Antibiotic Resistance, Virulence and Biofilm Forming Capacity of *Acinetobacter baumannii* Isolated from Goranchatbari Sub-Catchment in Dhaka City

By

Nayeema Haque

ID- 22176001

A thesis submitted to the Department of Mathematics and Natural Sciences in partial fulfilment of the requirements for the degree of Master of Science in Biotechnology

Department of Mathematics and Natural Sciences

BRAC University

August 2023

Declaration

It is hereby declared that

1. The thesis submitted is my/our own original work while completing 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 which has been accepted, or submitted, for any other degree or diploma at a university or other institution.

4. I/We have acknowledged all main sources of help.

Sincerely Yours,

……..

Nayeema Haque

ID-22176001

Enrolling Semester- Spring 2022

Department of Mathematics and Natural Sciences

BRAC University, Dhaka, Bangladesh

Approval

The thesis "Antibiotic Resistance, Virulence and Biofilm Forming Capacity of *Acinetobacter baumannii* Isolated from Goranchatbari Sub-Catchment in Dhaka City" submitted by Nayeema Haque of Spring, 2022 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Masters of Science in Biotechnology on 6/8/2023.

Examining Committee:	
Supervisor (External)	_____ **Dr. Zahid Hayat Mahmud** **Scientist and Head,** **Laboratory of Environmental Health, icddr,b**
Supervisor (Internal) (Member)	_____ **Iftekhar Bin Naser** **Associate Professor, Biotechnology** **Department of Mathematics of Natural Sciences, BRAC University**
Program Director (Member)	_____ **Dr. Munima Haque** **Associate Professor, Biotechnology** **Department of Mathematics of Natural Sciences, BRAC University**
External Examiner (Member)	_____
Departmental Head (Chair)	_____ **Prof. A.F.M. Yusuf Haider** **Chairperson,** **Department of Mathematics and Natural Sciences, BRAC University**

Ethics Statement

I am Nayeema Haque, student of M.Sc., Department of Mathematics and Natural Sciences, BRAC University, do hereby declare that the thesis on **"Antibiotic Resistance, Virulence and Biofilm Forming Capacity of *Acinetobacter baumannii* Isolated from Goranchatbari Sub-Catchment in Dhaka City"** is an original and authentic record of my research work carried out by me for the degree of Master of Science in Biotechnology, under the joint supervision and guidance of Dr. Zahid Hayat Mahmud, Scientist and Head, Laboratory of Environmental Health, Health System and Population Studies Division, International Centre for Diarrheal Disease Research, Bangladesh (icddr,b) and Dr. Iftekhar Bin Naser, Associate Professor, Biotechnology, Department of Mathematics and Natural Sciences, BRAC University, Dhaka, Bangladesh.

It has not been submitted by me for any other degree.

Sincerely Yours,

……..

Nayeema Haque

ID-22176001

Enrolling Semester- Spring 2022

Department of Mathematics and Natural Sciences

BRAC University, Dhaka, Bangladesh

Abstract

The presence of ESBL producing *Acinetobacter baumannii* is a major concern for various hospital and community healthcare settings, often being linked to an increased incidence of nosocomial infections. The biofilm formation ability of *A. baumannii* enables it to be a persistent pathogen in these environments Furthermore, there is some evidence that environmental transport of *Acinetobacter* spp. plays a role in the spread of clinically relevant *Acinetobacter* strains in the environment. The objective of this study is to isolate ESBL positive *A. baumannii* from environmental samples, molecular confirmation of the presence of major ESBL and virulence genes, determination of antibiotic susceptibility profile and biofilm formation assay of the ESBL producing isolates. In this study we investigated the molecular characterization of ESBL forming *A. baumannii* in aquatic environment. Between April and December 2022, with 3 rounds of sample collected on 3 different seasons a total of 21 samples were collected from environmental sources and finally the study was conducted using 56 ESBL producing *A. baumannii* isolates. ESBL *A. baumannii* was isolated from those samples and the isolates were subjected to antibiotic susceptibility, PCR analysis for presence of resistance and virulence genes, and lastly biofilm formation assay. A total of 56 ESBL producing *A. baumannii* were isolated among them, 71.4% were positive for bla_{TEM} gene, 5.4% were positive for bla_{SHV} gene. Among the virulence factors, 76.8% isolates harbored the *pgaB* gene, 69.6% *bfmS*, 64.3% *csuE*, 62.5% *ompA*, 28.6% *kpsMII*, 26.8% *ptk* and *epsA* genes, 10.7% *fimH* and 5.4% *bap* gene. In regard to antibiotic susceptibility testing, 8.9% of the isolates were resistant to cefotaxime, 5.4% to cotrimoxazole and piperacillin/tazobactam, 1.8% tetracycline. On the other hand, 91.1% of the isolates were intermediately resistant to cefotaxime and 87.5% to ceftriaxone. Out of all the 56 isolates one isolate was found to be multi-drug resistant (MDR). In case of biofilm formation, at a temperature of 25ºC, 1.78% isolates were found to be strong biofilm formers, 7.14% isolates were moderate biofilm formers, 66.07% were weak biofilm formers. At a temperature of 37ºC, 1.78% isolates were moderate biofilm formers, 76.78% isolates weak biofilm formers. This study suggests that the ESBL producing *A. baumannii* is spreading from the healthcare settings to the environments at a rapid speed in Bangladesh, acting as a potential reservoir for AMR bacteria. The information may have a profound effect in contributing to the improvements in public health care policies, which are a necessity to combat the spread of antibiotic resistant strain in the environment.

Keywords: *A. baumannii*, Environmental Samples, Biofilm, ESBL, Virulence Factor

Dedication

Dedicated to my family for their love and support

Acknowledgment

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Sincerely,

Nayeema Haque

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List of Abbreviation

ESBL	Extended Spectrum ß- Lactamase
ATCC	American Type Culture Collection
CFU	Colony Forming Unit
CLSI	Clinical and Laboratory Standards Institute
DNA	Deoxyribonucleic acid
PCR	Polymerase Chain Reaction
KPC	*Klebsiella pneumoniae* Carbapenamase
ompA	Outer membrane protein
epsA	Extra polysaccharide protein
WHO	World Health Organization
TE	Tris EDTA
TBE	Tris Borate EDTA
bla	ß-lactamase
MDR	Multidrug Resistant
OD	Optical Density
bp	Base pair
spp.	Species (plural)
sp.	Species (singular)
AMR	Antimicrobial Resistance
XDR	Extensively Drug Resistant
icddr,b	International Center For Diarrheal Disease Research, Bangladesh

Chapter One
INTRODUCTION

1.0 Introduction

The development of antibiotic resistance in bacteria has emerged as a significant global concern for public health in the past few decades. The Centre for Disease Control and Prevention (CDC) documented that only in the U.S each year more than 2.8 million antimicrobial infections occur and consequently more than 35,000 casualties occur (Kadri, 2020). The reported number of deaths due to resistant infection is at least 700,000 globally. Also, the fact that the actual number is most likely to a significantly higher due to poor reporting and surveillance. This puts into perspective of how life threatening the scenario has become. The crisis has worsened specially after 2020 due to the COVID-19 pandemic (Tanne, 2022). If the problem of antibiotic resistance remains unresolved by the year 2050, it is projected that public health will be severely affected, leading to a staggering number of over 10 million deaths annually (Murray et al., 2022). Resistance can occur due to several biochemical and physiological factors. One of the main reasons for the absence of progress in the prevention and management of resistance development is the lack of basic understanding on the mechanisms that contribute to the establishment and spread of resistance in the antimicrobial agents. The major issues behind this crisis are the mishandling of existing antibiotics and the slow development of new ones. The widespread of antibiotic resistance will jeopardize the health care system and soon a post-antibiotic age will come where infections are a luxury to treat. The emergence of antibiotic resistance poses a threat not only to public health but also to the economic stability of healthcare systems and national security (Ventola, 2015).

One of the primary mechanisms by which bacteria develop antibiotic resistance is through the production of specific enzymes, such as β-lactamases, which can break down the four-atom β-lactam ring found in β-lactam antibiotics. The Extended Spectrum Beta-Lactamases (ESBL) has emerged due to a mutation in the β-lactamase enzymes. ESBLs are a rapidly evolving group of beta-lactamases that are mediated by plasmids. They can hydrolyze a wide range of antibiotics i.e., penicillin, 1^{st}, 2^{nd}, 3^{rd} generation cephalosporins and aztreonam (Malloy & Campos, 2011). Being plasmid mediated it has played a role in the high-speed spread form one organism to another. The extensive use of the 3^{rd} generation cephalosporins (e.g. cefotaxime, ceftriaxone, ceftazidime) are responsible for the emergence of the ESBLs (Kaur & Singh, 2018). There are three primary ESBL enzymes, mainly *bla*$_{\text{CTX-M}}$, *bla*$_{\text{SHV}}$ *and bla*$_{\text{TEM}}$, which share an approximate 25% homology (Paterson & Bonomo, 2005). This high resistance to aminoglycosides, fluoroquinolones,

ureidopenicillins, and 3rd generation cephalosporins has led to the increased use of Carbapenems to treat these infections (Kaur & Singh, 2018).

Gram-negative bacteria, particularly *A. baumannii*, are frequently linked with the presence of extended-spectrum β-lactamases (ESBLs). *A. baumannii* is an opportunistic pathogen that is commonly found in healthcare facilities like hospitals. Antibiotic resistance is brought on by a class of potent Metallo Beta Lactamases (MBLs) enzymes known as carbapenemases. *A. baumannii* has been found to possess four groups of these enzymes, namely IMP-like, SIM-1, NDM-type, and VIM-like carbapenemases. Genetic analysis of *A. baumannii* isolates carrying metallo-beta-lactamases (MBLs) has shown the presence of genes such as bla_{SIM}, bla_{IMP} and bla_{VIM}, which encode these enzymes (Poirel et al., 2011). ESBLs are encoded by genes such as TEM-type, SHV-type, and CTX-M-type, which are responsible for conferring phenotypical resistance to penicillin and third-generation cephalosporins (Safari et al., 2015).

The Centers for Disease Control and Prevention (CDC) reports that multidrug-resistant (MDR) *A. baumannii* is associated with approximately 7,300 infections and 500 deaths annually. This bacterium is particularly notable as a pathogen responsible for hospital-acquired pneumonia, wound infections, and sepsis (Centers for Disease Control and Prevention, 2013). In 2017, the World Health Organization (WHO) designated *A. baumannii* with the highest priority level of critical when it issued its overall priority list of antibiotic-resistant bacteria. This classification was primarily based on the inadequate treatment alternatives currently available or under development for combating infections caused by this bacterium (Shrivastava et al., 2018). Treatment of *A. baumannii* infections are challenging because of the rise of multi-drug, pan drug, and extended drug-resistant strains of *A. baumannii* that are resistant to practically all classes of antibiotics. *A. baumannii* is an opportunistic pathogen that primarily affects critically adverse patients in intensive care units (ICUs) and causes a variation of infections. *A. baumannii* has been connected more frequently to constant wound infections, particularly in burn victims and injuries sustained in combat. Hospital-acquired infections triggered by *A. baumannii* encompass a range of conditions, including ventilator-associated pneumonia, wound infections, skin infections, urinary-tract infections (UTIs), bloodstream infections and secondary meningitis (Morris et al., 2019). *A. baumannii* acquired in healthcare settings (nosocomial) has been recognized as a causative agent of wound infections in individuals affected by both natural and man-made calamities (Dijkshoorn

et al., 2007). *A. baumannii* has frequently been isolated from injuries of martial personnel serving in Afghanistan or Iraq (Calhoun et al., 2008). Ventilator-associated pneumonia and bloodstream infection (bacteremia) are the most prevalent nosocomial infections linked with *A. baumannii*. These infections are known to have high morbidity and mortality rates.

Apart from their multi-drug resistance, *A. baumannii* also possesses the capability to form biofilms on both living (biotic) and non-living (abiotic) surfaces. Additionally, they have the ability to colonize skin epithelial cells, which poses a challenge to the efficacy of current treatments for wound infections. Biofilms are aggregates of bacteria on surfaces and is an important colonization strategy, leading to the occurrence of various diseases and difficulty in eradication (Beloin et al., 2008). The growth in biofilms has been shown to enhance the survival of bacterial population in hospital settings, leading to an heightened incidence of nosocomial infections (Lebeaux et al., 2014; Percival et al., 2015). The resistance to antimicrobial agents has been shown to be enhanced up to a 1000-fold as compared to planktonic cells. This increased resistance is thought to be due to reduced drug penetration and presence of an extra polymeric substrate layer which can act as a physical barrier. This leads to persistence of biofilms on surfaces which are difficult to treat, even upon the use of multiple antibiotics (Singla et al., 2013)

1.1 Background of the Study

The genus *Acinetobacter* is liable for many nosocomial outbreaks worldwide. It is reported that the intensive care unit (ICU) mortality rate due to *Acinetobacter* infection is around 40% (Alsan & Klompas, 2010). In the recent years, more than 210 different β-lactamases have been identified within this genus (W.-H. Zhao & Hu, 2012). Different Oxacillinases (OXA) enzyme families have their origin in *Acinetobacter* spp. This set of enzymes are the cause of resistance to new developed β-lactam antibiotics in *Acinetobacter baumannii* (Poirel & Nordmann, 2006). As a result, *Acinetobacter baumannii* has been regarded as one of the most challenging nosocomial pathogens. The WHO rated *Acinetobacter* to be the group in utmost crucial need of new antibiotics followed by *Pseudomonas* and carbapenem-resistant Enterobacteriaceae (Evans & Amyes, 2014; Santajit & Indrawattana, 2016). Occurrence and sensitivity of *Acinetobacter* spp. in medical settings is well reported, however their distribution and pattern of resistance in the environment remains inadequately documented (Maravić et al., 2016). There is evidence indicating that the

environmental movement of *Acinetobacter* contributes to the dissemination of clinically relevant *Acinetobacter* strains in aquatic environments. Conversely, in clinical settings, there is a constant influx of new strains that have the potential to exhibit distinct infectious characteristics (Wilharm et al., 2017). With the widespread of resistance among the clinical novel strains, the environment is in constant risk of getting exposed to it.

1.2 Objective of the Study

The primary goal of this study is to identify and detect *A. baumannii* strains in environmental samples that are capable of producing ESBLs. The objectives are given below:

- Isolation of ESBL positive *A. baumannii* from environmental samples
- Confirmation of major ESBL genes by PCR.
- Molecular detection of virulence genes.
- Determination of antibiotic susceptibility profile of the ESBL isolates.
- Biofilm formation assay of all the ESBL producing isolates.

Chapter Two

LITERATURE REVIEW

Literature Review

2.1 The Organism: *A. baumannii*

2.1.1 Historical Background

In 1911, a microbiologist from the Netherlands named Beijerinck M.W. isolated a bacterium called *Micrococcus calcoaceticus* from soil using a calcium acetate-enriched minimal medium (Howard et al., 2012) However, in 1954, Brisou and Prevott introduced the genus *Acinetobacter* to distinguish this bacterium from other motile organisms within the *Achromobacter* genus (Brisou & Prevot, 1954). The acceptance of the genus *Acinetobacter* came after a comprehensive study conducted by Baumann et al. in 1968. This study involved the examination of several organisms, including *Micrococcus calcoaceticus, Alcaligenes hemolysans, Mima polymorpha, Moraxella lwoffi, Herellea vaginicola, and Bacterium anitratum*. Baumann's study concluded that the organisms under investigation belonged to a single genus, and it was not possible to further classify them into different species based on phenotypic characteristics. The publication by Baumann in 1968 on the genus *Acinetobacter* was subsequently recognized by the sub-committee on the Taxonomy of Moraxella and Allied Bacteria, solidifying the acceptance and recognition of the *Acinetobacter* genus (Lessel, 1971).

In 1986, Bouvet and Grimont discovered inconsistencies when using phenotypic tests to identify the genus and species of *Acinetobacter*. This was due to the fact that *Acinetobacter* members possess different catabolic pathways, allowing them to adapt to a wide range of substrates (Bouvet & Grimont, 1986). To address this issue, DNA hybridization studies were introduced as a more reliable method to distinguish between species. These studies focused on DNA-DNA relatedness, classifying groups with over 70% similarity as genomic species. Presently, there are 32 recognized genospecies within this genus. The *Acinetobacter calcoaceticus – A. baumannii* (ACB complex) encompasses four genospecies: genospecies 1 (*A. calcoaceticus*), genospecies 2 (*A. baumannii*), genospecies 3 (*A. pittii*), and genospecies 13TU (*A. nosocomialis*) (Peleg et al., 2008). Among these, *A. baumannii* holds the greatest clinical significance as it is associated with hospital epidemics and nosocomial infections (Cerqueira & Peleg, 2011).

2.1.2 Taxonomy

The taxonomy of *A. baumannii* is as follow:

Domain: Bacteria
Phylum: Proteobacteria
Class: Gammaproteobacteria
Order: Pseudomonadales
Family: Moraxellaceae
Genus: *Acinetobacter*
Species: *A. baumannii*

2.1.3 Morphology, Physiological and Biochemical Characteristics

Acinetobacter is a type of bacteria that is classified as Gram-negative, catalase-positive, oxidase-negative, non-motile, and non-fermenting coccobacilli. Even though *Acinetobacter* is typically regarded as non-motile, there have been reports of observed differences in motility in response to factors such as illumination, (Mussi et al., 2010), quorum sensing (Clemmer et al., 2011), and iron chelation (Eijkelkamp et al., 2011). While the exact mechanisms of movement in *Acinetobacter* species are not definitively established, there is evidence to suggest that they may exhibit twitching motility on semi-solid surfaces rather than other forms of motility such as gliding, sliding, swimming, or swarming. However, additional study is needed to fully comprehend and confirm these observations (Eijkelkamp et al., 2011). One characteristic that complicates the identification of *Acinetobacter* is its resistance to de-staining during Gram staining, leading to misidentification as Gram-positive. Presently, no specific metabolic tests are available to clearly distinguish *Acinetobacter* from previous non-fermenting Gram-negative bacteria (Peleg et al., 2008). The negative oxidase test is a commonly employed method for rapid presumptive detection to distinguish *Acinetobacter* from other non-fermenting organisms with similar characteristics. This test helps differentiate *Acinetobacter* by determining the absence of the enzyme oxidase in the bacterial species being tested.

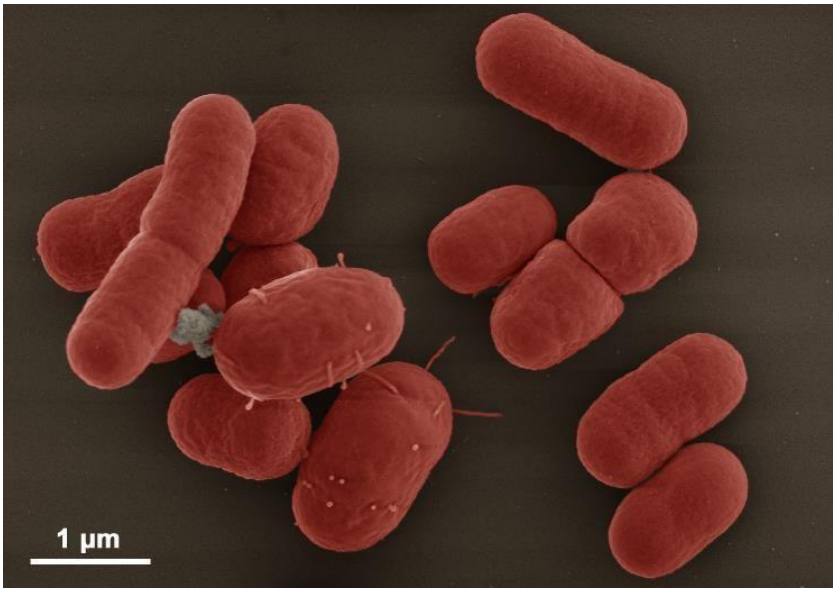


Figure 2.1: Scanning electron microscopy of *A. baumannii* (Retrieved from: © Gudrun Holland)

Acinetobacter is a bacterium that can readily grow on uncomplicated microbiological media. The colonies exhibit specific features such as a smooth, dome-shaped appearance with a pale yellow to grayish color and a distinct, unbroken side (Doi et al., 2009). In case of the *A. calcoaceticus-A. baumannii* complex, the colonies can reach a size of 1.5 to 3 mm in diameter after overnight culture, like Enterobacteriaceae species. On the other hand, most other *Acinetobacter* species generate smaller colonies (Peleg et al., 2008). While most *Acinetobacter* species can grow at normal room temperature, the pathogenic species *A. baumannii* thrives particularly well at 37°C, which is the typical body temperature. When recovering *Acinetobacter* isolates from composite samples, an enrichment medium like Leeds selective medium is commonly employed. (Doi et al., 2011). In the research conducted by Baumann et al. (1968), it was observed that Acinetobacter strains obtained from both environmental and clinical samples could be effectively cultured under aerobic conditions using an acidic enrichment medium supplemented with acetate and nitrate as a carbon and nitrogen source, respectively. This cultivation approach facilitated the growth of *Acinetobacter* strains in the laboratory setting. A distinguishing characteristic of *A. baumannii* is its possession of a thick cell wall, which offers protection against dry conditions and provides enhanced tolerance to variations in temperature, pH, and nutrient availability (Vila et al., 2007).

2.1.4 Natural Habitat

Different species of the *Acinetobacter* genus are commonly found in diverse environments, including soil and surface water samples (Baumann, 1968). However, not all species naturally inhabit the environment. (Peleg et al., 2008). Reports implies that *A. baumannii*, in particular, is not commonly found in the environment and has a low occurrence in the community (Dijkshoorn et al., 2005). Nonetheless, it has been detected in vegetables, fish, meat, and soil. (Houang et al., 2001). *A. baumannii* is seldom found as part of the normal fecal flora in individuals within the community, nor is it typically a part of the normal microflora on the skin. (Dijkshoorn et al., 2005), as a pathogenic bacterium, it exhibits a specific affinity for moist tissues like mucous membranes (Sebeny et al., 2008). *A. baumannii* is found normally in human clinical samples (Dijkshoorn et al., 2007). *A. baumannii* has been identified as a significant concern in field hospitals due to its infection of the environment and the subsequent transmission of infections in healthcare settings (Scott et al., 2007). One remarkable characteristic of *A. baumannii* is its capability to persist on dry surfaces for extended periods enhances its potential for nosocomial spread (Fournier et al., 2006).

A. baumannii has been observed to infect the skin, nostrils, and throat of healthy individuals. (Montefour et al., 2008). Unclean hands of hospital staff have been identified as a frequent path for *A. baumannii* transmission within the hospital surroundings (Jalalpour and Abdol, 2012). Once colonized, *A. baumannii* strains can persist in patients' bodies for weeks, underscoring the importance of strict personal hygiene and cleanliness to prevent pathogen transmission (Dijkshoorn et al., 2007). Hence, practicing rigorous personal sanitation is crucial in avoiding the transmission of this pathogen (Allegranzi et al., 2009). In addition to humans, research suggests that body lice, particularly in homeless individuals, can serve as a potential reservoir for *A. baumannii*, as 22% of tested lice samples were found to carry the bacterium. The hospital environment serves as a significant source of *A. baumannii*, as it possesses the ability to thrive under diverse temperature and pH conditions within healthcare settings, as well as its resilience against unfavorable factors such as desiccation, nutrient scarcity, and disinfectant treatments, contribute to its persistence (Gaddy & Actis, 2009). *A. baumannii* achieves its preservation in the hospital environment through the formation of biofilms on non-living surfaces. These biofilms provide protection against disinfectants, allowing the pathogen to persist for extended periods and

serve as a constant cause of infection (Pour et al., 2011). Various surfaces commonly found in healthcare settings, such as tabletops, bed rails, sinks, door handles, floors, mattresses, and pillows, have been associated as probable sources of *A. baumannii* (Wilks et al., 2006). Contaminated medical instruments, including catheters, sutures, and ventilators, have also been reported as sources of *A. baumannii* in hospitals (H.-W. Lee et al., 2008). The use of such devices can lead to ensuing colonization of the bacterium in different parts of the body, including the respiratory tract, blood, pleural fluid, urinary tract, surgical wounds, skin, and eyes of patients (Lorente et al., 2004). Therefore, the preservation of *A. baumannii* in the clinical settings is considered a crucial issue contributing to the transmission of infections in nursing homes and other healthcare settings (Rastogi et al., 2007).

2.2 Clinical Significance

A. baumannii is swiftly becoming a prominent multidrug-resistant bacterium and is often regarded as the archetype of antibiotic-resistant bacteria. Clinicians in intensive care units (ICUs) face significant challenges due to the severity of clinical conditions and the prevalence of multidrug resistance. *Acinetobacter* is commonly present in the hospital environment, colonizing the skin and mucous membranes of patients, and contributing to the spread of healthcare-associated infections. This bacterium possesses three key factors that contribute to its resilience: resistance to major antimicrobial agents, resistance to desiccation, and resistance to disinfectants (Seifert & Dijkshoorn, 2008).

2.2.1 Urinary Tract Infections

A. baumannii is not commonly responsible for urinary tract infections (UTIs), with only a 1.6% occurrence in UTIs acquired in ICU settings, primarily associated with catheter-related infections or colonization. However, there has been a gradual rise in the incidence of UTIs caused by *A. baumannii* (Weinstein et al., 2005). The presence of medical instruments like endotracheal tubes, intravascular catheters, ventricular catheters, or urinary catheters often creates an opportunity for opportunistic bacteria to colonize these sites (Joly-Guillou, 2005). It is not uncommon for *A.*

baumannii to cause uncomplicated UTIs in individuals who are otherwise healthy and not in a healthcare setting. (Peleg *et al*., 2008).

2.2.2 Meningitis

Acinetobacter is not commonly associated with meningitis, but there is a growing recognition of post-neurosurgical *A. baumannii* meningitis as a significant and concerning condition, with reported mortality rates reaching as high as 64% (García -Garmendia et al., 2001). Sporadic cases have been observed after neurosurgical trial (S. Chen et al., 2005). A concerning occurrence of Acinetobacter meningitis was reported children with leukemia following the processing of intrathecal methotrexate. Tragically, three children lost their lives due to the use of improperly sterilized needles. Several risk aspects for meningitis were identified, including the existence of a persistent linking between the brain ventricles and the external environment, ventriculostomy, cerebrospinal fluid (CSF) fistula, prolonged use of indwelling ventricular catheters exceeding 5 days, and the use of antimicrobial agents. These factors contributed to the susceptibility of the affected individuals to Acinetobacter meningitis. Turkey has seen a significant frequency of multidrug-resistant *Acinetobacter* causing meningitis in neurosurgical patients. (Metan et al., 2007). Proper therapy selection guided by local pathogen surveillance, along with an effective infection control program, is crucial in neurosurgical wards.

2.2.3 Suppurative Infections

Currently, the prevalence of *Acinetobacter* infections affecting the skin and soft tissues has become a significant concern. One reason for this may be that *Acinetobacter* species are the only Gram-negative bacteria naturally found on the human skin, as discussed by Seifert et al. (1997). According to Gaynes and Edwards (2005), *Acinetobacter* is responsible for approximately 2.1% of skin and soft tissue infections acquired in intensive care units (ICUs). Managing and eliminating this pathogen from burns units poses substantial challenges (Trottier et al., 2007). In the context of combat fatalities from Iraq or Afghanistan, *A. baumannii* is commonly isolated from wound infections (Peleg *et al*., 2008). Although it is frequently encountered in patients with open tibial

fractures, its low pathogenicity at this specific site allows for complete eradication. (Johnson et al., 2007).

2.2.4 Bloodstream Infections

A. baumannii accounted for 1.3% of all nosocomial bloodstream infections in the United States, making it the 10th most common causative agent (Peleg *et al*., 2008). In terms of bloodstream infections, *A. baumannii* was more frequently associated with infections acquired in the intensive care unit (ICU) compared to non-ICU wards (1.6% versus 0.9%, respectively). The crude mortality rate for bloodstream infections caused by *A. baumannii* varied between 34.0% and 43.4% in the intensive care unit (ICU) setting and 16.3% outside the ICU. Among ICU patients, *A. baumannii* bloodstream infections ranked third in terms of crude mortality rate, with higher rates observed only in infections caused by *P. aeruginosa* and *Candida* spp. It is noteworthy that *A. baumannii* infections typically occurred relatively late during hospital stays, with an average of 26 days from the time of admission (Peleg *et al*., 2008). The exact cause of the high crude mortality rate associated with A. baumannii bloodstream infections remains uncertain. It is unclear whether the occurrence of these infections in patients with existing critical illnesses contributes to the high mortality rate, or if the organism itself plays a significant role in the attributable mortality. Furthermore, between January 2002 and August 2004, a total of 102 cases of A. baumannii bloodstream infections were reported in healthcare facilities providing treatment to U.S. military members injured in Iraq or Afghanistan. (Peleg *et al*., 2008). Recent reports indicate that *A. baumannii* demonstrates resistance to a range of antibiotics, and importantly, *A. baumannii* bloodstream infection has a worse prognosis compared to bloodstream infections caused by other isolates (N.-Y. Lee et al., 2010).

2.2.5 Nosocomial (Hospital-acquired) Infections

Nosocomial infections, also known as hospital-acquired infections or healthcare-associated infections (HAI), refer to infections that are not present upon hospital admission but are acquired during the hospital stay (Monegro et al., 2022).

A. baumannii, an opportunistic pathogen, is known to cause a variety of infections, primarily affecting seriously ill patients in intensive care units (ICUs). These infections encompass ventilator-associated pneumonia, skin and soft tissue infections, wound infections, urinary tract infections (UTIs), secondary meningitis, and bloodstream infections (Morris et 15 al., 2019). In addition to nosocomial settings, *A. baumannii* has been observed to cause wound infections in individuals injured during both natural and man-made disasters. This highlights the pathogenic potential of *A. baumannii* outside of healthcare environments and its ability to contribute to wound infections in diverse settings (Dijkshoorn et al., 2007). *A. baumannii* has also been implicated in wound infections among individuals injured during natural or man-made disasters, particularly in military personnel deployed to Iraq or Afghanistan. Ventilator-associated pneumonia and bloodstream infections are the most prevalent and carry significant morbidity and mortality rates (Seifert et al., 1995).

The transmission of nosocomial *A. baumannii* infections can occur through various means. Host-related risk factors that contribute to susceptibility include major operation or trauma (particularly burn trauma), immunosuppression, advanced age, and prematurity in newborns (Dijkshoorn et al., 2007). Factors related to exposure include prior or prolonged hospital or ICU stays, residency in units with endemic *A. baumannii*, and exposure to contaminated clinical devices (Dijkshoorn et al., 2007). Medical treatment-related risk aspcets include mechanical ventilation, the presence of medical instruments, the frequency of invasive techniques, and former antimicrobial therapy (Dijkshoorn et al., 2007).

2.2.5 Community-Acquired Infection

While *A. baumannii* is generally considered a nosocomial pathogen, it can also be a notable cause of community-acquired pneumonia in tropical regions. In these areas, *A. baumannii*-associated pneumonia has been linked to a high mortality rate, reaching up to 64%. Such infections often occur in persons with underlying disorders like alcoholism, diabetes mellitus, smoking, and chronic lung disease, which can increase their susceptibility to *A. baumannii* pneumonia (Dexter et al., 2015). The exact contribution of host factors versus bacterial virulence factors in the development of these infections remains uncertain (Morris et al., 2019).

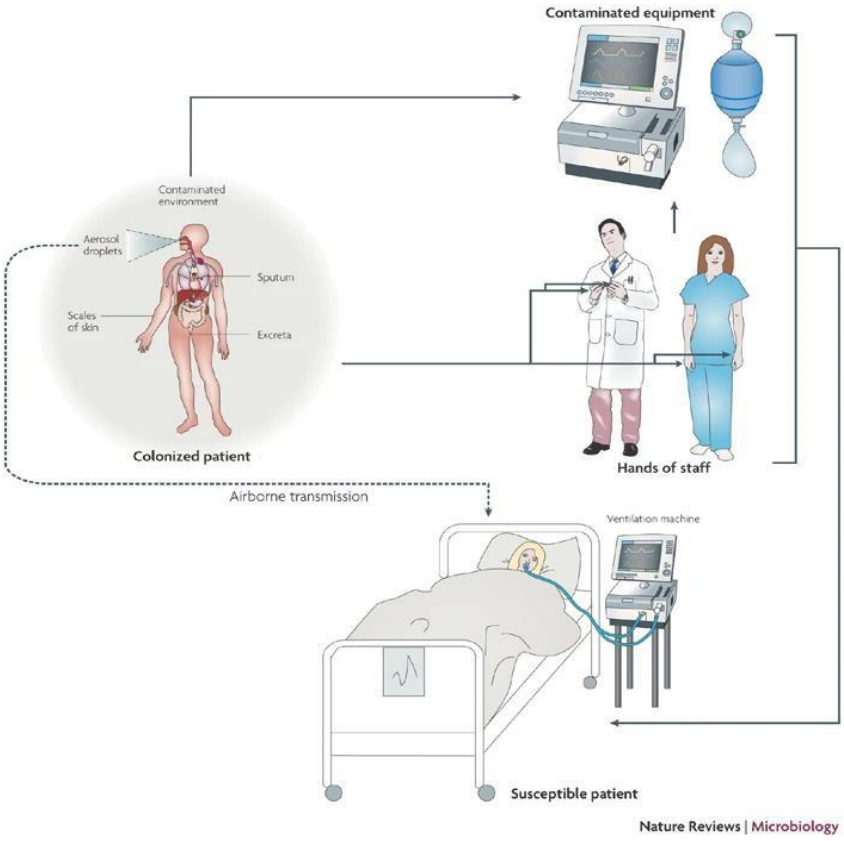


Figure 2.2: Different modes for transmission of nosocomial (hospital-acquired) infections caused by *A. baumannii.* Figure adapted with permission from Dijkshroon et. al., (2007); copyright 2022 Springer Nature.

2.2.6 Other Manifestations

Endocarditis caused by *Acinetobacter* is a rare but severe condition with a high mortality rate, predominantly observed in hospitalized patients who have underlying risk factors. *Acinetobacter* species can also lead to infections such as endophthalmitis or keratitis, which may be linked to the use of contact lenses or following eye operation (Corrigan et al., 2001). There have been reported cases of *Acinetobacter* infections arising invasive ocular procedures. Although ocular infections are uncommon, recent research has identified a potential association between *Acinetobacter* and adverse ocular reactions in individuals using extended-wear soft contact lenses. (Corrigan *et al*., 2001). Additionally, there are documented instances of *A. baumannii* causing perceptual and orbital cellulitis, including a case involving a 39-year-old woman (Miller, 2005). Another notable case involves a 3-month-old infant at Pereira Rossell Pediatric Hospital in Uruguay, who

experienced bloody diarrhea associated with a strain of A. haemolyticus producing Shiga toxin-2 (Grotiuz et al., 2006).

2.3 Pathogenicity of *Acinetobacter* Infections and Virulence

The precise mechanisms underlying the pathogenesis of *A. baumannii* infections are not yet entirely elucidated, and there is ongoing research to further understand its colonization, infection, and systemic transmission. Various potential mechanisms have been proposed, emphasizing special functions in colonization, infection, and the spread of epidemics. These mechanisms involve adherence to host cells, resistance to desiccation, disinfectants, and antibiotics, the formation of biofilms, quorum sensing, as well as the induction of inflammatory responses and cytotoxicity. Further studies are required to fully comprehend the intricate interplay between *A. baumannii* and its host during infection (Dijkshoorn et al., 2007). One critical early step in the colonization and infection process is the attachment of the bacteria to epithelial cells. A study by Choi et al. (2008) demonstrated that *A. baumannii* exhibits the ability to attack epithelial cells, although the extent of invasion may differ depending on the specific type of host cells involved. Research has indicated that respiratory tract epithelial cells are more vulnerable to invasion by *A. baumannii* compared to other types of epithelial cells. This suggests a potential preference of *A. baumannii* for respiratory epithelial cells when it comes to invasion.

2.3.1 *Acinetobacter* Adhesins and Fimbriae

During the initial stage of infection, the microorganism's capacity to infiltrate the host and commence an infection depends on its capability to adhere to and persist on the mucosal surfaces of the host. Adherence to these surfaces is crucial for the establishment of infection by providing the microorganism with access to host cells and resources necessary for survival and proliferation. Bacterial adhesins, such as fimbriae (pili), capsular polysaccharides, and cell wall components, play a crucial role in facilitating the attachment of bacteria to various surfaces. (Bergogne-Bérézin et al., 2008). *A. baumannii* demonstrates a natural ability to adhere to bladder tissue, similar to apathogenic strains of *Escherichia coli* (Sepulveda et al., 1998). Type 1 fimbriae-expressing *A. baumannii* strains exhibit erythrocyte agglutination independent of D-mannose. Under

transmission electron microscopy, fimbrial structures can be observed on *A. baumannii*, and the strains' haemagglutinating activity remains unaffected by D-mannose or D-galactose. (Sepulveda *et al*., 1998). Adherence of *A. baumannii* to human bronchial epithelial cells occurs through two distinct patterns: dispersed adherence, where individual bacteria attach to the cell surface, and clustered adherence, forming microcolonies in specific regions of the cell. Scanning electron microscopy reveals thin fimbria-like extensions firmly anchored to the cell membrane surface. There is no significant difference in adherence between outbreak and non-outbreak strains (Y.-T. Lee et al., 2009). The presence of a chaperone usher secretion system is crucial for fimbriae formation, enabling attachment to plastic surfaces and subsequent biofilm development (Tomaras et al., 2003). There is a proposed association between the PER-1 gene, which encodes a beta-lactamase enzyme, and the adhesion of *Acinetobacter* strains to Caco-2 cells (Sechi et al., 2004).

2.3.2 Cell surface Hydrophobicity and Enzymes

A. baumannii exhibits notable cell hydrophobicity, which aids in its adherence to host cells and evasion of phagocytosis. This characteristic also facilitates its effective attachment to plastic or polymer surfaces like catheters (Doughari et al., 2011). The hydrophobicity of *A. baumannii* is attributed to features such as a rough cell surface and protein protrusions on the cell surface (Phuong et al., 2009). Additionally, certain cell surface enzymes have been identified as contributors to hydrophobicity in *A. baumannii*. Studies have indicated that *A. baumannii* isolates obtained from catheters and tracheal devices demonstrate higher hydrophobicity compared to environmental samples (Boujaafar et al., 1990).

2.3.3 Toxic Slime Polysaccharides

According to Hoštacká (2002), *Acinetobacter spp*. can produce toxic slime polysaccharides. These polysaccharides, consisting of glucose building blocks, glucuronic acid, D-mannose, L-rhamnose, and D-glucose, are synthesized during the exponential growth phase of *A. baumannii*. The slime polysaccharides not only exhibit toxicity towards neutrophils, but also impede their movement and phagocytic function. Furthermore, *A. baumannii* capsular polysaccharides aid in avoiding the host's immune response and facilitate its survival in serum. (Rumbo et al., 2014). Generally, strains

of *Acinetobacter* that produce slime are associated with higher levels of virulence compared to non-producing strains.

2.3.4 Outer Membrane Protein

Outer membrane proteins (Omp) play a significant role in the pathogenicity of various Gram-negative bacteria, including certain strains of *Acinetobacter*, particularly *A. baumannii*. Among these proteins, Outer membrane protein A (OmpA, 38 kDa) has been detected in multiple *Acinetobacter* strains (Dijkshoorn, et al., 2007). In the context of infection, *A. baumannii* OmpA interacts with eukaryotic cells and subsequently translocates into the nucleus, leading to cell death. (Dijkshoorn et al., 2007). Furthermore, OmpA of *A. baumannii* has been found to induce early apoptosis and delay necrosis in dendritic cells. OmpA also contributes to biofilm formation, surface motility, and serum resistance in *A. baumannii* (McConnell et al., 2013). Another outer membrane protein, Omp33-36, has been recognized as crucial for the virulence of *A. baumannii*. It induces apoptosis in host cells by activating caspases and modulating autophagy (Rumbo et al., 2014). Additionally, cell surface components like adhesins and fimbriae (pili) have proved to be responsible in the attachment of *A. baumannii* to both host cells and inanimate surfaces (Eijkelkamp et al., 2013).

2.3.5 Surface and Mitochondrial Porins

Porins are proteins found in the lipid bilayer membrane of bacterial cells that function as channels for transporting molecules across the membrane. Their roles can vary depending on the bacterial species and include maintaining cellular structure, facilitating bacterial conjugation, binding to bacteriophages, and contributing to antimicrobial resistance (Braun, 2008). These porins form pores in the outer membrane, allowing small molecules to enter the bacterial cell. They are also essential components of efflux pumps, which help remove toxic substances from the cell. Research has shown that in *A. baumannii*, porins, in conjunction with efflux pumps, create a significant barrier that hinders the uptake of antibiotics, thereby promoting antibiotic resistance.

2.3.6 Siderophores

Obtaining iron for growth within a host is a critical factor in the virulence of bacteria. Siderophores are proteins produced by bacteria that bind to iron and assist in its uptake. *Acinetobacter* produces siderophores called acinetobactins, which help convert insoluble forms of iron into soluble chelates when iron availability is limited (Mihara et al., 2004). *A. baumannii*, in particular, relies on siderophores to acquire ferric ions under conditions of low iron, which is essential for its virulence. Research has demonstrated that the acinetobactin-mediated iron acquisition system in *A. baumannii* causes damage to human epithelial cells and leads to death in *Galleria mellonella* caterpillars and contaminated mice. Furthermore, clinical strains of *A. baumannii* have been found to grow in environments with limited iron and secrete iron-regulated catechol siderophores into their surrounding culture medium (Actis et al., 1993).

2.3.7 Glycoconjugates

In *A. baumannii*, the presence of lipooligosaccharide (LOS) contributes to resistance against drugs and desiccation (Boll et al., 2015). The capsular polysaccharide plays a protective role by preventing complement-mediated killing of cells (Russo et al., 2010). Additionally, glycoproteins have been found to enhance the formation and stability of biofilms, thereby contributing to virulence. Moreover, glycosylated type IV pili have been suggested to aid in immune evasion by defending the antigenic protein from detection by antibodies (Piepenbrink et al., 2016).

2.3.8 Phospholipase

A. baumannii possesses two phospholipase C (PLC) enzymes and three phospholipase D (PLD) enzymes, each with unique hydrolytic properties targeting phosphatidylcholine, a component of eukaryotic membranes (Fiester et al., 2016). The presence of PLD is crucial for the penetration of epithelial cells, resistance against human serum, and pathogenesis in vivo (Stahl et al., 2015). On the other hand, PLC plays a significant role in the haemolytic and cytolytic activities exhibited by *A. baumannii* about host cells (Fiester et al., 2016).

2.3.9 Iron Acquisition System

A. baumannii has developed an efficient iron acquisition system to alter to the iron-limited host environment where iron is predominantly bound to heme (Sheldon & Skaar, 2020). This system consists of eight gene clusters, each serving a specific function. One cluster is implicated in direct iron acquisition, two clusters facilitate heme uptake, and five clusters are responsible for the biosynthesis and utilization of siderophores (Sheldon & Skaar, 2020). Siderophores are specialized proteins that have a high affinity for iron and participate with host cells for its acquisition (Wandersman & Delepelaire, 2004). *A. baumannii* produces ten distinct types of siderophores, containing acinetobactin, baumannoferrins, and fimsbactins. (Sheldon & Skaar, 2020). Amid these, acinetobactin has been greatly researched and its structural characteristics have been well-documented (Eijkelkamp et al., 2011).

2.3.10 Efflux Pumps

Bacterial efflux systems are essential for the survival and virulence of gram-negative pathogens. These systems, which span the bacterial membrane, possess diverse substrate specificity and function to expel potentially harmful compounds from the periplasm to the extracellular environment. Efflux pumps play critical roles in gram-negative bacteria by facilitating the expulsion of bile salts, antimicrobial fatty acids and peptides, and actively secreting virulence aspects like siderophores (Morris et al., 2019). There are six families of bacterial efflux pumps known to date: the ATP binding cassette (ABC) family, the major facilitator superfamily (MFS), the resistance-nodulation-division (RND) family, the multidrug and toxin extrusion (MATE) family, the small multidrug resistance (SMR) family, and the drug/metabolite transporter (DMT) superfamily. These families encompass a diverse array of efflux systems, each with its own functional characteristics and mechanisms of action (Morris et al., 2019).

2.3.11 Host- Pathogen Interactions

Host-pathogen interactions in disease progression involve various factors such as strain virulence, host characteristics, and bacterial load. These interactions have significant implications for the infected host and seem to have adverse impacts on their health. A noteworthy observation is that

many genes responsible for resistance to antibiotics, heavy metals, and antiseptics in *A. baumannii* are believed to have originated from other highly pathogenic organisms (Fournier et al., 2006). A comparative analysis between the genomes of *A. baumannii* strain 17978 and the non-pathogenic *species A. baylyi* has identified 28 gene clusters specific to *A. baumannii*, out of which 16 are potentially associated with virulence (Smith et al., 2007). One notable gene cluster is the *csu* polycistronic operon, consisting of five genes. Some of these genes exhibit homology to proteins involved in chaperone functions and pilus assembly in Gram-negative bacteria (Tomaras *et al*., 2003). Studies have demonstrated that the formation of biofilms in *A. baumannii* is phenotypically linked to the production of exopolysaccharides and the formation of pili. These factors contribute to the development and stability of biofilms, which can enhance the pathogenic potential of *A. baumannii* (Tomaras *et al*., 2003). The ability of *A. baumannii* to produce biofilms on abiotic surfaces in hospital environments can be explained by its adherence to human bronchial epithelial cells and erythrocytes, which exhibit similar pilus-like structures. Quorum sensing, a regulatory mechanism observed in many Gram-negative organisms, has also been shown to control various virulence mechanisms. In *Acinetobacter*, the presence of N-acyl homoserine lactones (AHSL) as quorum sensing molecules suggests their function in the autoinduction of virulence factors (Joly Guillou, 2005). Diverse varieties of AHSL molecules have been detected, including 3-oxo substituted variants with acyl chains of varying lengths, 3-unsubstituted AHSL (except for C4), and 3-hydroxyl AHSL (C6, C8, and C10) (Gonzalez et al., 2001). Besides biofilm production, the production of exopolysaccharides is thought to confer protection against host defenses. Exopolysaccharides serve as a physical barrier that shields the bacteria from immune system components, making it more difficult for host defenses to eliminate the pathogen. This protective mechanism helps enhance the survival and persistence of the bacteria within the host environment (Joly Guillou, 2005). Toll-like receptor (TLR) signaling participates in a crucial part in the innate immune response to *A. baumannii* (Erridge et al., 2007). Studies utilizing mouse models have demonstrated that mice deficient in Toll-like receptor 4 (TLR4) exhibit increased bacterial counts, higher levels of bacteria in the bloodstream (bacteremia), impaired responses in terms of cytokines/chemokines, and delayed lung inflammation when compared to mice with intact TLR signaling (wild-type mice). These findings highlight the crucial role of TLR4 in recognizing and responding to bacterial infections, and the impact of TLR4 deficiency on the immune response and host defense mechanisms. *A. baumannii* lipopolysaccharide (LPS) has been recognized as a

significant stimulatory factor that triggers immune responses. CD14, a receptor protein, responsible for the binding of *A. baumannii* LPS to Toll-like receptor 4 (TLR4). This interaction between CD14 and TLR4 facilitates the detection of *A. baumannii* LPS by the immune system, leading to the initiation of immune responses against the bacterium (Knapp et al., 2006). Notably, human cells have shown that TLR2 is also a significant signaling pathway for the effective endotoxic potential of *A. baumannii* LPS, eliciting strong inflammatory responses similar to those caused by E. coli LPS. (Knapp *et al*., 2006). Mouse-derived monoclonal antibodies targeting *A. baumannii* outer membrane proteins (OMPs) expressed under iron-depleted conditions have demonstrated bactericidal and opsonizing effects by blocking iron uptake. Experimental infections conducted in well-defined animal models have provided valuable insights into disease processes. However, it's important to interpret the results of these studies cautiously, as they may not fully represent the complexities of natural infection contexts (Erridge *et al*., 2007). The findings from these studies indicate that *A. baumannii* endotoxin can trigger a potent inflammatory response during infection. Additionally, mouse-derived monoclonal antibodies specifically targeting *A. baumannii* outer membrane proteins (OMPs) expressed under conditions of iron depletion have demonstrated both bactericidal and opsonizing effects by obstructing iron uptake. Deliberately induced infections in well-defined animal models have proven valuable in acquiring insights into disease processes. However, it is crucial to interpret the results of experimental infections with caution, as they may not fully reflect the complexities of natural infection contexts (Joly-Guillou &Wolff, 2008).

2.4 Antibiotic

Since the deployment of the first antibiotic, salvarsan, in 1910, the field of antibiotics has undergone significant advancements, revolutionized advanced medicine and significantly extended the regular human life expectancy by approximately 23 years. The breakthrough of penicillin in 1928 marked the beginning of a golden age in natural product antibiotic exploration, reaching its pinnacle in the mid-1950s. The introduction of antibiotics into clinical practice is widely regarded as one of the most remarkable medical breakthroughs of the 20th century. Not only have antibiotics played a crucial role in treating infectious diseases, but they have also facilitated numerous modern clinical treatments, including cancer treatment, organ transplants, and

open-heart surgery. The impact of antibiotics on medicine has been profound, shaping the course of healthcare and improving the quality of life for millions of people worldwide.

There are five major antibiotic mechanisms. All of which are described below.

2.4.1 Interference with Cell Wall Synthesis

Penicillin and cephalosporin are classified as beta-lactam antibiotics, and their antimicrobial activity is attributed to their ability to target enzymes crucial for the synthesis of the bacterial cell wall's peptidoglycan layer. These antibiotics work by inhibiting the action of penicillin-binding proteins (PBPs), which are responsible for cross-linking the peptide chains within the peptidoglycan structure. By blocking the activity of these enzymes, penicillin and cephalosporin disrupt the assembly and integrity of the bacterial cell wall, leading to cell lysis and ultimately killing the bacteria. Their ability to interfere with peptidoglycan synthesis makes them effective against a extensive scale of bacterial infections and contributes to their widespread use in clinical practice (Leach et al., 2007).

2.4.2 Inhibition of Protein Synthesis

The oxyazolidinone family of antibiotics, which is the newest addition to the antibiotic arsenal, acts by interacting with the A site of the bacterial ribosome. By doing so, they prevent the binding of aminoacyl-tRNA molecules to this site, thereby inhibiting the initiation of protein synthesis. Tetracyclines, on the other hand, interfere with protein synthesis by binding to the 30S component of the bacterial ribosome. This binding weakens the connection between the ribosome and tRNA molecules, impairing the elongation phase of protein synthesis. Macrolide antibiotics bind to the 50S ribosomal subunit, thereby obstructing the elongation of nascent polypeptide chains during protein synthesis. Chloramphenicol acts by binding to the 50S ribosomal subunit as well, but its mechanism of action is different. It inhibits the peptidyl transferase process, which is responsible for the formation of peptide bonds during protein synthesis. Aminoglycosides attach to the 30S ribosomal subunit, preventing the initiation of protein synthesis and leading to the production of faulty proteins. Their binding interferes with the accurate reading of the genetic code and can result in the synthesis of non-functional or toxic proteins. Each of these antibiotic classes targets a

specific component of the ribosome and disrupts different stages of protein synthesis, ultimately inhibiting bacterial growth and promoting bacterial cell death (Leach et al., 2007).

2.4.3 Interference in Nucleic Acid Synthesis

Rifampicin is an antibiotic that acts by inhibiting DNA-directed RNA polymerase, an essential enzyme involved in the transcription process. By targeting this enzyme, rifampicin effectively disrupts the synthesis of RNA from DNA templates, thereby inhibiting bacterial gene expression (Strohl, 1997).

2.4.4 Inhibition of a Metabolic Pathway

Sulfonamides, such as sulfamethoxazole, and trimethoprim are antibiotics that target different steps in the folate synthesis pathway, which is essential for the production of nucleotides used in DNA and RNA synthesis. Sulfonamides work by mimicking a component of folic acid, a precursor molecule in the folate synthesis pathway. They competitively inhibit the enzyme dihydropteroate synthase, which is responsible for incorporating the precursor molecule into the folate synthesis pathway. By inhibiting this enzyme, sulfonamides disrupt the production of folate, which is necessary for the synthesis of nucleotides. This inhibition ultimately interferes with bacterial DNA and RNA synthesis, leading to the inhibition of bacterial growth (Strohl, 1997).

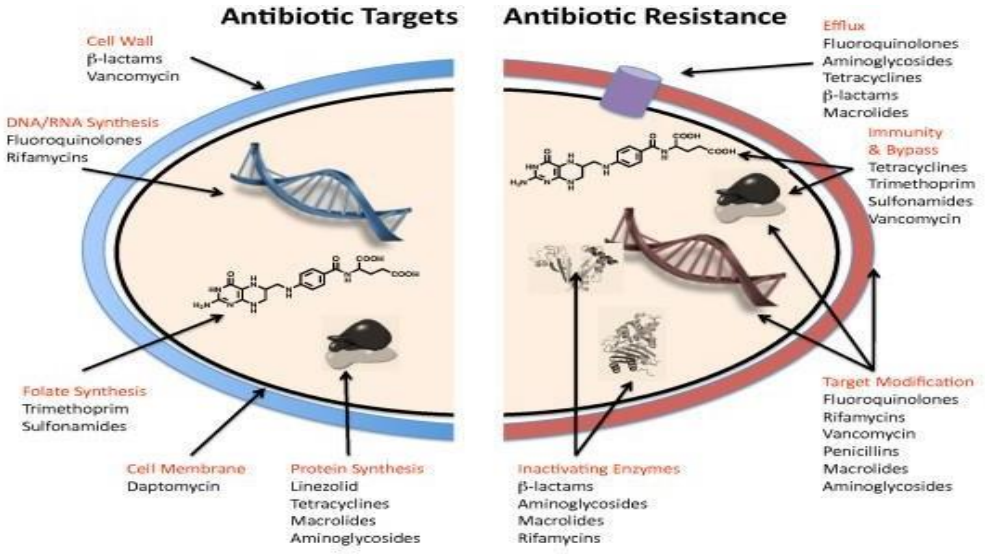


Figure 2.3: Antibiotic target vs resistance mechanism (Wright, 2010).

2.4.5 Disorganization of the Cell Membrane

Gram-positive bacteria have a cytoplasmic membrane, while Gram-negative bacteria have an inner membrane, and these membranes are the primary targets for certain antibiotics. Polymyxins are thought to work by enhancing the permeability of bacterial membranes, causing the release of cellular contents and ultimately cell death. On the other hand, daptomycin, a cyclic lipopeptide, rapidly kills bacteria by compelling to the cytoplasmic membrane in a calcium-dependent manner. This binding leads to the formation of membrane complexes, causing the efflux of potassium ions from the bacterial cell and resulting in cell death (Straus & Hancock, 2006).

2.4.6 Antibiotic Resistance

According to the World Health Organization (WHO), when bacteria, viruses, fungi, and parasites do not react to the treatments that used to be effective against them previously, it is referred to as antimicrobial resistance (AMR) (Antimicrobial Resistance, 2021). Specifically, antibiotic resistance is a type of AMR that appears when bacteria become unaltered by antibiotics that previously had an impact on them. In other words, bacteria develop the capacity to resist the impacts of antibiotics that were once capable of treating their infections effectively.

2.4.7 Categories of Antibiotic Resistance Pattern

The collaboration between the European Centre for Disease Prevention and Control (ECDC) and the Centers for Disease Control and Prevention (CDC) has resulted in the establishment of definitions for three distinct classifiaction of resistance patterns (Magiorakos et al., 2012).

Multidrug-resistant (MDR): MDR was defined as acquired non-susceptibility to at least one agent in three or more antimicrobial categories.

Extensively drug-resistant (XDR): XDR was defined as non-susceptibility to at least one agent in all except two or fewer antimicrobial categories i.e., bacterial isolates remain susceptible to only one or two categories.

Pan drug-resistant (PDR): PDR was defined as non-susceptibility to all agents in all antimicrobial categories.

2.4.8 Mechanisms of Antibiotic Resistance

Antimicrobial resistance refers to the loss of effectiveness of medicines, including antibiotics and antineoplastics, in treating diseases or conditions. It is comparable to dose failure or drug tolerance, where the intended antibacterial effect is not achieved. The term is commonly used when discussing pathogen resistance, specifically when it has developed through acquired mechanisms. Multidrug-resistant organisms are those that have developed resistance to multiple drugs (Fisher & Mobashery, 2010). Bacterial strains can have a variety of resistance mechanisms, which are described below.

2.4.9 Biochemical Aspect of Antibiotic Resistance

2.4.9.1 Through Hydrolysis

Enzymes produced by resistant bacteria can hydrolyze certain chemical bonds, such as amides and esters, present in many antibiotics. This enzymatic action leads to the inactivation of the antibiotics. ESBLs (extended-spectrum β-lactamases) are enzymes that specifically target and cleave these susceptible bonds. Antibiotics like penicillins, third generation cephalosporins (e.g., ceftazidime, cefotaxime, and ceftriaxone), and aztreonam are susceptible to inactivation by ESBLs. However, cephamycins (cefoxitin and cefotetan) and carbapenems are not affected by ESBL-mediated inactivation (Bonnet, 2004).

2.4.9.2 Through Redox Process

The process of antibiotic oxidation or reduction is not commonly exploited by pathogenic bacteria. However, there are a few instances where this strategy has been observed. For example, tetracycline antibiotics can be oxidized by the TetX enzyme. In addition, the bacterium *Streptomyces virginiae* is known to produce the streptogramin antibiotic virginiamycin M1, which

belongs to type A streptogramins. Notably, this bacterium has evolved a mechanism to protect itself from the antibiotic's action. It achieves this by converting a crucial ketone group located at position 16 of virginiamycin M1 to an alcohol. This modification alters the structure of the antibiotic, rendering it less effective against the producing bacterium and allowing it to survive in the presence of the antibiotic. These examples highlight rare cases where bacteria utilize oxidation or reduction processes to modify or neutralize antibiotics (W. Yang et al., 2004).

2.4.9.3 Antibiotic Inactivation through Group Transfer

Transferases are a distinct and extensive group of enzymes that play a considerable role in antibiotic resistance. These enzymes can deactivate antibiotics, such as aminoglycosides, chloramphenicol, streptogramin, macrolides, or rifampicin, by chemically modifying them through the addition of various groups (such as adenylyl, phosphoryl, or acetyl groups) to the periphery of the antibiotic molecule. This modification alters the structure of the antibiotic, rendering it unable to bind to its target effectively. Different chemical reactions, including O-acetylation, N-acetylation, O-phosphorylation, O-nucleotidylation, O-ribosylation, O-glycosylation, and thiol transfer, are employed in the resistance process. Importantly, these covalent modification reactions require specific co-substrates such as ATP, acetyl-CoA, NAD+, UDP-glucose, or glutathione to occur, and therefore, they are typically restricted to the cytoplasmic environment (Shaikh et al., 2015).

2.4.9.4 Antibiotic Inactivation through Target Modification

Another significant mechanism of resistance is the alteration of the antibiotic's target site, which leads to a decreased ability of the antibiotic to bind and inhibit the target. Through genetic mutations, alterations in the target site can occur, resulting in reduced susceptibility to the inhibitory effects of the antibiotic while still allowing normal cellular function to continue. This mechanism enables the bacteria to escape the intended action of the antibiotic and survive in the presence of the drug (Spratt, 1994).

2.4.10 Genetics of Antibiotic Resistance

2.4.10.1 Antibiotic Resistance via Mutations

Antibiotic resistance can be acquired through various biochemical pathways, with a significant proportion of them dependent on mutational events. When mutations occur in the genes encoding the recipients of antibiotics it plays a major role in conferring resistance. For instance, mutations in the genes RpoB and DNA-topoisomerases can lead to resistance against rifampicin and fluoroquinolones, respectively (Ruiz, 2003). In addition to target alterations, mutations can also affect the expression of proteins involved in antibiotic absorption or efflux systems. For example, in the bacterium *Pseudomonas aeruginosa*, mutations can result in lower expression or absence of the OprD porin, which restricts the permeability of the cell wall to carbapenem antibiotics (Wolter et al., 2004).

2.4.10.2 Antibiotic Resistance via Horizontal Gene Transfer

The horizontal transfer of genetic material is a major means by which antibiotic resistance spreads. Genes conferring antibiotic resistance can be transferred between bacteria through processes like conjugation, transformation, and transduction. Over the past 15 years, there has been a notable emergence of β-lactamase enzymes known as extended-spectrum β-lactamases (ESBLs), which are capable of resisting a wide range of β-lactam antibiotics, including cephalosporins (excluding carbapenems). One specific ESBL variant, CTX-M-15, was initially discovered in E. coli but has since spread to other bacteria within the Enterobacteriaceae family. This ESBL is commonly associated with a specific lineage known as the uropathogenic clone ST131. CTX-M-15 is often found on highly mobile plasmids of the IncFII type, and it is frequently linked to a mobile genetic element called IS26. Individuals with prolonged renal or liver dysfunction and those who travel to high-risk areas are at an increased risk of infection by antibiotic-resistant bacteria carrying CTX-M-15 (Nordmann et al., 2011).

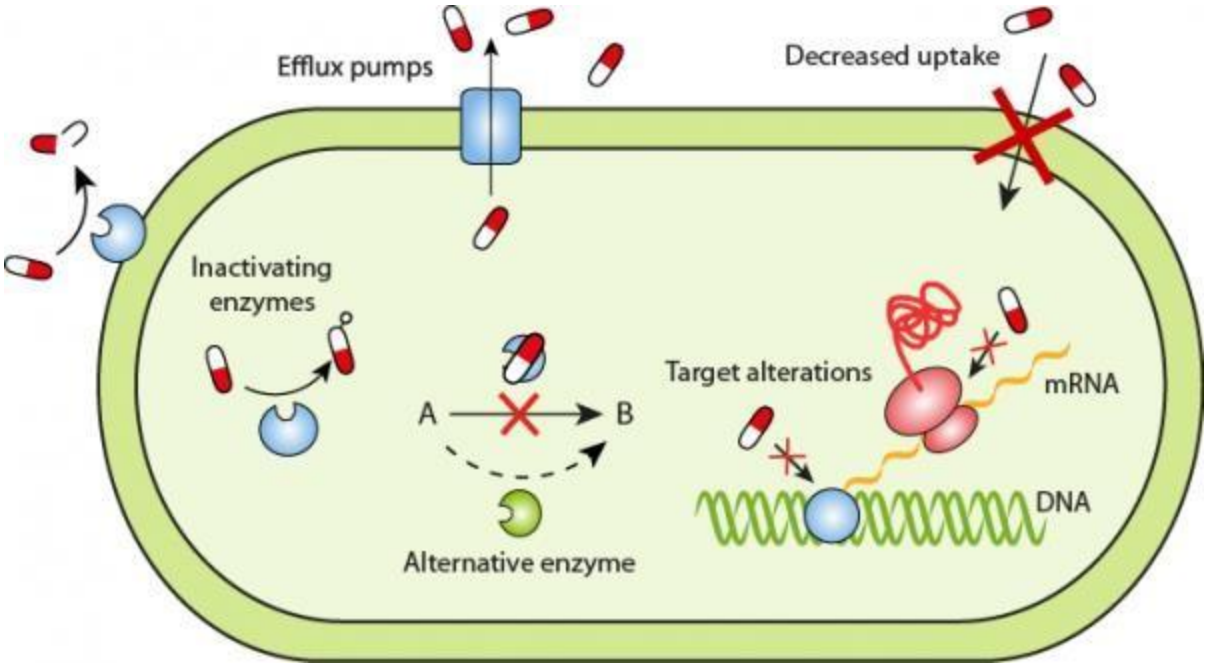


Figure 2.4: Antibiotic resistance mechanisms (Yuen, 2018)

2.5 Antibiotic Resistance in *A. baumannii*

Before the 1970s, a variation of antibiotics, with β-lactams, aminoglycosides, and tetracyclines, were effective in treating *Acinetobacter* infections. However, the emergence of *A. baumannii* with an extensive scale of intrinsic and acquired resistance determinants has led to resistance against all established antibiotics. This has created significant challenges in treating *A. baumannii* infections. (Peleg et al., 2008). There are variances in antimicrobial susceptibility between *A. baumannii*, It is recognized to be the most resistant species among the *Acinetobacter* genus, exhibiting differences in antimicrobial susceptibility (Van Looveren et al., 2004). The acronym ESKAPE is used to group high-frequency multidrug-resistant (MDR) bacteria, including *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, A. baumannii, Pseudomonas aeruginosa,* and *Enterobacter spp*., which encompasses *A. baumannii*. These ESKAPE pathogens are responsible for the majority of healthcare-associated infections and have the ability to evade the effects of antimicrobial agents (Navidinia, 2016). In 2017, the World Health Organization (WHO) identified a list of pathogens requiring urgent global action. Carbapenem-*resistant A. baumannii* (CRAB) has been labeled by the WHO as a critical priority (priority 1) pathogen on the list of

antibiotic-resistant bacteria, guiding research and development efforts for new drugs (Tacconelli et al., 2018).

2.6 β-lactamases

Resistance to β-lactam antibiotics is often mediated by enzymes known as β-lactamases, which are produced by bacteria (Kyriakidis et al., 2021). These enzymes have the ability to hydrolyze or break down β-lactam antibiotics, rendering them ineffective. β-lactamases can be classified into four classes, namely class A, B, C, and D, based on their sequence motifs and hydrolytic mechanisms. Classes A, C, and D are known as serine β-lactamases (SBLs) and rely on a serine residue in their active site. Class B β-lactamases, on the other hand, are zinc-dependent enzymes called metallo-β-lactamases (MBLs) (Massova & Mobashery, 1998). Class D β-lactamases, also known as oxacillinases or carbapenem-hydrolyzing class D β-lactamases (CHDLs), are particularly important in conferring resistance to carbapenems, as they can inactivate a broad range of β-lactam antibiotics (Antunes & Fisher, 2014).

2.6.1 Extended-spectrum beta-lactamases (ESBLs)

ESBLs, or extended spectrum β-lactamases, are enzymes produced by many pathogenic bacteria that have the ability to hydrolyze extended spectrum cephalosporins. This renders antibiotics like ceftazidime, ceftriaxone, cefotaxime, and oxyimino-monobactam ineffective against these bacteria (Paterson & Bonomo, 2005). However, ESBL-producing strains remain susceptible to carbapenems and cephamycins. The activity of ESBLs can be suppressed by clavulanic acid and tazobactam. Gram-negative bacteria, including Enterobacteriaceae and *Pseudomonas aeruginosa*, commonly harbor ESBLs, with the TEM-1 β-lactamase being the most prevalent (Bradford, 2001). TEM-1 β-lactamase is the most prevalent ESBL. TEM-1 can also hydrolyze penicillin and first-generation cephalosporins. TEM-2 is a derivative of TEM-1 with a single amino acid substitution (Bois et al., 1995). Different β-lactamase enzymes exhibit distinct phenotypes due to amino acid changes, which alter their structure and function. TEM, SHV, and OXA enzymes often display substitutions at specific amino acid positions (Bradford, 2001). The mixture of different amino acids results in diverse phenotypes of β-lactamase enzymes, with varying abilities to hydrolyze

third-generation cephalosporins and increased resistance to β-lactamase inhibitors (Winokur et al., 2001). The use of certain oximino-β-lactam antibiotics, and first-generation cephalosporins and penicillin, has been associated with the emergence of ESBL alterations. (Blazquez et al., 2000). Excessive use of β-lactam antibiotics has selected for ESBL-producing strains with numerous phenotypes and structural alterations in porins such as Omp, leading to resistance against cephalosporins and additional antimicrobials (Shakib et al., 2011) to acquire resistance to cephalosporins and other antimicrobials (Bradford et al., 1994). Plasmids carrying ESBL genes may also confer resistance to aminoglycosides and cotrimoxazole (Villa et al., 2000). Quinolone resistance is more common in ESBL producing strains, according to studies, while the mechanism of co-resistance is yet unknown (Paterson et al., 2000). ESBLs have become increasingly notable in the field of medicine due to their facility to generate strains that are resistant to cephalosporins, which are normally used as first-line antibiotics for various diseases. Prompt identification and treatment of infections caused by ESBL-producing strains are crucial, as delays can lead to higher rates of illness and death. The medication choices for strains that produce ESBL are constrained as they frequently exhibit resistance to various drugs, including aminoglycosides and fluoroquinolones. The prevalence of ESBL-producing strains is on the rise, leading in heightened morbidity, mortality, and healthcare expense. Extended spectrum-lactamase producing *A. baumannii* can be transmitted between humans and animals, and can also contaminate and infect aquatic environments, posing risks to those exposed. ESBL-producing *A. baumannii* have been found in vegetables in many parts of the world, and there have been reports of their presence in wild animals and the environment on multiple continents.

2.6.1.1 Types of ESBLs

There are more than 350 different ESBL variants, of which the well described ones are given below.

2.6.1.1.1 TEM

TEM-1 and TEM-2 are examples of TEM-type extended spectrum β-lactamases (ESBLs). In 1965, a strain of *E. coli* isolated from a patient named Temoneira in Athens, Greece led to the discovery of TEM-1 (Datta & Kontomichalou, 1965). TEM-1 exhibits enhanced breakdown of ampicillin

compared to carbenicillin, oxacillin, or cephalothin, but it does not affect extended-spectrum cephalosporins. Its activity can be reduced by clavulanic acid. TEM-2 shares a similar hydrolytic profile with TEM-1 but has a more active native promoter and a different isoelectric point. TEM-13 shows an identical hydrolytic profile to TEM-1 and TEM-2. It's important to note that TEM-1, TEM-2, and TEM-13 are not considered ESBLs (Jacoby & Medeiros, 1991). In 1984, TEM-3 was identified in *K. pneumoniae* in France and was initially referred to as CTX-1 due to its activity against cefotaxime. TEM-3 differs from TEM-2 by two amino acid changes. (Brun-Buisson et al., 1987). Currently, there are over 100 TEM-type β-lactamases, with the exception of TEM-1 and TEM-2, all of which are ESBLs. *E. coli* and *K. pneumoniae* are the most common bacteria carrying TEM-type ESBLs, but they can also be found in other Gram-negative bacteria, as well as various species of Enterobacteriaceae. Among Non-Enterobacteriaceae, TEM-type ESBLs are most frequently found in *P. aeruginosa* (Brun-Buisson et al., 1987).

2.6.1.1.2 SHV

SHV-type extended spectrum β-lactamases (ESBLs) are often observed in clinical isolates and are more common than other types of ESBLs (Jacoby, 1997). The acronym SHV stands for sulfhydryl variable, reflecting the belief that the activity of SHV enzymes could be inhibited by p-chloromercuribenzoate in a substrate-dependent manner, with variations depending on the specific substrate being tested (SYKES & BUSH, 1982). Unlike TEM-type β-lactamases, SHV-1 produces a smaller number of derivatives within the SHV family of β-lactamases. This gene is often carried on plasmids in most strains due to a serine-to-glycine substitution at position 238, along with a lysine-to-glutamate substitution at position 240 in certain strains. The serine residue at position 238 is crucial for the hydrolysis of ceftazidime, while the lysine residue at position 240 is important for cefotaxime hydrolysis. Over 100 SHV variants have been identified worldwide. SHV-type ESBLs are found in a broad range of bacteria, including Enterobacteriaceae, *Pseudomonas aeruginosa*, and *Acinetobacter* spp. (Huang et al., 2004).

2.6.1.1.3 CTX-M

Tzouvelekis described CTX-M for the first time in 2000 (Tzouvelekis et al., 2000). The designation CTX-M β-lactamase refers to the enzyme's capacity to metabolize cefotaxime

(Bonnet, 2004). CTX-M-type β-lactamases exhibit differential hydrolytic activity towards various antibiotics. They demonstrate higher efficiency in hydrolyzing cephalothin compared to benzylpenicillin, and they hydrolyze cefotaxime more effectively than ceftazidime. While MIC analysis is commonly used to test bacterial resistance to ceftazidime, certain CTX-M-type β-lactamases have been identified as resistant to this antibiotic despite the testing (Poirel et al., 2002). Furthermore, Aztreonam resistance has been identified as being varied. Cefepime can be hydrolyzed by CTX-M-type β-lactamases sulbactam and clavulanate (Bush et al., 1993). There is no point mutation in CTX-M, unlike TEM and SHV enzymes, and it is thought that CTX-M was initially found from the chromosomal *Kluyvera spp* after which it was transformed to plasmid (Radice et al., 2002). There have been 128 different varieties of CTX-M reported so far, which are divided into five categories: CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, and CTX-M-25. *Salmonella spp*., as well as other *Enterobacteriaceae*, are known to harbor them (Bradford et al., 1998).

2.6.1.1.4 OXA

The initial identification of OXA-type ESBLs occurred in *Pseudomonas aeruginosa* isolates originating from a specific hospital in Ankara, Turkey. A novel derivative of OXA-10 (designated as OXA-28) was discovered in a *Pseudomonas aeruginosa* strain from France. Additionally, in *Pseudomonas aeruginosa* isolates from France, a new ESBL called OXA-18 and an extended-spectrum variant of the narrow-spectrum OXA-13 β-lactamase (referred to as OXA-19) were identified. The name "OXA-type" β-lactamases is derived from their ability to hydrolyze oxacillin. These β-lactamases exhibit hydrolysis rates of cloxacillin and oxacillin that surpass 50% of the hydrolysis rates observed for benzylpenicillin (Bush et al., 1995). They have been identified in a number of gram-negative bacteria, but they're most frequent in *Pseudomonas aeruginosa* (Weldhagen et al., 2003). Indeed, the most common OXA-type β-lactamase, OXA-1, has been found in 1 to 10% of *E. coli* samples (Livermore, 1995). The majority of OXA-type β-lactamases do not possess the ability to effectively hydrolyze extended-spectrum cephalosporins, and therefore they are not classified as ESBLs. Specifically, OXA-10 exhibits poor hydrolysis activity towards cefotaxime, ceftriaxone, and aztreonam, resulting in reduced susceptibility to these antibiotics in many bacterial species. Other OXA ESBLs include OXA-11, OXA-14, OXA-16, OXA-17, OXA-19, OXA-15, OXA-18, OXA-28, OXA-31, OXA-32, OXA-35, and OXA-45.

These enzymes confer resistance to cefotaxime and, in certain cases, to ceftazidime and aztreonam (Toleman et al., 2003). Resistance to all β-lactam antibiotics can be easily acquired by producing a carbapenem-hydrolyzing metalloenzyme and an aztreonam-hydrolyzing OXA enzyme simultaneously (Toleman et al., 2003).

2.6.1.1.5 Toho β-lactamase

Toho-1 and Toho-2 are β-lactamases that share structural similarities with CTX-M β-lactamases. These enzymes were named after Tokyo's Toho University School of Medicine Omori Hospital, where a child was hospitalized with an infection caused by Toho-1 β-lactamase-producing Escherichia coli. Similar to other CTX-M-type β-lactamases, Toho-1 and Toho-2 exhibit higher hydrolytic activity against cefotaxime compared to ceftazidime (Ma et al., 1998).

2.6.1.1.6 PER

Only about 25%–27% homology exists between PER-type ESBLs and known TEM- and SHV-type (Bauernfeind et al., 1996). Penicillin and cephalosporins are efficiently hydrolyzed by PER-1 β-lactamase, but it is vulnerable to clavulanic acid inhibition. PER-1 was first discovered in *P. aeruginosa* (Neuhauser et al., 2003), and later in *S. enterica* serovar *Typhimurium* and *Acinetobacter* isolates (Vahaboglu et al., 2001). In Turkey, PER-1 was detected in 46 percent of nosocomial *Acinetobacter* spp. isolates and 11 percent of *P. aeruginosa* isolates (Vahaboglu et al., 1997). PER-2 has been found in *S. enterica* serovar *Typhimurium*, *E. coli*, *K. pneumoniae, Proteus mirabilis*, and *Vibrio cholerae* O1 El Tor, and shares 86 percent homology with PER-1 (Petroni et al., 2002).

2.6.1.1.7 GES -type β – lactamases

A *K. pneumoniae* isolate from a newborn kid who had recently been moved from French Guiana to France was the first isolate in which GES-1 was found (Poirel et al., 2002). GES-1 is inhibited by β-lactamase inhibitors and can hydrolyze penicillins and extended-spectrum cephalosporins, but not cephamycins or carbapenems. GES1 was designated as an ESBL because its enzymatic properties are comparable to those of other class A ESBLs.

2.6.1.1.8 VEB-1, BES-1, and other ESBL type β – lactamases

BES-1, CME-1, VE-B-1, PER, SFO-1, and GES-1 are only a few of the odd enzymes that are recognized as ESBLs (Bradford, 2001). These unique enzymes are not commonly found; details on these enzymes are discussed elsewhere (Naas et al., 2008).

2.6.1.1.9 New Delhi Metallo-β-Lactamase (NDM-1)

Carbapenems, also known as metallo-β-lactamases (MBLs), are a type of enzyme that utilize at least one zinc ion to hydrolyze β-lactam antibiotics. Among these enzymes, New Delhi metallo-β-lactamase (NDM-1) is unique as a broad-spectrum β-lactamase (belonging to class B carbapenems) capable of inactivating all β-lactams, with the exception of aztreonam. However, it is important to note that most NDM-1 producers also possess β-lactamases (such as CTX-M and CMY-type ESBLs) that hydrolyze aztreonam, rendering them resistant to all β-lactam antibiotics (Yong et al., 2009). NDM-1 was called after New Delhi, India, after *Klebsiella pneumoniae* and *Escherichia coli* bacteria were discovered from a Swedish patient who had received medical treatment there (Yong et al., 2009). Imipenem, Meropenem, ertapenem, gentamicin, amikacin, tobramycin, and ciprofloxacin resistance were discovered in NDM-1 producers, although isolates were susceptible to colistin (MICs 4 mg/L) and tigecycline (MICs 1 mg/L) (Kumarasamy et al., 2010).

2.6.2 ESBLs in Bangladesh

According to a study conducted in Bangladesh, approximately 3.5 percent of Gram-negative clinical isolates were found to be producing NDM-1, a type of carbapenems enzyme associated with broad-spectrum antibiotic resistance (Islam et al., 2012). Another investigation in hospitals of Bangladesh revealed that ESBL genes belonging to the $bla_{\text{CTX-M-1}}$ group were present in all isolates. Additionally, bla_{TEM}-type and $bla_{\text{OXA-1}}$-type genes were found in 82.5 percent and 47.5 percent of the isolates, respectively (Talukdar et al., 2013). In a separate research study focusing on wastewater, water from the neighborhood, and tap water samples, it was found that 51 percent of the isolates tested positive for the blaNDM-1 gene, with the most common bacterial species harboring this gene being *Klebsiella pneumoniae* (44 percent), followed by *Escherichia coli* (29 percent), *Acinetobacter* spp. (15 percent), and *Enterobacter* spp. (9 percent). These bacteria also

showed positive results for other antibiotic resistance genes, including *bla*$_{\text{CTX-M-1}}$ (80%), *bla*$_{\text{CTX-M-15}}$ (63%), *bla*$_{\text{TEM}}$ (76%), *bla*$_{\text{SHV}}$ (33%), *bla*$_{\text{CMY-2}}$ (16%), *bla*$_{\text{OXA-48}}$-like (2%), *bla*$_{\text{OXA-1}}$ (53%), and *bla*$_{\text{OXA-47}}$-like (60%) (Islam, 2021). These findings highlight the prevalence of antibiotic resistance genes among bacterial isolates in Bangladesh, posing challenges for effective treatment of infections in the region.

2.7 Biofilm Formation among *A. baumannii*

Biofilm is a complex substance that consists of microorganisms adhered to surfaces and enclosed within a hydrated polymeric matrix. This matrix is composed of polysaccharides, proteins, and nucleic acids, forming three-dimensional structures (Sauer et al., 2007). *A. baumannii,* a bacterium commonly found in healthcare settings, is capable of forming biofilms that enable its persistence in the environment and protect it from disinfectants, making it a continuous source of infection. Additionally, biofilm production by *A. baumannii* contributes to its resistance against antibiotics. The capacity of *A. baumannii* to adhere to both living and non-living surfaces facilitates the production of biofilms, which play a crucial role in the pathogen's persistence and development of antibiotic resistance in medical settings (Gaddy and Actis, 2009). factors influence the synthesis of biofilms in *A. baumannii*, including the availability of nutrients, surface structures like pili, omp, quorum sensing, and the presence of DNA and polysaccharides (Cerqueira and Peleg, 2011). Pili in *A. baumannii* are encoded by the *csu/BABCDE* chaperone-usher assembly system, that is regulated by a two-component regulatory system controlled by genes *bfmS* and *bfmR*. These factors collectively participate in the formation and regulation of biofilms in *A. baumannii*, impacting its persistence and antibiotic resistance (Luo et al., 2015). Transposon mutagenesis revealed that *csuA* and *csuE* are essential for bacterial attachment, biofilm formation and pili synthesis (Tomaras et al., 2003). Same researchers conducted a different study which revealed that the inactivation of the *bfmR* response regulator resulted in the absence of *csu* gene expression. Consequently, there was a complete loss of pili production and biofilm formation in *A. baumannii*. In contrast, mutation in the *bfmS* gene only partially impaired biofilm synthesis, proposing that it has a less meaningful role in the process. Another protein known as biofilm-associated protein (Bap) was also found to be associated with biofilm production in *A. baumannii*. Mutation of the Bap gene led to a substantial reduction in biofilm thickness, with a decrease of over 50% compared

to the wild-type strain (Loehfelm et al., 2008). Also, OmpA is crucial for the production of strong biofilms, specifically on polystyrene surface (Gaddy et al., 2009). Exopolysaccharides, specifically poly-β-(1-6)-N-acetylglucosamine (PNAG), were identified as crucial components for the structural integrity of *A. baumannii* biofilms produced under stressful conditions. These exopolysaccharides are necessary for maintaining the stability and cohesion of the biofilm structure (Choi et al., 2009). Further, PNAG is believed to play a role in cell-to-cell adhesion (Cramton et al., 1999). In addition to the causes mentioned earlier, quorum sensing, which is a well-known cell-to-cell communication mechanism in bacteria, has been found to play a role in regulating biofilm formation in *A. baumannii*. Specifically, the quorum sensing molecule 3-hydroxy-C12-HSL, produced by the autoinducer synthase encoded by the gene *abaI*, is crucial for the later stages of biofilm production, particularly on abiotic surfaces. Quorum sensing allows *A. baumannii* cells to coordinate their behavior and synchronize the formation of biofilms, contributing to the overall development and maturation of the biofilm structure (Niu et al., 2008). Many scientists have perceived that the clinical isolates of *A. baumannii* are strong biofilm formers (Gurung et al., 2013). In addition to its role in nosocomial spread, biofilm formation in *A. baumannii* has significant implications for antibiotic resistance and virulence. Clinical samples of multidrug-resistant (MDR) *A. baumannii* have been found to exhibit robust biofilm formation, which is associated with increased adherence to human bronchial epithelial cells. This heightened biofilm production contributes to the organism's capacity to persist and survive in the host, as well as its resistance to antibiotics (Gordon & Wareham, 2010). Therefore, it is imperative to target and inhibit biofilm production in *A. baumannii* as a critical approach for controlling its transmission within healthcare settings, while also mitigating its antibiotic resistance and virulence.

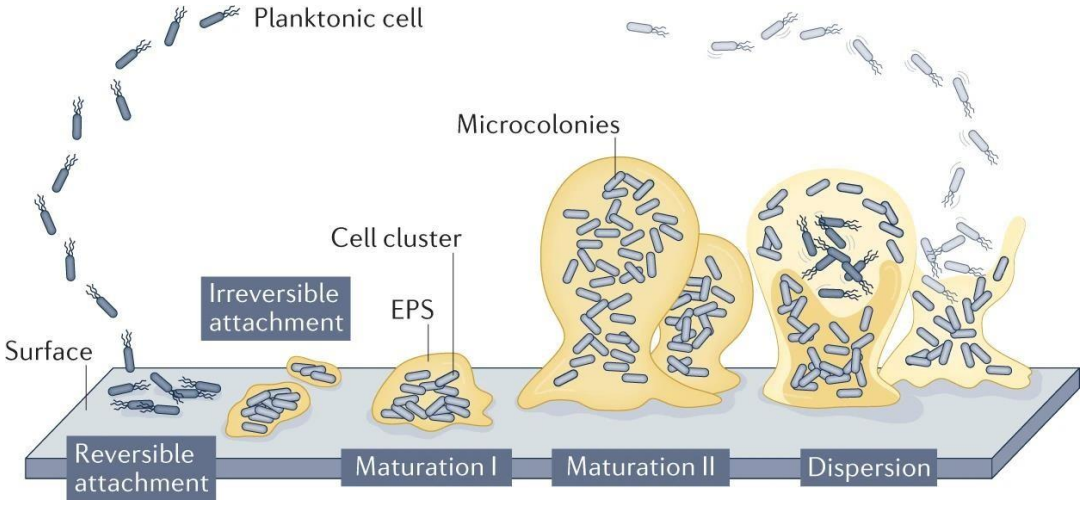


Figure 2.5: The original five-step model of biofilm development. Figure adapted with permission from Sauer et al, (2022); copyright 2022 Springer Nature.

2.7.2 Biofilm and Increased Antibiotic Resistance

E. coli has been identified as the most prevalent cause of urinary tract infections (UTIs), while *A. baumannii* also plays a major role in UTI cases. Many *A. baumannii* isolates have shown resistance to regularly used antibiotics i.e., ampicillin, amoxicillin-clavulanic acid, norfloxacin, cefuroxime, ceftriaxone, and co-trimoxazole. Risk factors for UTIs include conditions like diabetes and kidney disease, as well as the use of intrauterine devices, which can exacerbate the infection and increase the complexity and treatment expense, as well as the risk of complications and mortality (Niranjan & Malini, 2014). Biofilm production aids complication in UTIs by providing a protective environment for bacterial cells, making them more resistant to antibiotics compared to free-floating (planktonic) cells. Biofilms are often associated with medical devices such as catheters, ventilators, and contact lenses, presenting a challenging problem to address. However, studies have shown that when cells from a disrupted biofilm are grown in a planktonic state, they regain antibiotic susceptibility (Zuroff et al., 2010). The association between biofilms and antibiotic resistance has been extensively studied in catheter associated UTIs. It has been observed that bacteria within biofilms exhibit higher resistance to antibiotics such as ampicillin, cefotaxime, norfloxacin, and nalidixic acid compared to non-biofilm bacteria. Furthermore, the presence of biofilms can also influence the effectiveness of antibiotics in preventing biofilm formation. For instance, ampicillin has been found to affect various stages of biofilm formation, inhibiting

attachment and early development, but not preventing mature biofilm formation within 72 hours after discontinuing the treatment. This highlights the importance of considering both multidrug resistance mechanisms including efflux pumps, and the biofilm matrix itself in understanding penicillin resistance development. (Ito et al., 2009). Overall, the presence of biofilms in UTIs contributes to antibiotic resistance and poses challenges for effective treatment. Understanding and addressing biofilm formation and its impact on antibiotic response are important in combating UTIs caused by bacteria like *A. baumannii.*

2.8 Epidemiology of *A. baumannii*

Initially, *Acinetobacter* spp. were considered opportunistic pathogens with low clinical significance. However, since the 1980s, there has been a notable surge in the occurrence and severity of *A. baumannii* infections, particularly affecting patients in ICUs (Y. Zhao et al., 2019). This increase has been observed globally, with notable prevalence in Europe (such as the UK, Germany, Italy, Spain) and the United States, often involving multidrug-resistant (MDR) strains (Akrami & Namvar, 2019). The frequency of nosocomial infections in Intensive Care Unit due to *A. baumannii* scales from 2% to 10% of all gram-negative bacterial infections in Europe and responsible for about 2.5% of them in the United States (Fournier et al., 2006). Person-to-person contact, and the acquisition of antimicrobial resistance also contribute to the incidence of *A. baumannii* infections (Akrami & Namvar, 2019). The prevalence of *A. baumannii* infections can be influenced by man to man contact and antimicrobial resistance acquired by the bacteria (Munoz-Price & Weinstein, 2008). Research guided in the US have shown that the prevelance of *Acinetobacter* nosocomial infections raises during the higher temperatures (Eber et al., 2011). Asian countries have reported high rates of MDR (82%) and extensively drug-resistant (XDR) (51.1%) *Acinetobacter* spp., with particularly high imipenem resistance rates in Thailand, Malaysia, and India exceeding 80% (Chung et al., 2011). The prevalence of *Acinetobacter* among nosocomial infections in Bosnia was 51.4%, in which 74.1% belonged to respiratory infections. In India, studies have reported carbapenem resistance rates of 26-38% among *Acinetobacter* isolates from ICUs (Custovic et al., 2014). The prevalence of *Acinetobacter* in nosocomial infections in Bosnia was found to be 51.4%, with respiratory infections accounting for 74.1% of the cases. Community-acquired *A. baumannii* infections are more commonly detected in tropical and

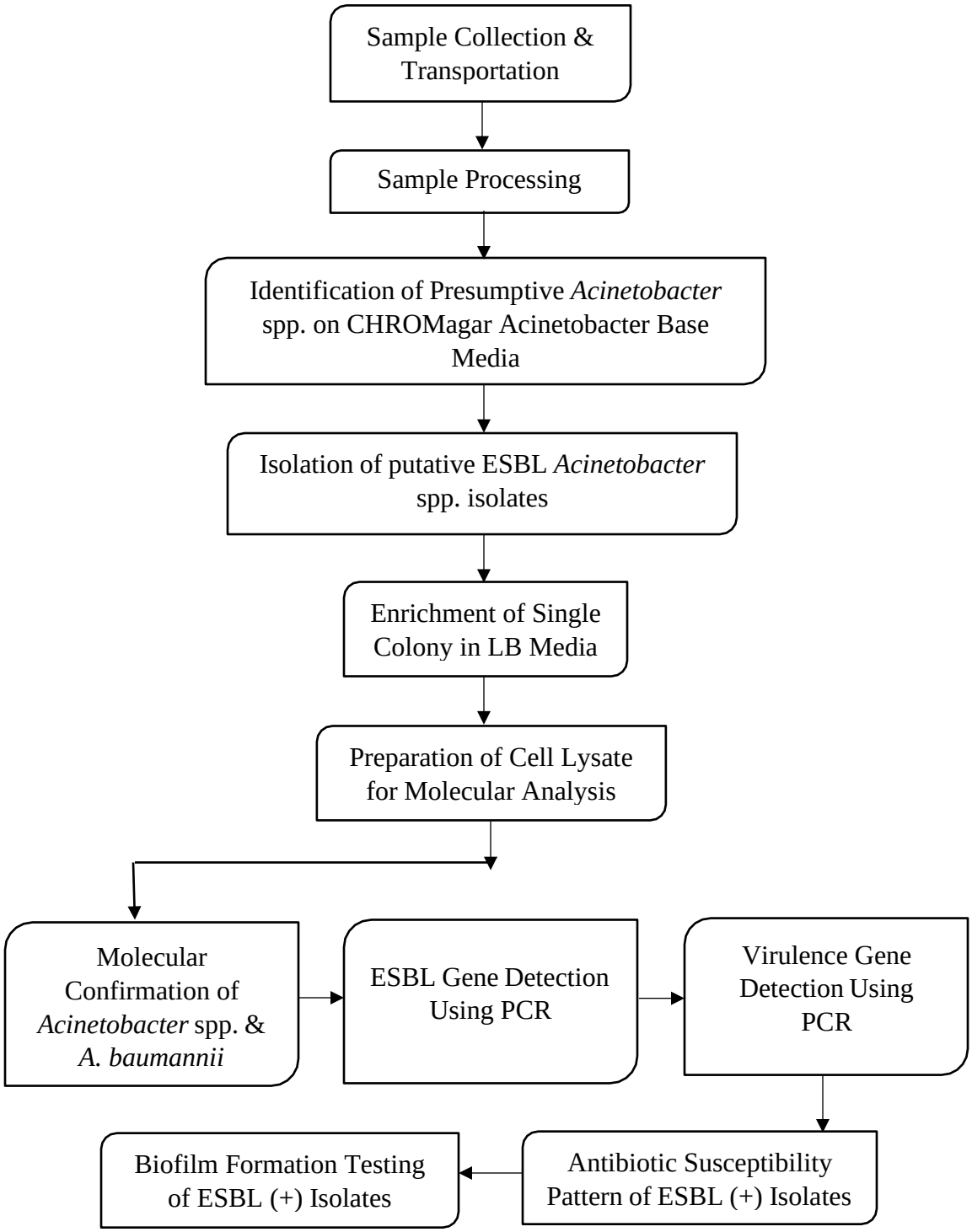
subtropical regions such as Singapore, Hong Kong, and Taiwan, often associated with rainy seasons (Davis et al., 2014). Carbapenem-resistant *A. baumannii* (CRAB) is a significant concern, accounting for approximately 65% of *A. baumannii* pneumonia cases in the United States and Europe (Zhao et al., 2019). The Baltic countries and southern/southeastern Europe have reported the highest percentages of resistance among *Acinetobacter spp*.

Chapter Three

MATERIALS & METHOD

3.0 Materials & Method

The entire study methodology is briefly given in the flowchart below



3.1 Sampling Site and Sample Collection

This study aimed to investigate the presence of pathogenic *A. baumannii* in the environments. Sampling was conducted with the approval of the Institutional Review Board (IRB) of icddr,b. The samples were collected from 7 points of the Goranchatbari Sub-catchment over 3 seasons; Pre monsoon (April, 2022), Monsoon (September, 2022) and Post monsoon (December, 2022). A total of 21 samples were collected over the three seasons. After collection, the samples were transported to the Laboratory of Environmental Health, icddr,b, Dhaka maintaining the cold chain according to the standard procedures in an insulated box with sufficient ice packs to maintain a temperature around within 4-10^{o}C. The samples were then processed within 8hrs of collection.

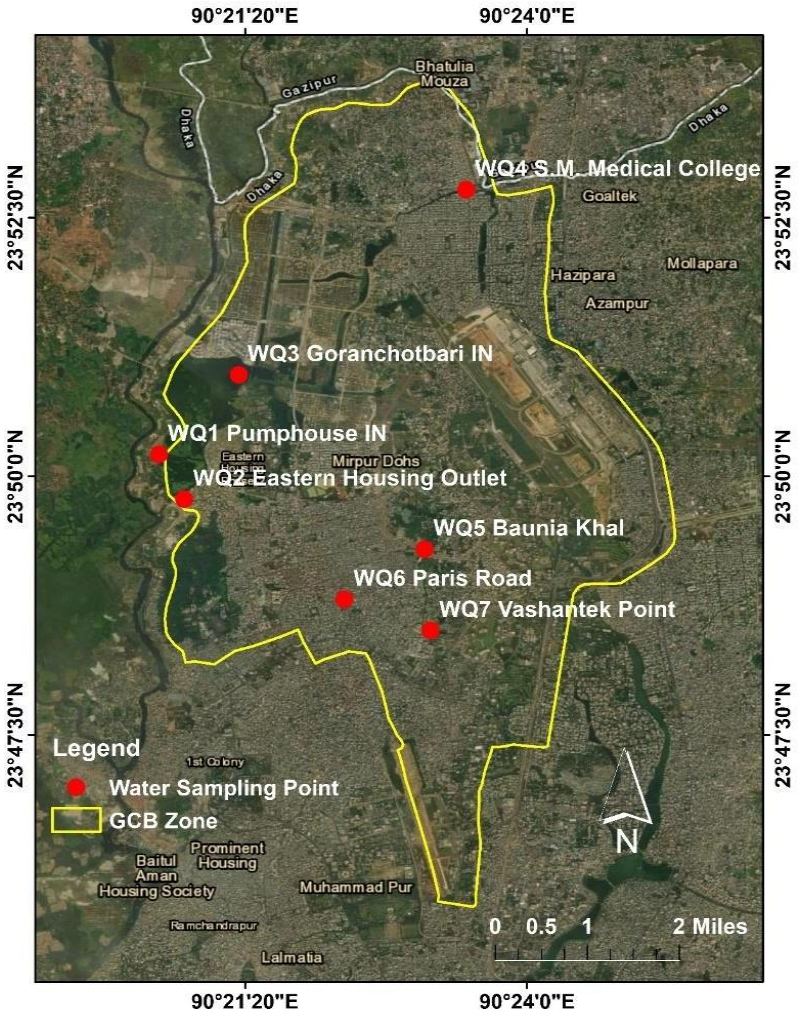


Figure 3.1: Locations of the sampling site

3.2 Sample Processing

For analysis, the samples were placed to allow them to reach room temperature and then they were subjected to serial decimal dilutions (10^{-1}, 10^{-2} and 10^{-3}) with sterile normal saline (0.85% NaCl). 100 µl of the diluted sample was then spread on CHROMagar™ Acinetobacter Base (CAB) agar plate. The plates were then incubated at 37 ± 0.5 ^{o}C for 18-24 hrs. Counts of the colonies from a countable dilution was then noted as CFU/100 ml.

3.2.1 Isolation and Identification of ESBL Positive *A. baumannii* on Selective Media

Following incubation, colonies with red color were considered as putative *Acinetobacter* spp. For further screening, maximum 10 colonies were picked randomly from each sample and patched on CHROMagar^{TM} ESBL media, one colony in one slot at a time, and incubated at 37°C for 18 h -24 h. Growth and distinctive colony morphology on these media confirm the production of extended-spectrum β-lactamases (ESBLs) by the isolates. Cream, opaque colonies on CHROMagar^{TM} ESBL is representative of ESBL producing *Acinetobacter* spp. Following the presence of growth, single colonies were then streaked onto CHROMagar™ Acinetobacter Base plate and incubated at 37 ± 0.5^{o}C for 18-24hrs to obtain discrete colonies again. The red colonies on CHROMagar™ Acinetobacter Base were further confirmation of *Acinetobacter* spp.

3.2.2 Preparation of Stock Culture for Further Analysis

After confirmation, single colony from CHROMagar™ Acinetobacter Base plate was picked and cultured into 3 ml of LB broth for enrichment in shaking incubator for 18h-24h at 37°C maintaining 120 rpm. A stock culture was prepared from enriched culture supplemented with 30% (v/v) glycerol (700µl enriched culture+300µl glycerol) and stored at -80°C for further analysis.

3.3 Bacterial Cell Lysate Preparation for Molecular Biological Analysis

3.3.1 Total cell DNA content isolation

The DNA from the samples were isolated using the boiling lysis method (Hossain et. al., 2021). The use of heat to break down the cell wall and cell membrane prior to cold shock exposure has been shown to yield relatively pure DNA that can be used for molecular analysis and routine exposure to heat and cold has been shown to aid in the isolation of bacterial DNA (Tell et al., 2003). During this procedure, one to two discrete colonies were scraped off the CHROMagar™ Acinetobacter Base plate containing the pure culture and inoculated into 3ml of LB broth, after which it was subjected to overnight incubation at 37^{o}C. Following incubation, 1.5ml of the culture was taken into a microcentrifuge tube and centrifuged at 13000 rpm for 5 minutes. The supernatant was then discarded, and the pellet was resuspended in 600µl of autoclaved DI water and mixed through repeated pipetting. The solution was then subjected to boiling at 100^{o}C for 10 minutes and then immediately cooled on ice for 10 minutes. Following the heat and cold treatment, the solution was centrifuged at 13000 rpm for 7-8 minutes, after which 100µl of the supernatant was transferred to a new microcentrifuge tube and stored at -20^{o}C until further use.

3.4 Detection of *Acinetobacter spp*. & *A. baumannii*

The total DNA content of the ESBL positive isolates was used for molecular detection of *Acinetobacter* spp. & *A. baumannii* according to the previously published protocol (Toledo-Arana et al., 2001, Tsai et al., 2018). For the reactions where available, *A. baumannii* NCTC 12156 acting as positive control was used. For this detection a multiplex PCR was performed using sequence specific primers. These genes were: *A. baumannii 16S–23S rRNA ITS*, the ITS region between the 16S and 23S rRNA genes is a good candidate for bacterial species identification (Tsai et al., 2018). The *recA* gene, is highly specific for the genus *Acinetobacter* (T.-L. Chen et al., 2007). The primer sequences are given in the **Table 3.11** (Primer Seq.). The reaction mixtures were prepared by mixing components given in **Table 3.1** (Multiplex). The PCR tube containing the reaction mixture was prepared in a laminar flow cabinet to provide a contamination free environment. Following preparation, the PCR tubes containing the reaction mixture and template DNA was capped and centrifuged briefly to spin down the contents. The PCR tubes were then placed in BIORAD

T100TM Thermal cycler (BIORAD, USA). PCR conditions used for each type of gene are given in **Table 3.2**. After performing all the cycles of PCR, tubes were stored at -20°C until furt her analysis.

3.4.1 Reaction mixture composition for PCR of *Acinetobacter* spp. *& A. baumannii*

PCR reaction mixture for 1 sample of total 25 µl volume.

Table 3.1 PCR reaction mixture for *Acinetobacter* spp. *& A. baumannii*

Serial No.	Reagent	Volume
1	Thermo Scientific™ DreamTaq™ Green PCR Master Mix (2X)	12.5 µl
2	P-Ab-ITS Forward (Concentration-0.2µm)	1.0 µl
3	P-Ab-ITS Reverse (Concentration-0.2µm)	1.0 µl
4	P-rA1 (Concentration-0.2µm)	1.0 µl
5	P-rA2 (Concentration-0.2µm)	1.0 µl
10	Template	2.0 µl
11	Nuclease Free Water	6.5 µl

3.4.2 PCR Conditions for *16S-23S-rRNA ITS* Gene and *recA* Gene

Table 3.2 PCR Conditions for *16S-23S-rRNA ITS* Gene and *recA* Gene

Process	Temperature (°C)	Time	Segment
Initial Denaturation	94 °C	5 minutes	Segment 1
Denaturation	95°C	30 seconds	Segment 2

Annealing	54°C	30 seconds	(30 Cycles)
Extension	72°C	30 seconds	
Final Extension	72°C	7 minutes	Segment 3

3.4.3 Post PCR detection of Amplified DNA through Agarose Gel Electrophoresis

The presence and subsequent amplification of the gene being examined is detected by resolving the PCR products in 1% agarose gel. The gel was made by dissolving 0.8gm agarose (Sigma) in 80ml of 0.5x Tris-borate EDTA (TBE) buffer over gentle heat to give a final concentration of 1% agarose. When the temperature cooled down to 50-60^{o}C, the gel was poured onto a gel casting tray fitted with appropriate combs. Following solidification, the gel was submerged in 0.5x TBE buffer in a gel electrophoresis tank. Then, 7µl of the products were loaded into each slot of the gel, with the first slot being filled with the DNA ladder. Electrophoresis was carried out at 80volts until the dye migrated 5-6 cm from the wells (after around 1.5 – 2.0 hrs.). The gel was finally removed carefully and placed in a staining (0.5 µg/ml EtBr) tray and stained for 15 minutes. Then the gel was de-stained for about 15 minutes in deionized water. The gel was then observed, and a photograph was taken on GelDoc Go Imaging System (BIORAD, USA).

3.5 Detection of *bla* genes by PCR

The total DNA content of the isolates was used to test the presence of *bla* gene groups, bla_{SHV}, bla_{TEM}, $bla_{CTX\text{-}M}$ and bla_{OXA} by polymerase chain reaction according to the previously published protocol (Fang et al., 2008). For the reactions where available, ESBL positive *E. coli* isolates acting as positive controls were used. For the first 4 gene groups (bla_{SHV}, bla_{TEM}, $bla_{CTX\text{-}M}$ and bla_{OXA}) a multiplex PCR was performed using sequence specific primers. The primer sequences are listed in **Table 3.11** (Primer Seq.). The reaction mixtures were prepared by mixing components given in **Table 3.3** (Multiplex). The PCR tube containing the reaction mixture was prepared in a laminar flow cabinet to provide a contamination free environment. Following preparation, the PCR tubes containing the reaction mixture and template DNA was capped and centrifuged briefly to spin

down the contents. The PCR tubes were then placed in BIORAD T100TM Thermal cycler (BIORAD, USA). PCR conditions used for each type of gene are given in **Table 3.4**. After performing all the cycles of PCR, tubes were stored at -20°C until furth er analysis.

3.5.1 Reaction mixture composition for PCR of *bla*$_{SHV}$, *bla*$_{TEM}$, *bla*$_{CTX-M}$ and *bla*$_{OXA}$

PCR reaction mixture for 1 sample of total 25 µl volume.

Table 3.3 PCR reaction mixture for *bla*$_{SHV}$, *bla*$_{TEM}$, *bla*$_{CTX-M}$ and *bla*$_{OXA}$ gene

Serial No.	Reagent	Volume
1	Thermo Scientific^{TM} DreamTaq^{TM} Green PCR Master Mix (2X)	12.5 µl
2	Primer *bla*$_{SHV}$ Forward (Concentration-0.2µm)	0.5 µl
3	Primer *bla*$_{SHV}$ Reverse (Concentration-0.2µm)	0.5 µl
4	Primer *bla*$_{TEM}$ Forward (Concentration-0.2µm)	0.5 µl
5	Primer *bla*$_{TEM}$ Reverse (Concentration-0.2µm)	0.5 µl
6	Primer *bla*$_{CTX-M}$ Forward (Concentration-0.2µm)	0.5 µl
7	Primer *bla*$_{CTX-M}$ Reverse (Concentration-0.2µm)	0.5 µl
8	Primer *bla*$_{OXA}$ Forward (Concentration-0.2µm)	0.5 µl
9	Primer *bla*$_{OXA}$ Reverse (Concentration-0.2µm)	0.5 µl
10	Template	2.0 µl
11	Nuclease Free Water	6.5 µl

3.5.2 PCR Conditions for *bla*$_{SHV}$, *bla*$_{TEM}$, *bla*$_{CTX-M}$ and *bla*$_{OXA}$ Gene

Table 3.4 PCR Conditions for *bla*$_{SHV}$, *bla*$_{TEM}$, *bla*$_{CTX-M}$ and *bla*$_{OXA}$ Gene

Process	Temperature (°C)	Time	Segment
Initial Denaturation	95°C	5 minutes	Segment 1
Denaturation	94°C	30 seconds	Segment 2 (30 Cycles)
Annealing	62°C	90 seconds	
Extension	72°C	1 minute	
Final Extension	72°C	10 minutes	Segment 3

3.6 Confirmation of Virulent Isolates

The isolates were investigated for the presence of the following biofilm related genes- *bap* (H.-W. Lee et al., 2008), *ompA* (Toledo-Arana et al., 2001), *csuE* (Braun & Vidotto, 2004), *epsA*, (Toledo-Arana et al., 2001), bla_{PER-1} (Tayabali et al., 2012), *bfmS* (H.-W. Lee et al., 2008), *ptk* and *pgaB* (Liou et al., 2014), *fimH*, and *kpsMII* (Bahador et al., 2013) was assessed using PCR according to the previously published protocol. 3 multiplex PCR were carried out for the ten virulence genes. For *epsA*, *bfms*, *fimH* and *csuE* a multiplex PCR was done. Next, for the bla_{PER-1}, *bap*, *ptk* and *pgaB* a multiplex PCR was performed using sequence specific primers. Lastly, for the two genes *ompA* and *kpsMII* another multiplex PCR using specific primers was conducted. The primer sequences and their PCR products are listed in **Table 3.11** (Primer Seq.). The reaction mixtures for the three multiplex PCR were prepared by mixing components given in **Table 3.5**, **Table 3.7** and **Table 3.9**. The PCR tube containing the reaction mixture was prepared in a laminar flow cabinet to provide a contamination free environment. Following preparation, the PCR tubes containing the reaction mixture and template DNA was capped and centrifuged briefly to spin down the contents. The PCR tubes were then placed in BIORAD T100TM Thermal cycler (BIORAD, USA). PCR conditions used for each multiplex PCR are given in **Table 3.6**, **Table 3.8** and **Table 3.10**. After performing all the cycles of PCR, tubes were stored at -20°C until further analysis.

3.6.1 Reaction mixture composition for PCR of *epsA, bfms, fimH* and *csuE*

PCR reaction mixture for 1 sample of total 25 µl volume.

Table 3.5 PCR reaction mixture for *epsA, bfms, fimH and csuE*

Serial No.	Reagent	Volume
1	Thermo Scientific™ DreamTaq™ Green PCR Master Mix (2X)	12.5 µl
2	Primer *epsA* Forward (Concentration-0.2µm)	0.5 µl
3	Primer *epsA* Reverse (Concentration-0.2µm)	0.5 µl
4	Primer *bfms* Forward (Concentration-0.2µm)	0.5 µl
5	Primer *bfms* Reverse (Concentration-0.2µm)	0.5 µl
6	Primer *fimH* Forward (Concentration-0.2µm)	0.5 µl
7	Primer *fimH* Reverse (Concentration-0.2µm)	0.5 µl
8	Primer *csuE* Forward (Concentration-0.2µm)	0.5 µl
9	Primer *csuE* Reverse (Concentration-0.2µm)	0.5 µl
10	Template	3.0 µl
11	Nuclease Free Water	5.5 µl

3.6.2 PCR Conditions for *epsA, bfms, fimH* and *csuE*

Table 3.6 PCR Conditions for *epsA, bfms, fimH and csuE*

Process	Temperature (°C)	Time	Segment
Initial Denaturation	94°C	5 minutes	Segment 1

Denaturation	94°C	1 minute	Segment 2 (30 Cycles)
Annealing	60°C	1 minute	
Extension	72°C	1 minute	
Final Extension	72°C	10 minutes	Segment 3

3.6.3 Reaction mixture composition for PCR of *bla*$_{PER-1}$, *bap, ptk, pgaB*

PCR reaction mixture for 1 sample of total 25 µl volume.

Table 3.7 PCR reaction mixture for *bla*$_{PER-1}$, *bap*, *ptk*, *pgaB*

Serial No.	Reagent	Volume
1	Thermo Scientific™ DreamTaq™ Green PCR Master Mix (2X)	12.5 µl
2	Primer *bla*$_{PER-1}$ Forward (Concentration-0.2µm)	0.5 µl
3	Primer *bla*$_{PER-1}$ Reverse (Concentration-0.2µm)	0.5 µl
4	Primer *bap* Forward (Concentration-0.2µm)	0.5 µl
5	Primer *bap* Reverse (Concentration-0.2µm)	0.5 µl
6	Primer *ptk* Forward (Concentration-0.2µm)	0.5 µl
7	Primer *ptk* Reverse (Concentration-0.2µm)	0.5 µl
8	Primer *pgaB* Forward (Concentration-0.2µm)	0.5 µl
9	Primer *pgaB* Reverse (Concentration-0.2µm)	0.5 µl
10	Template	3.0 µl
11	Nuclease Free Water	5.5 µl

3.6.4 PCR Conditions for *bla*$_{PER-1}$, *bap, ptk, pgaB*

Table 3.8 PCR Conditions for *bla*$_{PER-1}$, *bap, ptk, pgaB*

Process	Temperature (°C)	Time	Segment
Initial Denaturation	94°C	5 minutes	Segment 1
Denaturation	94°C	1 minute	Segment 2 (30 Cycles)
Annealing	56°C	1 minute	
Extension	72°C	1 minute	
Final Extension	72°C	10 minutes	Segment 3

3.6.5 Reaction mixture composition for PCR of *ompA* and *kpsMII*

PCR reaction mixture for 1 sample of total 25 µl volume.

Table 3.9 PCR reaction mixture for *ompA* and *kpsMII*

Serial No.	Reagent	Volume
1	Thermo Scientific™ DreamTaq™ Green PCR Master Mix (2X)	12.5 µl
2	Primer *ompA* Forward (Concentration-0.2µm)	0.5 µl
3	Primer *ompA* Reverse (Concentration-0.2µm)	0.5 µl
4	Primer *kpsMII* Forward (Concentration-0.2µm)	0.5 µl
5	Primer *kpsMII* Reverse (Concentration-0.2µm)	0.5 µl
6	Template	2.0 µl
7	Nuclease Free Water	8.5 µl

3.6.6 PCR Conditions for *ompA* and *kpsMII*

Table 3.10 PCR Conditions for *ompA* and *kpsMII*

Process	Temperature (°C)	Time	Segment
Initial Denaturation	94°C	5 minutes	Segment 1
Denaturation	94°C	1 minute	Segment 2 (30 Cycles)
Annealing	58°C	1 minute	
Extension	72°C	1 minute	
Final Extension	72°C	10 minutes	Segment 3

3.6.8 Primer Sequences

Table 3.11 List of Primers Used in This Study

	Target Gene	Primer Name	Sequence (5' → 3')	Product Size (bp)	Reference
Species Specific Primers	*16S-23S-rRNA ITS*	Ab-ITS-F	CATTATCACGGTAATTAGTG	208	(Tsai et al., 2018)
		Ab-ITS-B	AGAGCACTGTGCACTTAAG		
	recA	P-rA1	CCTGAATCTTCTGGTAAAAC	425	(T.-L. Chen et al., 2007)
		P-rA2	GTTTCTGGGCTGCCAAACATTAC		
ESBL Primers	bla_{SHV}	SHV-F	CTTTATCGGCCCTCACTCAA	237	(Fang et al., 2008)
		SHV-R	AGGTGCTCATCATGGGAAAG		

	bla$_{TEM}$	TEM-F	CGCCGCATACACTATTCTCAGAATGA	445	(Fang et al., 2008)
		TEM-R	ACGCTCACCGGCTCCAGATTTAT		
	bla$_{CTX-M}$	CTX-M-F	ATGTGCAGYACCAGTAARGTKATGGC	593	(Fang et al., 2008)
		CTX-M-R	TGGGTRAARTARGTSACCAGAAYCAGCGG		
	bla$_{OXA}$	OXA-F	ACACAATACATATCAACTTCGC	813	(Fang et al., 2008)
		OXA-R	AGTGTGTTTAGAATGGTGATC		
Virulence Gene Primers	*epsA*	epsA-F	AGCAAGTGGTTATCCAATCG	451	(Toledo-Arana et al., 2001)
		epsA-R	ACCAGACTCACCCATTACAT		
	ompA	ompA-F	CGCTTCTGCTGGTGCTGAAT	531	(Toledo-Arana et al., 2001)
		ompA-R	CGTGCAGTAGCGTTAGGGTA		
	bla$_{PER-1}$	bla$_{PER-1}$-F	ATGAATGTCATTATAAAAGC	927	(Tayabali et al., 2012)
		bla$_{PER-1}$-R	AATTTGGGCTTAGGGCAAGAAA		

	bap	bap-F	TACTTCCAATCCAATGCTAGGGAGGGTACCAATGCAG	1225	(H.-W. Lee et al., 2008)
		bap-R	TTATCCACTTCCAATGATCAGCAACCAAACCGCTAC		
	bfmS	bfmS-F	TTGCTCGAACTTCCAATTTATTATAC	1428	(H.-W. Lee et al., 2008)
		bfmS-R	TTATGCAGGTGCTTTTTTATTGGTC		
	ptk	ptk-F	GGCTGAGCATCCTGCAATGCGT	597	(Liou et al., 2014)
		ptk-R	ACTTCTGGAGAAGGGCCTGCAA		
	pgaB	pgaB-F	AAGAAAATGCCTGTGCCGACCA	490	(Liou et al., 2014)
		pgaB-R	GCGAGACCTGCAAAGGGCTGAT		
	fimH	fimH-F	TGCAGAACGGATAAGCCGTGG	870	(Bahador et al., 2013)
		fimH-R	GCAGTCACCTGCCCTCCGGTA		
	kpsMII	kpsMII-F	GCGCATTTGCTGATACTGTTG	272	(Bahador et al., 2013)
		kpsMII-R	CATCCAGACGATAAGCATGAGCA		
	csuE	csuE-F	ATGCATGTTCTCTGGACTGATGTTGAC	976	(Braun &

		csuE-R	CGACTTGTACCGTGACCGTATCTTGATAAG		Vidotto, 2004)

3.7 Antibiotic Susceptibility Testing

The antibiotic susceptibility pattern was tested for all the isolates following the Kirby-Bauer disk diffusion method which is recommended by the Clinical and Laboratory Standards Institute guidelines (CLSI, 2020). For the antimicrobial susceptibility testing commercially available disks (Thermo Scientific™ Oxoid™) were used and the antibiotic susceptibility pattern was detected for 11 antibiotic agents. The following antibiotic disks were used- Piperacillin/Tazobactam (TPZ, 110 μg), Cotrimoxazole (SXT, 25 μg), Cefotaxime (CTX, 30 μg), Ceftriaxone (CRO, 30 μg), Cefepime (FEP, 30 μg), Amikacin (AK, 30μg), Meropenem (MEM, 10 μg), Imipenem (IMP, 10 μg), Gentamicin (CN, 10 μg), Tetracycline (TE, 30 μg), Ciprofloxacin (CIP, 5 μg),

3.7.1 Procedure

The antibiotic disks were firmly placed on Mueller Hinton agar medium (Difco, MD, USA) which had been inoculated with fresh suspension of *A. baumannii* culture. For inoculation, isolated colonies of *A. baumannii* were picked from streaked agar plates and used to prepare an inoculum suspension of 1.5×10^{8}CFU/mL cell density which was standardized using a 0.5 McFarland solution. Following inoculum preparation, a sterile cotton swab was immersed and swirled several times in the suspension and while removing was pressed gently against the inner wall of the tube. The inoculation was then done by streaking the swab on the dried surface of the Mueller Hilton agar medium. To ensure even distribution, a repetitive streaking pattern was used. Withing 3-5minutes, antibiotic disks were placed on the streaked agar plates. The plates were incubated at 37^{o}C for 18h (±2hrs) and then investigated for the presence of clear zones due to growth inhibition. The diameter of the zone of inhibition was recorded in millimeter along with a 6mm disc diameter.

3.7.2 Interpretation

According to the diameter of the zone of inhibition, the isolates were categorized as susceptible, intermediate, or resistant as per CLSI guidelines were categorized as susceptible, intermediate or resistant as per CLSI guidelines (**Table 3.12)**.

Table 3.12 Zone of Diameter Interpretation for *A. baumannii*

Groups	Antibiotics	Sensitive	Intermediate	Resistant
3G Cephalosporin	Cefotaxime	≥23	15-22	≤14
3G Cephalosporin	Ceftriaxone	≥21	14-20	≤13
4G Cephalosporin	Cefepime	≥18	15-17	≤14
Sulfonamides	Cotrimoxazole	≥16	11-15	≤10
2G Fluoroquinolone	Ciprofloxacin	≥21	16-20	≤15
Carbapenem	Meropenem	≥18	15-17	≤14
Carbapenem	Imipenem	≥22	19-21	≤18
Aminoglycoside	Gentamicin	≥15	13-14	≤18
Aminoglycoside	Amikacin	≥15	13-14	≤18
Tetracycline	Tetracycline	≥15	12-14	≤11
Antipsedomonal penicillin + beta lactamase inhibitor	Piperacillin/Tazobactam	≥21	18-20	≤17

3.8 Biofilm Formation Assay

The biofilm formation capability of the isolates was detected using the quantitative adherence assay (Nirwati et al., 2019). For each isolate, an inoculum culture was prepared by inoculating a single colony in Lurie Bertani broth which was incubated overnight at 37^{o}C. Following overnight incubation, 2µl of the suspension was inoculated in a sterile 96 well flat bottom polystyrene microtiter plate (Costar, USA) which contained 198µl of fresh LB and for each run, negative controls containing uninoculated LB were used. The plates were then incubated at 25^{o}C and 37^{o}C for 48hours. Following incubation, the plates were washed gently with 200 µl of PBS and dried in an inverted position. The biofilm mass was then stained with 200µl of 0.1% crystal violet solution for 15 minutes. The wells were washed again with 200µl of distilled water to remove the unbound crystal violet and dried in an inverted position. Lastly, the stained biofilm mass was then dissolved by filling the wells with 200µl of 5% isopropanol acid. The optical density (OD) measurement was then performed for the microtiter plate using an ELISA plate reader (BioTek, USA) at a wavelength of 590nm.

3.8.1 Interpretation

The isolates were categorized either as strong, moderate, weak or non-biofilm former based on previously published protocol (Nirwati et al., 2019). From the negative values, the optical density cut-off value (ODc) was established. The ODc value is three standard deviations (SD) above the mean OD of the negative control, that is ODc=average OD of negative control-3×Standard deviation (SD) of negative controls. The isolates with with OD $\leq$ ODc are termed as non-biofilm producer. On the other contrary, the isolates with ODc < OD $\leq$ 2× ODc are categorized as weak biofilm producer, whereas 2×ODc < OD $\leq$ 4×ODc and OD> 4×ODc are categorized as moderate and strong biofilm producer respectively.

Chapter Four

RESULTS

4.0 Results

The present study was designed to isolate ESBL producing *A. baumannii*, the major pathogen responsible for nosocomial infections. The samples were collected from 7 points in the Goranchatbari Sub-catchment in Dhaka city, and their molecular characterization was conducted through various methods.

4.1 Sample Processing, Isolation and Identification of ESBL positive *A. baumannii*

4.1.1 Sample Processing & Isolation

100µl of each sample was spread on CHROMagar^{TM} Acinetobacter Base Media (CAB). The plates were then incubated at 37 ± 0.5 ^{o}C for 18-24hrs. Following incubation, the results were recorded for red-colored colonies which are indication of *Acinetobacter* spp. on CAB agar (**Figure 4.1)**. A total of 20 samples out of 21 presume to have *Acinetobacter* spp. The distribution of *Acinetobacter* spp. from different sampling point in each season is given in **Figure 4.2.** The prevalence of presumptive *Acinetobacter* spp. seems higher during the pre-monsoon season.

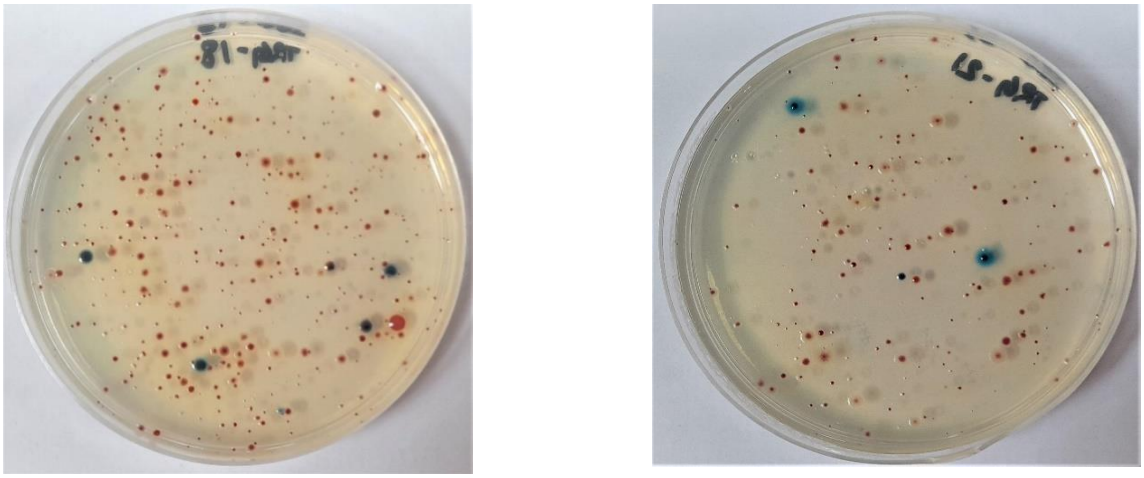


Figure 4.1: Red colonies on CAB indicative of *Acinetobacter* spp.

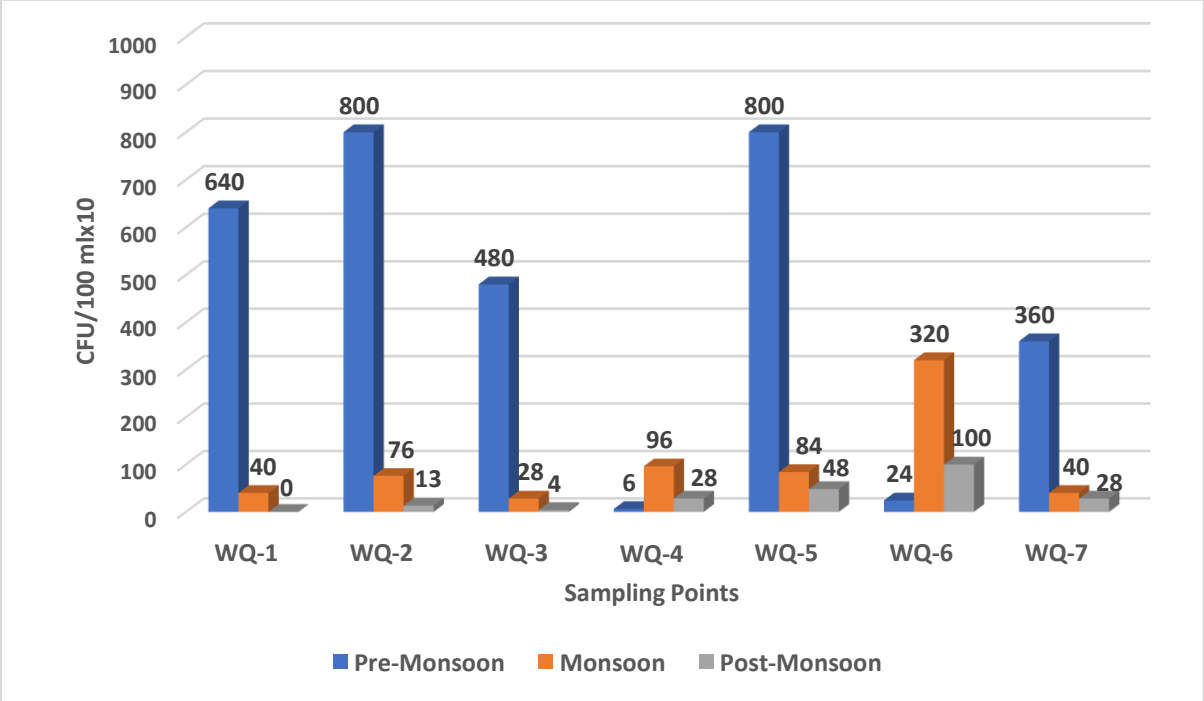
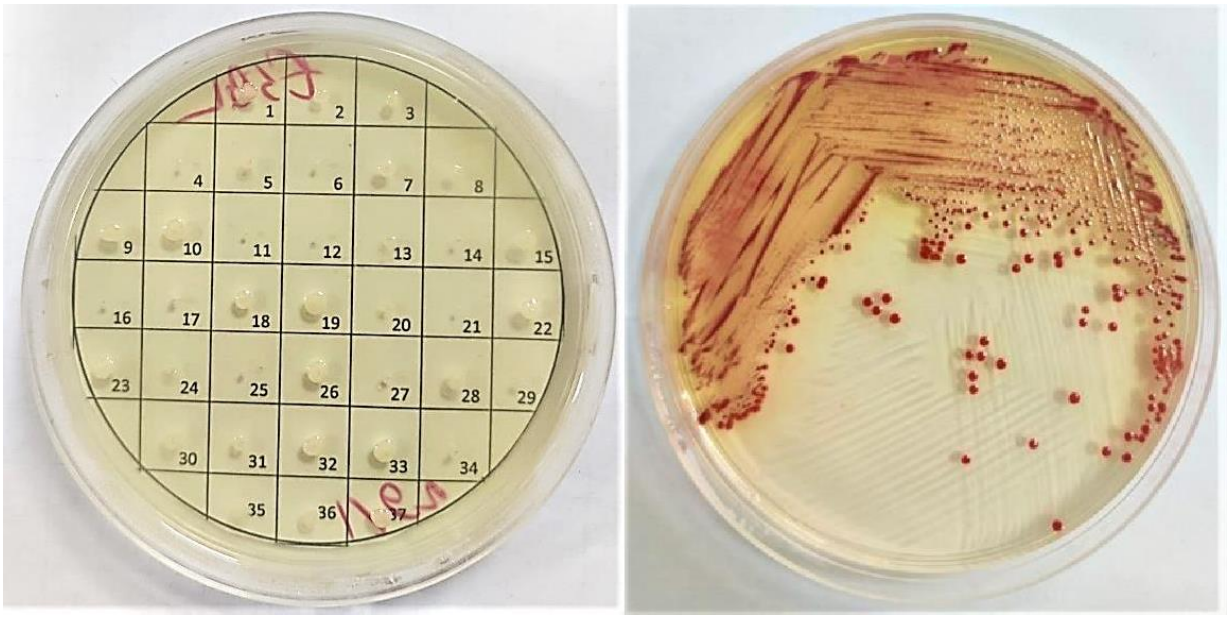


Figure 4.2: The distribution of presumptive *Acinetobacter* spp. from different sampling point in each season

4.1.2 Isolation and Identification of ESBL producing *Acinetobacter* spp.

The red-colored colonies on CAB agar were suspected to be *Acinetobacter* spp. A maximum of 10 colonies from each sample were inoculated into CHROMagar^{TM} ESBL media through the patch inoculation to phenotypically identify ESBL producing *Acinetobacter* spp. Cream, opaque colonies on the media plates were representative of ESBL producing *Acinetobacter* spp. All the isolates were assigned IDs using numerical values and the data was stored in a log sheet. Among the randomly selected 208 colonies a total of 117 isolates was found to be ESBL producers. Representative plate of ESBL positive presumptive *Acinetobacter* spp. is given in **Figure 4.3.**



(A) **(B)**

Figure 4.3: (A) *Acinetobacter* spp. on CHROMagar™ ESBL agar plate. The colonies from CAB were inoculated into the agar media plate, and slot 9,10,18, 19, 26, 32 and 33 indicates ESBL producing *Acinetobacter* spp. due to their growth and distinctive colour. (B) *Acinetobacter* spp. on CHROMagar™ Acinetobacter Base Plate

4.2 Prevalence of Presumptive ESBL *Acinetobacter* spp. Influenced by Season

The prevalence of presumptive ESBL producing *A. baumannii* based on season was studied upon. Here, during the pre-monsoon era, the number of presumptive ESBL *Acinetobacter* spp. found from different location were WQ-1: 20%, WQ-2: 0%, WQ-3: 44%, WQ-4: 61.54%, WQ-5: 42.86%, WQ-6: 45.45%, WQ-7: 20%. In the monsoon season, the number of presumptive ESBL *Acinetobacter* spp. found from different location were WQ-1: 40%, WQ-2: 60%, WQ-3: 30%, WQ-4: 30%, WQ-5: 80%, WQ-6: 80%, WQ-7: 50%. In the post monsoon season, WQ-1: 0%, WQ-2: 80%, WQ-3: 90%, WQ-4: 80%, WQ-5: 90%, WQ-6: 90%, WQ-7: 90% of the isolates were found. The percentage of the presumptive ESBL producing *Acinetobacter* spp. is given in the **Figure 4.4.**

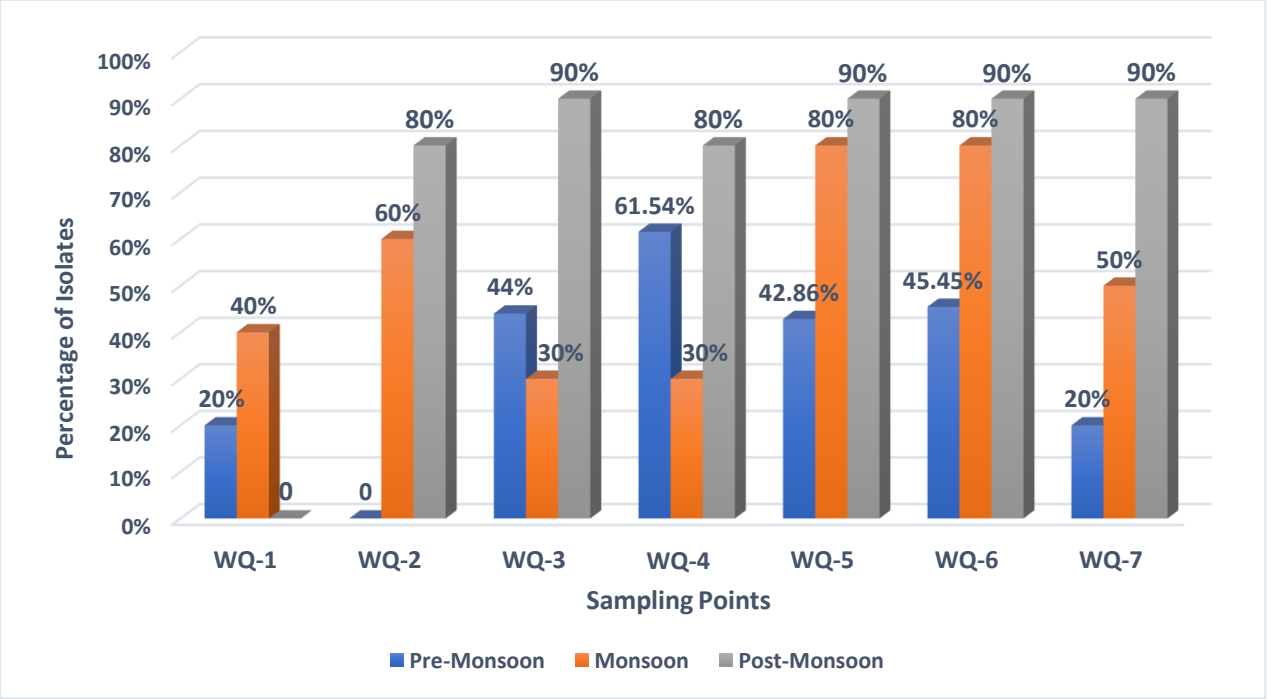


Figure 4.4: The percentage of presumptive ESBL producing *Acinetobacter* spp. isolates from different seasons

4.3 Detection of *Acinetobacter spp*. & *A. baumannii* by PCR

All the 117 ESBL producing isolates were screened for molecular confirmation of *Acinetobacter* spp. based on *recA* gene (T.-L. Chen et al., 2007) and *A. baumannii* based on *16S-23S-rRNA ITS* gene (Tsai et al., 2018). Among all the isolates, 76 of them were positive for *Acinetobacter* spp. based on the presence of *recA* gene and 56 of them were positive for *A. baumannii* based on the presence of *16S-23S-rRNA ITS* gene. The gel image for the PCR reaction is given in the **Figure 4.5.** Here, the band size of the amplified product of *recA* gene is 425 bp and *16S-23S-rRNA ITS* gene is 208 bp.

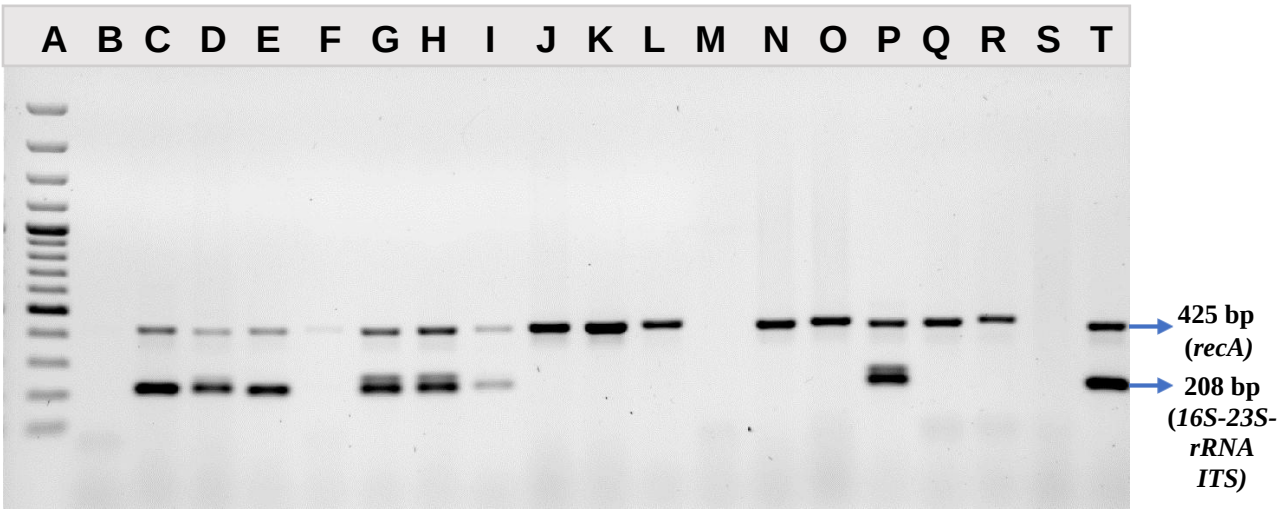


Figure 4.5: Bands obtained from agarose gel electrophoresis of PCR amplicon from *Acinetobacter* spp. and *A. baumannii* confirmation reaction. *recA* gene is specific for *Acinetobacter* spp and the band size is 425 bp. *16S-23S-rRNA ITS* gene is specific for *A. baumannii* and the band size is 208 bp. Lane-A contains 100 bp DNA ladder, whereas Lane-B is the negative control and Lane-C represent positive controls. Lane-E to T represent samples of *Acinetobacter* spp. being tested

4.3.1 Prevalence of *A. baumannii* Influenced by Season

The influence of the season on *A. baumannii* was studied upon and during the pre-monsoon era among 29 *Acinetobacter* spp. 10 (34.48%) of them were *A. baumannii.* In the monsoon period among 28 *Acinetobacter* spp. all 28 (100%) of them were *A. baumannii.* Lastly, in the post monsoon season, 18 (73.07%) of them were *A. baumannii out* of 26 *Acinetobacter* spp. The ratio of *A. baumannii* among *Acinetobacter* spp. is given in the **Figure 4.6.**

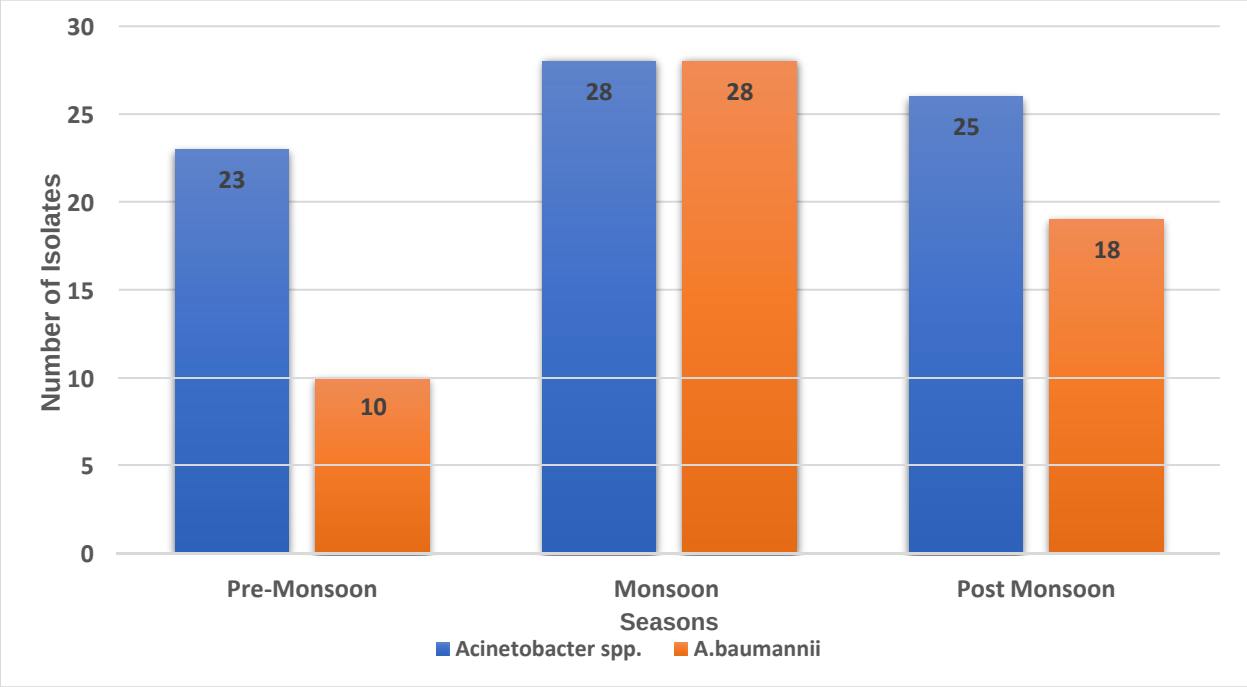


Figure 4.6: Prevalence of *A. baumannii* influenced by seasons

4.4 Detection of *bla* Genes by PCR

All the 56 isolates of *A. baumannii* were screened for ESBL producing genes by PCR. Among the isolates, 40 (71.4%) were positive for bla_{TEM}, 3 (5.4%) were positive for $bla_{SHV,}$ and none of the isolates tested positive for bla_{OXA} and $bla_{CTX\text{-}M}$. The gel image of the PCR and percentage of the presence of genes among the isolates are given in **Figure 4.7 and Figure 4.8** respectively. Here, the band size of the amplified product of bla_{TEM} and bla_{SHV} gene is 445 bp 237 bp respectively.

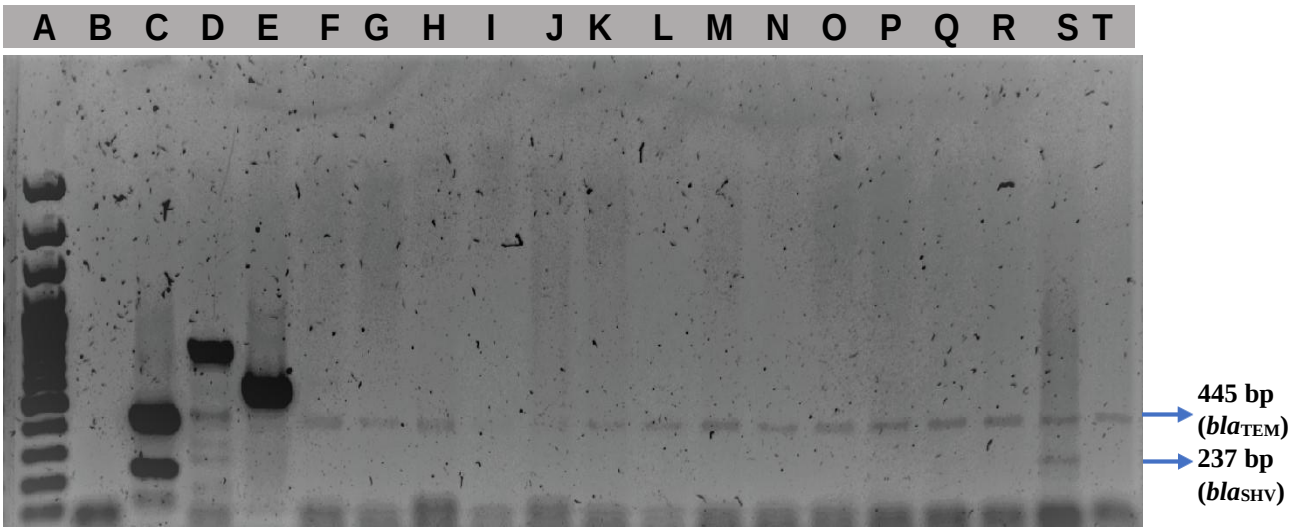


Figure 4.7: Bands obtained from agarose gel electrophoresis of PCR amplicon from ESBL Multiplex Reaction. Here, the presence of *bla*$_{TEM}$ and *bla*$_{SHV}$ is confirmed as the amplicon size for the genes are 445 bp and 237 bp respectively. Lane-A contains 100 bp DNA ladder, whereas Lane-B is the negative control and Lane-C, Lane-D and Lane-E represent positive controls. Lane-F to T represent samples of *Acinetobacter* spp. being tested.

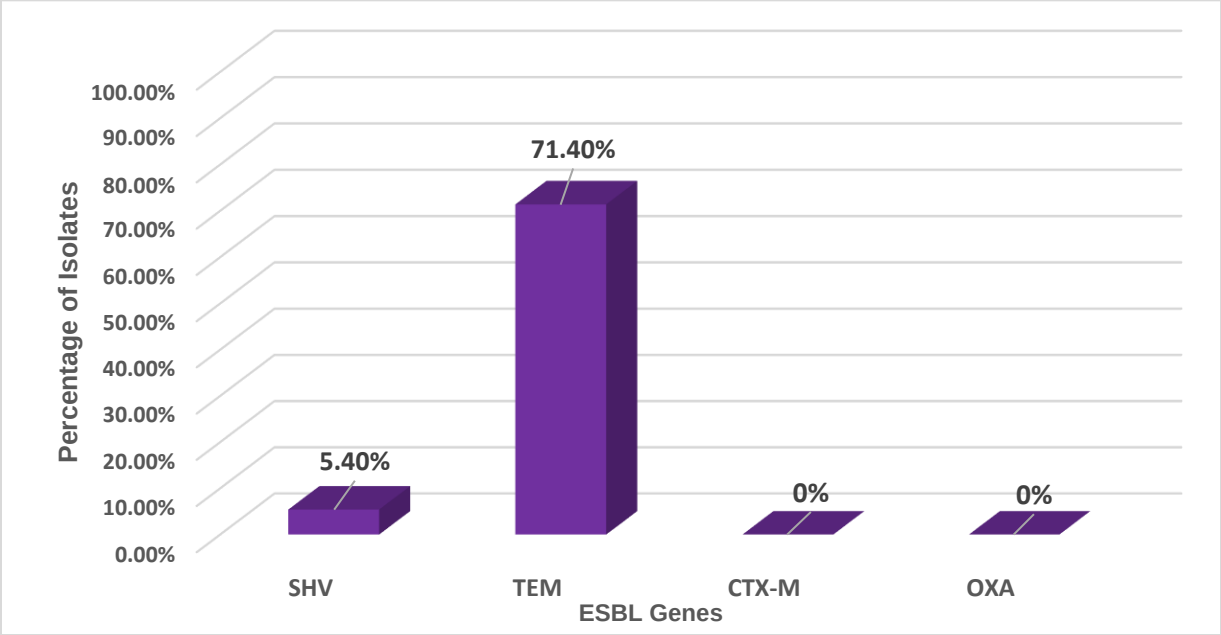


Figure 4.8: Prevalence of *bla* genes in ESBL producing *A. baumannii*

4.5 Detection of Virulence Genes by PCR

All the isolates (n=56) were tested for presence of virulence genes and biofilm related genes by using PCR. Among the isolates 15 (26.8%) of them were positive for *espA*, 39 (69.9%) positive for *bfms*, 36 (64.3%) of them had the presence of *csuE*, 6 (10.7%) of them were positive for *fimH*, 3 (5.4%) of them were positive for *bap*, 15 (26.8%) of them were positive for *ptk*, 43 (76.8%) of them were positive for *pgaB*, 35 (62.5%) of them had the presence of *ompA*, 16 (28.6%) of them were positive for *kpsMII* and none of them were positive for $bla_{\text{PER-1}}$. The gel image of the Multiplex PCR for *epsA, bfmS, fimH, csuE* and $bla_{\text{PER-1}}$, *bap, ptk, pgaB* and *ompA, kpsMII* are given in **Figure 4.9, 4.10 and 4.11** respectively. The total number of isolates containing virulence genes is given in **Figure 4.12**

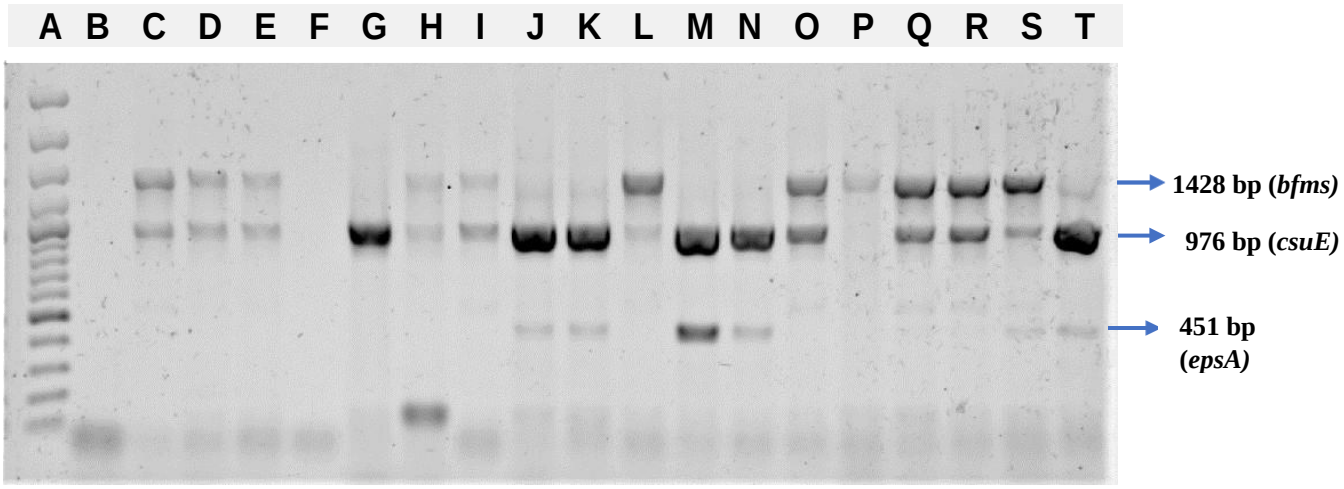


Figure 4.9: Bands obtained from agarose gel electrophoresis of PCR amplicon from multiplex virulence gene reaction (1). The presence of the genes *bfms, csuE, epsA* were confirmed by the amplicon product size of 1428 bp, 976 bp and 451 bp respectively. Lane-A contains 100 bp DNA ladder, whereas Lane-B is the negative control. Lane-C to T represent samples of *Acinetobacter* spp. being tested

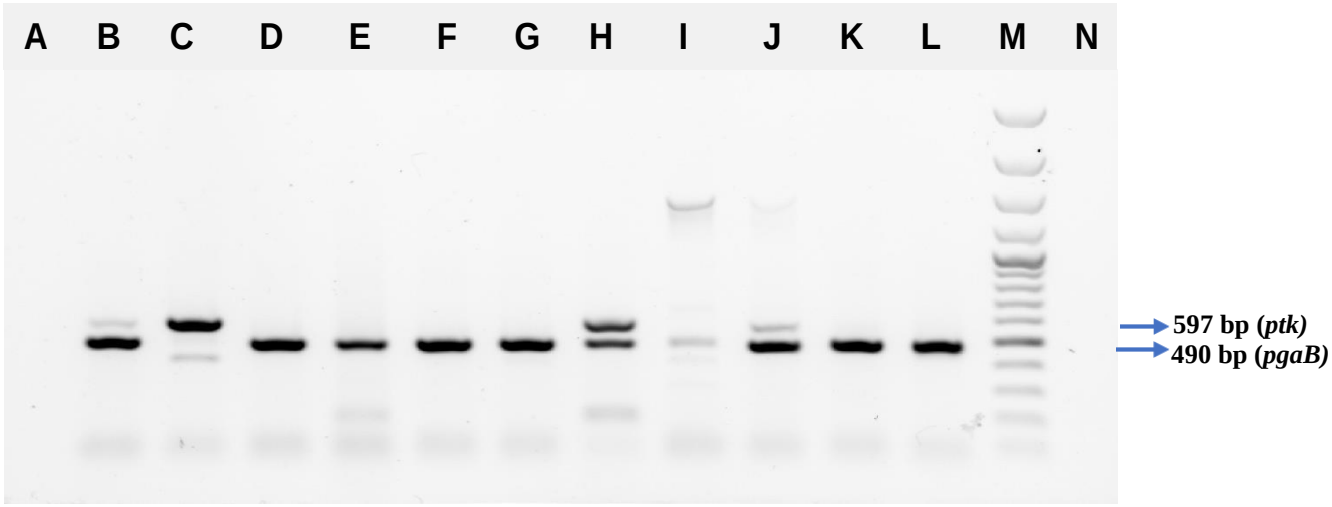


Figure 4.10: Bands obtained from agarose gel electrophoresis of PCR amplicon from Multiplex virulence gene reaction (2). The presence of the genes *ptk* and *pgaB* were confirmed by the amplicon product size of 597 bp and 490 bp respectively. Lane-M contains 100 bp DNA ladder, whereas Lane-A is the negative control. Lane-C to L represent samples of *Acinetobacter* spp. being tested

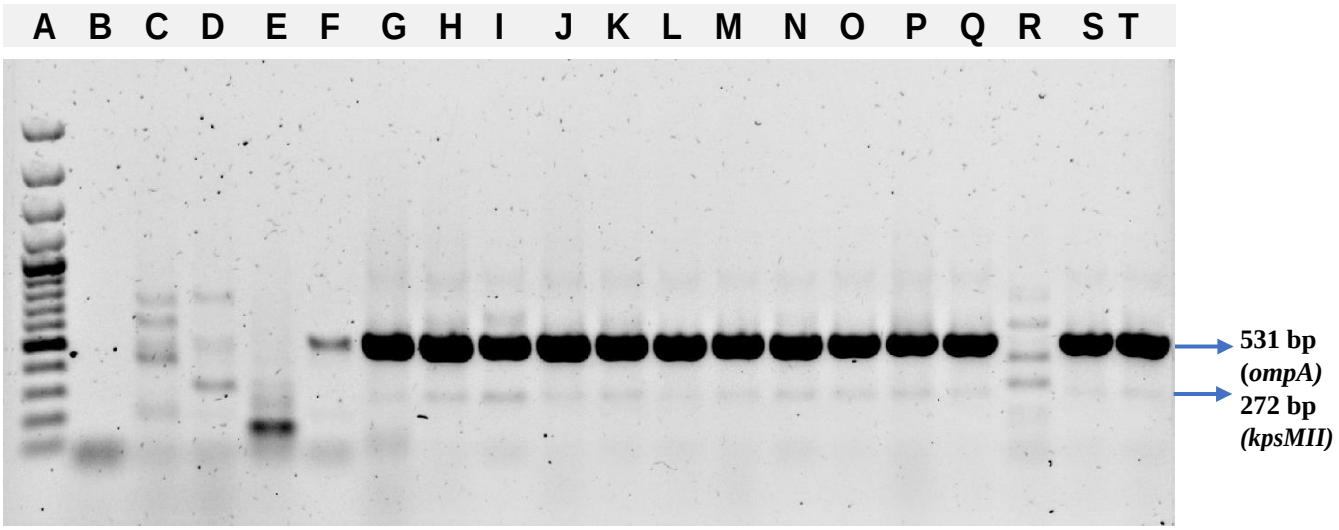


Figure 4.11: Bands obtained from agarose gel electrophoresis of PCR amplicon from multiplex virulence gene reaction (3). The presence of the genes *ompA* and *kpsMII were* confirmed by the amplicon product size of 531 bp and 272 bp respectively. Lane-A contains 100 bp DNA ladder, whereas Lane-B is the negative control. Lane-C to L represent samples of *A. baumannii.* being tested

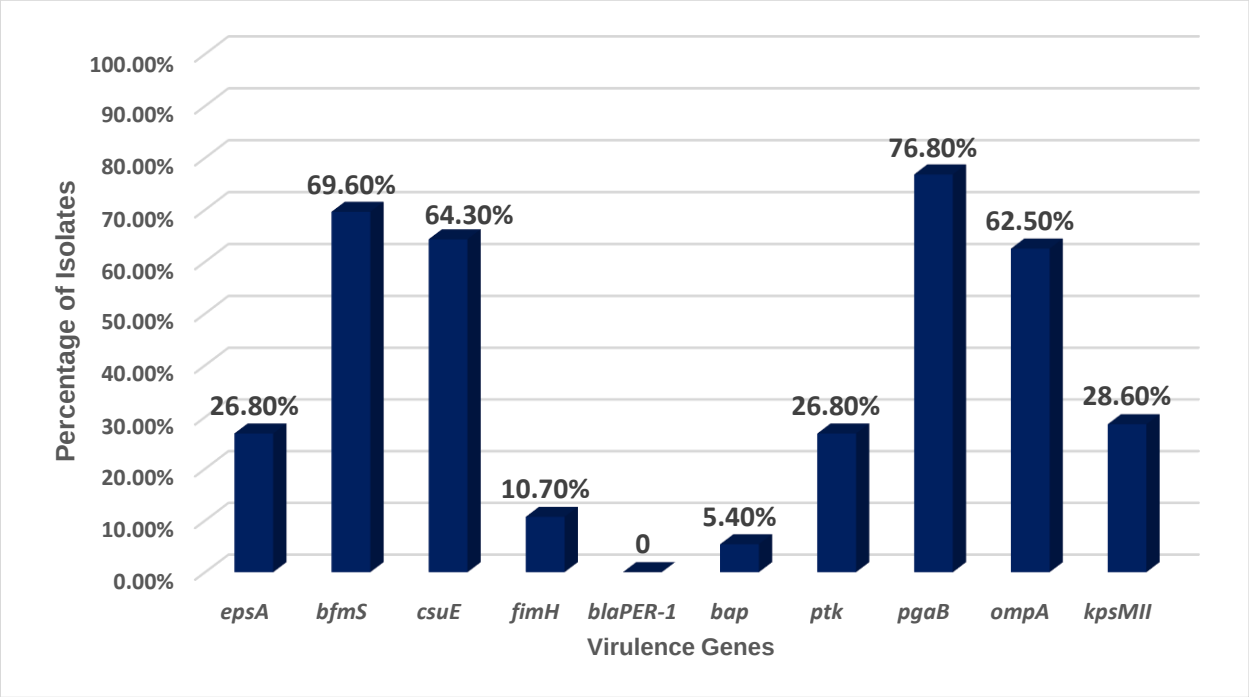


Figure 4.12: Percentage of *A. baumannii* isolates containing virulence genes

4.6 Antibiotic susceptibility pattern of ESBL forming *A. baumannii*

The of the Antimicrobial susceptibility testing was to determine the drug resistance characteristics of the ESBL forming *A. baumannii*. Due to the excessive use of antibiotics in hospital and healthcare settings, it is likely that the isolates may be multidrug resistant. Antibiotic susceptibility pattern was conducted for 56 ESBL producing *A. baumannii*. Among the isolates, around 8.9% (5/56) were resistant and 91.1% (51/56) were intermediate to cefotaxime, 5.4% (3/56) were resistant to cotrimoxazole, 5.4% (3/56) were resistant and 46.4% (25/56) were intermediate to piperacillin-tazobactam, 1.8% (1/56) were resistant and 3.6% (2/56) were intermediate to tetracycline, 87.5% (49/56) of them were intermediate to ceftriaxone, 3.6% (2/56) were intermediate to cefepime and none of the isolates were resistant to imipenem, meropenem, gentamicin, amikacin and ciprofloxacin. The information regarding resistance patterns is given in **Figure-4.14.**

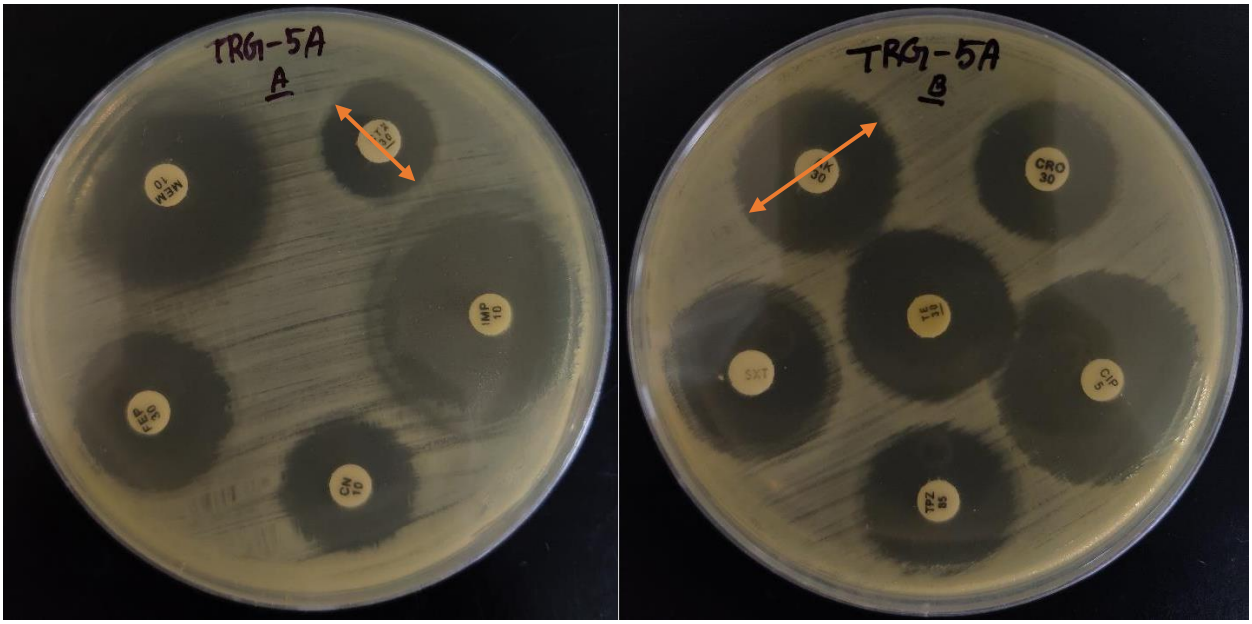


Figure 4.13: Two representative MHA plate of Antibiotic susceptibility testing for one of the isolates. Arrow indicates measuring of zone of inhibition.

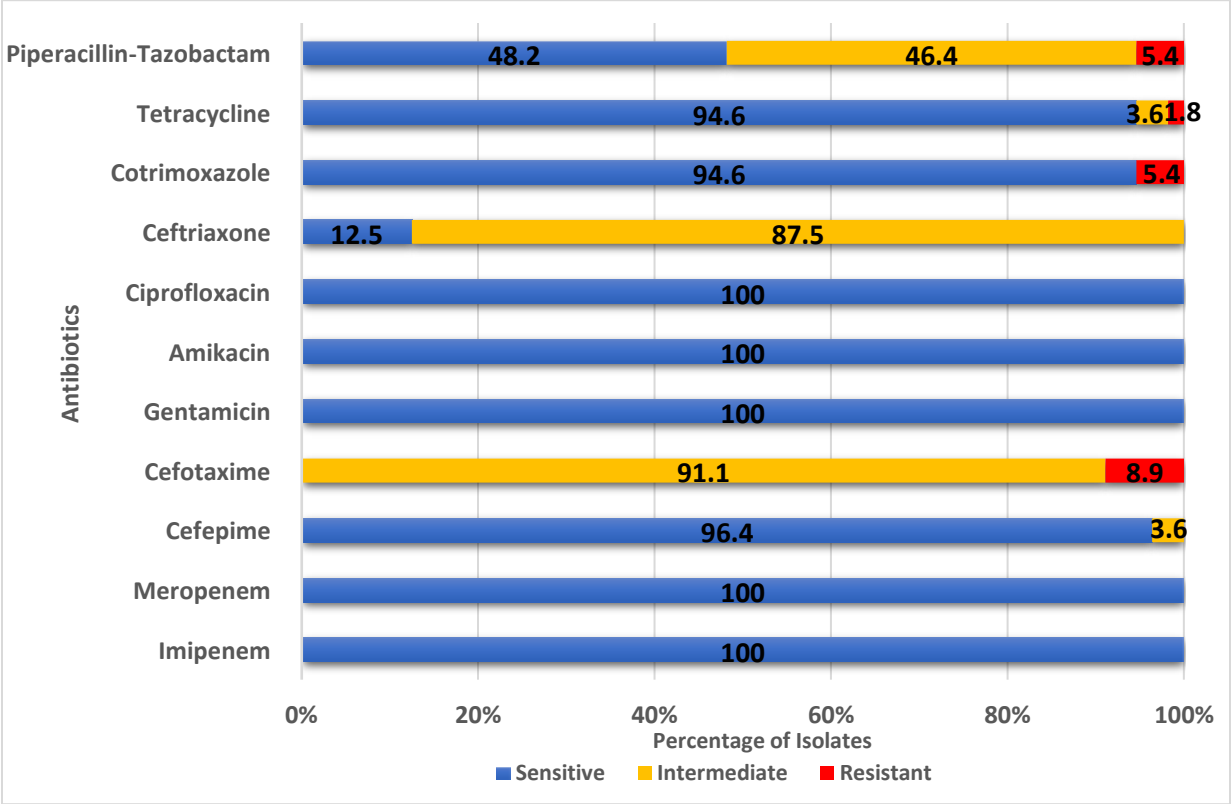


Figure 4.14 Antibiotic resistant patterns of ESBL producing *A. baumannii*

4.7 Biofilm forming capability of ESBL forming *A. baumannii*

The biofilm formation assay was performed for all the ESBL producing *A. baumannii*. Among the isolates tested, temperature variation was observed with incubation temperatures of 25ºC and 37ºC. In regard to 25ºC, a total of 1 (1.78%) isolates were strong bio film producer, 4 (7.14%) isolates were moderate biofilm producer, 37 (66.07%) isolates were weak biofilm producer and 12 (21.42%) isolates were no biofilm producers. In regard to 37ºC, a total of 1 (1.78%) isolates were moderate biofilm producer, 43 (76.78%) isolates were weak biofilm producers and 11 (19.64%) isolates were no biofilm producers and there was no strong biofilm producer. The biofilm formation capability according to temperature are illustrated in **Figure 4.15** and **Figure 4.16.**

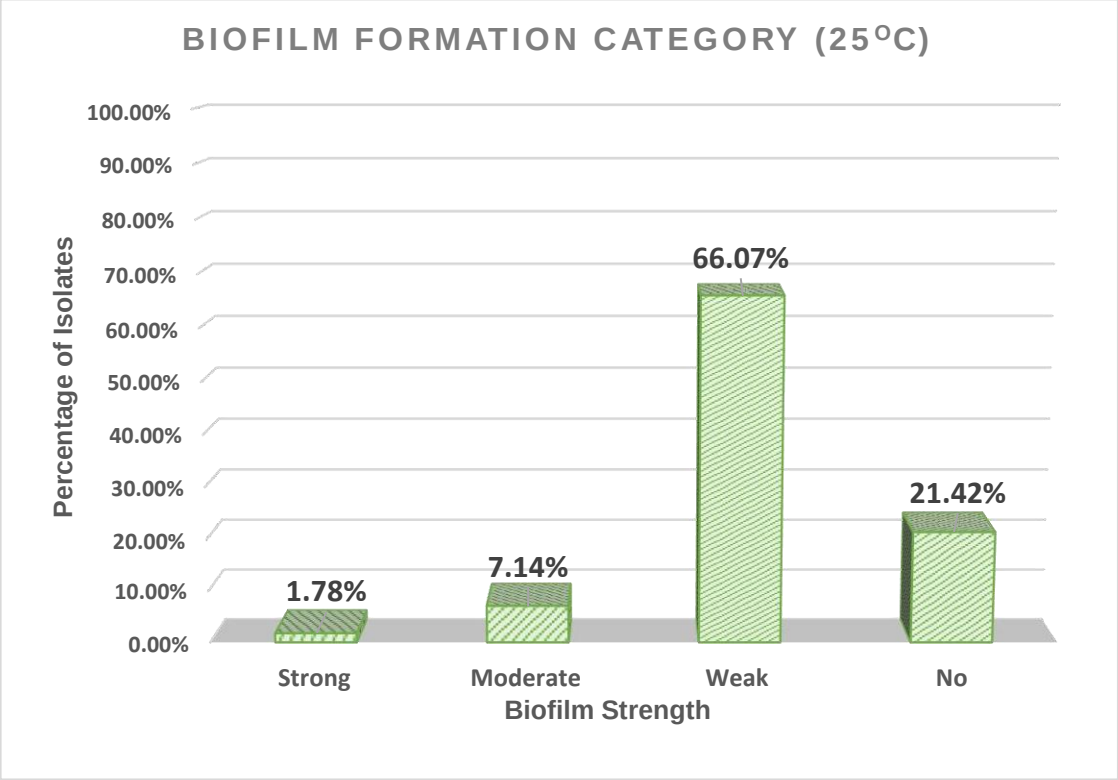


Figure 4.15: Percentage of *A. baumannii* isolates producing biofilm at 25º C

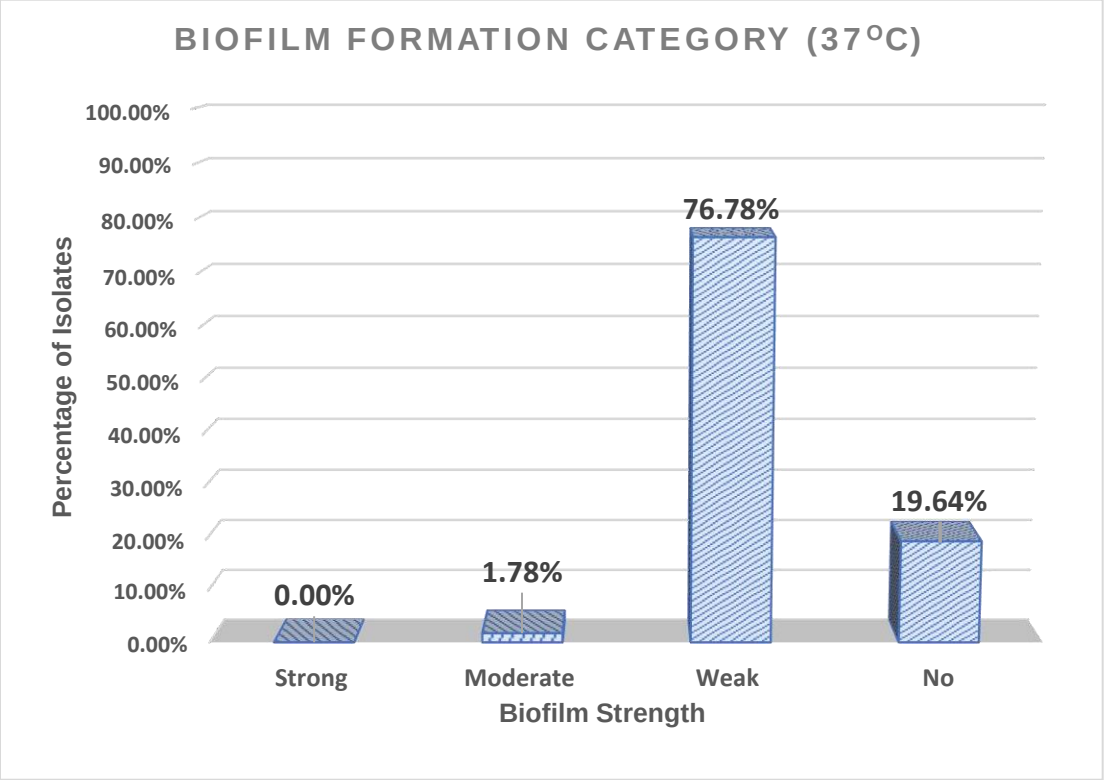


Figure 4.16: Percentage of *A. baumannii* isolates producing biofilm at 37° C

Chapter Five
DISCUSSION

5.0 Discussion

A. baumannii is a notable human pathogen, especially in relation to infections contracted in hospitals, which have a high risk of causing death (Esterly et al., 2011). Additionally, *A. baumannii* can form biofilms on various surfaces, both living and non-living (Gaddy & Actis, 2009). The capacity of *A. baumannii* to endure in healthcare environments is a crucial factor in facilitating the spread of infections in nursing homes and hospital settings (Rastogi et al., 2007). Over the past few years, this pathogen has developed resistance to a mainstream of existing antibiotics, leading to the emergence of multidrug-resistant (MDR), extensively drug-resistant (XDR), and pandrug-resistant (PDR) strains (Peleg et al., 2008). This has raised concerns among healthcare professionals due to the reduced choices for controlling *A. baumannii* infections, particularly considering it can cause a extensive series of infections such as pneumonia, bacteremia, meningitis, and urinary tract infections (Peleg et al., 2008).

This present study aimed to investigate the phenotypic and molecular characteristics of antibiotic resistance and virulence factors associated with ESBL producing *A. baumannii* isolated from Goranchatbari, the largest sub-catchment of Dhaka City. Out of 21 samples tested we found the prevalence of ESBL *Acinetobacter* spp. in 20 samples. A total of 117 presumptive isolates of ESBL *Acinetobacter* spp. was selected, whilst 76 of them were confirmed as *Acinetobacter* spp. and 56 of them were confirmed as *A. baumannii*. Overall, 56 ESBL producing isolates were subjected to gene confirmation using PCR, antibiotic susceptibility testing, biofilm formation assay. We used selective and differential chromogenic CAB agar for the presumptive isolation of *Acinetobacter* spp. As the CAB agar is selective for *Acinetobacter* spp., we had to determine the presence of *A. baumannii* by species specific PCR. For the molecular confirmation of *Acinetobacter* spp., the primer P-rA1 and P-rA2 was used which targets a highly conserved region of the *recA* gene specific for *Acinetobacter* spp. (T.-L. Chen et al., 2007). For the detection of *A. baumannii* primer P-Ab-ITS-F and P-Ab-ITS-B was used which targets 16S-23S ribosomal DNA intergenic spacer region of *A baumannii* (Tsai et al., 2018). In other studies, for the detection of *A. baumannii* bla_{OXA51}-like carbapenemase gene was targeted (Ghaima, 2018; Safari et al., 2015). The bla_{OXA51}-like carbapenemase gene is intrinsic to *A. baumannii*. Previous report shows that this gene is present in *A. baumannii* only but not in other 22 species of *Acinetobacter* (Turton et al., 2006).

However, Y.-T. Lee et al. (2009), first reported the presence of the *bla*$_{OXA51}$-like carbapenemase gene in a clinical isolate of *Acinetobacter* genomic species 13TU (Y.-T. Lee et al., 2009).

This study also aimed to see the seasonal distribution of *A. baumannii*. The initial count showed that the number of presumptive *Acinetobacter* colonies CFU/100 ml was higher in the pre-monsoon season. However, after molecular confirmation it was seen that, *A. baumannii* was most prevalent in Monsoon Season (September 2022). The findings implied that this pathogen appeared more commonly during this time period in aquatic environments. This observation is in accordance with the results of previous studies (Fernando et al., 2016; Tsai et al., 2018). Moreover, another study suggested that, during the past decade, the prevalence of this pathogen in hospitals was higher between July and October than between January and June which is also similar to our findings (McDonald et al., 1999).

A. baumannii possess several mechanisms for the inactivation of antibiotics. These include the production of ESBLs, Carbapenemase and Metallo β-lactamases (Pfeifer et al., 2010). Therefore, this current study aimed to investigate the prevalence of ESBL encoding genes (*bla*$_{CTX-M}$, *bla*$_{SHV}$, *bla*$_{TEM}$ and *bla*$_{OXA}$) in *A. baumannii* isolated from environmental samples. Among the ESBLs the main groups causing majority of hospital acquired infections are *bla*$_{CTX-M}$, *bla*$_{SHV}$ and *bla*$_{TEM}$ (Bora et al., 2014). Our study found that, among the 56 *A. baumannii* isolates, *bla*$_{TEM}$ (71.4%) was the most prevalent gene harbored among the isolates followed by *bla*$_{SHV}$ (5.4%). None of the isolates carried any *bla*$_{CTX-M}$ or *bla*$_{OXA}$ genes. Even though few studies have been conducted on ESBL producing *A. baumannii* isolated from the environmental samples where the presence of *bla*$_{TEM}$ was low, our findings are in agreement to previous studies conducted on clinical samples where *bla*$_{TEM}$ was the most prominent *bla* genes in *A. baumannii* followed by *bla*$_{SHV}$ (Ghaima, 2018).

It is known that, the distribution of virulence genes (*bap, epsA, bfmS, omp*A and *csu*E) are involved in the biofilm formation of clinical *A. baumannii* isolates with multidrug (Ghasemi et al., 2018; Sung, 2018; Thummeepak et al., 2016). In this study, PCR was used to determine the presence of biofilm related genes. It was seen that, 76.8% of the isolates harbored *pgaB* gene. The *pgaABCD* is the operon responsible for the production of poly-β-1,6-Nacetylglucosamine (PNAG) which plays a significant role in the formation of biofilm (Choi et al., 2009). Also, 69.6% of the isolates contained *bfm*S and 64.5% of the isolates were positive for *csu*E gene. The *bfmS* gene is a part of a two-component system that regulates the CsuA/BABCDE pilus usher-chaperone assembly

system (Tomaras et al., 2008) and *csu*E, is a member of the usher-chaperone assembly system, which mediate attachment and biofilm formation (C.-H. Yang et al., 2019). Among the isolates 62.5% of them carried the *omp*A gene, which is considered to be associated with drug resistance in *A. baumannii* (Smani et al., 2014). The isolates also contained other biofilm related genes such as, *kpsMII* (28.6%), *epsA* (26.8%), *ptk* (26.8%), *fimH* (10.7%) and *bap* (5.4%). None of the isolates contained $bla_{\text{PER-1}}$. In a previous report there was a high frequency presence of *csuE* (100%), followed by *pgaB* (98%), *epsA* and *ptk* (95%), *bfmS* (92%) and *ompA* (81%) (Zeighami et al., 2019). Even though our study found low presence of *epsA* and *ptk* gene it was in agreement with the high presence *pgaB, csuE, bfmS* and *ompA* gene. The high prevalence of *ompA* was also found in previous studies conducted in Thailand, Korea and China (Liu et al., 2016; Sung, 2018; Thummeepak et al., 2016). Liu et al. (2016), reported similar findings of the absence of $bla_{\text{PER-1}}$ gene among the isolates (Liu et al., 2016). Some other studies also reported the very low presence of $bla_{\text{PER-1}}$ gene (Ghasemi et al., 2018; Sung, 2018; Zeighami et al., 2019).

Widespread dissemination of *A. baumannii* infections resistant to beta-lactams antibiotics specially to the third generation of cephalosporins and carbapenems, has become a global concern (Pfeifer et al., 2010). It has become significantly hard to treat patients at the health care units. With the widespread of resistance among clinical samples we wanted to know the resistance pattern in the environmental samples. Among the tested antibiotics, 8.9% of the isolates were resistant to cefotaxime, 5.4% of them were resistant to cotrimoxazole and piperacillin/tazobactam, 1.8% of them were resistant to tetracycline. 91.1% of them were intermediately resistant to cefotaxime, 87.5% of them were intermediately resistant to ceftriaxone, 46.4% were intermediately resistant to piperacillin/tazobactam. Also, 100% of the isolates were susceptible to Imipenem, Meropenem, Gentamicin, Amikacin, Ciprofloxacin. Even though all the 56 isolates were ESBL producers as they were tested on the CHROMagar^{TM} ESBL plate, only 8.9% of the isolates were resistant to a 3rd generation cephalosporin. As the CHROMagar^{TM} ESBL plate contains a few 3rd generation antibiotics it can be assumed that, the other isolates were resistant to a different antibiotic that has not been tested in the antibiotic susceptibility test. This finding is in contrast to the clinical samples where 96.6% strains of *A. baumannii* were found resistant towards ceftazidime and 94.8% strains were resistant towards cefepime. 60.3% and 68.1% of the isolates were resistant to imipenem and meropenem respectively (Kaur & Singh, 2018). Previous studies conducted at India, Saudi Arabia and Nigeria also showed the high resistant towards cephalosporins (Nwadike et al., 2014; Rynga

et al., 2015; Said et al., 2014). Moreover, only one isolate was resistant to 3 antimicrobial categories (i.e., cefotaxime, cotrimoxazole and tetracycline). Thus, falling under the multi drug resistant (MDR) class. This is also in contrast to a study conducted on environmental *A. baumannii* isolates where none of them were found to be multi drug resistant *A. baumannii* (Tsai et al., 2018). The outcome of this study indicates that the environmental *A. baumannii* isolates differs from the clinical *A. baumannii* isolates, based on the multidrug-resistant outcome. However, it is still a great concern that, around 90% of the isolates are intermediately resistant to 3rd generation cephalosporins. From our study we can assume that, the clinical samples might be disseminating and in the near future isolates from the aquatic environment may also become multi drug resistant.

The biofilm formation rate of *A. baumannii* is 5-24% higher than the other species of *Acinetobacter* (Sung, 2018). Previous studies have reported the relation between biofilm formation and antibiotic resistance in *A. baumannii* (Qi et al., 2016). In our study, the biofilm assay was conducted at both 25^{o} and 37^{o} C. At 25^{o} C, 1.78% of the isolates were strong biofilm producer, 7.14% of them were moderate biofilm producer, 66.07% of the isolates were weak biofilm producer and 21.42% of the isolates were no biofilm producers. In regard to 37^{o}C, a total of 1.78% isolates were moderate biofilm producer, 76.78% isolates were weak biofilm producers and 19.64% of the isolates did not produce any biofilm. This finding indicates that the *A. baumannii* isolated from the environmental samples shows lower tendency to form biofilm compared to the clinical samples reported before where 58% of the isolates were strong biofilm producer and 42% of the isolates were moderate biofilm producers (Zeighami et al., 2019).

Even though the emergence and spread of AMR in the environment is significantly less compared to the clinical isolates, it will not be long before the situation changes. Moreover, *A. baumannii* can transmit through free-flowing water leading to the spread of this pathogen to other aquatic environments (Tsai et al., 2018). Therefore, if the use of antibiotics is not restricted multi drug resistant *A. baumannii* can spread to other environmental sources and can cause immense threat.

Chapter Six
CONCLUSION

6.0 Conclusion

This study was aimed to characterize ESBL producing *A. baumannii* isolates isolated from environmental sources. Among the isolates, ESBL gene bla_{TEM} and bla_{SHV}, were most prevalent. A significant number of ESBL producing isolates also tested positive for the presence different virulence genes, with high prevalence of *pgaB, ompA, csuE* and *bfmS*. All the isolates were subjected to antibiotic susceptibility testing, and around 90% of them were intermediately resistant to cefotaxime and ceftriaxone (3^{rd} Generation Cephalosporin). The biofilm formation capability was tested for all the isolates, and among them, different rates of biofilm formation was observed for the environmental isolates.

These results indicate the prevalence of ESBL producing *A. baumannii* in environmental sources. Immediate actions are necessary to effectively tackle the transmission of these pathogens and address the emergence of resistance. It is crucial to promote and ensure the completion of optimal treatment for prevention and control. In the future, it would be beneficial to conduct research to monitor the prevalence of ESBL *A. baumannii* in various environmental sources. Additionally, studies investigating plasmid profiles and performing conjugation experiments can provide insights into the dissemination of resistance and virulence factors among both pathogenic and environmental strains. These efforts hold the potential to yield valuable information on reducing antimicrobial resistance (AMR).

Chapter Seven

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7.0 References

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