aç *et al.*, 2013; Ovrutsky *et al.*, 2013; Ghaderifar *et al.*, 2018). Inoculated plates were tightly sealed with parafilm to avoid desiccation and aerobically incubated at 37^0C (Douesnard-Malo and Daigle, 2011; Muchesa *et al.*, 2014). Upon incubation, *Acanthamoeba* spp. growth was monitored daily for up to 2 weeks (Booton et al. 2010; Ovrutsky et al. 2013; Lorenzo-morales et al. 2015; Marciano-Cabral et al. 2014). To confirm *Acanthamoeba* spp., growth, representative colonies from the amoeba feeding tracks were suspended in normal saline and observed under a light microscope (Booton et al. 2010; Chomicz et al. 2015; Panda et al. 2015). *Acanthamoeba* spp. from positive plates were confirmed using Eosin and Methylene Blue stains as photomicrographs were taken (El-Sayed and Hikal, 2015). To purify *Acanthamoeba* spp. cultures from bacterial contaminants, small pieces of agar were cut from positive plates and sub-cultured on new NNA agar plates overlaid with heat killed *Escherichia coli* and 100µg/ml gentamycin (Bleasdale *et al.*, 2009; Ovrutsky *et al.*, 2013; Yousuf, Siddiqui and Khan, 2013; Lorenzo-Morales, Khan and Walochnik, 2015; Guimaraes *et al.*, 2016). The subcultures were monitored daily for up to 2 weeks to confirm whether there was growth or

not and identification done as for the primary *Acanthamoeba* spp. cultures (Booton et al. 2010; Ovrutsky et al. 2013; Lorenzo-morales et al. 2015; Marciano-Cabral et al. 2014).

3.5.4 DNA extraction of *Pseudomonas* sp. from *Acanthamoeba* spp. isolates

Extraction of total *Pseudomonas* sp. genomic DNA from known positive control of *Pseudomonas* sp., ATCC 27853, obtained from UoN Medical Microbiology laboratory, and from *Acanthamoeba* spp. isolates was done separately following the procedures described by Paulo (2015) and Aljanabi et al (1997). Pure colonies of *Pseudomonas* sp., ATCC 27853, were suspended in 2ml sterile normal saline in tubes, while pure *Acanthamoeba* spp. isolates were suspended in 2ml sterile PAS saline in separate tubes. Sterile salt homogenizing buffer (0.4M NaCl 10mM Tris-HCL pH 8.0 and 2mM EDTA pH 8.0) was used to homogenize *Pseudomonas* sp., ATCC 27853, and *Acanthamoeba* spp. isolates suspensions separately. After homogenization, 40µl of 20% SDS (2% final concentration) and 8 µl of 20 mg/ml protenase K (400µg final concentration) were pipetted into vials and mixed well. The well mixed samples were incubated at 65^0 C for 1 hour after which 300 µl of 6M NaCl (NaCl saturated water) was pipetted into individual samples and vortexed at the highest speed for 30 seconds. Centrifugation followed at 10000 x g for 30minutes. The supernatants from each sample was pipetted into clean tubes and the deposits resuspended with the same volume of isopropanol and then incubated at -20 ^{0} C for 1 hour. After incubation, the samples were centrifuged at 1000 x g at 4 ^{0} C. The supernatants was discarded and the remaining pellet washed in 70% ethanol and dried. Finally, 100 µl of sterile double distilled water was used to resuspend the pellet.

3.5.5 PCR for detection of *Pseudomonas* genomic DNA

Polymerase chain reaction of *Pseudomonas* genomic DNA was performed as described by Paulo (2015) and Spilker et al (2004). For detection, the following primers, PA-GS-F (5'-GACGGGTGAGTAATGCCTA-3') and PA-GS-R (5'-CACTGGTGTTCCTTCCTATA-3') were used to amplify a 618 base pairs fragment. A total volume of 25 µl was used for amplification. The mixture constituted 30 ng DNA, 10 pmol of each of forward and reverse primers, 5 pmol dNTP, reaction buffer (50 mM KCl2, 10 mM Tris–HCl), 1.5 mM MgCl2, and 1 U of Platinum Taq DNA Polymerase (Invitrogen TM). For DNA amplification, the thermocycler (Thermo Hybid) was set to different temperatures to allowing for the different cycling steps to be carried out followed as demonstrated in Table 2.

Table 2: PCR amplification steps

	Temperature	**Time (minutes)**	**Cycling steps**	
Step 1	94ºC	1 min	Initial denaturation	
Step 2	94ºC	1 min	Denaturation	
Step 3	58ºC	1 min	Annealing	35 cycles
Step 4	72ºC	2 min	Extension	
Step 5	72ºC	7 min	Final extension	
Step 6	4ºC	Pause		

PCR amplification cyclic steps at different temperatures and timings: Initial denaturation, denaturation, annealing, extension and final extension.

3.5.6 Agarose gel electrophoresis

Electrophoresis of the amplified products was run on agarose gel as described by Sambrook and Russell (2006) and Kafkas et al (2012). A mold was formed by sealing the edges of a clean dry glass plate using tape. Enough electrophoresis buffer Tris-borate-EDTA (TBE) was prepared for both the electrophoresis tank and for the casting gel. A 1.5% agarose was prepared by weighing 15 grams of agarose and dispensed into a clean glass bottle. Then 1 litre of the TBE buffer was added and swirled to mix. After capping the bottle loosely, the slurry was heated in a bath of boiling water until the agarose dissolved. Using insulated gloves the dissolved gel was transferred into 55^0 C water bath to cool. After cooling 0.5ug/ml ethidium bromide was added and swirled thoroughly to mix well. A comb was placed at about 1mm above the plate to form slots for samples in the gel. The warm agarose was poured into the mold to form a 5 mm thick layer. Care was taken to avoid introducing air bubbles between or under the teeth of the comb. The gel was then allowed to form polymers completely for 45 minutes at room temperature. The set gel was overlaid with a small amount of TBE buffer and the comb removed with caution. The TBE buffer was then poured off, and the tape removed. The mould was mounted in the electrophoresis tank and the TBE buffer added to about 1mm above the gel. *Pseudomonas* sp. ATCC 27853, and *Acanthamoeba* spp. DNA extracts were mixed with 0.20 volume of 6x gel loading buffer in separate tubes respectively. Disposable micropipettes were used to slowly load size standards into the right and left side wells of the submerged gel. Similarly, *Pseudomonas* sp. ATCC 27853 DNA extract, was loaded into the well after the right size standard. DNA extracts from *Acanthamoeba* spp. isolates were loaded into subsequent wells up to the left size standard, the gel tank lid closed, and electrical leads attached to facilitate DNA migration from the positive anode (red). A 1.5 V/cm voltage was applied and bubbles on the anode confirmed correct connection and an active electrophoresis process. The gel

was run until the DNA samples migrated a sufficient distance. Then electric current was switched off and gel removed from the gel tank. Migrated bands were examined under ultra violet light and photographs of the observed bands were taken. *Pseudomonas* sp. ATCC 27853 positive control bands were used to locate and identify amplified genomic DNA of *Pseudomonas* sp. extracted from *Acanthamoeba* spp. isolates.

3.5.6 Quality Assurance

All the requirements as outlined below, to obtain quality, relevant and reliable test results were observed during the pre-analytical, analytical and post analytical stages of the project. At the pre-analytical stage there was proper documentation and labeling of all collected swabs per surface and equipment, with delivery being carried out immediately to UoN Medical Microbiology laboratory for processing and culture. At the analytical stage of the project, all equipment that were used for culture such as incubators, autoclaves, ovens and pipettes were accessed for proper working condition by checking whether appropriate maintenance and calibration had been done before the start of the project. Cultures were carried out under aseptic conditions in safety cabinets running both negative and positive controls in parallel. Personal protective clothing such as laboratory coats and gloves were worn during experiments to avoid contamination.

Culture media sterility and test of fitness for intended use were confirmed before carrying out any cultures. All contaminated material and culture plates were autoclaved before disposal to avoid contamination. At post analytical stage, correct and legible documentation of results was done and stored in soft and hard copies. Upon completion of the present study, the report findings and recommendations were disseminated to Medical Microbiology UoN, KNH ICU departments and in both local and international conferences.

3.6 Data Handling and Analysis

Data was collected as hard copy forms and then entered in Excel before being exported to the Statistical Package for Social Sciences (SPSS) Version 23 for statistical analysis. Descriptive statistics computed were frequencies and proportions. At univariate and bivariate level, χ^2 test parametric test was used to test for associations. *P* -value of less than 0.05 at 95% confidence interval was regarded as statistically significant in all tests. The results were presented as tables, graphs, a pie chart and photomicrographs.

3.6.1 Data management

Data was also collected in electronic format and stored in a pass-word protected computer. This was only accessible to the principal investigator. Hard copies of the data were kept in a locked cabinet only accessible to the principle investigator. No data on any individual patient was collected.

3.6.2 Variables

The primary outcome in this study was the isolation of *Acanthamoeba* spp. on surfaces and equipment at KNH ICU and the secondary outcome was *Pseudomonas* sp. detected within *Acanthamoeba* spp. isolates. The independent variables were the different surfaces and equipment identified for swab collection. The dependent variables were the *Acanthamoeba* spp., *Pseudomonas* sp. and bacterial isolates obtained in the study.

3.7 Ethical Considerations

The proposal was submitted to the Kenyatta National Hospital - UoN Ethics and Research Committee (KNH-ERC) for review and was approved (P303/06/2017) prior to inception of the study and the approval letter issued (attached, Appendix 1). A letter of no objection to carrying out the study was granted by the Head of KNH ICU (Study Registration Number CCU40/06/2017) on behalf of the institution and the accompanying Registration certificate (attached, Appendices 2 and 3). Since the proposed study involved collection of swabs on surfaces and equipment at KNH ICU for culture with no direct involvement of human subjects, personal data or use of human specimens, there was no consent required.

3.8 Dissemination of findings

The study findings were disseminated to the Microbiology Department, UoN and KAVI – Institute of Clinical Research, UoN through a journal club on 9th August 2019, and to KNH ICU on 14th August 2019. The study abstract was accepted for a poster presentation (Appendix 5) at the 4th Africa International Biotechnology and Biomedical Conference (AIBBC) in Mombasa, Kenya on 27th and 28th August 2019. A manuscript is being prepared and will be submitted for publication in a peer- reviewed open-accessed journal.

3.9 Funding

This study was supported by the Kenyatta National Hospital Research Fund, funding number KNH/R&P/23G/3 (Appendix 4) and the principal investigator as a Master of Science (MSc.) research project at UoN.

CHAPTER FOUR: RESULTS

4.1 Summarized *Acanthamoeba* species culture, bacterial culture and PCR results

All available surfaces and equipment at KNH ICU that could potentially act as formites were identified and selected for swab collection. The number of swabs collected per surface and equipment is depicted in Table 3

Table 3: Number of swabs collected per selected surface and equipment at KNH ICU

Swab location	Number of swabs collected
Sinks	21
ECG	21
Patients lockers	21
Bed rails	21
Ventilator setting screen	16
Suction machines	13
Door handles	9
Floors	6
Drip stands	6
Soap containers	6
Telephone handles	3
Nurses desk	2
Central monitor screen	2
Defibrillator	2
Resuscitation trolley	2
Walls	2
Total	153

Varied number of swabs collected per selected surface and equipment at KNH ICU

A total 153 swabs were collected in duplicate to give a total of 306 swabs. Of the 306 swabs, a batch of 153 swabs were cultured on NNA media for *Acanthamoeba* spp. isolation while the other batch of 153 swabs were cultured on BA and MAC for bacteria isolation. Table 4 displays a summary of *Acanthamoeba* spp. cultures, subcultures and PCR results. The proportion of swabs that had *Acanthamoeba* spp. isolates was 93.5% (143/153). *Acanthamoeba* spp. growth intensity was categorized as no growth, light growth, moderate growth and heavy growth as exhibited in Table 5. Subsequent sub-cultures were done on 96/153 (62.7%) of the primary *Acanthamoeba* spp. cultures. Of the 96 sub-cultured plates, only 22 (22.9%) plates had positive *Acanthamoeba* spp. growth. Polymerase chain reaction test to detect *Pseudomonas* sp. genomic DNA was conducted on all the 22 positive *Acanthamoeba* spp. subcultures. *Pseudomonas* sp. genomic DNA was detected in almost half or 10 (45.5%) of the PCR tested *Acanthamoeba* spp. subcultures. Of the 153 swabs cultured for bacterial isolation, 126 (82.4%) had bacterial growth. There were 168 different bacterial isolates identified in the study of which 18 (10.7%) were *Pseudomonas* spp. Table 6 and Figure 10 show a summary on bacterial isolates results.

Table 4: Primary *Acanthamoeba* spp. culture, purification subcultures and PCR test for the detection of *Pseudomonas* sp. genomic DNA results

Swab location	No. of Swabs cultured	No. of culture plates with *Acanthamoeba* spp. growth	No. of *Acanthamoeba* spp. purification subcultures	No. of cultures positive for *Acanthamoeba* spp. upon subculture	No. of subcultures tested for *Pseudomonas* sp. genomic DNA by PCR	No. of subcultures positive for *Pseudomonas* sp. genomic DNA by PCR
Sinks	21	21 (100.0%)	13 (61.9%)	6 (46.2%)	6 (46.2%)	4 (66.7%)
ECG* machine	21	21 (100.0%)	12 (57.1%)	1 (0.08%)	1 (0.08%)	1 (100.0%)
Bed rails	21	20 (95.2%)	16 (76.2%)	1 (0.63%)	1 (0.63%)	0 (0.0%)
Patient lockers	21	19 (90.5%)	11 (52.4%)	1 (9.1%)	1 (9.1%)	0 (0.0%)
Ventilators	16	16 (100.0%)	11 (68.8%)	2 (18.2%)	2 (18.2%)	1 (50.0%)
Suction machines	13	11 (84.6%)	9 (69.2%)	4 (44.4%)	4 (44.4%)	2 (50.0%)
Door handles	9	6 (66.7%)	7 (77.8%)	1 (0.14%)	1 (0.14%)	1 (100.0%)
Floor	6	5 (83.3%)	3 (50.0%)	0 (0.0%)	-	-
Drip stands	6	6 (100.0%)	3 (50.0%)	0 (0.0%)	-	-
Soap containers	6	6 (100.0%)	5 (83.3%)	3 (60%)	3 (60.0%)	1 (33.3%)
Telephone handles	3	3 (100.0%)	1 (33.3%)	1 (100.0%)	1 (100.0%)	0 (0.0%)
Walls	2	2 (100.0%)	1 (50.0%)	0 (0.0%)	-	-
Resuscitation trolleys	2	2 (100.0%)	1 (50.0%)	0 (0.0%)	-	-
Nurses desks	2	2 (100.0%)	0 (0.0%)	-	-	-
Central monitor screens	2	2 (100.0%)	2 (100.0%)	1 (50%)	1 (50.0%)	0 (0.0%)
Defibrillators	2	1 (50.0%)	1 (50.0%)	1 (100.0%)	1 (100.0%)	0 (0.0%)
Totals	**153**	**143 (93.5%)**	**96 (62.7%)**	**22 (22.9%)**	**22 (22.9%)**	**10 (45.5%)**

***ECG= Electrocardiography**

Acanthamoeba spp. growth was positively associated with the swab location; P = 0.008 (χ^2 =71.160 df 45). All swabs collected from sinks, ECG machine, telephone handles, drip stands, soap containers, walls, ventilators, resuscitation trolleys and nurses' desks had 100% growth of *Acanthamoeba* spp. The least growth of *Acanthamoeba* spp. was obtained from defibrillators swabs at 50% followed by door handles swabs at 66.7%.

4.1.1 Photomicrographs of *Acanthamoeba* spp. trophozoites and cysts with distinguishing identification features obtained from the study

Acanthamoeba spp. trophozoites and cysts were identified based on their distinguishing characteristics on wet saline preparations and different staining techniques as shown in Figure 6 and Figure 7.

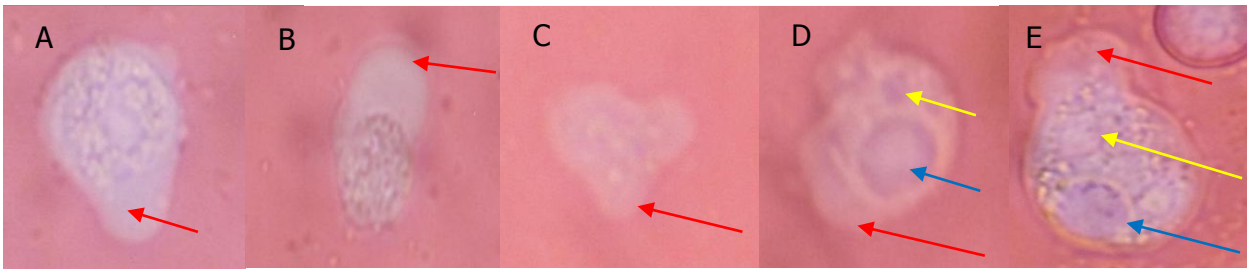

Figure 6: Eosin stained *Acanthamoeba* spp. trophozoites displaying prominent identification features
(A to E) Trophozoites of *Acanthamoeba* spp. with acanthapodia (red arrows) used for locomotion and food uptake; (D and E) Trophozoites with a large food vacuole (blue arrows) and a nucleus (yellow arrows)

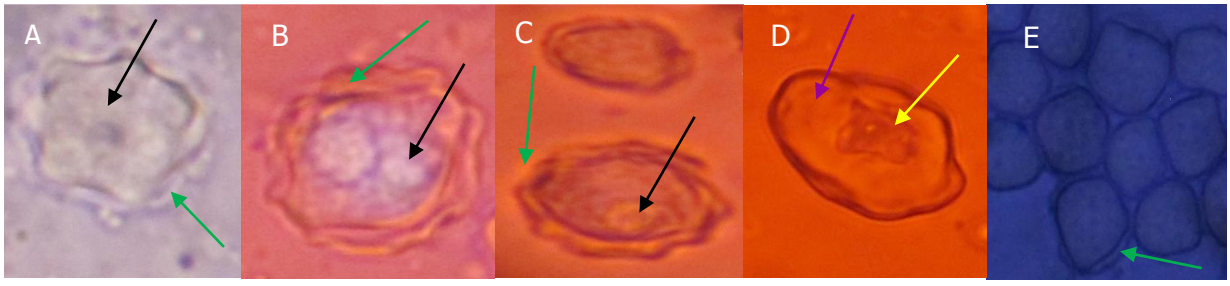

Figure 7: *Acanthamoeba* spp. cysts showing distinguishing features on different staining techniques as observed with light microscope

(A) Cyst on wet saline mount; (B, C and D) Eosin stained cysts and (E) Methylene blue stained cysts. Irregular wrinkled outer layer (green arrows); double wall enclosing the Ectocyst (purple arrow) and the inner Endocyst polygonal in shape (yellow arrow) and Nucleus (black arrows).

4.1.2 *Acanthamoeba* spp. growth intensity

The prevalence of *Acanthamoeba* spp. isolates was 93.5% (143/153) in the present study. *Acanthamoeba* spp. growth intensity was grouped into different categories depending on the number of trophozoites, cysts or both seen under light microscope. Of the 9 cultured swabs from door handles, 3 (33.3%) had no growth of *Acanthamoeba* spp. Light growth (+) was recorded on 68 (44.4%) of all positive *Acanthamoeba* spp. cultures. Nearly all swabs cultured from drip stands 5/6 (83.3%) registered light growth of *Acanthamoeba* spp. Moderate growth (++) of *Acanthamoeba* spp. was observed on 50/153 (32.7%) cultures and almost half of the swabs cultured from ECG 10/21 (47.6%) and suction machine 6/13 (46.2%) with *Acanthamoeba* spp. growth were in this category. The heaviest growth (+++) of *Acanthamoeba* spp. was recorded on 25/153 (16.3%) of all the *Acanthamoeba* spp. cultures. Soap containers 4 (66.7%) were the majority in the heavy growth category followed by sinks 8 (38.1%) (Table 5 and Figure 8).

Table 5: Tabular representation of *Acanthamoeba* spp. growth intensity

Swab location	Number of swabs cultured	No of swabs with *Acanthamoeba* spp. growth	No growth (negative)	Light growth (+)	Moderate growth (++)	Heavy growth (+++)
Sinks	21	21 (100.0%)	0 (0.0%)	8 (38.1%)	5 (23.8%)	8 (38.1%)
ECG	21	21 (100.0%)	0 (0.0%)	7 (33.3%)	10 (47.6%)	4 (19.0%)
Bed rails	21	20 (95.2%)	1 (4.8%)	10 (47.6%)	7 (33.3%)	3 (14.3%)
Patient lockers	21	19 (90.5%)	2 (9.5%)	13 (61.9%)	5 (23.8%)	1 (4.8%)
Ventilators	16	16 (100.0%)	0 (0.0%)	6 (37.5%)	7 (43.8%)	3 (18.8%)
Suction machines	13	11 (84.6%)	2 (15.4%)	4 (30.8%)	6 (46.2%)	1 (7.7%)
Door handles	9	6 (66.7%)	3 (33.3%)	2 (22.2%)	4 (44.4%)	0 (0.0%)
Soap containers	6	6 (100.0%)	0 (0.0%)	1 (16.7%)	1 (16.7%)	4 (66.7%)
Drip stands	6	6 (100.0%)	0 (0.0%)	5 (83.3%)	1 (16.7%)	0 (0.0%)
Floor	6	5 (83.3%)	1(16.7%)	4 (66.7%)	1 (16.7%)	0 (0.0%)
Telephone handles	3	3 (100.0%)	0 (0.0%)	3 (100.0%)	0 (0.0%)	0 (0.0%)
Central monitor screens	2	2 (100.0%)	0 (0.0%)	0 (0.0%)	1 (50.0%)	1 (50.0%)
Walls	2	2 (100.0%)	0 (0.0%)	1 (50.0%)	1 (50.0%)	0 (0.0%)
Defibrillators	2	1 (50.0%)	1 (50.0%)	0 (0.0%)	1 (50.0%)	0 (0.0%)
Resuscitation trolleys	2	2 (100.0%)	0 (0.0%)	2 (100.0%)	0 (0.0%)	0 (0.0%)
Nurses desks	2	2 (100.0%)	0 (0.0%)	2 (100.0%)	0 (0.0%)	0 (0.0%)
Totals	**153**	**143 (93.5%)**	**10 (6.5%)**	**68 (44.4%)**	**50 (32.7%)**	**25 (16.3%)**

The intensity of *Acanthamoeba* spp. growth was associated with the swab location; P=0.008 (χ^2 =71.160 df 45).

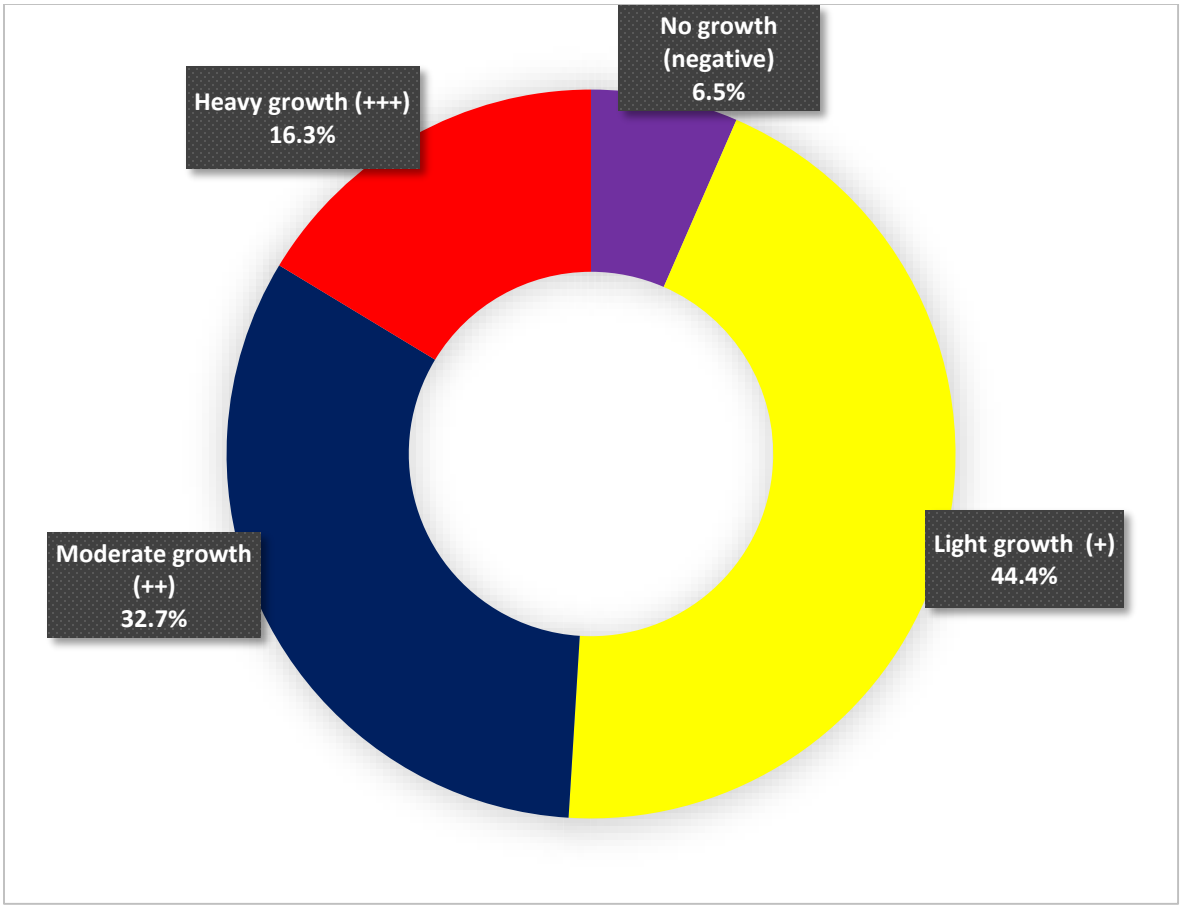

Figure 8: A pie chart displaying percentage intensity of *Acanthamoeba* spp. growth

Acanthamoeba spp. growth intensity was graded based on the number of *Acanthamoeba* spp. trophozoites, cysts or both observed under light microscope. No growth (Negative) = No trophozoite, cysts or both seen in all microscopic fields; light growth (+) = 1 to 3 trophozoites, cysts or both seen in all microscopic fields; moderate growth (++) = 1 to 3 trophozoites, cysts or both seen per field and heavy growth (+++) = more than 3 *Acanthamoeba* trophozoites, cysts or both seen per microscopic field.

4.2 Molecular detection of *Pseudomonas* sp. genomic DNA from *Acanthamoeba* spp. isolates

The PCR test was performed on a total of 22 positive *Acanthamoeba* spp. subcultures to detect *Pseudomonas* sp. genomic DNA harbored within *Acanthamoeba* spp. isolates. The distribution and results of the 22 PCR tested samples are displayed in Table 6. *Pseudomonas* sp. genomic DNA within *Acanthamoeba* spp. isolates was detected from almost half or 10/22 of the PCR tests. Of the 10 positive PCR tested samples, 4 were from *Acanthamoeba* spp. positive subcultures obtained from sinks, 2 from suction machines, 1 from among the ECG, door handles, ventilators and soap containers. However, there was no significant association (P=0.590; χ^2=13.160 df 15) between swab location and detection of *Pseudomonas* sp. genomic DNA. Figure 9 shows a Gel electrophoresis image for the detection of *Pseudomonas* sp. genomic DNA after PCR amplification.

Table 6: Detection of *Pseudomonas* sp. genomic DNA from *Acanthamoeba* spp. isolates by PCR technique

Swab location	No. of Swabs cultured	No. of subcultures tested for *Pseudomonas* sp. genomic DNA by PCR	No. of subcultures positive for *Pseudomonas* sp. genomic DNA by PCR
Sinks	21	6 (46.2%)	4 (66.7%)
ECG* machine	21	1 (0.08%)	1 (100.0%)
Bed rails	21	1 (0.63%)	0 (0.0%)
Patient lockers	21	1 (9.1%)	0 (0.0%)
Ventilators	16	2 (18.2%)	1 (50.0%)
Suction machines	13	4 (44.4%)	2 (50.0%)
Door handles	9	1 (0.14%)	1 (100.0%)
Floor	6	-	-
Drip stands	6	-	-
Soap containers	6	3 (60.0%)	1 (33.3%)
Telephone handles	3	1 (100.0%)	0 (0.0%)
Walls	2	-	-
Resuscitation trolleys	2	-	-
Nurses desks	2	-	-
Central monitor screens	2	1 (50.0%)	0 (0.0%)
Defibrillators	2	1 (100.0%)	0 (0.0%)
Totals	**153**	**22 (22.9%)**	**10 (45.5%)**

Pseudomonas sp. genomic DNA was detected in more than half or 2/3 of *Acanthamoeba* spp. positive subcultures obtained from sinks.

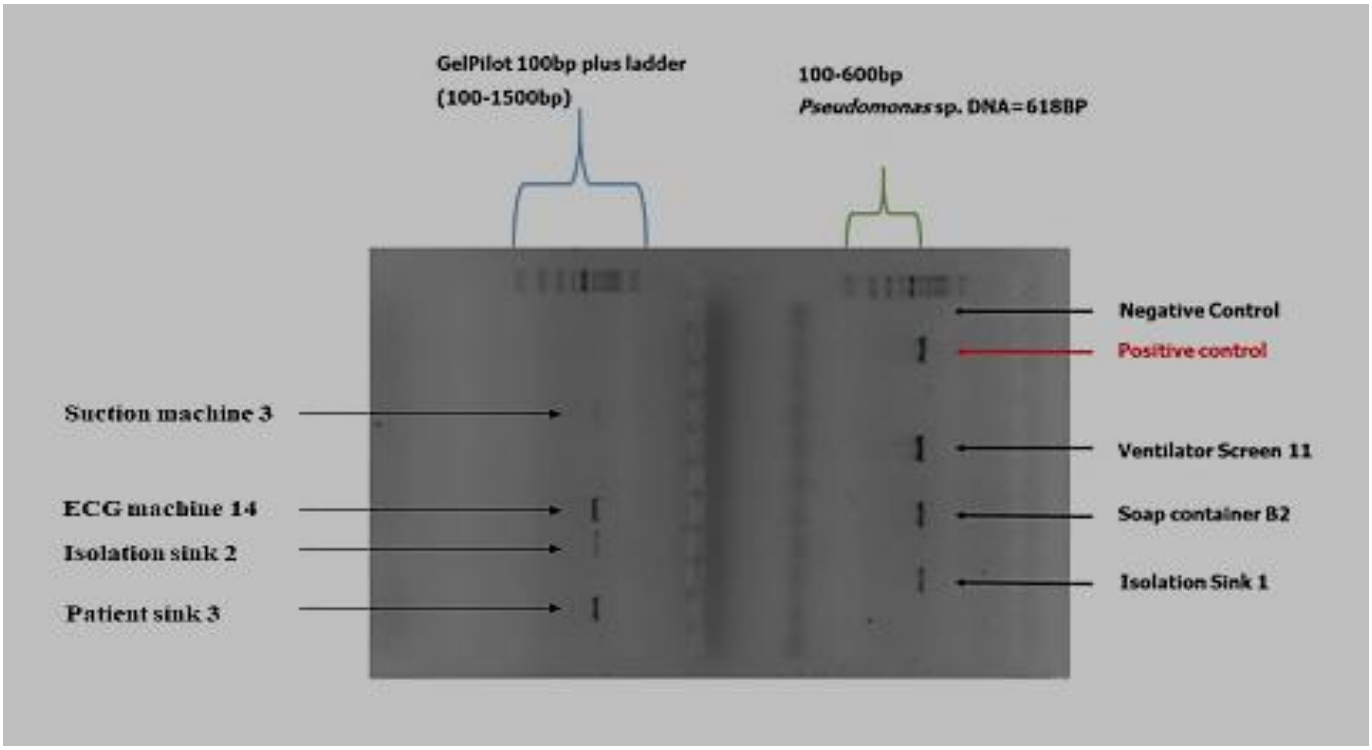

Figure 9: Gel electrophoresis image for *Pseudomonas* sp. genomic DNA detection by PCR
Molecular weight marker QIAGEN GelPilot 100bp plus ladder (100 -1500bp) (cat.no.239035) was used to locate and identify amplified *Pseudomonas* sp. genomic DNA (618 base pairs) extracted from *Acanthamoeba* spp. isolates. *Pseudomonas aeruginosa* (ATCC 27853) was used as a positive control

4.3 Bacterial cultures

The second batch of 153 swabs collected from selected surfaces and equipment at KNH ICU were cultured on BA and MAC agar for the isolation and identification of contaminating bacteria. Of the 153 cultured swabs, 126 (82.4%) swabs had bacterial growth. All swabs collected from floors, walls, central monitor screens and defibrillators had 100% bacterial growth. Almost all, 20/21 (95.2%) swabs collected from sinks, ECG, patient lockers had bacterial growth while 12/13 (92.3%) of swabs collected from suction machines had bacterial growth followed by ventilator 13/16 (81.3%), bed rails 17/21 (81.0%), door handles 7/9 (77.8%), drip stand 3/6 and nurses desks 1/2 both (50%). Finally telephone handles 1/3 (33.3%) had bacterial growth. However, all swabs from soap containers had no bacterial growth. A total of 168 bacterial isolates were obtained of

which, *Staphylococcus aureus* 68 (44.4%) was the most prevalent isolate followed by Coagulase negative staphylococcus 42 (27.5%). *Klebsiella* spp. isolates were 19 (12.4%) while *Pseudomonas* spp. isolates were 18 (11.8%). The least isolated bacteria were *Escherichia coli* and *Serattia* spp. both having 1 (0.7%) isolate each. Table 7 shows bacterial isolates in relation to swab location.

Table 7: A summary of bacterial isolates in relation to swab location

Swab location	Number of swabs cultured	Number of swabs with bacterial isolates	*Pseudomonas* sp.	*Klebsiella* sp.	*Staph aureus*	Cons*	*Enterococcus feacalis*	*Escherichia coli*	*Micrococcus* Sp.	*Serattia* Sp.	*Bacillus* Sp.
Sinks	21	20 (95.2%)	12 (57.1%)	9 (42.9%)	1 (4.8%)	2 (9.5%)	3 (14.3%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (4.8%)
ECG	21	20 (95.2%)	1 (4.8%)	1 (4.8%)	12 (57.1%)	9 (42.9%)	0 (0.0%)	0 (0.0%)	4 (19.5%)	0 (0.0%)	0 (0.0%)
Bed rails	21	17 (81.0%)	1 (4.8%)	1 (4.8%)	13 (61.9%)	5 (23.8%)	0 (0.0%)	0 (0.0%)	2 (9.5%)	0 (0.0%)	0 (0.0%)
Patient lockers	21	20 (95.2%)	3 (14.3%)	2 (9.5%)	10 (47.6%)	13 (61.9%)	1 (4.8%)	0 (0.0%)	2 (9.5%)	0 (0.0%)	1 (4.8%)
Ventilators	16	13 (81.3%)	1 (6.3%)	2 (2.5%)	7 (43.8%)	4 (25.0%)	0 (0.0%)	0 (0.0%)	1 (6.3%)	1 (6.3%)	0 (0.0%)
Suction machines	13	12 (92.3%)	0 (0.0%)	3 (23.1%)	11 (84.6%)	2 (15.4%)	0 (0.0%)	1 (7.7%)	2 (15.4%)	0 (0.0%)	0 (0.0%)
Door handles	9	7 (77.8%)	0 (0.0%)	0 (0.0%)	6 (66.7%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (1.1%)
Floors	6	6 (100.0%)	0 (0.0%)	0 (0.0%)	1 (16.7%)	4 (66.7%)	1 (16.7%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Drip stands	6	3 (50.0%)	0 (0.0%)	1 (16.7%)	1 (16.7%)	2 (33.3%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Soap containers	6	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Telephone handles	3	1 (33.3%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (33.3%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Walls	2	2 (100%)	0 (0.0%)	0 (0.0%)	2 (100%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Resuscitation trolleys	2	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Nurses desk	2	1 (50.0%)	0 (0.0%)	0 (0.0%)	1 (50.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Central monitor screen	2	2 (100.0%)	0 (0.0%)	0 (0.0%)	2 (100.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Defibrillators	2	2 (100.0%)	0 (0.0%)	0 (0.0%)	1 (50.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Totals	153	126 (82.4%)	18 (11.8%)	19 (12.4%)	68 (44.4%)	42 (27.5%)	5 (3.3%)	1 (0.7%)	11 (7.2%)	1 (0.7%)	3 (2.0%)

*** Cons = Coagulase negative Staphylococcus. Staph = Staphylococcus**

The proportion of bacterial isolates per swab location. There was an association ($P<=0.0001$; $\chi^2=58.882$; $df=15$) between type of bacterial isolate and the swab location

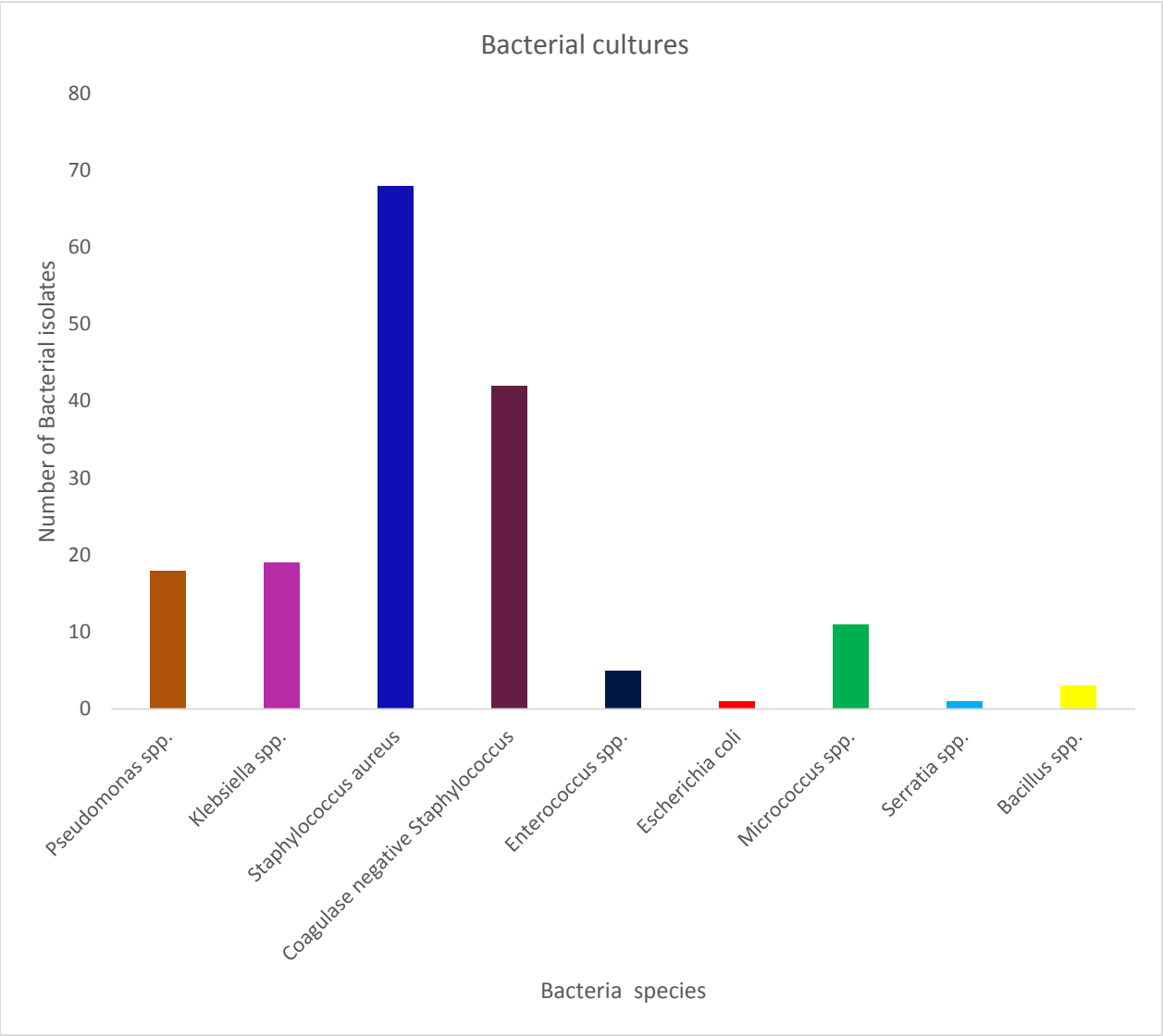

Figure 10: Bar graph representing bacterial isolates in the study

A total of 168 bacterial isolates were obtained. Of the 168 bacterial isolates, 68 (44.4%) were *Staphylococcus aureus*, 42 (27.5%) Coagulase negative staphylococcus, 19 (11.3%) *Klebsiella* spp. and 18 (10.7%) *Pseudomonas* spp. among others.

4.3.1 Proportions comparison of *Pseudomonas* spp. obtained on bacterial culture to *Pseudomonas* sp. genomic DNA detected within *Acanthamoeba* spp. isolates by PCR in relation to swab location

The proportion of *Pseudomonas* spp. isolated on culture was positively associated with swab locations ($P<0.001$; $\chi^2= 51.305$; df 15). The highest proportion of *Pseudomonas* spp. was isolated from sinks 57.1% (12/21) followed by patient lockers 14.3% (3/21) while most other sites had no *Pseudomonas* spp. isolates. Moreover, the proportion of *Pseudomonas* sp. genomic DNA detected within *Acanthamoeba* spp. isolates was 45.5% (10/22) of which almost half or (4/10) were detected from *Acanthamoeba* spp. isolates from sinks. However, there was no significant association ($P=0.590$; $\chi^2=13.160$; df 15) between the detected *Pseudomonas* sp. genomic DNA within *Acanthamoeba* spp. isolates and swab location. Table 8 illustrate the proportion comparison between *Pseudomonas* spp. from bacterial culture and *Pseudomonas* sp. genomic DNA detected within *Acanthamoeba* spp. by PCR.

Table 8: *Pseudomonas* spp. isolates upon culture and *Pseudomonas* sp. genomic DNA detected within *Acanthamoeba* spp. isolates per swab location

Swab location	No. of swabs cultured	No of swabs with *Pseudomonas* sp. on bacterial culture	No of swabs with *Pseudomonas* sp. genomic DNA detected within *Acanthamoeba* spp.
Sinks	21	12 (57.1%)	4/6 (66.7%)
ECG	21	1 (4.8%)	1/1 (100%)
Bed rails	21	1 (4.8%)	0/1 (0.0%)
Patient lockers	21	3 (14.3%)	0/1 (0.0%)
Ventilators	16	1 (6.3%)	1/2 (50%)
Suction machines	13	0 (0.0%)	2/4 (50%)
Door handles	9	0 (0.0%)	1/1 (100%)
Floors	6	0 (0.0%)	-
Drip stands	6	0 (0.0%)	-
Soap containers	6	0 (0.0%)	1/3 (33.3%)
Telephone handles	3	0 (0.0%)	0/1 (0.0%)
Walls	2	0 (0.0%)	-
Resuscitation trolleys	2	0 (0.0%)	-
Nurses desk	2	0 (0.0%)	-
Central monitor screen	2	0 (0.0%)	0/1 (0.0%)
Defibrillators	2	0 (0.0%)	0/1 (0.0%)
Totals	153	18 (11.8%)	10/22 (45.5%)

Proportion comparison between *Pseudomonas* spp. isolated from surfaces and equipment to *Pseudomonas* sp. genomic DNA detected within *Acanthamoeba* spp. isolates implies that sinks (57.1%, 66.7%), ECG (4.8%, 100%) and ventilators (6.3%, 50%) are locations most likely to harbor *Acanthamoeba* spp. that are likely to graze on *Pseudomonas sp*. a known ARB.

CHAPTER FIVE: DISCUSSION

5.1 OVERALL DISCUSSION

For the first time in Kenya, this study documents positively identified isolates of *Acanthamoeba* spp. by light microscopy upon culture and to the best of our knowledge this is the first research work on *Acanthamoeba* spp. in Kenya. Though FLA such as *Acanthamoeba* spp. are universal contaminants of virtually all environments, their existence in hospital environment pose an explicit risk of expedient infections in immunocompromised patients causing diseases such as GAE, sinusitis and skin lesions while the non-hospitalized and immunocompetent may acquire AK and PAM (Qvarnstrom *et al.*, 2006; Barnard, 2015; Panda *et al.*, 2015; Shokri *et al.*, 2016; Taravaud, Loiseau and Pomel, 2017; Souza, 2018). Hospitalized patients are also predisposed to nosocomial infections through exposure to ARMs within FLA (Lim and Webb, 2005; Weber, Raasch and Rutala, 2017).

Unfortunately, most health care workers are not sensitized on the risk factors and possible Acanthamoeba- associated disease outcomes and hence associated nosocomial infections are likely to go unsuspected, undetected or misdiagnosed (Sente *et al.*, 2016). Another concern is that, although hospital ICUs have a restricted flow of people and have put into place scrupulous cleaning strategies, the isolation of *Acanthaomeba* spp. from these area is an indicator that efforts to control contamination are still not effective enough against *Acanthamoeba* spp. This can lead to continuous proliferation of FLA and subsequent release of pathogenic ARMs into the environment (Costa *et al.*, 2010).

The present study was conducted at KNH ICU using swabs collected from selected surfaces and equipment aimed at culture and isolation of *Acanthamoeba* spp. and subsequent detection of

Pseudomonas sp. genomic DNA within the isolated *Acanthamoeba* sp. From the literature, there were no similar studies in relation to sample selection and sample size determiation. Two closely related hospital based studies were, one on the characterization of *Acanthamoeba* spp. isolates from dust samples at a public hospital in Brazil and the other one on *Acanthamoeba* spp. containing endosymbiotic *Chlamydia* isolated from smear samples collected on floors, drainage and sinks at communal hospital areas in Japan (Costa *et al.*, 2010; Fukumoto *et al.*, 2016). The majority of the hospital-FLA related studies used different samples for the isolation and detection of either FLA or FLA and their endosymbiotic bacteria. Samples commonly used included hospital water from distribution systems, dental unit water, biofilms on hospital showers, hemodialysis water and clinical cornea scrapings (Thomas *et al.*, 2005; Trabelsi *et al.*, 2010; Hassan *et al.*, 2012; Fukumoto *et al.*, 2016; Sente *et al.*, 2016; Muchesa *et al.*, 2017; Ng *et al.*, 2017).

The present study identified *Acanthamoeba* spp. trophozoites based on the described morphological features in literature such as the characteristic active motility, the prominent acanthapodia which are spine-like projections on the trophozoite surface used for locomotion and food uptake, a large food vacuole and a nucleus. *Acanthamoeba* spp. cysts were also identified by their irregular wrinkled outer layer, a prominent double wall enclosing an ectocyst, a polygonal shaped endocyst and a nucleus as shown in Figures 6 and 7. These identifying features were in agreement with features reported and displayed by various researchers (Khan, 2006; Liang *et al.*, 2010; Duarte *et al.*, 2013; Yousuf, Siddiqui and Khan, 2013; Al-Ghamdi, 2016; Gad and Al-herrawy, 2016; Behnia *et al.*, 2017; Karakavuk *et al.*, 2017; Vijayakumar, 2018; Wang *et al.*, 2018).

The proportion of swabs with *Acanthamoeba* spp. isolates in the present study was 93.5% (143/153). There was a positive association observed between the intensity of *Acanthamoeba* spp. growth and the swab location (P=0.008; χ^2 =71.160; df 45). This association can be confirmed by the heavy intensity of *Acanthamoeba* spp. growth observed on swabs obtained from soap containers 66.7% (4/6), sinks 38.1% (8/21) and ECG 19.0% (4/21). These results are in agreement with 99.1% (221/223) *Acanthamoeba* spp. isolates obtained from water matrices samples in Spain (Izquierdo *et al.*, 2013; Montalbano *et al.*, 2015). The high proportion of *Acanthamoeba* spp. reported in this study could be attributed to accumulation of bacterial mat and dirt on the surfaces and on equipment noted during sample collection, especially on sinks and bed rails. As such *Acanthamoeba* spp. was isolated from all swabs collected from all sinks (21/21) and nearly all swabs collected from bed rails (20/21). This explanation is supported by Sente and others (2016) who noted that *Acanthamoeba* sp. is attracted to areas with abundant bacteria and high organic matter for nutrition and organic molecules necessary for growth and development (Sente *et al.*, 2016; Balczun and Scheid, 2017). This further explains the high prevalence of *Acanthamoeba* spp. on swabs collected from soap containers, sinks, ECG and bed rails. In contrast, a study conducted in the Middle East obtained a prevalence of 34.4 % (31/90) *Acanthamoeba* spp. isolates from pond water. The authors suggested that the reduced prevalence of *Acanthamoeba* spp. in pond water could have been due to the frequent replacement of pond water with fresh water which in turn destroy biofilms and reduces bacteria that would be a source of nutrients for FLA (Ghaderifar *et al.*, 2018).

Several studies have reported that FLA graze on microorganisms such as bacteria, fungi algae and even viruses for nutrition and hence a reduction in the microorganism population can subsequently translate into low FLA prevalence (Fabres *et al.*, 2016). Varying prevalences of *Acanthamoeba*

spp. isolates have been reported globally owing to differences in isolation methods, varied sample sources and divergent geographical locations (Montalbano *et al.,* 2015; Muchesa *et al.,* 2017). Examples of such prevalences are, 48% (45/94) *Acanthamoeba* spp. isolates obtained from hospital drinking water in Iran and 41.9 % (13/31) from tap water in Rio Grande do Sul, Brazil (Bagheri *et al.*, 2010; Winck, Caumo and Rott, 2011). In Africa, studies have also reported varied prevalence of *Acanthamoeba* spp. from different samples using culture method. These are such as, Trabelsi *et al,* (2010) in Tunisia 69% (135/196) from dental unit water, Sente and colleagues (2016) in Uganda 33% (107/324) and 42.9% (36/84) from environmental and tap water respectively, Hassan and colleagues (2012) in Egypt 42.9% (30/70) from hemodialysis and dental unit samples, while South African researchers, Muchesa *et al*, (2013) 12.8% (22/150) from waste water and Barnard (2015) 11.3 % (7/63) from biofilm samples.

The medical literature has documented different effects on the interactions between FLA and ARMs including the existence of concealed niches of microorganisms, transfer of pathogens across hosts, genetic exchange spheres, ARMs enhanced pathogenicity and virulence, ARMs training against macrophages, increased drug resistance, protection against harsh conditions and their contribution to nosocomial infections burden despite stringent infection control measures (Horn, 2001; Khan and Siddiqui, 2014; Barnard, 2015; Fabres *et al.*, 2016; Balczun and Scheid, 2017; Biehl *et al.*, 2017; Rubenina *et al.*, 2017). Driven by the quest to carry out more studies on the effects of FLA and ARMs interactions and their possible impact on public health, FLA endosymbiotic studies have been on the increase in the past 10 years (Kara *et al.,* 2015; Montalbano *et al.,* 2015). Similarly, this study also sought to confirm a possible endosymbiotic relationship between *Acanthamoeba* spp. and *Pseudomonas* sp., a known ARM, from swabs collected from selected surfaces and equipment at KNH ICU. The present study narrowed down

to focus on the detection of *Pseudomonas* spp. from *Acanthamoeba* spp. since both are ubiquitous in nature and the chances of coexistence are high (Gon *et al.*, 2012; Paulo, 2015; Moradali, Ghods and Rehm, 2017). Secondly, previous KNH ICU studies reported *Pseudomonas* spp. as one of the most common etiologic agents of nosocomial infections with high inherent resistance to drugs and infection control disinfectants (Ngumi, 2006; Njoki, 2009; Inyama *et al.*, 2011).

In the present study, *Pseudomonas* sp. genomic DNA was detected from *Acanthamoeba* spp. isolates by PCR technique. To obtain pure *Acanthamoeba* spp. cultures for PCR, all the primary *Acanthamoeba* spp. cultures were subcultures on fresh NNA to purify *Acanthamoeba* spp. isolates from bacterial and fungal contamination. Of the primary *Acanthamoeba* spp. isolates, 62.7% (96/153) were subcultured and only 22.9% (22/96) subcultures were positive for *Acanthamoeba* spp. There was a considerable drop in the proportion of positive *Acanthamoeba* spp. subcultures which could have been due to the fact that majority of the positive *Acanthamoeba* spp. plates were frozen for storage after the primary cultures for about one month before subcultures were performed. There is a possibility therefore that majority of the *Acanthamoeba* spp. trophozoites died during storage. Of the 22 PCR tested samples, *Pseudomonas* spp. genomic DNA was detected from 45% (10/22) samples. These results are in agreement with results by Ioviena *et al* (2011) in a study on the detection of bacterial endosymbionts in clinical *Acanthamoeba* spp. isolates where the most prevalent endosymbiotic bacterium was *Pseudomonas* spp. at 59.1% (13/22) in comparison to other bacteria.

Shahan and colleagues (2015), in a study in Sudan pointed out that FLA and bacteria interaction are complicated and that the outcome of these associations are determined by the nature of the bacteria, whether the bacteria exists intracellularly, extracellularly or facultatively. The researchers

pointed out that extracellular bacteria that bear Type III secretory systems such as *Pseudomonas aeruginosa* kill FLA such as *Acanthamoeba* spp. (Shanan *et al.,* 2015). A similar observation was made by Kara *et al* (2015) who suggested that bacterial invasion and survival within FLA is enhanced by effector proteins secreted by Type III or Type IV secretory systems expressed by the majority of ARBs. The released effector proteins manipulate FLA defense system in the favour of the producing ARB. Such has been noted in *P. aeruginosa* which express Type III secretory system that releases effector proteins capable of lysing FLA (Kara *et al.*, 2015). This could also have been the reason why there was a drop on the number of positive *Acanthamoeba* spp. subcultures from primary *Acanthamoeba* spp. cultures presuming that effector proteins released by *Pseudomonas* spp. could have lysed majority of *Acanthamoeba* spp.

The detection of *Pseudomonas* sp. genomic DNA within *Acanthamoeba* spp. isolates confirms a potential health risk to patients at KHN ICU should these patients acquire ARM-related nosocomial infections. This is because the mechanisms used by ARMs to evade amoebic killing would similarly be used to evade macrophage killing in the patients (Thomas *et al.*, 2009; Balczun and Scheid, 2017). In addition, in the event that drug resistant bacteria coexist with naïve bacteria within the same *Acanthamoeba* sp. host, then the *Acanthamoeba* sp. host would facilitates drug resistant genes transfer and hence the increase in drug resistant bacteria population (Bertelli and Greub, 2012; Fukumoto *et al.*, 2016). To the best of our knowledge this is the first study in Kenya that has confirmed the co-existence of *Pseudomonas* spp. with *Acanthamoeba* spp. A similar study was conducted in South Africa on the coexistence of FLA with bacteria in selected South African hospitals water distribution systems by Muchesa and Colleagues (2017). *Serratia* spp., *Sphingomonas paucimobilis*, *Delftia acidovorans* and *Comamonas testosteroni* were among the endosyombionts detected. Though these bacteria are part of water-systems microbial flora in

hospitals, they have been linked to nosocomial infections of the eyes, urinary and respiratory tracts and blood. Muchesa *et al* (2017) suggested that, FLA could act as survival and proliferation niches from where pathogens are disseminated to the immunosuppressed patients in hospitals. There was no significant association between swab location and detection of *Pseudomonas* sp. genomic DNA by PCR (P=0.590; χ^2=13.160; df 15) however, the current study still raises the same concern for the ICU patients.

The present study also sought to speculate on bacterial contamination on selected surfaces and equipment at KNH ICU that could predispose to nosocomial infections in the unit. A total of 168 bacterial isolates were obtained of which, 40.5% (68/168) were *Staphlococcus aureus*, 25% (42/168) coagulase negative *Staphylococcus*, 11.3% (19/168) *Klebsiella* spp. and 10.7% (18/168) were *Pseudomonas* spp. among others. These results are in agreement with a previous study on incidence of nosocomial infections at KNH ICU that reported 13.6% *Pseudomonas aeruginosa* among other bacteria (Inyama *et al.*, 2011). On the contrary, Ngumi (2006) had reported 25.8% *Pseudomonas aeruginosa* in an independent study at KNH ICU. However, these results are slightly higher than those obtained by Altayyar *et al* (2016) who reported 6% (12/200) *Pseudomonas aeruginosa* isolates from hospital environment in south Libya. A noted concern also, was the high proportion of both *Acanthamoeba* spp. and bacterial isolates from equipment that are directly used on patients at KNH ICU. For example, swabs collected from ECG machines, ventilators, resuscitation trolleys and central monitor screens had 100% *Acanthamoeba* spp. growth and 92.3% (12/13) from suction machines. Similarly, the proportion of bacterial isolates obtained from swabs collected from the same sites was high, for example 100% (2/2) from central monitor screens, 95.2% (20/21) from ECG, 92.3% (12/13) from suction machines, 81.3% (13/16) from ventilators and 50% (3/6) from drip stands. Only swabs collected from resuscitation trolleys had no bacterial

growth. This portrays a high level of *Acanthamoeba* spp. and bacterial contamination in the unit that may predispose patients to nosocomial infections from both *Acanthamoeba* spp. and bacterial contaminants. This is in agreement with a suggestion made by Altayyer *et al* (2016) that the level of hospital surface and equipment contamination is directly proportional to the rate of nosocomial infections. The present study therefore supports a proposal by Davane *et al* (2014) that continuous and proper infection control measures and hospital environment surveillance for pathogenic microorganisms and FLA contamination should be prioritized in efforts to lower the high incidence of nosocomial infections.

There was a positive association between bacterial isolates and swab location ($P<0.001$; ($\chi^2 = 58.882$; df=15). This association alluded to the notion that bacterial isolates were likely to be isolated from specific contaminated surfaces and equipment at KNH ICU. There was a higher likelihood of bacterial growth on the floors, walls, central monitor screens and defibrillators since all swabs collected from these sites had bacterial growth, followed by the sinks, ECG and patient lockers that had 95.2% (20/21) bacterial growth. Suction machines had 92.3% (12/13) bacterial growth, followed by the ventilator, 81.3% (13/16), bed rails, 81.0% (17/21) and door handles, 77.8% (7/9). Most of these sites had abundant *Acanthamoeba* spp. growth possibly due to heavy accumulation bacteria and dirt as noted during sample collection that must have served as nutrition supply for *Acanthamoeba* spp. growth. An interesting finding was on soap containers which had no bacterial growth but had 100% (6/6) *Acanthamoeba* spp. growth. The reason behind this observation could be that either *Acanthamoeba* spp. had fed on all bacteria on the soap container for nutrition which in return enhanced *Acanthamoeba* spp. growth or the soap contents could have contained bactericidal chemicals that killed all the bacteria but spared *Acanthamoeba* spp. A comparison of the distribution of *Pseudomonas* sp. isolated on bacterial cultures to *Pseudomonas*

sp. genomic DNA detected within *Acanthamoeba* spp. isolates implied that sinks (57.1%, 66.7%), ECG (4.8%, 100%) and ventilators (6.3%, 50%) were locations most likely to harbor *Acanthamoeba* spp. which would in turn graze on *Pseudomonas sp*. one of the known ARM.

The majority of the bacteria isolated in the current study are known etiological agents of nosocomial infections (Blot *et al.*, 2005; Ngumi, 2006; Inyama *et al.*, 2011; Anaissie, 2017). In the present study, the proportion of *Pseudomonas* spp. isolated from selected sites at KNH ICU was 10.7% (18/168) of the total bacterial isolates. Of concern is that, should the infection control at KNH ICU put efforts towards decontaminating this proportion of *Pseudomonas* spp. there would still be a proportion of *Pseudomonas spp*. existing within *Acanthamoeba* spp. in the environment that will not be effectively eliminated. Unfortunately still, the infection control disinfectants in current use in many health facilities are not effective against FLA such as *Acanthamoeba* spp. which are left in circulation within the hospital environment (Costa *et al.*, 2010; Iqbal, Siddiqui and Khan, 2014). These become hidden sources of survival, proliferation and dissemination of more pathogenic microorganisms despite efforts put to reduce contamination (Costa *et al.*, 2010). Consequently, nosocomial infections may prevail as has been in the world today. On a broad perspective, results from the present study are aimed at shedding light on the existence of these hidden reservoirs of pathogenic ARMs (Siddiqui and Khan, 2013).

5.2 LIMITATIONS

The present study would have been more profitable in advising policy change in infection prevention and control at KNH if genomic DNA of the most common and persistent pathogens isolated from KNH ICU would have been analyzed. These pathogens include *Acinetobacter baumannii*, *Klebsiella* sp., *Enterobacter* sp., *Escherichia coli*, *Proteus* sp. *and Staphylococcus*

areaus. Other tests such as the detection of antibiotic resistance genes would have assisted this study in demonstrating the possible gene transfer between ARMs from the environment and within FLA that could be contributing to the major challenge of antimicrobial resistance facing the world today. These suggestions were not possible in the proposed study due to financial constraints. *Acanthamoeba* spp. subcultures were delayed due to unexpected delay in the shipment of NNA from the United States which might have led to a drop in the proportion of positive *Acanthamoeba* spp. growth after subculture. This also prolonged the study period than expected.

CHAPTER 6: CONCLUSION AND RECOMMENDATIONS

6.1 CONCLUSION

The present study for the first time in Kenya isolated *Acanthamoeba* spp. one of the FLA from swabs obtained from selected sites at KNH ICU. The study also confirmed the existence of *Pseudomonas* sp. within *Acanthamoeba* spp. isolates using PCR. Results from this study may inform the infection control bodies in Kenya and beyond on the importance of incorporating anti-FLA disinfectants for hospital infection control measures in efforts to reduce the incidence of nosocomial infections. Infection control targets against FLA will also halt the possible effects of the interaction between FLA and ARMs such as *Pseudomonas* spp. Free living amoeba such as *Acanthamoeba* spp. should be considered as hidden reservoirs for survival, proliferation and dissemination points of pathogens across hospitals environments. Therefore, such masked reservoirs should be explored extensively through research in search for answers to the high incidence of nosocomial infections despite the use of high-level infection control measures and the unexplained surge of multidrug and pan drug resistance that has been a global challenge.

6.2 RECOMMENDATIONS

The following recommendations were made based on the present study findings.

1. More studies should be conducted to shed more light on the effects of FLA endosymbiotic relationships with different microorganisms.
2. Many more improved methods of isolation and detection of FLA and the related endosymbionts should be explored to generate for more reliable results.

3. Infection control policies should be augmented to include disinfectants that target FLA especially in hospital settings as the world is currently grappling with the challenging unexplained escalation in the incidence of nosocomial infection and antimicrobial resistance.
4. Regular training on best antiseptic techniques should be conducted especially for medical personnel who handle patients to limit FLA and ARMs contamination across hospital environments and across patients.
5. Creating awareness and sensitization among medical personnel on possible FLA and ARMs nosocomial infections is paramount.
6. Exploration of presence of FLAs and ARMs in patients' specimens especially in those with infections of unknown etiology should be considered.

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APPENDIX 1: ETHICAL APPROVAL LETTER

UNIVERSITY OF NAIROBI
COLLEGE OF HEALTH SCIENCES
P O BOX 19676 Code 00202
Telegrams: varsity
Tel:(254-020) 2726300 Ext 44355

KNH-UON ERC
Email: uonknh_erc@uonbi.ac.ke
Website: http://www.erc.uonbi.ac.ke
Facebook: https://www.facebook.com/uonknh.erc
Twitter: @UONKNH_ERC https://twitter.com/UONKNH_ERC

KENYATTA NATIONAL HOSPITAL
P O BOX 20723 Code 00202
Tel: 726300-9
Fax: 725272
Telegrams: MEDSUP, Nairobi

KENYATTA NATIONAL HOSPITAL APPROVED 29 AUG 2017 KNH/UoN-ERC P.O. BOX 20723-00202 NRB

Ref: KNH-ERC/A/253

29th August , 2017

Zipporah Macharia
Reg. No.H56/80959/2015
Dept. of Medical Microbiology
School of Medicine
College of Health Sciences
University of Nairobi

Dear Zipporah

Revised research proposal – *Acanthamoeba*-associated *Pseudomonas* species in Kenyatta National Hospital Critical Care Unit (P303/06/2017)

This is to inform you that the KNH- UoN Ethics & Research Committee (KNH- UoN ERC) has reviewed and **approved** your above proposal. The approval period is from 29th August , 2017 – 28th August, 2018.

This approval is subject to compliance with the following requirements:

a) Only approved documents (informed consents, study instruments, advertising materials etc) will be used.
b) All changes (amendments, deviations, violations etc) are submitted for review and approval by KNH-UoN ERC before implementation.
c) Death and life threatening problems and serious adverse events (SAEs) or unexpected adverse events whether related or unrelated to the study must be reported to the KNH-UoN ERC within 72 hours of notification.
d) Any changes, anticipated or otherwise that may increase the risks or affect safety or welfare of study participants and others or affect the integrity of the research must be reported to KNH- UoN ERC within 72 hours.
e) Submission of a request for renewal of approval at least 60 days prior to expiry of the approval period. (*Attach a comprehensive progress report to support the renewal*).
f) Submission of an *executive summary* report within 90 days upon completion of the study. This information will form part of the data base that will be consulted in future when processing related research studies so as to minimize chances of study duplication and/ or plagiarism.

For more details consult the KNH- UoN ERC website **http://www.erc.uonbi.ac.ke**

Protect to discover

APPENDIX 2: KNH-ICU RESEARCH APPROVAL LETTER

Telegram:" MEDSUP," Nairobi
Tel.: 2726300-9
E-mail: knhadmin@knh.or.ke

KENYATTA NATIONAL HOSPITAL
P. O. Box 20723-00202-KNH
Fax 2725272
NAIROBI

REF.: KNH/ ANAE/ATTCH. R/39/VOL.II/09

DATE: 22ND AUGUST, 2017

Head of Research Program
Kenyatta National Hospital
P. O. BOX 20723-00202
KNH

Dear Sir

RE: ZIPPORAH WANJIKU MACHARIA-H56/80959/2015

The above named is proposing to carry out a research in our Critical Care Unit on ***Acanthamoeba* – associated *Pseudomonas* species in Kenyatta National Hospital Critical Care Unit** (P303/06/2017) .

This letter is to let you know that the Unit has no objection in her carrying out the research which will go a long way in helping the hospital in controlling the nosocomial infections.

Kindly accord her the necessary assistance to carry out this research study.

Thank you

Dr. H. Opere
HOD- Anaesthesia/Theatre

APPENDIX 3: STUDY REGISTRATION CERTIFICATE

KNH/R&P/FORM/01

KENYATTA NATIONAL HOSPITAL
P.O. Box 20723-00202 Nairobi

Tel.: 2726300/2726450/2726565
Research & Programs: Ext. 44705
Fax: 2725272
Email: knhresearch@gmail.com

Study Registration Certificate

1. Name of the Principal Investigator/Researcher
   ZIPPORAH WANJIKU MACHARIA
2. Email address: Zippywanjiku58@gmail.com Tel No. 0722935685
3. Contact person (if different from PI) SAMUEL KITHUGI NJOGU
4. Email address: samuel_njogu@yahoo.com Tel No. 0725914410
5. Study Title
   Acanthamoeba - associated Pseudomonas species in Kenyatta National Hospital Critical Care Unit
6. Department where the study will be conducted Kenyatta National Hospital Critical Care Unit
   (Please attach copy of Abstract)
7. Endorsed by Research Coordinator of the Department where the study will be conducted.
   Name: ..... Signature ..... Date .....
8. Endorsed by Head of Department where study will be conducted.
   Name: DR. HEZRON O. OTIENO Signature ..... Date 06-09-2017
9. KNH UoN Ethics Research Committee approved study number P305/08/2017
   (Please attach copy of ERC approval)
10. I ZIPPORAH WANJIKU MACHARIA commit to submit a report of my study findings to the Department where the study will be conducted and to the Department of Research and Programs.
    Signature ..... Date 6/9/2017
11. Study Registration number (Dept/Number/Year) CHS 140/106/2017
    (To be completed by Research and Programs Department)
12. Research and Program Stamp

All studies conducted at Kenyatta National Hospital **must** be registered with the Department of Research and Programs and investigators **must commit** to share results with the hospital.

Version 2: August, 2014

APPENDIX 4: KNH RESEARCH GRANT NOTICE OF AWARD

KENYATTA NATIONAL HOSPITAL
P.O. Box 20723-00202 Nairobi

Tel.: 2726300/2726450/2726565
Research & Programs: Ext. 44705
Fax: 2725272
Email: knhresearch@gmail.com

KNH/R&P/23G/3/13 **Date: 15th December 2017**

Zipporah Macharia
Dept. of Medical Microbiology
School of Medicine

Dear Zipporah,

RE: KNH RESEARCH GRANT NOTICE OF AWARD

It is our great pleasure to inform your study titled ***"Acanthamoeba- associated pseudomonas species in Kenyatta National Hospital Critical Care Unit."*** is approved for funding.

Laboratory tests will be procured through the supply chain Department.

Any publications resulting from this award will acknowledge Kenyatta National Hospital.

We look forward to working with you in implementing this study. Please do not hesitate to contact the department if you have any questions or issues.

Congratulations.

Dr. John Kinuthia
Head, Research and Programs

APPENDIX 5: RESEARCH DISSEMINATION POSTER

Acanthamoeba - associated *Pseudomonas* Species at Kenyatta National Hospital Intensive Care Unit

Macharia Z. W[1], Kariuki H. N[1], Masika M.M[1], Mureithi M.W[1], Mburugu M[1], Gitau W[1], Mwororo J[2].

[1]University of Nairobi, Nairobi, Kenya. [2]Kenyatta National Hospital, Nairobi, Kenya.

Introduction

- Free living amoeba (FLA) such as *Acanthamoeba* species are ubiquitous protozoa that graze on bacteria, viruses and fungi
- Amoeba resistant microorganisms (ARMs) such as *Pseudomonas* species resist amoebic killing
- *Pseudomonas* species is implicated in nosocomial infections
- ARMs may also cause nosocomial infections and perpetuate spread of antimicrobial resistance genes

Objectives

This study was aimed at culture and isolation of *Acanthamoeba* spp. from selected sites at KNH ICU and subsequent molecular detection of *Pseudomonas* sp. genomic DNA within *Acanthamoeba* spp. isolates

Methodology

Study Design
- Descriptive cross-sectional study

Study sites
- KNH ICU

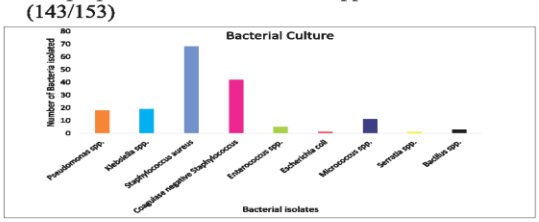
Sampling methods
- Convenient sampling methods
- Sample size guided by KNH infection control protocol
- Total samples =153 swabs in duplicates = 306

Specimen collection
- Swabs collected from selected sites at KNH ICU: **page saline** transport media
- Swabs were labeled appropriately and transported to UoN Microbiology lab

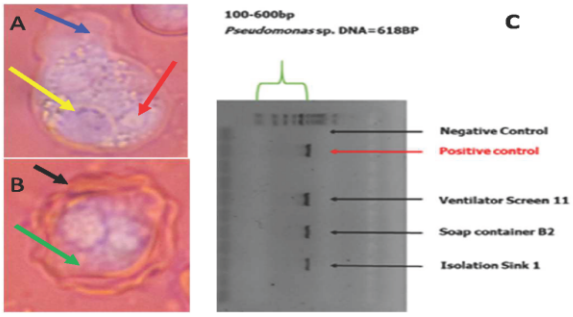
Culture, isolation, identification and PCR
- *Acanthamoeba* spp. culture **NNA + heat killed E. coli**
- Incubation at 37 °C for up to 2 weeks
- *Acanthamoeba*spp. identification
- *Acanthamoeba* spp. purification PCR
- Bacterial culture

Results

- 153 swabs were collected in duplicates obtaining 306 swabs from selected sites at KNH ICU
- *Acanthamoeba* spp. culture was performed on the first batch of 153 swabs
- PCR detection of *Pseudomonas* sp. genomic DNA was tested from 22 *Acanthamoeba* spp. subcultures
- The proportion of *Acanthamoeba* spp. isolates was 93.5% (143/153)



Of the 168 bacterial isolates, 68 (40.5%) were *Staphylococcus aureus*, 42 (25%) coagulase negative Staphylococcus, 19 (11.3%) *Klebsiella* spp. and 18 (10.7%) *pseudomonas* spp.



(A) *Acanthamoeba sp.* trophozoites with acanthapodia (blue arrow) large food vacuole (yellow arrow) and a nucleus (red arrow)
(B) *Acanthamoeba sp.* cyst with double wall enclosing ectocyst (black arrow) & endocyst (green arrow)
(C) *Pseudomonas* sp. genomic DNA within *Acanthamoeba* spp. was detected in 45.5% (10/22) PCR tests

- *Acanthamoeba* spp. growth intensity was associated with swab site dirt and bacterial mat accumulation P=0.008

Conclusion

- *Acanthamoeba* spp. was isolated from selected sites at KNH ICU for the first time in Kenya
- *Pseudomonas* sp. an ARM co-existence with *Acanthamoeba* spp. was also confirmed

Recommendation

- More research on the interaction between FLA and ARMs should be conducted especially in Kenya
- Possible contribution of ARMs to nosocomial infections and spread of antimicrobial resistance genes in the hospital environments should be investigated

Acknowledgement

Funding: Kenyatta National Hospital Research & Programs
Appreciation: Kenyatta National Hospital Intensive Care Unit, Department of Medical Microbiology University of Nairobi, supervisors, study participants and family

Corresponding Author

Zipporah Wanjiku Macharia
Email: zippywanjiku58@gmail.com
Mobile Number: +254722935685

APPENDIX 6: PLAGIARISM REPORT

Turnitin Originality Report

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