Isolation of *Acinetobacter baumannii* From Patient and Hospital Environment: Analyzing Their Antibiotic Resistance, Serum resistance & Biofilm Formation

By

Mst. Maskera Jinnah

19226010

Kaniz Fatema

19126005

A thesis submitted to the Department of Mathematics and Natural Sciences in partial fulfillment of the requirements for the degree of

Bachelor of Science in Microbiology

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Declaration

It is hereby declared that

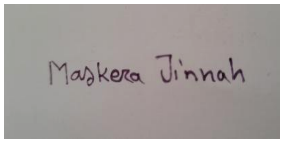
1. The thesis submitted titled "**Isolation of *Acinetobacter baumannii* from Patient and Hospital Environment: Analyzing Their Antibiotic Resistance, Serum Sensitivity & Biofilm Formation"** is our original work while completing our degree at BRAC University.

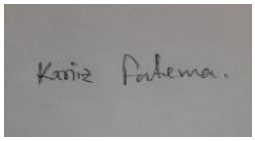
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.

3. The thesis does not contain material that has been accepted, or submitted, for any other degree or diploma at a university or other institution.

4. We have acknowledged all main sources of help.

Student's Full Name and Signature:





Mst Maskera Jinnah
19226010

Kaniz Fatema
19126005

Approval

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1. Mst. Maskera Jinnah (19226010)
2. Kaniz Fatema (18236011)

Summer 2023 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Bachelor of Science in Microbiology on 6th june 2024.

Examining Committee:	
Supervisor: (Member)	_____ Fahim Kabir Monjurul Haque, PhD Associate Professor, Microbiology Program, Department of Mathematics and Natural Sciences , Brac University
Program Director: (Member)	_____ Nadia Sultana Deen, PhD Associate Professor, Microbiology Program, Department of Mathematics and Natural Sciences, Brac University
Departmental Head: (Chair)	_____ A F M Yusuf Haider, PhD Professor and Chairperson, Department of Mathematics and Natural Sciences Brac University

Ethics Statement

The study was conducted in accordance with the Declaration of Helsinki, and ethical approval was obtained from the Institutional Review Board of Rajshahi Medical College and Hospital. Informed written consent was collected from each study participant prior to data collection. For the convenience of the participants, the consent form was prepared in both Bengali and English.

Abstract:

The *Acinetobacter baumannii* is a gram-negative coccobacillus also known as opportunistic bacterial pathogens currently creating great concern in clinical aspects due to their capacity to endure for extended periods of time in the environment and ability to cause multi-drug resistant infections. The objective of the study was to identify *A. baumannii* from hospital environments and admitted patients in hospital to analyze their antibiotic resistance, serum resistance and biofilm formation. Total 450 samples were collected from different wards of Rajshahi Medical College and Hospital. Among them we obtained 53 isolates of *A. baumannii* confirmed by polymerase chain reaction by targeting of the blaOXA-51 gene. From 53 isolates, 20 were from patient's samples (7 endotracheal aspirates, 4 blood, 5 wound swab, 2 throat swab and 2 catheter tube) and 33 isolates were from environment's samples (bed sheet, surface of furniture, nebulizer machine, floor, nurse's hand swab, food cart, medicine cart and trolleys). Subsequently, an antibiotic susceptibility test was done. Isolates from patient specimens were resistant to gentamicin (90%), amikacin (90%), cefepime, piperacillin-tazobactam (85%), ceftazidime(85%), and tetracycline (80%). A significant proportion of the isolates, 70%, displayed resistant against levofloxacin, imipenem, meropenem. Most importantly, 90% of all patient isolates were MDR. On the other hand, hospital environment's isolates were resistant against ceftazidime(100%), imipenem(87.9%,), piperacillin-tazobactam (78.8%), and cefepime(78.8%). A significant proportion of the isolates, 69.7% and 66.7% were resistant against meropenem and gentamicin. Among them 80% of all isolates were MDR. The result of the serum bactericidal assay showed that almost 31% of isolates were serum resistant and 35% were sensitive and 34% were intermediate. Isolates from the environment's samples were 10% more resistant than the isolates from the patient's samples. Among environment's isolates 36% isolates were resistant whereas among patient's isolates only 26% were resistant. According to quantitative biofilm formation results, among 33 environmental isolates 17% of isolates formed strong biofilm, 14% formed moderate film, and 30% formed weak and 39% isolates did not form biofilm.

Keywords: *Acinetobacter baumannii*; multidrug resistance; environmental samples; patient samples.

Dedication

I, Mst. Maskera Jinnah, would like to dedicate my thesis to my Family.

I, Kaniz Fatema, would like to dedicate this thesis to my beloved Mother, without her, I am nothing.

Acknowledgement

To start, we express our gratitude to the Almighty Allah for granting us the opportunity and ability to complete this study in good health and sound mind. Our profound gratitude and appreciation is due to our esteemed supervisor.

Department of Mathematics and Natural Sciences, BRAC University, his steadfast oversight, constructive criticism, knowledgeable counsel, contagious enthusiasm for pursuing new lines of inquiry, and constant encouragement due to which we were able to grow and learn as a student and a person. Our Laboratory Officers- Shamima Akhter Chowdhury has always graced us with help whenever we needed for which we are grateful. And of course, the incomparable generosity and guidance of our Research Assistant Maftuha Zahra cannot be forgotten. Throughout the project, she has been by our side- assisting us, teaching us, figuring out how to set up our experiments, how to interpret our data and always being available for us no matter day or time. We thank you for facilitating us with knowledge and wisdom that we will remember for a long time.

Lastly, but not the least we would like to express our gratefulness to lab attendants Ashik-E Khuda, Tanzila Alam, and office attendant Nadira Yeasmin for their unwavering help during these couple of months at the laboratory and everyone who assisted us and engaged with us in the laboratory.

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List of Abbreviations:

AGE Agarose Gel Electrophoresis

AMR Antimicrobial Resistant

AMEs Aminoglycoside-modifying enzymes

AST Antimicrobial Susceptibility Test

bp Base pair

CFU Colony Forming Unit

CLSI Clinical Laboratory Standards Institute

DNA Deoxyribonucleic Acid

ECDC European Centre for Disease Prevention and Control

EDTA EthylenediamineTetraacetic Acid

EPS Extracellular Polymeric Substances

FH Factor H

HCAI Health Care Acquired Infections

HGT Horizontal Gene Transfer

HCW Health care workers

ICU Intensive Care Unit

LPS Lipopolysaccharides

MBLs Metallo-β-lactamases

MCT Microcentrifuge Tubes

MDR Multidrug-Resistant

MHA Mueller–Hinton agar

MIC Minimum Inhibitory Concentration

NHS Normal Human Serum

NA Nutrient Agar

OXA Oxacillinase

PCR Polymerase Chain Reaction

PBS Phosphate Buffered Saline

RMCH	Rajshahi Medical College and Hospital
Rrna	Ribosomal RNA
rpm	Revolutions Per Minute
T1N1	Tryptone Salt Agar
TE	Tris-EDTA
TBE	Tris-Borate EDTA
UV	Ultraviolet

CHAPTER 1

Introduction

1.1 Background:

Nosocomial infection is an infectious disease which was nonexistent during the time of admission that occurs after the discharge of the patients and also can appear during healthcare delivery for other diseases (Khan et al., 2015). Nosocomial infection causes very serious health problems in hospitals worldwide. In developing countries such as Bangladesh, those infections have been creating concern for both the health care providers, patients and their families. It can increase the cost of the health care services which conclude extra cost to treat infection (Alrubaiee et al., 2017). Most of the noticeable nosocomial infections are urinary tract infections, respiratory pneumonia, and surgical site wound infections, bacteremia, gastrointestinal and skin infections (Agrawal, et al., 2006). According to the European Centre for Disease Prevention and Control (ECDC) estimated that around 4131000 patients are attacked by health care acquired infections (HCAI) in Europe every year that come for the treatment in hospitals. The estimation of nosocomial infections incidence in the USA hospitals were 4.5% in 2002, and around 9.3 infections per 1000 patient-days and 1.7 million affected patients.

Acinetobacter baumannii is gram-negative coccobacilli that have the ability to cause serious multi-drug resistant infections. It causes between 2-10% of all hospital infections caused by gram-negative bacteria. (Antunes et al., 2014). Numerous opportunistic illnesses, including septicemia, pneumonia, endocarditis, meningitis, skin and wound infections, and urinary tract infections have been linked to *A. baumannii* (Zurowska et al., 2008). Over the past 30 years, there has been a major shift in the taxonomic status of the genus *Acinetobacter*. *A. baumannii*, its most significant representative, has become one of the most threatening pathogens for health care providers worldwide. In the present antibiotic era, its remarkable ability to induce or adopt resistance has driven its clinical significance and made it one of the dangerous infectious organisms, particularly during the last few years (Peleg et al., 2008). Also, it is capable of persisting for extended periods of time and may propagate readily in hospital settings. These characteristics may indicate its inclination to start protracted epidemics (Shamsizadeh et al., 2017). Due to cross-transmission in the hospital setting, *A. baumannii* can spread through either indirect interaction with contaminated places and equipment or

direct contact with sick individuals (Kilic et al., 2008). Understanding the origins and reservoirs of nosocomial infectious agents is essential for the prevention and control of hospital infections. The identification of *A. baumannii* sources within hospital settings enhances our understanding of potential pathways for *A. baumannii* transmission that would help to make possible the implementation of more suitable control strategies to prevent the increase in *A. baumannii* infections (Shamsizadeh et al., 2017).

CHAPTER 2

Literature Review

2.1 Reservoir

The prevalence of *A. baumannii* in Hospital Environment is very critical. Research team of Swati Sharma investigated the *A. baumannii* populations in the intensive care unit and their impacts on colonization pressure and transmission. A six-month prospective monitoring was carried out where swab samples from patient, Health care workers' (HCWs) surrounds, hospital sewage, and surveillance cultures were all collected. Using both genotypic and phenotypic approaches, *A. baumannii* was identified (Sharma et al., 2022). In hospitals, *A. baumannii* is commonly detected on floors, equipment, and environmental areas. It has the ability to persist for extended periods of time and develop resistance factors (Cruz-López et al., 2022).

While it has long been debated whether *A. baumannii* exists in non-hospital settings, the application of molecular techniques in recent years has made it possible to prove that the organism is present in a variety of environmental settings, including soil, waste water, human lice, pets, slaughter animals, and human carriage (Eveillard et al., 2013). It is commonly known that one of the finest habitats for microorganisms to flourish is soil and water since it gives them the nutrients they need to survive and allows them to pick up new genes from nearby microbes of the same or other genus (Byrne-Bailey et al., 2009).

2.2 Antibiotic resistance of *Acinetobacter baumannii*

Antimicrobial resistance, often known as antibiotic resistance (AMR), has become a significant global concern for healthcare systems. Because of its arising and ever-increasing resistance, *A. baumannii* creates a worldwide threat to human health as well as a therapeutic challenge. (Kyriakidis et al., 2021). There are reports that *A. baumanni*i isolates are resistant to almost every antibiotic currently in use. The combination of improper and excessive antibiotic use, an inadequate antibiotic stewardship program, and the bacteria's inherent capacity to evolve in response to novel environmental challenges and accumulate new resistance mechanisms has turned *A. baumannii* into the superbug. Because of the bacterium's

genetic makeup, *A. baumannii* is a natural transformant that can quickly evolve resistance to antibiotics and for which there is very little effective treatment. *A. baumannii* implies an intricate set of resistance mechanisms against antibiotics such as efflux pumps, genetic changes, and the enzymatic breakdown of antibiotics as defense mechanisms against the effects of antimicrobials (Jalali et al., 2022).

The review of the published literature on antibiotic resistance of *A. baumannii* in Iran by Moradi revealed that antibiotic resistance is increasing over the years. From 2001 to 2007, rate of resistance to all antibiotics was high whereas the rate of resistance was low for carbapenem, lipopeptides, and aminoglycosides compared to the others. On the other hand, between 2010 to 2013 the rate of resistance has increased specially against carbapenem (imipenem and meropenem). (Moradi et al., 2015).

There are multiple mechanisms behind antimicrobial resistance of *A. baumannii* such as beta-lactamases synthesis, outer membrane protein, aminoglycoside-modifying enzymes and horizontal gene transfer etc. Details are descried bellow:

Beta-lactamases synthesis

The synthesis of beta-lactamases, which are enzymes that can hydrolyze beta-lactam medicines like cephalosporins and penicillins, is one of the primary mechanisms of antimicrobial resistance (AMR) in *A. baumannii* (Poirel et al., 2003). reported in the Journal of Antimicrobial Chemotherapy that these enzymes are becoming more common in *A. baumannii* strains, which is why beta-lactam antibiotic treatment is becoming less and less effective.The main factor contributing to carbapenem resistance is the existence of oxacillinases (OXA), which are β-lactamases of the Ambler class D. More than 400 OXA enzymes that are encoded by genes situated on chromosomes or plasmids have been identified. *A. baumannii* strains were also found to contain strains of other β-lactamases, including class A, class B (metallo-β-lactamases, or MBL), and class C (AmpC) (Monem et al., 2020).

Outer Membrane Proteins

Proteomic analysis says that another mechanism of AMR in *A. baumannii* is protein diversity of *A. baumannii* MDR strains may be associated with the emergence of resistance traits, particularly outer membrane proteins, which are implicated in cellular drug uptake or efflux. Many differences in OMP expression are frequently linked to the formation of an antibiotic resistance level (Monem et al., 2020). Mutant strains lacking OmpA (ΔompA and OmpA-like domain deletion) exhibit instability in their outer membrane and greater susceptibility to antibiotics, such as cephalosporins and penicillins (Kyriakidis et al., 2021).

Aminoglycoside-modifying enzymes

Aminoglycoside resistance is mostly caused by enzymes called aminoglycoside-modifying enzymes, or AMEs. Aminoglycoside-modifying enzymes (AMEs) that reduce the ability of AG to bind, modify the target site of 16S rRNA methyltransferases, and restrict the absorption of AG once permeability is lost or efflux pumps are overactive (Kyriakidis et al., 2021). Three enzymes such as acetyltransferases, adenylyltransferase, and phosphotransferases—normally exist on transposable elements are mostly responsible for mediating such resistance by altering aminoglycosides.There have been reports of various AME in *A. baumannii.* According to reports, a gene encoding APH (3')-VI phosphotransferase is linked to amikacin resistance. Moreover, AME aac (6')-Iad contributes significantly to amikacin resistance in Japanese Acinetobacter species (Lin, 2014).

Horizontal gene transfer

The horizontal gene transfer-mediated acquisition of resistance genes in *A. baumannii* is another mechanism of AMR. According to a 2007 study published in the journal Clinical Microbiology and Infection, *A. baumannii* strains have the ability to scavenge resistance genes from other bacterial species via plasmids, rendering them resistant to a broad spectrum of antibiotics. (Journal of Clinical Microbiology, 2007).

2.3 Serum resistance

Serum resistance is the ability of microbes to evade the host immune system's bactericidal effect. In the case of *A. baumannii*, serum resistance mechanisms are of particular interest due to their implications for virulence and treatment strategies.

Role of Complement-Mediated Bactericidal Activity: the serum contains potent antibodies and complement proteins that collectively exert bactericidal activity against invading pathogens (Hood & Skaar, 2012). This complement-mediated bactericidal activity is a crucial component of the host's innate immune response (Ricklin et al., 2010). Many bacteria have evolved sophisticated strategies to evade complement-mediated killing, including the exploitation of complement inhibitors and regulatory proteins (Blom & Ram, 2008). By hijacking these regulatory mechanisms, pathogens such as A. baumannii can evade immune detection and clearance.

A. baumannii Serum Resistance Mechanisms: Certain strains of A. baumannii have been implicated in severe infections, including bacteremia with high mortality rates, attributed in part to their ability to resist the bactericidal action of normal human serum (NHS) (Gaddy & Actis, 2009). Studies have identified outer membrane protein A (OmpA) as a key player in A. baumannii serum resistance (Kim et al., 2009). OmpA facilitates binding to Factor H (FH), a critical regulator of the complement cascade, thus conferring serum resistance. In addition to its role in immune evasion, OmpA-mediated serum resistance has been associated with enhanced virulence traits in A. baumannii, including increased adherence to and invasion of host cells (Lee et al., 2006). A study shows A. baumannii isolates revealed a heterogeneity in their susceptibility to human serum. Three isolates displayed a marked resistance, exhibiting a survival rate exceeding 50% after a 3-hour incubation. Conversely, the remaining four isolates demonstrated a pronounced sensitivity, with a survival rate below 20% after the same incubation period (King et al., 2009).

2.4 Biofilm

Biofilm formation by *A. baumannii i*s a complex process driven by various factors and mechanisms. The bacterial lifestyle known as biofilms consists of diverse protein matrices, nucleic acids, polysaccharides, and bacterial microcolonies that are distributed through water channels to form dynamic community settings (Hall-Stoodley et al., 2004).

Initially, *A. baumannii* attaches to surfaces through adhesion mechanisms, which can involve surface proteins and pili. Once attached, the bacteria start to produce and secrete extracellular polymeric substances (EPS), which include polysaccharides, proteins, and DNA. These EPS form a protective matrix around the bacterial cells, providing structural support and enhancing resistance to environmental stresses. (Dufour et al., 2010). A cell-to-cell communication system mediated by signaling molecules also called quorum sensing, plays a crucial role in coordinating biofilm formation. (Saipriya et al., 2020). As *A. baumannii* cells multiply and reach a critical density, they release signaling molecules that trigger changes in gene expression, promoting the production of EPS and other biofilm-related factors. (Gedefie et al., 2021). Biofilm formation confers several advantages to *A. baumannii*, including increased resistance to antibiotics, disinfectants, and host immune defenses. Moreover, biofilms allow *A. baumannii* to persist in diverse environments, such as hospital surfaces, medical devices, and water sources, facilitating its transmission and dissemination.

A study published in BMC Infectious Diseases in 2019 investigated A. baumannii isolates from intensive care unit (ICU) patients in India. All isolates were biofilm producers, with 58% demonstrating strong biofilm formation ability (Zeighami et al., 2019). Another study published in Archives of Iranian Medicine in 2019 investigated A. baumannii isolates from burn wound infections in Iran. The researchers found that over 70% of theisolates were strong biofilm producers (Ranjbar & Farahani, 2019).

2.5 Objectives: This study was aimed to isolate and characterize *A. baumannii* from hospital environments and admitted patients in the Rajshahi Medical College and Hospital and to analyze the pattern of antibiotic resistance, serum resistance and biofilm formation.

CHAPTER 3:
Material and methods

3.1 Samples collection

Hospital environmental samples were collected from Rajshahi Medical College and Hospital using sterilized cotton swabs following standardized protocols and samples were transported to Microbiology Laboratory of BRAC University through Amies transport media maintaining a cold chain. Clinical samples from hospital admitted patients were collected directly with the help of professionals following European commission's guidelines 2020 and all samples were transferred to the microbiology laboratory of Rajshahi Medical College maintaining cold chain. Total 450 samples (250 from environment and 200 from patient) were collected between June and December 2023. In this study, patient samples were collected from hospitalized patients of RMCH. These samples included urine, blood, wound swab, stool, catheter tube, and endotracheal aspirates. Whereas hospital environmental samples included swabs of the bed sheet, furniture, nebulizer machine, floor, nurse's hand swab, food cart, medicine cart and trolleys of ICUs and different wards (medicine, burn, surgery, orthopedics, pediatric, geology) of Rajshahi Medical College and Hospital.

3.2 Isolation of *A. baumannii*

A. baumannii isolates of hospital environmental samples were recovered from Amies transport media by spreading them onto Leeds culture media. Clinical swab samples were directly spread onto Leeds *Acinetobacter* agar base (Himedia). On the other hand, blood samples were firstly inoculated into tryptic soy broth (TSB) for enchichment, further they were spread onto selective (Leeds) media. All samples were cultured on Leeds Acinetobacter agar base (Himedia) by following streak plating method and incubated overnight at 44°C. . *A. baumannii* isolates of both clinical and hospital environment samples were isolated by following different standard microbiological techniques including morphological, biochemical reactions and cultural properties (Washington et al., 2006). In accordance with manufacturer's information, suspected colonies of *A. baumannii* were selected for molecular confirmation (PCR).

3.3 DNA extraction

The boiling process was chosen to extract DNA of isolates. We started by labeling sterile microcentrifuge tubes (MCT) with our isolate IDs. For every MCT, 150 ul of Tris-EDTA (TE) buffer was diluted. Next, a loopful of bacterial colonies from our NA subculture plates were collected and dissolved in the TE buffer using an inoculating loop. The cells were then homogeneously mixed throughout the buffer by vortex. The boiling source, a dry heating block with a temperature setting of 100°C, was employed. After rising temperature, the MCTs were put in the heating block's wells and allowed to boil for 15 minutes at 100°C. Following the end of this stage, the MCTs were removed from the block and placed in a centrifuge, where they were spun for 6 minutes at 14000 rpm. After 6 minutes, the MCT has a clear liquid containing DNA known as supernatant and the pellet at the bottom that contains cell debris and components. The supernatant was then moved to a different MCT that was labeled and kept at -20°C until PCR was conducted.

3.4 Identification of *A. baumannii* by PCR:

The boiling method was applied to extract DNA from bacteria. According to this method, single suspected colonies were selected and added to microcentrifuge tubes (MCT) with 200 µl of TE buffer and suspended with the help of a vortex mixer. Then tubes were then boiled for ten minutes at 100°C. The tubes were centrifuged for eight minutes at x13000 rpm, after which the pellet was disposed of and the supernatant was moved to a fresh tube.

In order to identify isolates of *A. baumannii*, PCR was performed using the primers specific for blaOXA-51 gene primers: OXA-51-F: 5'TAATGCTTTGATCGGCCTTG-3' and OXA-51-R: 5'-TGGATTGCACTTCATCTTGG-3'). The 15 µl volume of PCR mixes contained 4.9 µl of nuclease-free water, 7.5 µl of Takara Bio's 2× emerald PCR master mix, 0.3 µl of each set of primers (10 µM), and 2 µl of DNA template. Utilizing an applied bio system (Thermo-Fisher) thermal cycler, the PCR program was set up as follows: 94°C for 5 minutes, then 30 cycles of 94°C for 1 minute, 55°C for 1 minute, 72°C for 1 minute, and a final extension at 72°C for 10 minutes. After PCR, 10 µl of PCR products were separated, and they were then electrophoresed with 110 Voltage for 60 min in gel composed of 2% agarose in TBE buffer with 0.5 µg/mL DNA ethidium bromide dye. UV illumination was used to

visualize the gels, and all of the pictures were kept. When a band found at the anticipated size of 353 bp for blaOXA-51 in the gel, it was deemed positive for *A. baumannii.*

3.5 Antimicrobial susceptibility testing

To determine the drug resistance pattern of *A. baumannii*, the Kirby-Bauer disc diffusion method was used to conduct an antibiotic susceptibility test. Turbidity was set to 0.5 McFarland by suspending colonies in 5 mL of 0.9% NaCL solution. Once confirmation of isolation is done, each sample was streaked onto nutrient Agar and let to incubate for the entire night in order to get ready for the Kirby Bauer disc diffusion test. In short, bacterial cultures were employed for the preparation of a solution, which was swabbed onto MHA agar plates after being compared to a McFarland standard of 0.5. Using sterile forceps, antibiotics were administered for *Acinetobacter species*. Following an 18–24 hours' incubation period at 37°C, the antibiotics' zone of inhibition was determined and interpreted according to CLSI guidelines 2023.

Antibiotic name	Antibiotic Class	Zone interpretation criteria
Imipenem (IMP)	Carbapenem	S>=22, I=19-21, R<=18
Meropenem (MRP)		S>=18, I=15-17, R<=14
Amikacin (AK)	Aminoglycosides	S>=17, I=15-16, R<=14
Gentamicin (CN)		S>=15, I=13-14, R<=12
Ciprofloxacin (CIP)	Fluoroquinolones	S>=21, I=16-20, R<=15
Levofloxacin (LE)		S>=17, I=14-16, R<=13
Ceftazidime (CAZ)	Cephalosporins	S>=18, I=15-17, R<=14
Cefepime (CPM)		S>=18, I=15-17, R<=14
Tetracycline (TE)	Tetracyclines	S>=15, I=12-14, R<=11
Doxycycline (DO)		S>=13, I=10-12, R<=9
Piperacillin-Tazobactam (TZP)	β lactam combination	S>=21, I=18-20, R<=17

Table 1: Antibiotics list that was used in AST

3.6 Serum resistance assay

The susceptibility of bacteria to human serum was determined by the method of Hughes and colleagues with slight modification. A single colony of bacteria was taken in 1 ml Nutrient Broth micro centrifuge tube and kept in 1-2 hours shaking incubator. After centrifuged it at 8000 rpm for 3-4 minutes, supernatant was discarded and 1 ml (0.9%) saline was deposited in the pellet. 20 ul of solution was taken in 180ul of fresh saline and the solution was measured by implying OD 600nm and the result range was between 0.08 to 0.1. Then, cells were diluted to 2 x 10 6 cells/ml in physiological saline (0.9%). From this dilution the bacterial suspension diluted up to 10 -7 in the saline. Twenty-five µl of bacterial suspensions of those dilutions and 75 µl of normal human serum were mixed. Twenty-five µl of bacterial suspensions and 75 µl of normal human serum (NHS) were put into microtiter trays, mixed, and incubated at 37 °C according to hour (0-3) at 37°C. Viability was determined immediately and after 3 h of incubation by plating on brain heart infusion agar for colony counts. Responses were graded as highly sensitive, intermediately sensitive, or serum resistant according to the system of Hughes and colleagues. Each strain was tested three times.

3.7 Quantitative biofilm formation assay

Three wells of a 96-well plastic tissue culture plate with a flat bottom were used for each isolates. 180 µl of Luria-Bertani (supplemented with 1% glucose) and 20 µl of the overnight culture (diluted to a final optical density 630 (OD630) = 0.08- 0.1) were added to each well. *A. Baumannii* was chosen as the positive control, while sterile Luria-Bertani supplemented with 1% glucose was employed as the negative control. Following an 18-hour incubation period at 37°C, each well underwent three PBS washes, a 1 hour drying process at 60°C, and a 15-minute staining period using 180µl of 2% Hucker's crystal violet. Following the solubilization of the dye associated to the adherent cells with 180 µl of 33% (v/v) glacial acetic acid, the absorbance was measured at OD 600. Every assay was carried three times. Three standard deviations over the mean OD of the negative control was the definition of the OD cut-off (ODc). No biofilm producers OD ≤ ODc, weak biofilm producers (ODc < OD ≤ 2×ODc), moderate biofilm producers (2×ODc< OD ≤ 4×ODc), and strong biofilm producers (4×ODc < OD) were the categories into which all the strains were categorized based on their ability to adhere.

CHAPTER 4

Results

4.1 Confirmation of *A. baumannii* using PCR and gel electrophoresis

Total 53 *A. baumannii* isolates have been isolated from 450 samples. 20 were from patient samples and 33 from the hospital environment.

In order to identify the species, PCR and agarose gel electrophoresis were used. The banding pattern in gel was observed using a UV transilluminator to determine the requisite band size. Using a 100 bp DNA ladder, a band was seen at the 353 bp region to identify isolates that tested positive for *A. baumannii*. 15 *A. baumannii* isolates were confirmed, as shown by the bands at the 353 bp level, in the picture below (fig1), which displays the positive isolates.

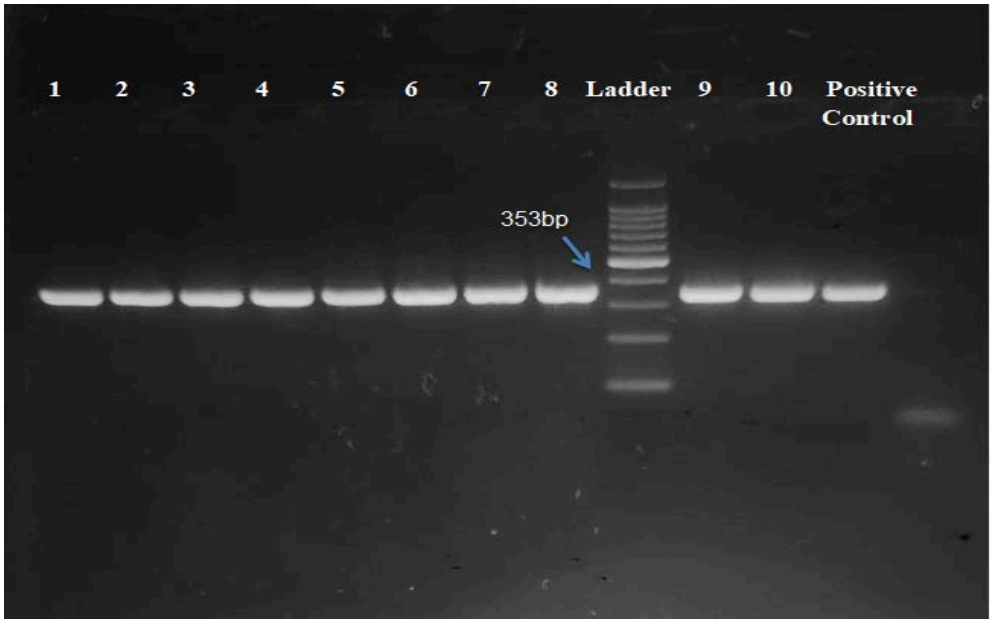

Figure 1: Agarose gel visualization of *A. baumannii* species identification via PCR by using a 100 bp DNA ladder

4.2: Confirmed *A. baumannii* found in hospital environment vs indoor patient:

A total of 450 samples were collected and 80 suspected of them subjected to PCR testing between June and December of 2023; 53 of those isolates were positive for *A. baumannii*, accounting for 11.77% of the sample size. Of these 53, 20 isolates came from patients and 33 from hospital environmental samples.

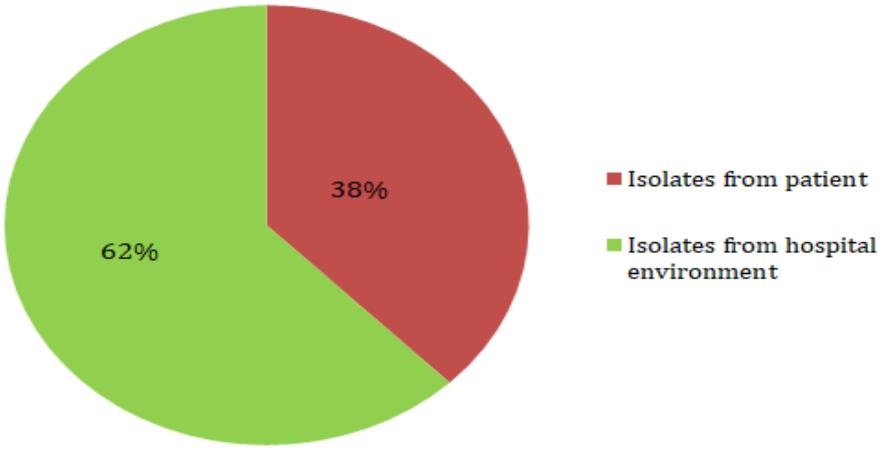

Figure 2: Hospital environment isolates vs indoor patient isolates

4.3: Distribution of clinical isolates among different departments of hospitals:

Among all 20 the patient samples, 45% from the ICU patient, 25% were from wound swabs collected from different surgical patients, 20% from medicine department patients, and 10% from the patient of the burn unit.

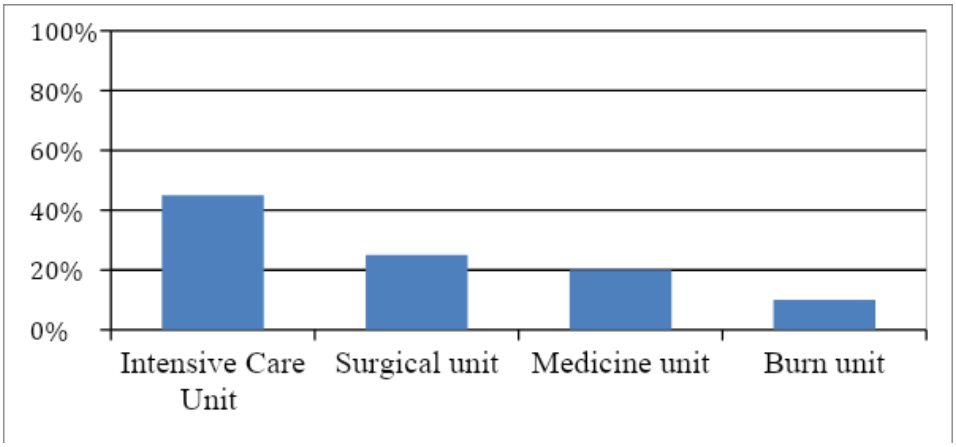

Figure 3: Clinical isolates among different departments of hospitals

4.4: Antibiotic Susceptibility Analysis:

Total 11 antibiotics were employed against each isolate to measure the zone of inhibition, and the data was interpreted using the range shown in Table 1 to determine whether the antibiotic was "sensitive," "intermediate," or "resistant." It was also found that satellite colonies were visible in several zones. Certain zones showed satellite colonies, which was also recognized carefully.

Figure 4*:* Antibiotic susceptibility testing of isolates against a panel of antibiotics.

Antibiotic resistance pattern of *A. baumannii* isolates:

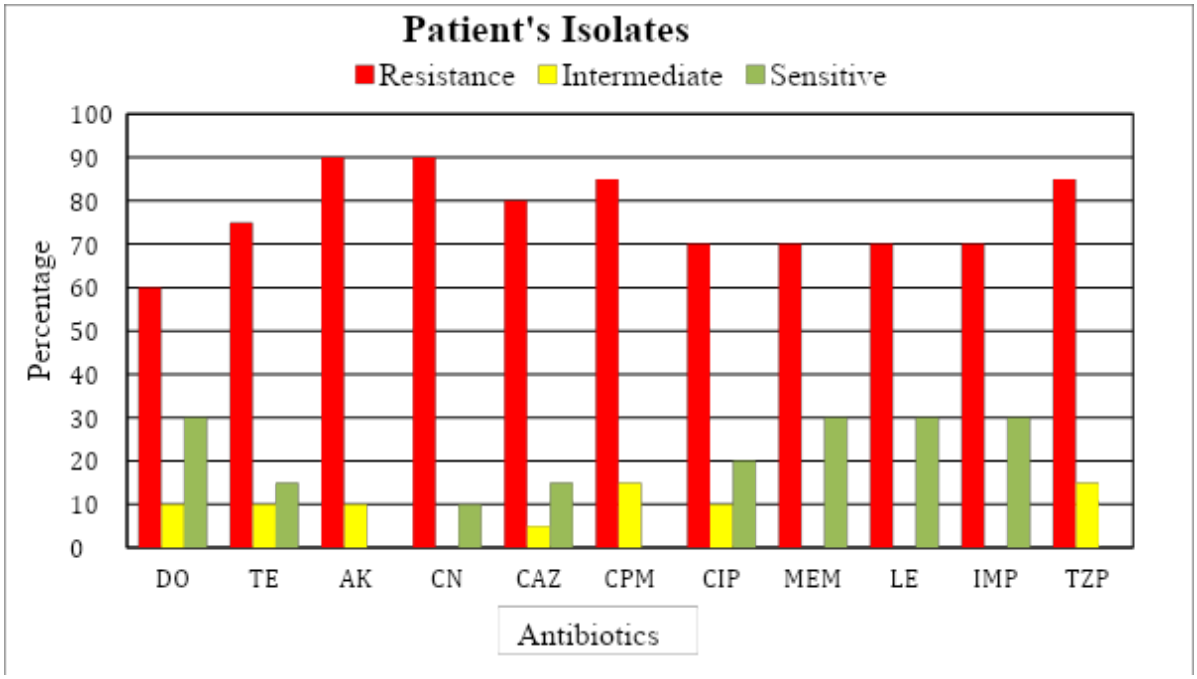

Figure 5: Antimicrobial susceptibility (AST) pattern against *Acinetobacter baumannii* isolates retrieved from patient's samples. doxycycline(DO), tetracycline(TE), amikacin(AK), gentamicin (CN), ceftazidime(CAZ), cefepime(CPM), ciprofloxacin(CIP), levofloxacin(LE), imipenem(IMP), meropenem(MRP), piperacillin-tazobactam(TZP).

The results of the antibiotic susceptibility test of patient isolates showed that 90% of isolates were resistant to amikacin (AK) and gentamicin (CN). When tested against cefepime, piperacillin-tazobactam (TZP), ceftazidime(CAZ), and tetracycline (TE), 85%, 85%, 80% and 75% of the isolates showed resistance, respectively. A significant proportion of the isolates, 70%, displayed resistant against levofloxacin(LE), imipenem(IMP), meropenem(MRP). Most importantly, 90% of all isolates were MDR.

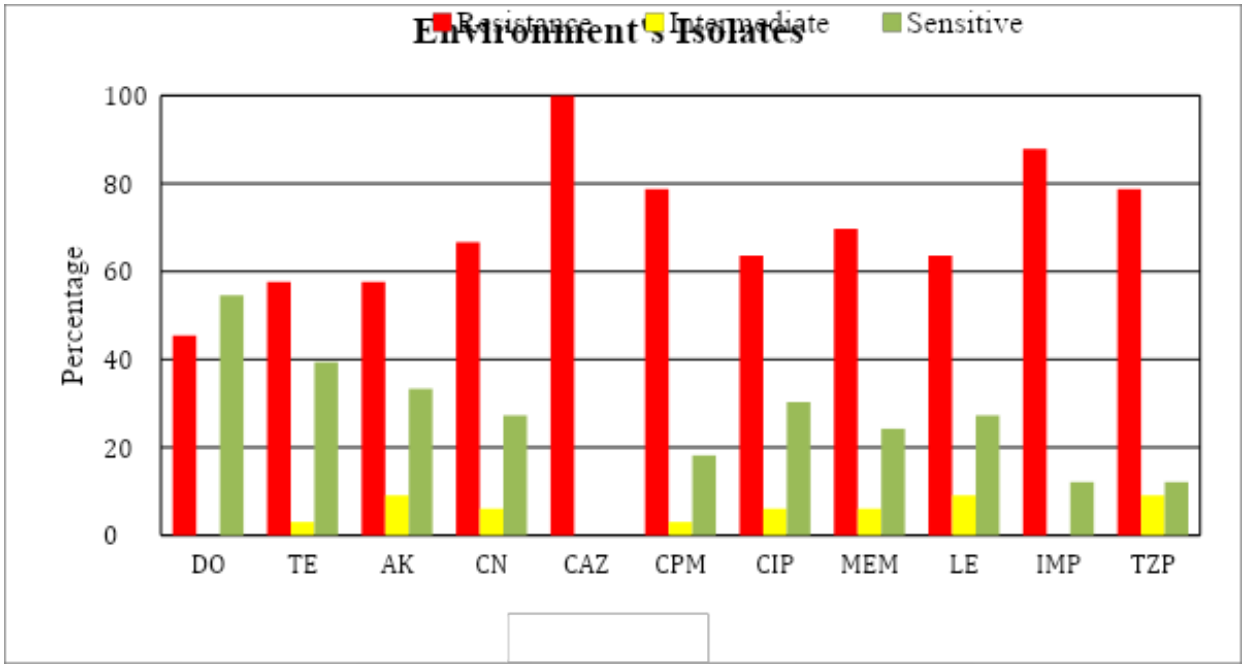

Figure 6: Antimicrobial susceptibility (AST) pattern against *Acinetobacter baumannii* isolates retrieved from hospital environment's samples. doxycycline(DO), tetracycline(TE), amikacin(AK), gentamicin (CN), ceftazidime(CAZ), cefepime(CPM), ciprofloxacin(CIP), levofloxacin(LE), imipenem(IMP), meropenem(MRP), piperacillin-tazobactam(TZP).

The results of the antibiotic susceptibility test of hospital environment's isolates showed that 100% of isolates were resistant to ceftazidime(CAZ). When tested against imipenem(IMP), piperacillin-tazobactam (TZP), and cefepime(CPM), 87.9%, 78.8%, and 78.8% of the isolates showed resistance, respectively. A significant proportion of the isolates, 69.7%, were resistant against meropenem(MRP). Then, 66.7 % isolates were resistance against gentamicin (CN). Most importantly, 85% of all isolates were MDR.

4.5: Serum resistance pattern:

The results of the serum bactericidal assay showed that 31% of isolates were serum resistant and 35% were sensitive and 34% were intermediate. Isolates from the environment's samples were 10% more resistant than the isolates from the patient's samples. Among environment isolates 36% isolates were resistant whereas among environment isolates only 26% were resistant.

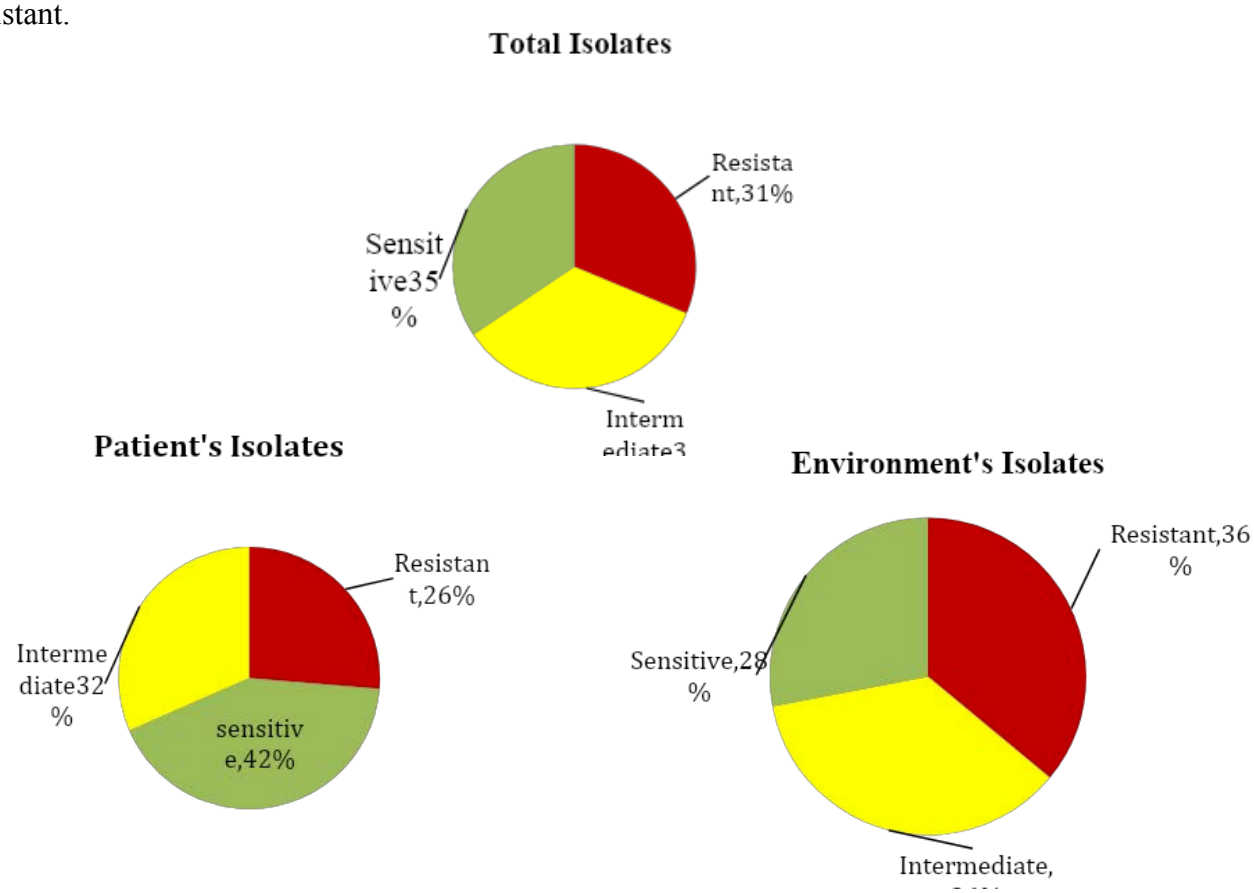

Figure 7: Serum bactericidal assay analysis against both patients and hospital environmental all 53 isolates.

4.6 Biofilm formation analysis:

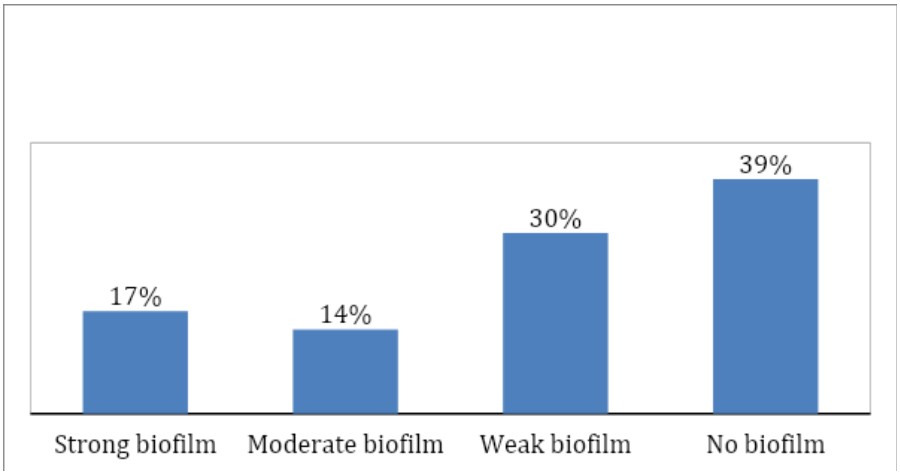

Figure 8: Evaluation of biofilm formation

On the basis of the ODc cut-off =0.23, the evaluation intensity of biofilm formation categorized as "no film", "weak film", "moderate film", and "Strong film" was done. Among total 33 environmental isolates 17% of isolates formed strong biofilm (4×ODc < OD), 14% formed moderate film (2×ODc< OD ≤ 4×ODc), and 30% formed weak (ODc < OD ≤ 2×ODc) and 39% isolates did not form biofilm. In total, 61% isolates were biofilm producers and 39% were non biofilm producers.

CHAPTER 5: DISCUSSION

In Bangladesh, the research on *A. baumannii* has primarily centered on ICU patients, neglecting its presence in the different wards and hospital environment. This lack of attention to them has resulted in a gap in knowledge of the cross contamination pattern between patient and different hospital settings and the pattern of its antibiotic resistance, serum resistance and biofilm formation. To fill this knowledge gap, in this study, we isolated 53 *A. baumannii* from hospital environments and admitted patients in hospitals from different wards between June to December 2023 aim to observe the antimicrobial resistance pattern of these isolates against antibiotics of various classes.

Out of 200 different patient specimens, 20 (10%) *Acinetobacter* isolates were identified. Similar results were reported by (Hanna et al., 2010) in Egypt, who showed that the isolation rate of *A. baumannii* from the clinical specimens including endotracheal aspirates, blood sample, Urine samples, Sputum samples, Pus samples etc was 10.6%. (Hanna et al., 2010)

Out of 250 different environment samples, 33 (13.2%) *Acinetobacter* isolates were identified. Similar findings were detected by (Farzana et al., 2022) in Bangladesh, who reported 10% (10 *A. baumannii* out of 100 samples) isolation rate of *A. baumannii* from different hospital environmental surfaces including bed rails, bed sheets, switchboards, sinks, blood pressure cuffs, ventilators, catheters, O2 masks, suckers, toilets, and sewage-drains (Farzana et al., 2022). However, these results disagreed with results of (Banerjee et al., 2005 and Hanna et al., 2010) that showed that isolation rate of *A. baumannii* from environmental samples were 23.33% and 22% respectively. (Banerjee et al., 2005 & Hanna et al., 2010). The difference could be explained by a distinct patient population with varying underlying illnesses related to time, temperature, weather, and environment.

According to the test result of antibiotic susceptibility test, out of 20 isolates from patient specimens 18 (90%) were multidrug resistant (MDR) showing resistance to three or more classes of antibiotics. The highest resistance was to gentamicin (90%) and amikacin (90%). The study of clinical samples of a hospital in Taiwan by (Liu et al., 2016) reported that 100% and 96% resistance against gentamicin and amikacin respectively. However, lower rates of resistance were reported against amikacin (66%) by (Somaia M. El-Shiekh et al., 2011) in

Egypt. In India, Prashanth and Badrinath (2004) piblished that 28.1% and 48.4% of *Acinetobacter* isolates were resistant to ceftazidime, and ciprofloxacin, respectively. In our study, the higher resistance rates were observed among ceftazidime (85%) and ciprofloxacin (70%). Variation of the resistance rate may be due to the over uses of the antibiotics, developing resistance mechanism by bacteria over this time period. Most importantly, in past years' antibiotic loses their potentiality to kill bacteria.

On the other hand, 85% of all isolates of hospital environments were MDR. 33(100%) isolates of hospital environment's isolates were resistant against ceftazidime. This result was in accordance with (Somaia M. El-Shiekh et al., 2011) that showed that ceftazidime was the most resistance antimicrobial agent against *A. baumannii* (100%). Against imipenem(IMP), piperacillin-tazobactam (TZP), and cefepime(CPM), 87.9%, 78.8%, and 78.8% of the isolates showed resistance, respectively. Lower rates of resistance were reported against imipenem (33%) in the study of hospital environment samples by (Enas A. Daef et al., 2013). Lack of studies of hospital environment *A. baumannii*, made it difficult to compare with others.

The results of the serum resistance assay showed that 31% of isolates were serum resistant, 34% were intermediate and 35% were sensitive. The study in 2009 showed 50% of *A. baumannii* were serum resistant after 3-hour incubation in NHS (King et al., 2009). Lower rates of resistance were observed in our study may be due to response of human immunity.

According to our biofilm formation results of 33 hospital environmental isolates, in total, 61% isolates were biofilm producers (17% strong biofilm, 14% moderate film, and 30% formed weak biofilm) and 39% were non biofilm producers. The study of Rodríguez‐Baño et al., found a similar result as ours that 63% isolates of *A. baumannii* formed biofilm in vitro, 33 (36%) did not form biofilm (Rodríguez-Baño et al., 2008).

In conclusion, *A. baumannii* is more challenging since it is becoming more resistant to drugs. It has an extensive record of creating biofilms, is closely associated with multi-drug resistance, and can spread transportable genetic material to other diseases that are clinically significant (Gallagher et al., 2020). Neglecting this organism can bring the moment when drastically change and spread out of them will cause significant threat to patient's physical, psychological, and financial well-being.

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