CLINICAL SIGNIFICANCE OF *ACINETOBACTER* SPECIES ISOLATED FROM THE UNIVERSITY TEACHING HOSPITAL, LUSAKA

BY

LEOCRISIA MWANAMOONGA

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UNIVERSITY OF ZAMBIA

DECLARATION

The contents of the dissertation are the author's own works. The dissertation has not previously been submitted for the award of degree to any University.

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Leocrisia Mwanamoonga

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Date: 19th April 2022

DEDICATION

This manuscript is dedicated to my son, my family and all my friends for understanding my busy schedules during the entire study period.

CERTIFICATE OF APPROVAL

This dissertation submitted by **Leocrisia Mwanamoonga** is approved as fulfilling part of the requirements for the award of the degree of Master of Science in One Health Laboratory Diagnostic Science (OHLD) at the University of Zambia.

Supervisor Name: ……..

Supervisor signature: ……..

Name and Signature of Examiners

Internal Examiners

1. Name: ……Signature: ……..Date: ……..……

2. Name: ……Signature: ……..Date: ……;…….……

3. Name: ……Signature: ……..Date: ……...……

ABSTRACT

Acinetobacter species have emerged as important pathogens globally responsible for various infections and are among the leading causes of hospital-acquired infections (HAI). *Acinetobacter* species has in the recent past been the causative agent of several infections such as sepsis, Ventilator-Associated Pneumonia (VAP), soft tissue infections (wound and infections), and Urinary Tract Infection (UTI) and meningitis. In this study, 60 *Acinetobacter* species were isolated, of which forty (40) were clinical specimens from patients with various conditions and 20 were environmental isolates. The environmental specimens were from surfaces and equipment in contact with the patients, such as beds, beddings, suction machines, ventilators, ward gowns and endotracheal tubes. The environmental sources contributed a significant proportion of *Acinetobacter* species isolated at 33.3% (at 95% CI: 21.1-45.6). The clinical isolates had a total of 40 isolates (66.7%). Among them, the admission ward showed the highest number of *Acinetobacter* species isolated (*n*=13), followed by the Paediatric Intensive Care Unit (PICU) (*n*=12), and the least number was from the Main Intensive Care Unit (MICU) (*n*=1).

From both the environmental and clinical isolates, the most frequently isolated species was *Acinetobacter baumannii* (*n*=43), with the least being *A. ursingii* (*n*=1). The *Acinetobacter* spp. had 50% (20) isolation in sepsis and 15% (6) meningitis. The resistance gene AdeB (efflux pump) was detected in 82.5% of the clinical isolates, and resistance to amikacin was observed at 1.6%. The isolates resistant to imipenem were observed at 17%., whilst the Oxa48 for carbapenem resistance was not detected in both the clinical and the environmental isolates. The study also showed that the isolates detected from the environmental isolates were also isolated from the clinical isolates.

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LIST OF ABBREVIATIONS

AIDS	Acquired Immunodeficiency Syndrome
AMR	Antimicrobial Resistance
CDC	Center for Disease Control and Prevention
CLOSE	Clinical &Laboratory Standard Institute
DNA	Deoxyribose Nucleic Acid
ESBL	Extended Spectrum Beta-Lactamase
GATE	Genome Analysis Tool Kit
HIV	Human Immunodeficiency Virus
ICU	Intensive Care Unit
ACC	Acinetobacter Carbapenemase
LTCF	Long Term Care Facilities
MDR	Multi Drug-Resistant
MIC	Minimum Inhibitory Concentration
MELVA	Multiple-Locus Variable-Number of Tandem Repeat Analysis
MLS	Multi Locus Sequence Typing
NDM-1	New Delhi metallo-beta Lactamase-1-gene
PCR	Polymerase Chain Reaction
UTI	Urinary Tract Infections
SNPs	Single Nucleotide Polymorphism
VAP	Ventilator-Associated Pneumonia

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

The genus *Acinetobacter* are ubiquitous, free-living, and saprophytic bacilli that can be obtained easily from the soil, water, food, and sewage. They survive very well in a wide range of environments. *Acinetobacter* species are Gram-negative coccobacilli, encapsulated and non-motile bacteria. *Acinetobacter* species display resistance mechanisms to all existing antibiotic classes and a prodigious capacity to acquire new resistance determinants. *A. baumannii* is the most commonly isolated clinical subspecies among the *Acinetobacter* spp. known to cause infections in humans and animals. It is becoming an increasingly important cause of nosocomial infections and is one of the six most important multi-resistant bacteria in hospitals worldwide (Antunes, Visca, & Towner, 2014). *A. baumannii, A. nosocomial and A. pittii* are clinically relevant nosocomial pathogens globally with similar phenotypic characteristics. These species are grouped as *A. calcoaceticus–A. baumannii* (Acb) complex (Tal-Jasper et al., 2016).

A. baumannii is known to cause several infections but most commonly causes nosocomial pneumonia, particularly ventilator-associated pneumonia (VAP), bacteraemia, and skin and soft tissue infections such as burns and wounds (Antunes et al., 2014). The increasing antimicrobial resistance (AMR) in *A. baumannii* poses a treatment challenge in already critically ill patients, leaving the most expensive and last resort antibiotics such as carbapenems as the only choice of treatment (De Francesco, Ravizzola, Peroni, Bonfanti, & Manca, 2013). Although the magnitude of AMR in *Acinetobacter* species has been studied in developing countries, there is a paucity of data in Zambia. *Acinetobacter* species (mostly *A. baumannii)* continue to be isolated at UTH, with a number of the isolates showing resistance

to a wide range of antibiotics. The isolates were mostly from patients in intensive care units (ICUs), the immunocompromised and those who have stayed for more than two weeks in the Hospital.

The environment and instruments are essential components that contribute to hospital-acquired infections (HAIs) during the stay of patients. As instruments are being used on patients, the probability of carrying the *Acinetobacter species* and transferring them to the patients is high. This may result in the patient getting infected. The spread of the organism to various parts of the body is highly likely to occur, causing infections that have shown challenges in attaining successful patient treatment.

1.2 Statement of the Problem

Antibiotics have been used in human and veterinary medicine for more than seven decades. They have contributed significantly to treating pathogenic bacteria and protecting human and animal health. However, the spread of antimicrobial-resistant microorganisms in recent decades is a significant challenge for humanity. In Zambia, there are a number of challenges that has led to the high numbers of infections due to antimicrobial resistance (AMR). This has mainly been attributed to misuse and abuse of antibiotics, self-prescription and not completing the course of antibiotics. In addition, the poor hygiene in hospitals has also contributed to the resistant strains as this allows organisms to propagate and in turn acquire and exchange resistance traits thereby infecting the patients. Acquired resistance to some antimicrobials is now widespread to such an extent that their value for treating certain life-threatening infections is being compromised. Additionally, the pipeline for the development of new antimicrobial drugs has contracted. This problem is compounded by the global genomic scope of the antimicrobial resistomes, such that antimicrobial resistance (AMR) spans a continuum from genes in pathogens found in the clinic to those of benign

environmental microbes, along with their proto-resistance gene progenitors. In addition, infections caused by AMR bacteria are associated with substantially higher morbidity and mortality rates than those caused by antimicrobial-susceptible bacteria. It is also unknown how antimicrobial-resistant bacteria and their resistomes spread between humans and their environment.

AMR is of significant concern in low-income countries due to misuse and over-use of antimicrobial agents, widespread availability of counterfeit or substandard drugs, and poor infection control measures (Elder *et al.*,2016). The scarcity of reliable and timely information may further limit epidemiological surveillance and effective stewardship efforts, particularly in sub-Saharan Africa.

The emergence and spread of AMR, especially Extended-Spectrum β-Lactamase-producing *Enterobacteriaceae* (ESBL-PE), is complex and considered to be a significant threat to extensive global health, economy and societal implications and poses a unique challenge to humanity, with 54 % being found to possess ESBL *Acinetobacter* species (Adegoke, Faleye, Singh, & Stenström, 2016). While only infrequently associated with respiratory disease, *Acinetobacter baumannii* is a common environmental organism with significant potential to cause bacteraemia, sepsis, urinary tract infections, lower respiratory tract infections and meningitis in immunocompromised. Of additional concern, *Acinetobacter* species acquire, accumulate, and transfer various AMR determinants. Therefore, it may represent a significant reservoir for resistance within the gut and may increase the risk of resistant infections in hospital environments (Cheesman *et al.*, 2017; Taitt *et al.*, 2017).

Laboratory records at the University Teaching Hospital (UTH) show a high isolation rate of *Acinetobacter* species. Most of these isolates are resistant to several readily available antibiotics and are commonly used in our hospital setting. However, the burden of *Acinetobacter* at UTH or other hospitals in Zambia is unknown.

There is also a paucity of data in our setting despite the observed high magnitude of AMR, which may be attributed to the extensive use of antibiotics in hospital settings. Possible consequences are treatment failures, which may promote the spread of resistant pathogens and prolong illness and hospital stay, thereby increasing treatment and hospital costs. These can lead to iincreased morbidity and mortality**.**

1.3 Significance of the Study

The genus *Acinetobacter* is known to cause hospital-acquired infections (HAIs). They are known to contribute to the morbidity and mortality of immune-compromised patients in high dependency wards. This could be attributed to resistance to the commonly used antibiotics. Although the burden of HAI and AMR in pathogens causing HAI have been done in developed countries and other parts of Africa, there is still very scanty data in Zambia. This study will provide evidence-based data such as the resistance similarity of isolates from patients with the ones from the hospital equipment, i.e. ventilators and other surfaces that patients come in contact with while in the hospital and the burden of AMR in pathogens causing HAI. The study's findings will be used to support the implementation of better therapeutic combinations and reduce the chances of infections by putting in place better disinfection protocols. The data from this study will also contribute to the formulation of antibiotic treatment guidelines for patients and strengthen the periodical infection prevention processes.

1.4 Research Question

What is the clinical significance of *Acinetobacter* species isolated from admitted patients and the ward environmental surfaces and instruments at the University Teaching Hospital in Lusaka, Zambia?

1.5 Objectives

1.5.1 General Objective

To evaluate the clinical significance of *Acinetobacter* species isolated from admitted patients and ward environmental surfaces and instruments at the University Teaching Hospital, Lusaka.

1.5.2 Specific Objectives

1.5.2.1 To determine the frequency and identify antimicrobial resistance profiles of *Acinetobacter* species isolated from clinical specimens (blood, cerebral spinal fluids (CSF), burns and wounds, body fluids, nasopharyngeal swabs and Sputum) and the ward environment and instruments.

1.5.2.2 To detect AMR genes from clinical and environmental isolates.

1.5.2.3 To determine the similarity of AMR profiles of clinical and environmental *Acinetobacter* species isolates.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 General Overview of *Acinetobacter* species

The genus *Acinetobacter* is a major cause of nosocomial infections; it is increasingly associated with various epidemics and has become a widespread concern in hospitals worldwide. Multi - antibiotic-resistant *Acinetobacter baumannii* is now recognized to be of great clinical significance (Almasaudi, 2018). Antimicrobial resistance is a significant challenge in medicine because more otherwise "normal" microorganisms have become implicated in different infections. Antimicrobial resistance is defined as the development by a disease-causing microbe, through mutation or gene transfer, of the ability to survive exposure to an antimicrobial agent that was previously an effective treatment. Established pathogens show new extended resistance to otherwise effective antibiotics, and many therapeutic options are being challenged. This problem is a global problem and affects all regions of the world.

Being widely distributed in the environment, *Acinetobacter* species are organisms that have shown to be mainly acquired during the stay of patients in the hospital. This has contributed to the number of isolates commonly isolated from patients and has led to advocating specific treatment protocols for *Acinetobacter* species as they easily acquire and may have intrinsic resistance to the otherwise normally susceptible organisms.

Hospital-acquired infections (HAIs) or nosocomial infections have increased in patients admitted to various hospitals worldwide (Khalawetektook, 2018). The success of the

formulations of the various antibiotics towards the treatment of bacterial infections has shown a different picture based on data compiled as a result of surveillance to illustrate the emergence of many genera of bacteria that are resistant to all antibiotics (Perez et al., 2007). *Acinetobacter* species have shown mechanisms of resistance and factors that make them able to confer resistance to a wide range of antibiotics (Perez et al., 2007).

Several cases of MDR *A. baumannii* have been reported from countries such as Palestine, United Arab Emirates, Bahrain, Saudi Arabia and Lebanon (Almasaudi, 2018). A retrospective study to evaluate the prevalence of multidrug-resistant bacteria that cause infections in patients in intensive care units (ICUs) of Riyadh Military Hospital, Saudi Arabia, showed that the most common organism isolated from intensive care unit patients was *A. baumannii* (Almasaudi, 2018). It has been estimated that about 4,150,000 deaths will be attributed to antimicrobial resistance (AMR) in Africa by 2050, second only to Asia (MO, 2017). It is particularly more severe in sub-Saharan Africa (MO, 2017).

Ventilator acquired pneumonia (VAP) continues to be one of the most recurrent intensive care unit (ICU) infections, with occurrence ranging from 6% - to 52 % and continues to be the primary cause of high morbidity, mortality and increased financial burden in ICUs (Royer et al., 2015). Molecular typing of the organisms was done using Pulsed Field Gel Electrophoresis (PFGE), and the data were analyzed using BioNumerics V. 4.0 (Applied Maths, Sint- Martens-Latem, Belgium)(Royer et al., 2015).

The burden/gravity of hospital-acquired infections in Zambia has not yet been studied or understood. At UTH, *Acinetobacter species* have been isolated from blood culture, pus

swabs, cerebrospinal fluid (CSF) and, in a few cases, urine samples. In Zambia, there is a paucity of data on *Acinetobacter* species.

2.2 Biology of *Acinetobacter* species

*Acinetobacter*s are Gram-negative, non-motile glucose-non-fermenting oxidase-negative and increasingly important opportunistic pathogens. The bacteria can survive for a long time in a solid and wide range of environments for a long time. This is mainly due to the simple nutritional requirements and pH values, high degree of resistance to disinfectants and antiseptics, ability to form biofilms on environmental surfaces, medical equipment such as catheters or respiratory equipment, and biotic surfaces. These features contribute to the nosocomial spread of *Acinetobacter* species (Dash et al., 2021)..

2.3 Transmission of *Acinetobacter* species

Acinetobacter species are mainly transmitted through exposure to contaminated hospital devices such as endotracheal intubations through inhalation. Direct contact with medical personnel that have been exposed to the organisms is also another way of transmission (Falagas, Vardakas, Kapaskelis, Triarides, & Roussos, 2015). Exposure to contaminated hospital equipment and direct contact with medical personnel that have been exposed to the organisms are the main routes through which acquisition of the organisms takes place (Sheikh, Savari, & Montazeri, 2020). There are mainly three species of clinical importance; *Acinetobacter calcoaceticus*, *Acinetobacter baumannii* and *Acinetobacter pittii* (Falagas et al., 2015)

2.4 Clinical Relevance

The increased occurrence of *Acinetobacter* species is because of improved medical (laboratory) procedures. It has also been noted that *Acinetobacter* has become resistant to several antimicrobial agents over the years(Nowak & Paluchowska, 2016). The other important features of the clinical relevance of the *Acinetobacter* species are high natural resistance and the ability to regulate the innate and acquire foreign antimicrobial resistance(Elder et al., 2016). *Acinetobacter baumannii* has been defined by the Infectious Diseases Society of America as one of the most significant hospital pathogens (Nowak & Paluchowska, 2016). The genus *Acinetobacter* belongs to the family *Moraxellaceae* and has at least 21 species, with *A. baumannii* as the most clinically significant. Even when there is a phenotypic identification system in place, differentiating the various species of *Acinetobacter* using molecular methods is essential to establish and understand the organisms better. There are more reliable methods of identification, which include amplified 16S rRNA gene restriction analysis (ARDRA) and Amplified Fragment Length Polymorphism (AFLP) (Sheikh et al., 2020). The *Acinetobacter* species cause several acute hospital infections. These include Ventilator-associated pneumonia, Bloodstream infections, Urinary tract infections, meningitis, skin and soft tissue infections, burn and surgical wound infection, endocarditis and osteomyelitis. The risk factors for *Acinetobacter* infection include trauma, surgical procedures, premature or old age, hospitalization, antimicrobial therapy including medical treatment, mechanical ventilation, urinary catheters, intravascular catheters and drainage tubes (Theis, Dasari, Vrana, Kurtin, & Dogan, 2013). *Acinetobacter* is responsible for 2–10% of all Gram-negative hospital infections, mainly affecting critically ill patients, particularly those hospitalized in intensive care units. Furthermore, *A. baumannii* healthcare-associated infections may increase the risk of mortality from 8% to 40% (Singhal, Kumar, Kanaujia, & Virdi, 2015).

2.5 Virulence factors

Virulence factors are bacteria-associated molecules acquired for a bacterium to cause disease. For *Acinetobacter* species, a few virulence factors have been identified to date. The virulence determinants responsible for the *Acinetobacter* species pathogenicity include lipopolysaccharide (LPS), capsular polysaccharides (CPS), outer membrane protein A (OmpA), and outer membrane vesicles (OMV), phospholipase D (PLD), and biofilm (Singhal et al., 2015). LPS has been shown to contribute to the surface carbohydrate residues in virulence. The endotoxins are involved in the stimulation of inflammatory signaling in human monocytic cells, based on TLR2 (toll-like receptor) and TLR4 receptors. Therefore, the pathology of *Acinetobacter* infections may be associated with exaggerated innate immune response to the LPS (Singhal et al., 2015). Other determinants implicated in the virulence of *A. baumannii* are capsular polysaccharides. *A. baumannii* survives bactericidal activity of the complement through the protective characteristic of the CPS. OmpA is one of the best-characterized *A. baumannii* virulence factors. It is responsible for the damage to the human airway cells *via* induction of apoptosis(Nogbou et al., 2021). This takes place by releasing the proapoptotic molecule cytochrome c and the apoptosis-inducing factor. The company facilitates adherence and invasion into epithelial cells. OMVs play a significant role in delivering virulence factors into host cells. This helps horizontal gene transfer and protects the cell from host immune response. The other virulence factors in *Acinetobacter* species are phospholipases(Cheesman et al., 2017). They are involved in the breakdown of the host cells' phospholipids. This, in turn, enhances bacterial invasion. Biofilm is the three-dimensional multicellular layer that is very important in infection. Its formation occurs on the host cells and inside medical devices, making it very important in infection (Khalawetektook, 2018).

2.6 Drug resistance

Acinetobacter species are known to be intrinsically resistant to many groups of antimicrobial agents (*e.g.* glycopeptides, aminoglycosides, cephalosporins, carbapenems, penicillins, macrolides, lincosamides, and streptogramins)(Alcántar-Curiel et al., 2019). These bacteria can develop resistance to all classes of antimicrobial agents used in the therapy. The process can be associated with genetic modifications resulting in membrane alterations, overexpression of efflux pumps (EP), overexpression of intrinsic antibiotic modifying enzymes, modifications of target sites for antimicrobial agents, and acquisition of novel resistance determinants (Sheikh et al., 2020). *A. baumannii* strains enhanced by the selective pressure of the hospital environment may gain resistance via mutational changes and horizontal gene transfer from other members of the species, genus, non-fermenters and/or *Enterobacteriaceae* family (Nowak & Paluchowska, 2016). Observed for a period of time, rapid accumulation of resistance determinants to multiple antimicrobials among *A. baumannii* strains resulted in eliminating penicillins, cephalosporins, aminoglycosides, quinolones, and tetracyclines as effective treatment options for many clinical isolates(D'Souza et al., 2019). Carbapenems have been seen to have good activity and low toxicity, making them the last resort in therapeutic options for *Acinetobacter* infections (Nowak & Paluchowska, 2016).

2.7 Laboratory Diagnosis of *Acinetobacter* infections

The laboratory is very important in the isolation, identification and susceptibility testing of *Acinetobacter* species(Khalawetektook, 2018). They grow on blood agar, chocolate agar and mackonkey agar. On mackonkey a differentiative media, they are non lactose fermenters. *Acinetobacter* are identified as gram negative coccobacilli and non fermentative(Kosack, Page, & Klatser, 2017). This is done by using biochemical tests including triple sugar iron, lysine iron agar, sulphur indole motility, Citrate agar and Urea agar. The analytical profile

index 20NE detection system (API 20NE) can also be used after 24 hour incubation http://patft.uspto.gov/netacgi/nph. other tests that are used include oxidase test, motility test and catalase test(Santajit & Indrawattana, 2016). These are done to confirm the isolate. Identification of *Acinetobacter* can also be done using automated procedures using the MALDI-TOF and the Vitek 2 compact(Ogbolu et al., 2020; Singhal et al., 2015). Identification of *Acinetobacter* can further be confirmed using molecular methods. This includes polymerase chain reaction (PCR), partial sequencing and whole genome sequencing (WGS)(Tal-Jasper et al., 2016).

In addition, susceptibility testing is done on the isolated organisms. Susceptibility testing is done using the disc diffusion method and the minimum inhibitory concentration method (MIC)(Khalawetektook, 2018). The MIC method is considered to be the gold standard between the two methods(Falagas et al., 2015).

Furthermore, PCR, partial sequencing and Whole genome sequencing are used to establish the presence of resistance genes in organisms(Cheesman et al., 2017; Nocera, Attili, & De Martino, 2021). This is done by using primers for the individual resistance genes that may be present in the organism(Chiang et al., 2011).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Site

3.1.1 Clinical and environmental sampling site

The study was conducted at the University Teaching Hospital (UTH). UTH is a tertiary referral and teaching Hospital in Lusaka Province with a bed capacity of approximately 2000 and comprises several outpatient clinics and wards. The hospital is the largest highly specialised referral facility in Zambiacomprising of the following five hospitals: Adult Hospital, Children's Hospital, Mother and Newborn Hospital, Eye Hospital and Cancer Disease Hospital (CDH). UTH has one Microbiology Laboratory, which provides diagnostic services to all the hospitals. The laboratory also national reference laboratory and serves as a public health laboratory responsible for pathogen confirmation and AMR surveillance. This work was conducted in this microbiology lab at UTH.

3.1.2 Study Frame

This was a facility-based cross-sectional study conducted at the University Teaching Hospital in Lusaka. The study involved investigating clinical samples [CSF, sputum, pus swabs (wounds and burns swabs), blood culture, urine, synovial fluid, pericardial fluid, peritoneal fluid and pleural fluid] received in the Microbiology Laboratory at University Teaching Hospital (UTH) from the UTH wards between July 2021 and December 2021. These samples were part of the routine diagnosis and patient care and were processed for *Acinetobacter* isolation. In order to maintain confidentiality, the samples were given study-specific numbers. Swabs from environmental ward surfaces and instruments were also processed and screened for *Acinetobacter* species. Clinical details were retrieved from the Laboratory

Information System (LIS). The clinical details are entered into the LIS during the Central Specimen Registration (CSR) registration process. The details are based on the Clinicians' notes on the request form.

3.1.3 Inclusion Criteria

All *Acinetobacter* species isolates from specimens [CSF, sputum, pus swabs (wounds and burns swabs), blood culture, urine, synovial fluid, pericardial fluid, peritoneal fluid and pleural fluid] received in the Microbiology Laboratory at UTH from January 2021 to December 2021. Environmental and instrument swabs from the ward surfaces where the patients are admitted to the UTH were included.

3.1.4 Exclusion Criteria

All polymicrobial growth on culture plates and *Acinetobacter* isolates from patients not admitted to UTH.

3.1.5 Sample Size Determination and Sampling Method: Clinical samples

All *Acinetobacter species* isolated as the only organism from samples received in the Microbiology Laboratory at the UTH were included in this study. The determination was based on the minimum number of positive isolates based on the 2017 and 2018 data received in the Microbiology Laboratory. The study targeted to obtain a minimum of 50 Acinetobacter isolates. This would be sufficient to observe any AMR pattern and genetic diversity if present (Nagelkerke et al., 2015). Therefore, we aimed to process as many samples coming to the Microbiology laboratory until this number was achieved. Based on the 2017 to 2018 UTH Microbiology Laboratory Reports, this would require processing an estimated number of about 250 samples, assuming a conservative 20% Acinetobacter spp. recovery rate(Emori, 1988; Khurshid et al., 2020).

3.2 Sample Size Determination and Sampling: Environmental Samples

The study aimed to collect swab samples from ward surfaces and all the equipment, from all the wards where the clinical samples were collected or where patients were admitted at the time to sample collection. We planned to sample at least 20 swabs from each of the following hospital wards: Paediatric ICU, Neonatal ICU, admission ward, surgical ward, medical ward and Main ICU, which would bring the total number of swabs to 140. Assuming an isolation frequency of 20%, this would translate into about 28 isolates,

3.3 Isolation and Identification of *Acinetobacter species*

3.3.1 Isolation of *Acinetobacter*:

Clinical samples: The study involved the isolation of *Acinetobacter* isolates from CSF, blood cultures, respiratory samples [(including sputum and bronchial alveolar lavage (BAL)], other body fluids and skin and soft tissue swabs.

Environmental samples (swabs from ward surfaces and instruments): The environmental swabs were from surfaces and equipment that came into contact with the patients from whom Acinetobacter species was isolated. This was used to determine the phenotypic and genotypic relatedness between clinical and environmental isolates. All the samples were sub-cultured on blood, chocolate and MacConkey agar (Oxoid) plates using sterile swabs for inoculation and wire loops for streaking. Blood and Chocolate agar plates were incubated for up to 18-24 hours at 35ºC in a 5% carbon dioxide incubator, while MacConkey agar plates were incubated aerobically at 35˚C for 18- 24 hours.

3.3.2 Phenotypic Identification

After incubation, MacConkey agar plates were examined for non-lactose fermenters (NLF) suggestive of Gram-negative bacteria. Gram staining was then performed to confirm them as Gram-negative bacteria and viewed under the microscope to confirm the Gram-negative coccobacilli (Pleomorphic) morphology. The suspected pure colonies were then subjected to biochemical testing on Triple Sugar Iron Agar, Lysine iron agar, Sulphur Indole Motility (SIM), Citrate agar and Urea agar. Oxidase and catalase tests were also performed. Presumptive *Acinetobacter* species were non-fastidious, non-hemolytic, and non-fermenting and gave a neutral reaction with no gas production in TSI and LIA. They were also urease negative, with no hydrogen sulphide production, indole negative, non-motile, nitrate production negative, catalase-positive, and oxidase negative. The Vitek 2 compact automated system with a Gram-Negative (GN) Vitek card and Polymerase Chain Reaction (PCR) were also used to confirm the identity of *Acinetobacter* species. The primers used to confirm the *Acinetobacter* species were *blaOXA23* and *blaOXA24*.

3.4 Determination of AMR Characteristics of A*cinetobacter species*:

Phenotypic Screening

3.4.1 Antimicrobial Susceptibility Testing

The confirmed *Acinetobacter* species isolates were subjected to antimicrobial susceptibility testing using the Kirby-Bauer disc diffusion method. The 0.5 McFarland was determined using the turbid meter after making a suspension using 2 ml normal saline and pure colonies of the organism. The organism was then spread onto the quality-controlled Muller Hinton agar (Oxoid) plate. Within 15 minutes, the selected 6mm antibiotic discs were placed onto inoculated Muller Hinton. A maximum of six antibiotic discs were placed on a single 120mm Muller Hinton plate. The Mueller Hinton agar plates were incubated aerobically at 37℃ for

24 hours. After incubation, the zones of inhibition were measured in millimetres and interpreted as susceptible, intermediate, or resistant, according to Clinical and Laboratory Standards Institute (CLSI) 2020 guidelines. The following antibiotics (Oxoid, Basingstoke, UK) were used; tetracycline, cotrimoxazole, cefotaxime, ceftriaxone, ciprofloxacin, amikacin, gentamicin, imipenem, cefepime, tobramycin, ceftazidime and piperacillin-tazobactam..All the antibiotics were quality controlled using *Acinetobacter* ATCC 19606 control strain.

The Minimum Inhibitory Concentration (MIC) was obtained from the Vitek 2 System, an automated system. A pure colony was suspended in 3ml normal saline equivalent to 0.5 Mcfarland standard. This was marked as tube one, used for identification of *Acinetobacter*. From tube one, 145µl was transferred to tube 2, containing 3ml of sterile normal saline. Tube 2 was then used for Antimicrobial susceptibility testing (AST). An identification card Gram-Negative (GN), was placed in tube one, and an AST card, AST GN86 card, is put in tube two.

3.4.2 Screening for Extended Spectrum β- Lactamase (ESBL) Producers

3.4.2.1 Use of Combination Discs/or Vitek 2 System

All isolates resistant to third-generation cephalosporins were subjected to the combination of Cefpodoxime/clavulanic acid, Cefotaxime/clavulanic acid and Ceftazidime/clavulanic acid combination discs. This was done in order to screen for ESBL producers. The Kirby-Bauer disk diffusion test on Mueller-Hinton agar used the 0.5 McFarland standard to give accurate susceptibility results. The suspension was then spread onto the quality-controlled Muller Hinton. Within 15 minutes, the selected 6mm antibiotic discs were placed onto inoculated Muller Hinton. A maximum of six discs were placed on a single 120mm Muller Hinton plate.

The isolates were first tested against cefpodoxime and cefotaxime (Oxoid) as indicator cephalosporins and ESBL production was confirmed phenotypically using combination discs (cefpodoxime/clavulanic acid cefotaxime/clavulanic acid) (Oxoid) according to CLSI guidelines (CLSI, 2018). The test was considered positive if the inhibition zone diameter was ≥ 5 mm larger with clavulanic acid than without.

3.4.3 Screening of Carbapenemase Producers

3.4.3.1 Use of E-test or Vitek 2 System

All isolates resistant to meropenem and/or imipenem were subjected to susceptibility testing using Minimum Inhibitory Concentrations (MICs) of Meropenem and/or Imipenem to detect resistance genes. The MIC (E-test) was performed as outlined in point 3.4.1.

3.4.4 Genotypic Identification (ID confirmation)

DNA Extraction (bacterial lysates) followed by PCR targeting Internal Transcribed Sequence (ITS) DNA Sequencing of ITS and BLAST ID in NCBI database was used. The Zymoclean™ Gel DNA recovery kit was used to purify PCR products according to the manufacturer's instructions.

3.5 Polymerase Chain Reaction

Pooled samples of *Acinetobacter* species were screened using PCR targeting *IMP, OXA-23, OXA-24, gryA, parC, adeB, tetE, tetG, OXA-Uni, NDM, VIM and Oxa-48* for resistance genes. The segments of these genomes were amplified by PCR using the primers for the genes used, and these are listed in Table 3.1 and conditions described by (Sobouti et al., 2020). This involved a reaction of 20µl with 10µl 2X one-taq master mix, 0.48µM forward and reverse primers, 5.08µl of nuclease-free water and 3µl of the template. The PCR

conditions involved initial denaturation at 95ºC for 1 minute followed by 35 cycles of denaturation at 95ºC for 30 seconds, annealing for 30 seconds, extension at 72ºC for 1-minute and a final extension step at 72ºC for 10 minutes.

3.5.1 Gel electrophoresis of PCR product

Gel electrophoresis was used to separate PCR products on a 1% agarose gel pre-stained with ethidium bromide in TAE buffer composed of 2M Tris base, 2M Acetic acid, 0.0005M EDTA and distilled water. The products were viewed using the Benchtop 3UV transilluminator. A sample was considered positive if a clear band of fragment size was seen compared to the 100bp molecular weight standard.

3.5.2 Purification of DNA

The Zymoclean™ Gel DNA recovery kit was used to purify PCR products according to the manufacturer's instructions. Briefly, 3X of ADB was added to the PCR product, incubated at r.t.p for 1-minute and then transferred to a spin column and centrifuged at 10000G for 1 minute. The flow-through was discarded, and 200µl DNA wash buffer was added and centrifuged 10000G for 1 minute. As in the previous step, the flow-through was discarded, and this step was repeated one more time. 30µl of DNA elution buffer was then added directly to the column, inserted in a new tube and centrifuged for 1 minute to elute and collect the purified PCR product.

3.5.3 Cycle sequencing reaction

The brilliant dye terminator cycle sequencing kit (Nimagen® Netherlands) was used to sequence the purified PCR product. The reaction volumes were as follows; 1µl brilliant dye, 3.5µl brilliant dye buffer, 0.5µM primer, 13.5µl nuclease-free water, and 1µl (template)

purified DNA. The thermal cycler conditions were as follows; initial denaturation at 96ºC for 45 seconds, then 25 cycles of denaturation at 96ºC for 10 seconds, annealing at 50ºC for 5 seconds, and extension at 60ºC for 4 minutes.

3.5.4 Ethanol precipitation and capillary electrophoresis

Excess buffers and dNTPs were removed from the cycle sequencing products using the ethanol precipitation method. Briefly, for each sample, 2µl of EDTA, 2µl of acetate and 90µl of absolute alcohol was added and incubated for 10 minutes in the dark. The mixture was then centrifuged at 15000 rpm for 20 minutes, and the supernatant was discarded. 200µl of 70% alcohol was added and then centrifuged for 5 minutes, and the resulting supernatant was discarded. After this, the samples were wrapped in aluminium foil and vacuum dried for 10 minutes and then dissolved in 20µl of highly ionized formaldehyde and finally denatured at 95ºC for 5 minutes. All purified and denatured samples were then subjected to capillary electrophoresis using the ABI 3500 genetic analyzer (Applied Biosystems).

3.6 Determination of AMR Characteristics of Acinetobacter species: Genotypic Screening

DNA Extraction was done using the heat treatment method. The extraction was done by first making a suspension of the organism in nuclease-free water. The suspension was put in a heat block with wells (water bath), and each well was filled with water up to 3/4 full. the Eppendorf tubes with organism suspension were put and heated to 95ºC for 10 minutes. They were then transferred to a refrigerated centrifuge for 5 minutes at 10G. The supernatant was discarded and the pellet reserved. To the pellet (sediment), 10µl of nuclease-free water was added, and the process from heating was repeated two more times. To the final sediment, 10µl of nuclease-free water was added, and these were the samples stored with DNA. This

was carried out for PCR detection of AMR and virulence genes using the heat extraction method. Primers for the genes used are listed in Table 3.1. This enabled PCR detection of Carbapenemase genes using the PCR for *blaOXA-24*, *blaOXA-23*, *blaOXA-48; blaIMP*, *blaVIM* (Bonnin, Nordmann, & Poirel, 2011). The detection of *tetE and tetG* (tetracycline resistance determining regions), *OXA-Uni, NDM* and *VIM* (Metallo-beta-lactamase resistance determining regions), *gyrA, parC,* (Fluoroquinolone resistance-determining regions) and AdeB to determine the presence of Efflux pumps regulation genes was done (Hujer *et al.*, 2006; Doi *et al.*, 2007).

3.7 Determination of Clinical Significance of Acinetobacter species

Signs and symptoms compatible with infections of the following sterile sites with *Acinetobacter* species being isolated as the predominant or only isolate and agreed with clinical findings: *Acinetobacter* species isolates from lower respiratory tract indicating infection (i.e. pneumonia backed by radiographical findings, etc.). For the blood (bacteraemia and sepsis) and cerebrospinal fluid (CSF) (meningitis), pure growth of *Acinetobacter* species indicated the presence of infection as these are sterile sites.

Table 3.1: Primers used for PCR

Gene	Forward	Reverse
Tet E	ATTGGCCTATCCTTGAA	TATGGTTCATGTCCGGTT
Tet G	CCTTAATTCCGGTATCAGAT	GGAATTCGTGCATCCATGTA
Bla OXA 48	GTTACTCGTATTCAACGCGATT	AGTGTATCGCGTTATATCG
VIM	CCTTATGTTAGGATAGGGG	TATGATTCCCGTAATAGCCT
NDM	TTAGGGCTAAGCTATGATA	GTACCTTGATTTGTATTA
IMP	CTACCGCAGCAGAGTCTTTG	AACCAGTTTTGCCTTACCAT
blaOXA23	TGTCATAGTATTCGTCGTT	TTCCCAAGCGGTAAA
blaOXA24	TTTGCCGATGACCTT	TAGCTTGCTCCACCC
gyrA	GCTGGCTAACGGTAACTC	GGCTTCAATGGGACTG
parC	CTGAACAGGCTTACTTGAA	AAGTTATCTTGCCATTCG
adeB	TACCGGTATTACCTTTGCCGGA	GTCTTTAAGTGTCGTAAAAGCCAC
16S rRNA	GGGGTCTTACTATTCTG	TTCCCTTCTCCTTTC

CHAPTER FOUR

4.0 RESULTS

4.1 Descriptive Results

4.1.1 Distribution of isolates

A total of 60 *Acinetobacter* species were isolated from different wards at the University Teaching Hospital (UTH). Forty were from the patients admitted for more than 48 hours from various wards, while 20 were environmental samples collected from surfaces around the patients and instruments used on them (Table 4.1). The environmental sources contributed 33.3% (at 95% CI: 21.1-45.6) of *Acinetobacter* species isolated (Table 4.1).

Table 4.1: Summary survey statistics indicating isolate distribution by source

Source of isolates	**Total number (60)**	**Percentage (%)**	**95% CI**
Environment	20	33.3	21.1 - 45.6
Admission Ward	13	21.7	10.9 - 32.4
Paediatric ICU	12	20	9.0 – 30,0
Neonatal ICU	3	5	0.0 - 10.7
Surgical Ward	5	8.3	1.1 - 15.5
Medical Ward	6	10	2.2 - 17.8
Main-ICU	1	1.7	0.0 – 5.0

Admission ward, Paediatric ICU (Paediatric Intensive Care Unit), Neonatal ICU(Neonatal Intensive Care Unit), Surgical ward (wounds and burns ward), Medical ward (All medical conditions), Main ICU(Main Intensive Care Unit)

The admission ward had the highest number of isolates at 21.7% (95% CI: 10.9- 32.4). Among the clinical isolates, the main ICU had the least *Acinetobacter* species isolated at 1.7% (95% CI: 0-5%) (Table 4.1).

The total number of environmental swabs collected was 67, out of which 20 pure *Acinetobacter* species were isolated. This gives an isolation rate of 30%, of which 25% (5/20) were from taps, 20% (4/20) from sinks, 15% (3/20) from suction machines and 10 % (2/20) from each of the following; beds, blankets ventilators and ward gowns. There were 6 taps sampled, and only 4 of them had *Acinetobacter* spp. The hand washing sinks sampled were 10, and 4 showed growth while 6 beds were sampled, and 2 had growth of *Acinetobacte*r spp. Three blankets and 3 ventilators were sampled, and 2 of each of the 3 showed growth of *Acinetobacter* spp. Further, six suction machines were sampled, and 3 had growth of *Acinetobacter*. Similarly, 6 ward gowns were also swabbed, and 2 showed growth of *Acinetobacter* spp. The sampled wards included: Paediatric ICU, Neonatal ICU, admission ward, surgical ward, medical ward and Main ICU. This shows 25%, 30%, 10%, 10%, 10% and 15% respectively. (Table 4.2). The highest environmental *Acinetobacter* species came from the NICU (30%), whilst the lowest was from the admission ward, surgical ward and medical wards (10%) (Table 4.2).

Table 4.2 Distribution of Environmental Isolates by swab surface and ward-type

	Site Swabbed	No. of isolates	Frequency
	Environmental	20	
Sites swabbed	Tap	5	25%
	Sink	4	20%
	Bed	2	10%
	Blanket	2	10%
	Ventilator	2	10%
	Suction Machine	3	15%
	Ward Gowns	2	10%
Wards representing the Environmental sample	Admission ward	2	10%
	Paediatric ICU	5	25%
	Neonatal ICU	6	30%
	Surgical Ward	2	10%
	Medical Ward	2	10%
	Main-ICU	3	15%

4.2 *Acinetobacter* species isolation based on clinical conditions

The patients from which Acinetobacter species were isolated were admitted for the following clinical conditions; sepsis, meningitis, burns, Congenital Heart disease (CHD), Deep vein thrombosis (DVT), severe pneumonia, infective endocarditis (IE), Pulmonary tuberculosis (PTB), status epilepticus and Urinary Tract Infection (UTI). The clinical isolates (40) were highest in patients admitted with sepsis at 50% (20/40), followed by meningitis at 10% (6/40), wound at 7% (4/40) and Burns at 3% (2/40) hydrocephalus. The distribution frequency for other conditions is shown in Figure 4.2.

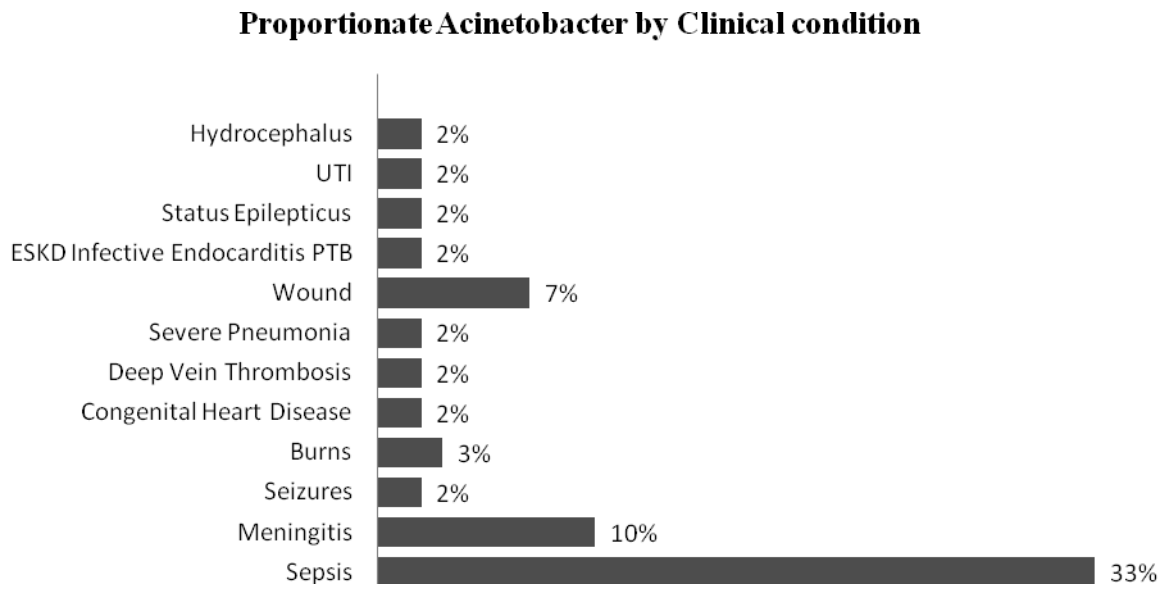

Figure 4.2: Distribution of Acinetobacter isolation by clinical condition.

4.3 Speciation of *Acinetobacter* using the VITEK

Six species were identified from the Acinetobacter genus: The most important in human infection, *A. baumannii*, was the most frequently isolated. The isolation rate was clinical isolates (*n*=10) and environmental isolates (*n*=13). *A. lwoffii* had 10 isolates, of which clinical isolates were 6 and environmental were 4. *A. johnsonii* had 4 isolates, of which 2 were clinical isolates, and 2 were environmental. There were 2 *A. junii* isolates, of which 1 was clinical and the other environmental. For *A. haemolyticus* and *A. ursingii*, each had 1 clinical isolate and nothing from the environment. The environmental isolates were 20 in total (*n*=13 *A. baumannii*; *n*=4 *A. lwoffii, n*=2 *A. johnsonii* and *n*=1 being *A. junii*) (Table 4.4).

4.4 Distribution of *Acinetobacter* species (Environment/Clinical)

The isolates were recovered from patients admitted to the University Teaching Hospital in different wards for more than 48 hours in this study. All the forty (40) isolates were hospital-

acquired as the patients were admitted for more than one week. Twenty isolates from patients with sepsis represented 50% of all the clinical isolates. (Table 4.2).

Paediatric Intensive Care Unit (PICU) showed the highest number of *Acinetobacter* species isolated (*n*=7) from patients with sepsis, whilst the admission ward had the second highest with (n=5) isolates due to sepsis (Table 4.3); this was followed by the medical ward (*n*=4), Neonatal Intensive care unit (NICU) (*n*=3) and surgical ward (*n*=1) (Table 4.3). For meningitis, Admission ward (*n*=3), PICU (*n*=2) and MICU (*n*=1). There was no meningitis associated *with Acinetobacter* species from the remainder of the wards.

Table 4.3: Distribution of *Acinetobacter* species isolates by the specimen source (ward type).

Clinical Details	Wards							
	Admission ward	PICU	NICU	Surgical	Medical	MENU	Enviro	Total
Sepsis	5	7	3	1	4	0	0	20
Meningitis	3	2	0	0	0	1	0	6
Burns	0	0	0	2	0	0	0	2
Congenital Heart Disease	0	1	0	0	0	0	0	1
Deep Vein Thrombosis	0	0	0	1	0	0	0	1
Severe Pneumonia	0	1	0	0	0	0	0	1
Wound	1	0	0	1	2	0	0	4
ESKD Infective Endocarditis PTB	1	0	0	0	0	0	0	1
Status Epilepticus	2	0	0	0	0	0	0	2
UTI	1	0	0	0	0	0	0	1
Hydrocephalus	0	1	0	0	0	0	0	1
Environment*	0	0	0	0	0	0	20	20

Environment*: Environmental samples were obtained from the ward tables, trays, beds, ventilators, suction machines and beddings. Clinical samples: from patients admitted to the various wards at UTH. Admission ward, PICU-Paediatric Intensive care Unit, NICU- Neonatal Intensive Care unit, Medial ward, Surgical ward(burns and wounds), MICU (Main Intensive Care Unit).

There were 2 isolates from the burns ward, and (*n*=1) was from the PICU with congenital heart disease whilst with deep vein thrombosis (*n*=1) from the surgical ward, (*n*=1) severe pneumonia from PICU, (*n*=4) *Acinetobacter* species isolated from patients with wounds

(*n*=1) Admission ward, (n=1) surgical, (*n*=2) medical). The End-stage kidney disease (ESKD) showed (*n*=1), and this was from the Admission ward (Table 4.3).

4.5 Distribution and Identification of specific *Acinetobacter* species by source/ward

Six *Acinetobacter* species were isolated during the study. Isolates of *Acinetobacter* species from sepsis included the following; (*n*=4 *A. baumannii* isolates); (*n*=2 *A. ursingii* and *n*=1 *A. lwoffii*). Isolates from meningitis were as follows; (*n*=3 *A. baumannii); (n*=2 *A. lwoffii*, and *n=1* was *A. ursingii).* All the isolates from the Medical, NICU, PICU and Surgical wards were *A. baumannii* (Table 4.4). Other *Acinetobacter* species were isolated from the Admission ward: (*n=2 A. johnsonii, n=1 A. haemolyticus* and *n=1 A. junii*) (Table 4.4).

Table 4.4: Distribution and Identification of specific *Acinetobacter* species by source

Ward	*A. baumannii*	*A. johnsonii*	*A. haemolyticus*	*A. junii*	*A. lwoffii*	*A. ursingii*
Admission ward	10	2	1	1	2	1
PICU	6	0	0	0	2	0
NICU	6	0	0	0	2	0
Surgical	1	0	0	0	0	0
Medical	6	0	0	0	0	0
MENU	1	0	0	0		0
Enviro	13	2	0	1	4	0

There were 4 *Acinetobacter* species isolated from patients with wounds, and all were *A. baumannii*. From burns 2, *Acinetobacter* species were isolated. One was *A. lwoffii*, and 1 was *A. baumannii* (Tables 4.3 and 4.4). The rest of the conditions (Congenital Heart disease, Deep vein thrombosis, severe pneumonia, seizures, Infective endocarditis, Epilepsy, Urinary Tract Infection(UTI) and Hydrocephalus) had one isolate each.

4.6 Susceptibility Profiles of *Acinetobacter* species

Table 4.5: Antibiotic Susceptibility patterns of *Acinetobacter* species

Antibiotics	Resistant (R) n (%)	Resistance 95% CI	Intermediate (I) n (%)	Susceptible n (%)
Tetracycline	59(98%)	94-100	0(0%)	1(1.6%)
Cefepime	35(60%)	47-73	4(6.7%)	19(31.7%)
Ciprofloxacin	34(57%)	44.6-70.6	2(3.3%)	23(38.3%)
Gentamicin	32(53%)	40-66	2(3.3%)	26(43%)
Co-trimoxazole	42(70%)	58-82	3(5%)	15(25%)
Ceftazidime	38(65%)	53-78	6(10%)	14(23%)
Ceftriaxone	14(23%)	32-72	6(10%)	7(12%)
Tobramycin	12(20%)	11.7-36	14(23%)	24(40%)
Cefotaxime	13(22%)	24.-62	8(13.3%)	9(15%)
Piperacillin/Tazobactam	12(20%)	16.6-48	8(13.3%)	17(28%)
Imipenem	10(17%)	7.3-28.2	3(5%)	43((72%)
Amikacin	1(2%)	0-60	1(2%)	4(7%)

Resistance was highest to tetracycline at 98%, followed by co-trimoxazole at 70% the ceftazidime at 65%. Cefepime, ciprofloxacin, gentamicin and ceftriaxone had 60%, 57%, 53% and 23%, respectively. The least resistance was observed to amikacin at 1.6% (Table 4.5).

4.6.1 Multidrug resistance profiles

The frequency of MDR (isolates resistant to three classes of antibiotics) of the clinical isolates showed resistance to aminoglycosides, fluoroquinolones and sulphonamides, translating into 75% (30/40) MDR. The isolates with four or more than four (4) classes (aminoglycosides, fluoroquinolones, sulhonamides, cephalosporins, carbapenems and tetracycline) fall under XDR, and 52.5% (21/30) were XDR (Table 4.6). The clinical and

environmental isolates were tested for extended beta Lactamase (ESBL) production, and 40% (16) of the clinical were ESBL positive, and 50%(10) environmental isolates were ESBL positive.

Table 4.6: Frequency of MDR in Acinetobacter

Parameter	Resistance to one or several classes of antibiotics (%)						Total
No. of classes of antibiotic	1	2	3	>3	4	>4	
No. of isolates of *Acinetobacter* spp	3(7.5)	7(17.5)	5(12.5)	30(75%)	4(10)	21(52.5)	40(100)

The PCR images (Figures 4.3 and 4.4) below show some gels with bands indicating a positive result. A band indicates that a particular resistance gene is present during the polymerase chain reaction in the isolates run.

4.7 Molecular results

4.7.1 Polymerase chain reaction

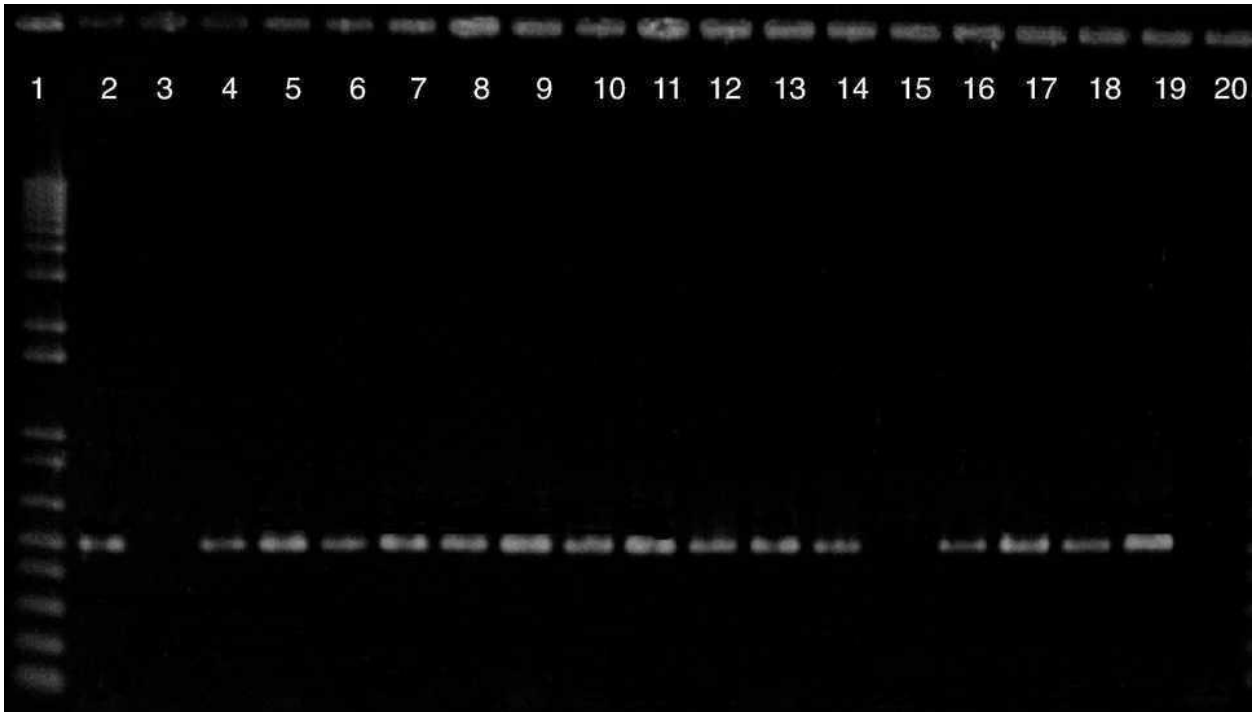

Figure 4.3: Detection of blaOXA-23 genes in Acinetobacter spp. Environmental isolates. Lines 1 DNA Ladder 100bp; blaOXA-23 positive control; line 2, Negative reaction control line3: 4-14 and 16-19: isolates carrying blaOXA-23; line 15 and 20: isolates not carrying blaOXA-23

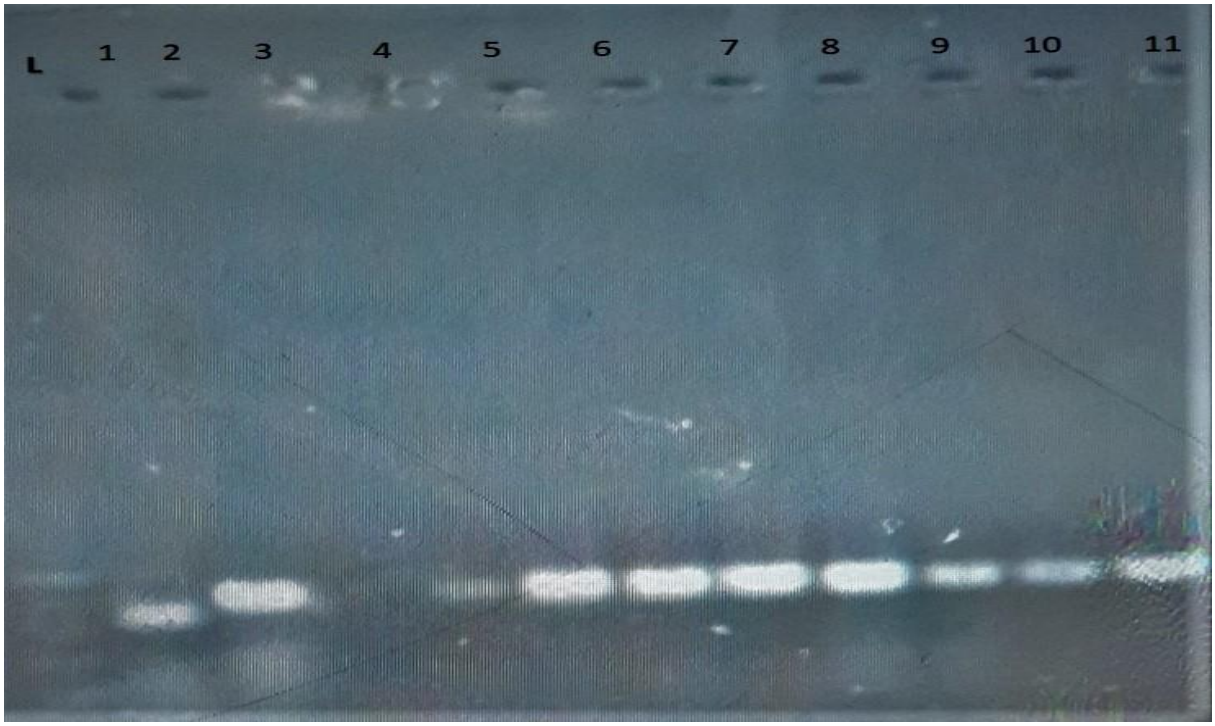

Figure 4.4: Agarose gel electrophoresis for fractionated Oxa-Uni gene for clinical samples. Bands were fractionated by electrophoresis on a 1% agarose gel and visualized under UV light after staining with Ethidium Bromide (L=ladder, lane 1` to 11 are results with 1,2,4,5,6,7,8,9,10 and 11 being positive, and 3 was negative.

The PCR images (Figures 4.3 and 4.4) above show some gels with bands indicating a positive result. A band indicates that a particular resistance gene is present during the polymerase chain reaction in the isolates run.

Table 4.8: Resistant gene determinants in Clinical and environmental isolates

Genes	Environmental Isolates	Clinical Isolates
	Positive	Positive
TetE	0(0%)	24(60%)
AdeB	20(100%)	33(82.5%)
GyrA	7(35%)	32(80%)
ParC	15(75%)	29(72.5%)
NDM	0(0%)	17(42.5%)
VIM	8(40%)	14(35%)
IMP	0(0%)	21(52.5%)
Oxa-Uni	3(15%)	13(32.5%)
Oxa23	6(30%)	14(35%)
Oxa24	6(30%)	10(25%)
Oxa48	0(0%)	0(0%)

All the environmental samples were negative for *NDM, IMP, Oxa 48, Tet E* and *Tet G*. *AdeB* was the highest among the resistance gene determinants tested, with 82.5% positivity in clinical isolates and 100% in environmental isolates. Resistance gene Oxa48 was not found in any of the clinical isolates. The *NDM* (New Dehli Metallo-β lactamases) was in 42.5% of clinical isolates. The *IMP* (a carbapenem resistance) gene had 52.5% clinical isolates. For the *Oxa-UNI* gene (Universal Oxacillinase), Oxa23, Oxa24, 15%, 30%, and 30% from the environment and 32. 5%, 35% and 25% resistance from clinical isolates, respectively. In environmental isolates, 15% were positive, while 32.5% of the clinical were positive. The *Oxa23* gene (oxacillinase) had 30% positive environmental isolates and 35% positive clinical isolates. For the *Oxa24* gene (Oxacillinase), 15% of the environmental isolates were positive. For the clinical isolates, 25% were positive for *the Oxa24* gene. All the clinical isolates were negative for *Oxa48*. The *GyrA* gene (gyrase) was 35% environmental and 80% clinical isolates, respectively, while the *parC* gene (Fluoroquinolone resistance) was 75% environmental and 72.5% clinical isolates. In addition, amino acid sequence analysis of the

parC gene showed a switch from serine to leucine on position 80, and no mutation on position 84 in both environmental (P7 and P9) and clinical (P1 and P3) isolates (Figure 4.6).

Figure 4.6: Amino acid multiple sequence alignment of the parC gene. A. baumanni (X95819.1) strain was used as a reference. P1 to P2correspond to study sequences.

CHAPTER FIVE

5.0 Discussion

The objective of this study was to evaluate the clinical significance and genotypic relatedness of *Acinetobacter* species isolated from admitted patients, ward environmental surfaces and instruments at the University Teaching Hospital, Lusaka. We isolated resistant *Acinetobacter* species from both the clinical specimen and the environment.

To the best of our present knowledge, this is one of the first few studies that has conclusively isolated and characterized *Acinetobacter* species at the University Teaching Hospital (UTH) from clinical and environmental specimens. Further, this study has been able to elucidate drug-resistant patterns of *Acinetobacter* species. In addition, the genetic relatedness of *Acinetobacter* species isolated from patients and the environment has been elaborated under this study.

This study isolated *Acinetobacter* from blood, cerebrospinal fluid (CSF), swabs (burns and wounds), urine, and respiratory samples. In a similar study

A. baumannii was isolated from blood, urine, swabs (pus and burns), CSF and respiratory samples (Du et al., 2019). They found that the isolates were resistant, with some resistance to carbapenems. Carbapenems are usually the last drug choice in the selection/use of antibiotics. Resistance to carbapenems would mean more resistant isolates; this may pose a challenge of treating infections leading to high mortalities. The isolation of *Acinetobacter* species from these samples poses serious concerns as the affected patients could either be

immunocompromised, have prolonged hospital stay, have undergone surgery or have acquired the organism through ventilation (in the case of Ventilator-associated pneumonia, VAP). All the 40 clinical isolates were Hospital-acquired as the samples were collected 48hrs post-admission. This is according to the description of nosocomial infection in the CDC guideline(Emori, 1988). The samples included in this study were from sterile sites. Sterile sites are the sites in the human body which do not have normal flora. Having isolated *Acinetobacter* species as a single organism, this could mean that the organism was the cause of the infection and could cause meningitis, sepsis, lower respiratory tract infection, UTI, and infection of wounds. *Acinetobacter* species is a nosocomial pathogen, according to the CDC guideline, which causes infections in immune-compromised patients (Emori, 1988). *Acinetobacter* is also clinically significant as it causes severe diseases, including sepsis, meningitis, cause septic wounds, UTI and pneumonia. Similar findings were observed by Olaniyi and co-workers and Parajuli and co-workers (Ayobami *et al.*, 2019, Parajuli *et al.*, 2017). They found that various *Acinetobacter* species were isolated from patients with sepsis, meningitis, wounds, UTI, septic wounds, and pneumonia, which is congruent with our study's findings.

Sixty (60) various *Acinetobacter* species were isolated. Forty (40) came from patients admitted to UTH for more than 48 hours across various wards, while twenty (20) were environmentally derived samples from surfaces and devices used on patients. This is an important finding as it shows that the isolates from patients who initially had no infection were present in the environment with similar AMR patterns and resistant genes. This indicates the high possibility of the clinical isolates coming from the hospital environment and medical devices. This finding is similar to a study done by Sheikh and co-workers (Sheikh et al., 2020). They also found that isolates from the environment were similar to

those from the clinical samples and these samples were from patients with sepsis, meningitis, pneumonia, wounds and UTIs.

Proportionately, environmental sources contributed 33.3% of the *Acinetobacter* isolates. This finding confirms that *Acinetobacter* species are predominant in the hospital environment and could pose a threat in contributing to HAI, as these species were similar to those found in clinical samples. This is similar to studies done by (Almasaudi, 2018). They found that the environmental isolates were similar to patient isolates. The study also demonstrated that the environmental and clinical isolates had the same source through the phylogenetic tree.

Regarding patient-derived *Acinetobacter* species, the admission ward accounted for 21.7%, followed by the paediatric ICU at 20%, with the medical ward accounting for 10%. The high recovery of the *Acinetobacter* species in the medical wards can be attributed to several factors, such as the lowered immunity and conditions that require prolonged admission. It could also be a result of prolonged use of antibiotics, age and poor hygiene of the environment (Sheikh et al., 2020).

The surgical wards had much lower isolation which may be alluded to disinfection frequency with 0.5% hypochlorite and 10 % Virkon. Unlike the medical wards, which are constantly congested, the surgical ward is usually cleaned and disinfected using 0.5% hypochlorite, hence a low recovery of the organism. The turnover of patients in the medical wards is also higher, making thorough cleaning and disinfection a challenge. Our results are similar to the findings by Jasper and Co-workers (Tal-Jasper et al., 2016); they showed that the medical

ward had a higher number of *Acinetobacter* species isolated compared to the other wards and that cleaning and disinfecting high turnover wards is generally a challenge.

The other wards were surgical, which accounted for 8.3%, whilst the Neonatal ICU contributed 5%.. The isolation of *Acinetobacter* species at 5% from the Neonatal ICU still pauses concerns as the affected patients have an underdeveloped immune system and, therefore, may not be able to clear the infection successfully. Acinetobacter having the ability to acquire resistance and is also intrinsically resistant, will propagate and be challenging to treat. This may result in high mortality. The Main ICU's least isolation rate was 1.7%. The low isolation from the MICU maybe because of the regular disinfection processes and also the fact that the inflow of people and patients is controlled. Our results are congruent with earlier studies that isolated *Acinetobacter* species from the hospital environment and clinical isolates (Ayobami *et al.*, 2019, Eve *et al.*, 2003, Sileem, Said and Meleha, 2017).

The isolates were recovered from patients admitted at UTH across wards for more than 48 hours in this study. This data was collected from the Laboratory Information system (LIS), and confirmation of the clinical details was made with the clinicians. Forty (40) were hospital-acquired as the samples were collected after 48 hours of post-admission. An infection is considered hospital-acquired only if the patient did not present with any clinical features of an illness upon admission (Emori, 1988). This regard implies patients from whom isolates had no clinical indications of *Acinetobacter* infection upon admission. This could mean that the infection was acquired from hospital environments. This is similar to Santajit and Indrawattana (Santajit & Indrawattana, 2016). Their study found out that the isolates were from patients admitted for more than 48 hours and that the *Acinetobacter* species were

isolated as single isolates agreeing with the definition of nosocomial infection as explained above.

Regarding the source of isolates with the highest isolation rate, 20 isolates came from patients with sepsis representing 50% of all the clinical isolates. Sepsis is a blood infection among diseases with high mortality rates, mainly when limited treatment options. Accordingly, based on these findings, sepsis was the highest contributing condition regarding *Acinetobacter* isolation, similar to findings in Tanzania by Moremi and co-workers (Moremi, Claus, Vogel, & Mshana, 2017). After taking into account, the same ward that contributed to the highest isolation rates based on sepsis, we found that the Paediatric Intensive Care Unit (PICU) had the highest number of *Acinetobacter* species isolated (*n*=7), followed by the admission ward (*n*=5). The surgical ward had the lowest isolation rate, with only one isolate. Other studies similar to our current study revealed similar findings pointing out that surgical wards are the least affected (Du et al., 2019). The PICU had the highest number of *Acinetobacter* isolated, given that the patients are young and their immunity is not fully developed. This also proves that the infection from the hospital environment and instruments were the primary sources of the organisms in this ward. This is similar to a study done in Italy by Nocera, Attili and De Martino, 2021. This makes them prone to infection and, therefore, the high numbers of the isolates compared to the other wards. This finding is similar to the findings done in Italy by (Nocera et al., 2021), who also found in their study that sepsis had the highest number of isolates in relation to other conditions.

Among the species isolated from the wards included *A. baumannii,* one of the WHO priority AMR pathogens (Tal-Jasper et al., 2016). *Acinetobacter baumannii* is among the ESKAPE (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumonia, A. baumannii,*

Pseudomonas aeruginosa, and *Enterobacter* species) pathogens, causing a wide range of infections. In addition to the high infection rates, the *A. baumannii* has been seen to be highly resistant. *A. baumannii* has exhibited both MDR and extensively drug-resistant (XDR). The prevalence of the XDR *A. baumannii* phenotype has increased exponentially worldwide (Tal-Jasper et al., 2016). The presence of XDR may be due to prolonged use of antibiotics, leading to treatment challenges that may result in death.

This study found that *Acinetobacter* species showed high resistance to tetracycline at 98%. Our findings are consistent with similar studies done across several hospitals in Baghdad City in Iraq by Khalawetektook and co-workers (Khalawetektook, 2018). Such high resistance findings mean that the treatment with these antibiotics, which are the first line (first choice), will be rendered irrelevant in the health care system. Our results further correlated with other earlier studies concerning the resistance profiles displayed by co-trimoxazole (70%, *n*= 42), ceftazidime (65%, *n*=38), cefepime, ciprofloxacin, gentamicin and ceftriaxone at 60% (35), 57% (34), 53% (32) and 23% (14), respectively (Soltani *et al.*, 2016, D'Souza *et al.*, 2019, Parajuli *et al.*, 2017, Antunes, Visca and Towner, 2014). The resistance percentages shown mean that these antibiotics will not work when administered. The susceptibility of *Acinetobacter* species to piperacillin-tazobactam, cefotaxime tobramycin and imipenem was similar to findings in Baghdad (Khalawetektook, 2018).

In this study, 40% of the clinical isolates were Extended-spectrum beta-lactamase (ESBL) positive, whilst 50% of the environmental isolates were positive. The ESBLs are enzymes that confer resistance to beta-lactam antibiotics. The beta-lactam antibiotics include penicillins and cephalosporins. This is an important finding as ESBL producing organisms are very hard to treat as many prescribed antibiotics, including beta-lactamase inhibitors like

penicillins (Benzylpenicillin, ampicillin) and cephalosporins (cefotaxime, ceftazidime, ceftriaxone, cefepime) will not be able to kill the bacteria. The presence of ESBL *Acinetobacter* species was found in similar studies in Saudi Arabia (Almasaudi 2018) and Seoul (D'Souza et al., 2019).. In both studies, it was discovered that there are strains that are ESBL producers among the Acinetobacter species, which contribute to the unsuccessful treatment of *Acinetobacter* infections. This may lead to the transfer of the ESBL to other organisms, which can cause infections which may result in death.

The MDR and XDR in *Acinetobacter* isolates were defined according to a new standardized international document by (Magiorakos et al., 2012) which defines the antimicrobial susceptibility results to all antimicrobial agents. The frequency of MDR clinical isolates that showed resistance to three or more antibiotics under this study included aminoglycosides, fluoroquinolones, and sulphonamides, which accounted for 75% of the resistant clinical isolates 30/40. These are drugs that are usually readily available (University Teaching Hospital Antibiotics guideline) and frequently prescribed to patients making the organism develop resistance. Therefore, the resistance to these antibiotics is an indication that the *Acinetobacter* species have since developed resistance to them over time because of their constant and prolonged use. This is similar to the study done in Baghdad (Khalawetektook, 2018). The findings in the study showed that resistance to antibiotics was high, and this was due to the natural acquisition of resistance by the Acinetobacter and antibiotic prolonged use.

The XDR of the clinical isolates in this study accounted for 52.5%. These findings are consistent with Sobouti and co-workers who had similar findings from their earlier study in Iran (Sobouti et al., 2020).

Table 5.1 shows the interpretation of MDR and XDR. The results elaborate what it means for the antibiotic to either be qualified as MDR or XDR. Resistance of an organism to cephalosporins, fluoroquinolones and aminoglycosides (with another class) at the same time means that the organism is MDR. MDR, including resistance to carbapenems, is XDR.

Table 5.1: Antimicrobial drug categories and agents proposed for the characterization of MDR and XDR Acinetobacter

Antimicrobial categories	Multidrug resistant (MDR)	Extensively drug-resistant(XDR)
Definitions	Resistance to at least Three classes of drugs: 1) Cephalosporins 2) Fluoroquinolones 3) Aminoglycosides	MDR Acinetobacter + Resistance to carbapenems
Therapeutic Options	Carbapenems Polymyxins	Polymyxins Tigecycline

In our current study, the *AdeB* gene, the multidrug efflux pump, was the highest gene conferring resistance among all the tested resistance gene determinants, expressing 82.5% positivity in clinical isolates and 100% in environmental isolates. The *AdeB* gene (the efflux pump conferring gene for aminoglycosides) has been associated with the resistance to aminoglycosides, including gentamicin, amikacin, tobramycin, neomycin and streptomycin. Among these, gentamicin is the most commonly used drug. A study conducted in Switzerland reported similar results consistent with our results (Nocera et al., 2021). There was no Oxa48 found in any of the clinical isolates. This observation was similar to the findings in another study (Huys, Cnockaert, Nemec, & Swings, 2005). The *GyrA* gene (gyrase) was expressed at 35% in environmental isolates, while it was conferred at 80% in clinical isolates.

Regarding the *ParC* gene (Fluoroquinolone resistance), it was expressed at 75% in environmental isolates, whilst in clinical isolates, it was conferred at 72.5%. When the amino acid sequence of the *parC* gene from isolates that showed resistance to fluoroquinolone was analyzed, only four out of nine had a switch from serine to leucine on position 80 and no mutation on position 84. This mutation is believed to confer fluoroquinolone resistance in *A. baumannii*, and whether the mutation is present or not, so long as the bacteria possess a mutation on the gyrase gene, it will be resistant to fluoroquinolone (Nogbou et al., 2021). The *NDM* (New Dehli Metallo-β lactamases) gene was found at 42.5% only in clinical isolates. Similarly, the *IMP* (a carbapenem resistance) gene had 52.5% and was only found in clinical isolates. For the *Oxa24* gene (Oxacillinase), 15% of the environmental isolates were positive. For the clinical isolates, 25% were positive for *the Oxa24* gene. All the clinical isolates were negative for *Oxa48*.

The similarity in AMR resistance profiles of the isolates found in the environment and those isolated from clinical samples suggest possible transmission of the organisms from the environment to patients. This was seen from the similarity of species from the environment and those from the clinical samples. This particular finding is similar to those observed in studies by De Francesco and co-workers and by Royer and their team in Italy and Brazil, respectively (De Francesco *et al.*, 2013, Royer *et al.*, 2015). De Francesco, Royer, and their teams found that the *Acinetobacter* species isolated from ventilators (hospital equipment) and other intubation gadgets were the same species found in patients within the same ward.

One of the limitations faced in this study was the lack of reagents to sequence the isolates. This limited our ability to compare the isolates at the genetic level.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

This study has observed AMR Acinetobacter species from both clinical specimens and the environment, which is of public health significance. The findings also showed that *Acinetobacter* species were isolated from patients with different conditions from different sample types. This has been demonstrated by the similarity in the species type and the resistance patterns from clinical and environmental isolates. This study has shown that *Acinetobacter* species are resistant to the most commonly used antibiotics (University Teaching Hospital Antibiotic guidelines).

6.1 RECOMMENDATIONS

In this study, *Acinetobacter* spp. were isolated, the resistance genes, including antimicrobial resistance, were detected, and it was shown that the isolates from the environment and the clinical samples were similar. This similarity of the isolates augments the need to direct efforts to reduce hospital-acquired infections, which may be more severe and life-threatening than the condition for which a patient was admitted. We, therefore, recommend that the treatment protocols, the disinfection of medical devices with 0.5% hypochlorite as an infection prevention procedure, should be implemented at regular intervals to reduce the burden of nosocomial infections. Given the relatively and comparatively broad spread nature of the *Acinetobacter* species isolated across different wards and environmental surfaces, innovative modalities such as rapid diagnostics and easy to use screening protocols need to be developed for nosocomial organisms. In addition, tight and strict adherence to antimicrobial stewardship, recommendations, and infection control measures to reduce resistance and patient-to-patient transmission of *Acinetobacter* species is paramount.

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