Molecular profiling, Antibiotic Susceptibility Patterns and Pathogenic Traits of ESBL Producing *Acinetobacter baumannii* Isolated from Wastewater Discharges from Goranchatbari Sub-Catchment area in Dhaka City

By
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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
May 2024

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Declaration

It is hereby declared that,

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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,

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I am Amanta Rahman, student of M.Sc., Department of Mathematics and Natural Sciences, BRAC University, do hereby declare that the thesis on **"Molecular profiling, Antibiotic Susceptibility Patterns and Pathogenic Traits of ESBL Producing *Acinetobacter baumannii* Isolated from Wastewater Discharges from Goranchatbari Sub-Catchment areas 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,

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Abstract

Acinetobacter baumannii is one of the six pathogens for which new antibiotic formulations must be developed, as recommended by the World Health Organization. In this study, 28 wastewater samples were collected from the Goranchatbari sub-catchment area in Dhaka city over several seasons. The samples were characterized for the presence of *Acinetobacter* spp. and the isolates were subjected to phenotypic testing for ESBL production, presence of major antibiotic resistance and pathogenic genes, biofilm production and antibiotic susceptibility testing. Furthermore, the isolates were phylogenetically clustered based on their ERIC profiles and correlation matrix. Out of 28 samples, 27 were positive for *Acinetobacter* spp. and a total of 106/249 (42.6%) representative isolates were positive for ESBL production. Out of these 106 isolates, 97 (91.5%) were genotypically confirmed to belong to the *Acinetobacter* spp. and of which, 72 (74.2%) were genotypically confirmed as *Acinetobacter baumannii*. Among the distribution of β-lactamase genes, bla_{TEM} was the most prevalent being present in 40/72 (55.6%) isolates, followed by bla_{SHV} in 3/72 (4.2%) isolates. With respect to the pathogenic genes, *pgaB* and *bfmS* were the most prevalent being present in 80.6% and 69.4% of isolates respectively. A large percentage of the isolates concomitantly carried one or more pathogenic genes with 1/72 (1.4%) and 6/72 (8.3%) of isolates carrying 7 and 6 pathogenic genes each respectively. The antibiotic susceptibility testing revealed a diverse range of resistance patterns with high levels of intermediate resistance being observed for cefotaxime and ceftriaxone. The biofilm formation screening revealed the widespread ability of *Acinetobacter baumannii* to form biofilm at different temperatures, with 35/72 (48.6%) of isolates forming strong biofilm at 37°C and 32/72 (44.4%) of isolates forming strong biofilm at 25°C. The phylogenetic clustering of the *A. baumannii* isolates resulted in the formation of 10 clusters at a 60% similarity index, and correlation matrix helped reveal important associations between genotypic and phenotypic traits. These results demonstrate the continued prevalence of *A. baumannii* within these environmental reservoirs and its ability to persist despite seasonal variations, prioritizing changes in environmental health policies that aim to reduce the widespread prevalence of these pathogens.

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

Dedication

Dedicated to my family for their love and support.

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I am grateful to Almighty Allah for bestowing His endless showers of blessings and giving me the ability to gain knowledge through which I can progress in life and keeping me healthy and helping me complete my research successfully.

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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
ERIC	Enterobacterial repetitive intergenic consensus
UTI	Urinary tract infection
icddr,b	International Center for Diarrheal Disease Research, Bangladesh

CHAPTER 1: INTRODUCTION

1.1 Background of the Study

In the recent years, the rise of antibiotic resistance in bacteria has become a critical global health concern, with at least 700,000 reported deaths attributed to resistant infections worldwide. This figure likely underestimates the true impact due to inadequate reporting and surveillance with the situation worsening after the COVID-19 pandemic (Tanne, 2022). Murray et al., 2022 predicted a grim future that without effective intervention, it is estimated that by 2050, antibiotic resistance could contribute to over 10 million deaths annually worldwide (Murray et al., 2022). The widespread prevalence of antibiotic resistance not only threatens the stability of healthcare systems but also if left unchecked could lead to a future where treating infections will become increasingly difficult, leading to significant societal and economic consequences (Ventola, 2015).

The genus *Acinetobacter* is a family of gram-negative bacteria, characterized by its strictly aerobic, catalase-positive, oxidase-negative, non-motile and non-fermenting coccobacilli morphology (Bergogne-Berezin & Towner, 1996). It thrives on simple microbiological media and is commonly found in various environments, including soil and surface water (Bergogne-Berezin & Towner, 1996; Peleg et al., 2008). However, not all species of *Acinetobacter* naturally reside in environmental habitats. Recent studies indicated that *A. baumannii* associated with human hosts is not typically part of the normal microbiota in humans or animals and has mostly been isolated from clinical samples (Dijkshoorn et al., 2007; Maravić et al., 2016). Due to the organisms' ability to colonize environmental surfaces in healthcare facilities and persistence for prolonged periods, it is a significant concern in nosocomial settings and healthcare-associated infections (Fournier et al., 2006).

The prevalence of *Acinetobacter* spp. in medical environments is well documented but their antimicrobial resistance patterns in natural settings remain poorly understood. This is of particular concern since in 2017 the World Health Organization (WHO) classified *A. baumannii* as the highest priority in its overall priority list of antibiotic-resistant bacteria (Morris et al., 2019). Evidence suggests that *Acinetobacter* strains can spread resistance traits among populations outside the clinical settings and there is a persistent risk of the resistant clinical isolates being exposed in the environment (Maravić et al., 2016). Lately, the detection of *A. baumannii* has increased drastically from environments such has municipal wastewater discharged from hospitals,

industrial or domestic effluents. Hospital effluent, in particular, is determined as the primary source of the clinically significant *A. baumannii* with confirmed cases of the presence of carbapenem-resistant *Acinetobacter* spp., including *A. baumannii,* in hospital wastewater (Music et al., 2017; Zhang et al., 2013). Nevertheless, despite the frequent exposure of these hospital waste into the water bodies, there has been a limited investigation to detect the presence of similar resistant isolates from water bodies which are mostly adjacent to hospitals and other clinical settings.

The emergence of *A. baumannii* strains resistant to multiple antibiotics poses a serious threat to public health. One of the primary ways bacteria become resistant to antimicrobials is through the use of enzymes like extended spectrum β-lactamases (ESBLs) (Shafiq et al., 2021). ESBLs, a type of class-A β-lactamases, can hydrolyze third generation antimicrobials including extended spectrum cephalosporins and monobactams (Ghafourian et al., 2015). There are three primary ESBL enzymes—CTX-M, SHV, and TEM, which are more commonly characterized within resistant bacteria isolated from environmental samples (Moniruzzaman et al., 2023). Within the TEM and SHV groups, only specific types are classified as ESBL due to their range of activity. However, all CTX-M variants are classified as ESBLs due to their broad spectrum activity against cefotaxime (Castanheira et al., 2021). The increasing prevalence of ESBL-producing *Acinetobacter baumannii* strains raises significant concerns, as it may lead to the limited availability of effect antibiotics for future for therapeutic approaches (Owlia et al., 2012).

Despite the extensive research on the epidemiology and resistance pattern of *A. baumannii* strains, there is a limited understanding regarding its pathogenicity traits and virulence patterns (L. C. S. Antunes et al., 2011). Various mechanisms have been proposed to emphasize its role in colonization, infection, and epidemic spread, however, the main concern is its ability to colonize and form biofilm on both biotic and abiotic surfaces, leading to chronic and persistent infections as well as antimicrobial resistance in *A. baumannii* (Zeighami et al., 2019). Studies reported the significant virulence factors associated with *A. baumannii* biofilm formation include outer membrane protein A (ompA), biofilm-associated protein (Bap), chaperon-usher pilus (Csu), extracellular exopolysaccharide (EPS), the two-component system (BfmS/BfmR), poly-β-(1,6)-N-acetyl glucosamine (PNAG), and quorum-sensing systems (Ghasemi et al., 2018; Thummeepak et al., 2016). Other studies have established a positive correlation between biofilm formation and antibiotic resistance in *A. baumannii* isolates (Qi et al., 2016). Since biofilm formation is attributed

to reduced drug penetration and an additional polymeric barrier (Gilbert et al., 2003; Wentland et al., 1996), antimicrobial resistance within biofilms increase substantially (Ceri, 1999). Consequently, the heightened resistance challenges treating persistent biofilm causing infections, even with multiple antibiotics (Singla et al., 2013).

Considering the knowledge gap persistent, this study aimed to detect and isolate extended-spectrum β-lactamase (ESBL) producing *A. baumannii* strains in the environment, assess molecular detection of virulence genes, antibiotic susceptibility profiling, and biofilm formation assay of the ESBL isolates. Additionally, molecular typing was conducted based on ERIC-PCR to characterize the genetic similarity among the virulent ESBL *A. baumannii* isolates.

1.2 Objective of the study:

The present study aimed to detect and isolate *A. baumannii* strains in the environment that exhibit extended-spectrum β-lactamase (ESBL) production. The study also includes molecular detection of virulence genes, antibiotic susceptibility profiling, and biofilm formation assay of the ESBL isolates. Additionally, molecular typing was also conducted based on ERIC-PCR in virulent *A. baumannii* isolates from the environmental samples.

CHAPTER 2:
LITERATURE REVIEW

2.1 The Organism: *Acinetobacter baumannii*

2.1.1 Historical Background

The Acinetobacter was introduced in 1954, by Brisou and Prevott to differentiate this bacterium from other motile organisms within the Achromobacter genus (Brisou, 1954). However, the acceptance of the genus *Acinetobacter* followed a comprehensive study conducted by Baumann et all in 1968, where various organisms were examined, leading to a conclusion that the organism in question belonged to a single genus, and was not possible to further classify into different species, based on their phenotypic characteristics. Baumann's publication in 1968 on the genus *Acinetobacter* received recognition from the sub-committee on the Taxonomy of Moraxella and Allied Bacteria, thereby confirming and establishing the acceptance of the *Acinetobacter* genus (Lessel, 1971).

However, some inconsistencies were discovered by Bouvet and Grinmont in 1986, in using the phenotypic tests for identifying *Acinetobacter* species, attributing to the fact that *Acinetobacter* members possess different catabolic pathways, allowing them to adapt to a wide range of substrates (Bouvet & Grimont, 1986). This led to the introduction of DNA hybridization studies as a more reliable method, focusing on DNA-DNA relatedness to classify groups with over 70% similarity as genomic species. At present, there are 32 recognized genospecies within the *Acinetobacter* genus, including those within the *Acinetobacter calcoaceticus – A. baumannii* (ACB complex), which encompasses four genospecies: *A. calcoaceticus, A. baumannii, A. pittii, and A. nosocomialis*. Among these, *A. baumannii* is particularly significant in clinical settings due to its association 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

Bacteria of the genus *Acinetobacter* are classified as Gram-negative, catalase-positive, oxidase-negative, non-motile, and non-fermenting coccobacilli. Despite being generally considered non-motile, there have been reports indicating variations in motility under certain conditions such as illumination, quorum sensing, and iron chelation (Clemmer et al., 2011; Mussi et al., 2010). While the precise mechanism of movement in *Acinetobacter* species are not fully understood, there is evidence suggesting potential twitching motility on semi-solid surfaces rather than other forms of motility like gliding, sliding, swimming, or swarming. However, additional study is required to validate these observations (Eijkelkamp et al., 2011). One challenge in identifying *Acinetobacter* is its resistance to de-staining, which can lead to its misidentification as Gram-positive. Currently, there are no specific metabolic tests available to reliably distinguish *Acinetobacter* from other non-fermenting Gram-negative bacteria (Peleg et al., 2008). However, the negative oxidase test is commonly used for rapid presumptive detection to differentiate *Acinetobacter* from other non-fermenting organisms with similar characteristics. This test helps identify *Acinetobacter* by confirming the absence of the oxidase enzyme in the bacterial species being tested.

Figure 2.1: Morphological traits of *Acinetobacter* spp. (*Healthcare-Associated Infections (HAIs)*, n.d.)

Acinetobacter is a type of bacterium that can easily grow on basic nutrient media. The colonies typically have a smooth, dome-shaped appearance and can range in color from pale yellow to gray (Doi et al., 2009). However, in case of *A.calcoaceticus - A. baumannii* complex, colonies can grow to a diameter of 11.5 to 3 mm overnight, similar to *Enterobacteriaceae* species, while other species

tend to produce smaller colonies (Peleg et al., 2008). Most *Acinetobacter* species can thrive in room temperature, however, the pathogenic species, *A. baumannii* thrives particularly well at the typical human body temperature at 37°C. Baumann et al. (1968) is a study discovered both environmental and clinical strains of *Acinetobacter* to be successfully cultured under aerobic conditions using acidic medium supplemented with acetate and nitrate as carbon and nitrogen sources respectively, facilitating laboratory growth of *Acinetobacter* strains (Baumann, 1968). Another notable characteristic of *A. baumannii* is its thick cell wall, providing protection against dry conditions, and increasing tolerance to harsh conditions, such as changes in temperature, pH, and nutrient availability (Vila et al., 2007).

2.1.4 Natural Habitat

Different species of *Acinetobacter* genus are commonly present in a range of environment, including soil surface and water samples, however, reports suggest that not all species naturally reside in the environment (Peleg et al., 2008). *A. baumannii*, specifically, is not frequently encountered in the environment and has a low prevalence in the community, regardless of previous reports of being detected in vegetables, fish, meat, and soil (Dijkshoorn et al., 2005; Houang et al., 2001). *A. baumannii* is rarely present in the normal fecal flora in individuals within the community, nor is it typically a part of the skin microflora; however, are commonly detected in human clinical samples (Dijkshoorn et al., 2005). As a pathogenic bacterium, it tends to target moist tissues like mucous membranes and has the capability to persist on dry surfaces for extended periods (Sebeny et al., 2008). This poses a significant concern in hospitals due to the ability to contaminate the environment and spread infection in the healthcare settings, increasing the risk of nosocomial transmission (Fournier et al., 2006).

The hospital environment serves as a significant source of *A. baumannii* due to its ability to survive under diverse conditions such as temperature and pH, and its resilience to factors like desiccation and disinfectants (Gaddy & Actis, 2009). *A. baumannii* persists by forming biofilms on surfaces, providing protection, and serving as a constant source of infection. 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, like catheters and ventilators, can also introduce *A. baumannii*

in hospitals leading to colonization in various parts of the body, contributing significantly to the transmission of infection in healthcare facilities (H.-W. Lee et al., 2008; Rastogi et al., 2007). Previous reports suggests that the possible routes for *baumannii* isolates to infect healthy individuals are though various routes including the skin, nostrils, and throat (Custovic et al., 2014). It has been established that *A. baumannii* strains can persist in patients for weeks and are often transmitted through unclean hands of healthcare staff in clinical environment. This highlights the importance of strict personal hygiene and cleanliness, signifying maintenance of rigorous personal sanitation practices in preventing the transmission of this pathogen (Dijkshoorn et al., 2005).

A. baumannii is rapidly emerging as a prominent multi-drug resistant bacterium, significantly contributing to antimicrobial resistance. Healthcare providers in clinical settings are constantly faced with significant hurdles due to the severity of patients' conditions and the prevalence of multidrug resistance (Dijkshoorn et al., 2007).

2.2 Clinical Significance

Acinetobacter is commonly found in hospital environments, colonizing patients' skin, and mucous membranes, contributing to the spread of healthcare-associated infections. According to past studies, approximately 2.1% of skin and soft tissue infections acquired in intensive care units (ICUs) and burn units are caused by strains of *Acinetobacter* (Trottier et al., 2007). The prevalence of *Acinetobacter* infections affecting the skin and soft tissues is a significant concern, partly because *Acinetobacter* species are the only Gram-negative bacteria naturally present on human skin (Seifert et al., 1995).

A. baumannii accounts for 1.3% of all nosocomial bloodstream infections in the United States, ranking as the 10th most common causative agent (Peleg et al., 2008). The crude mortality rate for *A. baumannii* bloodstream infections varies between 34.0% and 43.4% in ICU settings and 16.3% outside the ICU, making it the third-highest cause of mortality among ICU patients, surpassed only by *P. aeruginosa* and *Candida spp.* (H.-W. Lee et al., 2008). *A. baumannii* bloodstream infections demonstrate resistance to various antibiotics and carry a worse prognosis compared to infections caused by other pathogens.

A. baumannii is not commonly associated with urinary tract infections (UTIs), however, there has been a gradual rise in the number of incidences. Previous incidences reports that 1.6% of UTIs caused by *A. baumannii* are acquired in ICU settings, linking to catheter-related infections or colonization (Weinstein et al., 2005). Catheters and other medical instruments like endotracheal tubes provides opportunities for these pathogenic organisms to colonize these sites, causing infection (Joly-Guillou, 2005). Additionally, outside healthcare settings, *A. baumannii* can also cause uncomplicated UTIs in otherwise healthy individuals.

Recently post-neurosurgical *A. baumannii* meningitis has also emerged as a concerning condition, with mortality rates reported as high as 64%, while *Acinetobacter* strains are not typically associated in causing meningitis (García-Garmendia et al., 2001; Y.-T. Lee et al., 2009). There have been some tragic incidence reports where improperly sterilized needles in children with leukemia has led to *Acinetobacter* related meningitis and fatalities. Risk factors for meningitis include persistent links between brain ventricles and the external environment, ventriculostomy, cerebrospinal fluid (CSF) fistula, prolonged use of ventricular catheters exceeding 5 days, and prior antimicrobial therapy, increasing susceptibility to *Acinetobacter* meningitis (Metan et al., 2007).

2.2.1 Nosocomial (Hospital-acquired) Infections

Nosocomial infections, also termed as hospital-acquired or healthcare-associated infections (HAI), occur when patients acquire infections during their hospital stay, rather than upon admission (Monegro et al., 2017). *A. baumannii,* an opportunistic pathogen, is notorious for causing a range of infections, predominantly affecting critically ill patients in intensive care units (ICUs). These infections include ventilator-associated pneumoniae, skin and soft tissue infections, wound infections urinary tract infections (UTIs), secondary meningitis, and bloodstream infections (Morris et al., 2019). However, ventilator-associated pneumoniae and bloodstream infections are the most prevalent, carrying significant morbidity and mortality rates. Apart from hospital settings, *A. baumannii* has been observed to cause wound infections in individuals during both natural and man-made disasters, highlighting its pathogenic potential beyond healthcare environments and its role in wound infections across diverse settings. Notably, *A. baumannii* has also been linked to

wound infections among individuals injured during natural disasters or deployed military personnel (Peleg et al., 2008; Seifert et al., 1995).

A. baumannii infections can be transmitted through various means, including host and medical treatment factors. Major surgeries or trauma, immunosuppression, advanced age, premature infants are few of the major factors contributing to susceptibility, alongside mechanical ventilation, medical instruments, invasive procedures, and previous history of antimicrobial therapy. Prolonged hospital or ICU stays, residency in units with endemic *A. baumannii*, and exposure to contaminated medical devices are few of the common factors that can increase the susceptibility to infections (Dijkshoorn et al., 2007).

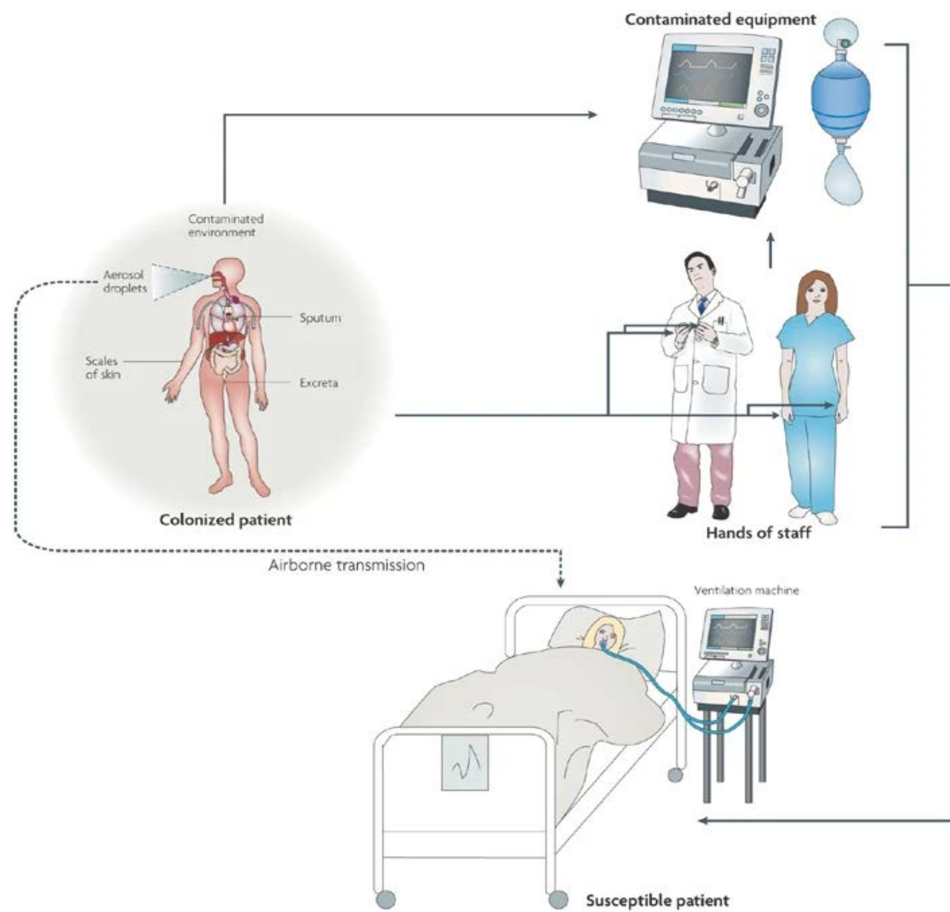


Figure 2.2: Different modes for transmission of nosocomial (hospital-acquired) infections caused by *A. baumannii* (Dijkshoorn et al., 2007).

2.2.2 Community-Acquired Infections

A. baumannii is commonly associated with hospital acquired infection, however it can also be a significant cause of community-acquired pneumoniae, particularly in tropical regions. In these areas, *A. baumannii* pneumoniae is linked to a high mortality rate, which can reach up to 64%, mostly affecting individuals with underlying conditions like alcoholism, diabetes mellitus, smoking, and chronic lung disease. Vulnerability to *A. baumannii* pneumonia increases in patients with chronic lung disease (Dexter et al., 2015). However, the exact contribution of host factors and bacterial virulence factors in the development of these infections remain uncertain (Morris et al., 2019).

2.3 Pathogenicity of *Acinetobacter* Infections and Virulence

The exact mechanisms driving *A. baumannii* infections are not fully understood yet, and there are ongoing research aiming to shed light on the potential mechanism on the functions in colonization, infection, and transmission of epidemics. These mechanisms include adherence to host cells, quorum sensing, biofilm formation, resistance to drying, disinfectants, and antibiotics, as well as the triggering of inflammatory responses and cytotoxicity. However, further studies are needed to fully understand the complex interactions between *A. baumannii* and its host during infection (Dijkshoorn et al., 2007). In a previous study conducted by Choi et al (2008), it is revealed that the initial stage of the bacterial colonization and infection is its ability to adhere to epithelial cells, although the degree of invasion may vary depending on the specific type of host cells involved (Choi et al., 2009). Studies suggest that respiratory tract epithelial cells may be particularly susceptible to invasion by *A. baumannii* compared to other epithelial cell types, indicating a potential preference of *A. baumannii* for respiratory epithelial cells during invasion.

2.3.1 *Acinetobacter* Adhesins and Fimbriae

In the beginning or primary phase of any infection, a microorganism's ability to adhere to and persist on the mucosal surfaces of the host is pivotal for initiating the infection. This adherence grants the microorganism access to host cells and resources necessary for its survival and proliferation. Various bacterial adhesins, including fimbriae (pili), capsular polysaccharides, and

cell wall components, are instrumental in facilitating the attachment of bacteria to different surfaces (Bergogne-Bérézin et al., 2008). *A. baumannii*, for instance, naturally possesses the capability to adhere to bladder tissue, akin to apathogenic strains of Escherichia coli. Notably, strains of *A. baumannii* expressing Type 1 fimbriae exhibit erythrocyte agglutination independently of D-mannose. Transmission electron microscopy has revealed the presence of fimbriae structures on A. baumannii, and these strains' hemagglutinating activity remains unaffected by D-mannose or D-galactose (Sepulveda et al., 1998). In the context of adherence to human bronchial epithelial cells, *A. baumannii* demonstrates 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 has shown thin fimbria-like extensions firmly anchored to the cell membrane surface during this process. Importantly, research indicates that there is no significant difference in adherence between outbreak and non-outbreak strains of *A. baumannii* (Y.-T. Lee et al., 2009). The chaperone usher secretion system plays a critical role in the formation of fimbriae in *A. baumannii*. This system facilitates attachment to various surfaces, including plastic surfaces, thereby promoting subsequent biofilm development. Biofilms, comprised of bacterial communities encased in a protective matrix, enhance resistance to antibiotics and host defenses (Tomaras et al., 2003). Additionally, there is a suggested association between the PER-1 gene, encoding a β-lactamase enzyme conferring resistance to β-lactam antibiotics, and the adhesion of *Acinetobacter* strains to Caco-2 cells, a type of human epithelial cell line. This potential link between antibiotic resistance and bacterial adhesion underscores the complex interplay between microbial virulence factors and host-pathogen interactions (Sechi et al., 2004). Study of the mechanisms by which *A. baumannii* adheres to host tissues and initiates infections is crucial for developing effective strategies to prevent and treat *Acinetobacter* infections, particularly in healthcare settings where these pathogens pose significant challenges.

2.3.2 Cell surface Hydrophobicity and Enzymes

A. baumannii shows exquisite cell hydrophobicity, a characteristic that contributes to its ability to adhere to host cells and evade phagocytosis, the process by which immune cells engulf and destroy pathogens. This hydrophobicity also mediates its effective attachment to plastic or polymer surfaces commonly found in medical devices such as catheters (Doughari et al., 2011). The

hydrophobic nature of *A. baumannii* is attributed to several features, including a rough cell surface and protein protrusions on the cell surface. These structural aspects enhance the interactions between *A. baumannii* cells and hydrophobic surfaces, promoting adherence and biofilm formation (Phuong et al., 2009). Moreover, specific cell surface enzymes have been identified as contributors to the hydrophobicity of *A. baumannii*. These enzymes likely modify the surface properties of the bacterium, further enhancing its hydrophobic characteristics. It has been reported that *A. baumannii* isolates obtained from catheters and tracheal devices exhibit higher hydrophobicity compared to environmental samples. This finding suggests that the ability to adhere to surfaces and form biofilms may be particularly pronounced in clinical isolates, potentially contributing to the persistence and virulence of *A. baumannii* infections associated with medical devices (Boujaafar et al., 1990). Assessment and understanding of the mechanisms underlying *A. baumannii* cell hydrophobicity is crucial for developing strategies to prevent and control infections caused by this opportunistic pathogen, especially in healthcare settings where medical devices play a significant role in patient care. By targeting the factors that contribute to *A. baumannii* adherence and biofilm formation, it might be possible to mitigate the impact of these infections and improve patient outcomes.

2.3.3 Toxic Slime Polysaccharides

The production of toxic slime polysaccharides by *Acinetobacter* spp., particularly *A. baumannii*, represents a significant aspect of its pathogenicity. These polysaccharides, composed of various sugars including glucose, glucuronic acid, D-mannose, L-rhamnose, and D-glucose, are synthesized during the exponential growth phase of the bacterium. Research by Hoštacká (2002) highlights the detrimental effects of these slime polysaccharides on the host immune response (Hoštacká & Klokočníková, 2002). Not only do they exhibit toxicity towards neutrophils, key immune cells involved in fighting bacterial infections, but they also impede the movement and phagocytic function of these cells. This interference with the host's immune defenses allows *A. baumannii* to evade clearance and establish infection more effectively.

Additionally, *A. baumannii* capsular polysaccharides play a crucial role in obstructing the host's immune response and promoting bacterial survival in serum. These polysaccharides create a protective barrier around the bacterium, shielding it from detection and destruction by the immune

system. This evasion strategy contributes to the bacterium's ability to persist and cause infections in the host (Rumbo et al., 2014).

Furthermore, there is a correlation between the production of slime polysaccharides and the virulence of Acinetobacter strains. Generally, strains that produce slime are associated with higher levels of virulence compared to non-producing strains. This suggests that the production of toxic slime polysaccharides is an important virulence factor *for Acinetobacter* spp., contributing to its pathogenicity and ability to cause severe infections.

2.3.4 Outer Membrane Protein

Outer membrane proteins (Omp) are integral components of the outer membrane of Gram-negative bacteria and play a significant role in their pathogenicity. In certain strains of *Acinetobacter*, particularly *A. baumannii*, OmpA has been identified as a key outer membrane protein with diverse functions. OmpA, a 38 kDa protein, has been detected in multiple strains of *Acinetobacter*. In the context of infection, *A. baumannii* OmpA interacts with eukaryotic cells and can translocate into the nucleus, ultimately leading to cell death (Dijkshoorn et al., 2007). Additionally, OmpA has been found to induce early apoptosis and delay necrosis in dendritic cells, which are crucial components of the immune system. Beyond its role in pathogenesis, OmpA contributes to biofilm formation, surface motility, and resistance to serum in *A. baumannii* strains (McConnell et al., 2013). Another outer membrane protein, Omp33-36, has also been recognized as crucial for the virulence of *A. baumannii*. Omp33-36 induces apoptosis in host cells by activating caspases, which are enzymes involved in programmed cell death, and modulating autophagy, a cellular process involved in degradation and recycling of cellular components (Rumbo et al., 2014). In addition to outer membrane proteins, cell surface components such as adhesins and fimbriae (pili) are also important for the attachment of *A. baumannii* to both host cells and inanimate surfaces. Adhesins facilitate the initial attachment of the bacterium to surfaces, while fimbriae play a role in adherence to host cells, thereby facilitating colonization and infection (Eijkelkamp et al., 2013). Overall, outer membrane proteins and cell surface components are critical for the pathogenicity of *A. baumannii*, mediating interactions with host cells, evasion of immune defenses, and biofilm formation. Understanding the roles of these proteins and components is essential for developing strategies to combat *A. baumannii* infections and mitigate their impact on human health.

2.3.5 Surface and Mitochondrial Porins

Porins are reported to be crucial components of the outer membrane in bacterial cells, including those of *Acinetobacter baumannii*. These proteins function as channels or pores, allowing the passage of small molecules across the lipid bilayer membrane. Their roles can vary among bacterial species and can include maintaining cellular structure, facilitating bacterial conjugation, interacting with bacteriophages, and contributing to antimicrobial resistance (Braun & Vidotto, 2004). In the case of *A. baumannii*, porins play a significant role in antimicrobial resistance by forming channels that regulate the passage of antibiotics and other molecules into the bacterial cell. When combined with efflux pumps, which are transport proteins that actively remove toxic substances, including antibiotics, from the bacterial cell, porins create a formidable barrier that hinders the uptake of antibiotics. It has been studied that alterations in porin expression or structure can contribute to antibiotic resistance in *A. baumannii*. For example, reduced expression or mutations in porin genes can lead to decreased permeability of antibiotics into the bacterial cell, reducing their efficacy. Additionally, porins may interact with efflux pumps to enhance the removal of antibiotics from the cell, further contributing to resistance (Braun & Vidotto, 2004).

2.3.6 Siderophores

Acquisition of sufficient amount of iron is crucial for the growth and virulence of bacteria, including *Acinetobacter* species. Siderophores, specialized molecules produced by bacteria, play a key role in iron acquisition by binding to iron and facilitating its uptake into the bacterial cell. *Acinetobacter* species, including *A. baumannii*, produce siderophores known as acinetobactins. These siderophores are particularly important for acquiring ferric ions (Fe3+) from the environment, especially under conditions of low iron availability. Acinetobactins help convert insoluble forms of iron into soluble chelates, making iron more accessible for bacterial uptake and utilization (Mihara et al., 2004). Research has demonstrated the significance of acinetobactin-mediated iron acquisition in the virulence of *A. baumannii*. Studies have also shown that the iron acquisition system involving acinetobactins can cause damage to human epithelial cells, which are a primary target during infection. Furthermore, experiments in *Galleria mellonella* caterpillars and mice contaminated with *A. baumannii* have revealed that this iron acquisition system contributes

to the bacterium's pathogenicity, leading to death in these host organisms. Clinical strains of *A. baumannii* have been found to grow in environments with limited iron availability, indicating the importance of siderophore-mediated iron acquisition in the bacterium's survival and proliferation in host environments. Additionally, these clinical strains have been shown to secrete iron-regulated catechol siderophores into their surrounding culture medium, further highlighting the role of siderophores in iron acquisition by *A. baumannii* (Actis et al., 1993). Thus, the production of siderophores such as acinetobactins is a critical virulence factor for *A. baumannii*, enabling the bacterium to acquire iron for growth and survival within the host.

2.3.7 Glycoconjugates

In Acinetobacter baumannii, various surface structures and components contribute to its resistance against drugs, desiccation, and evasion of the host immune response. Lipooligosaccharide (LOS) is one such component that plays a role in resistance against drugs and desiccation. LOS is a glycolipid found in the outer membrane of *A. baumannii* and has been implicated in conferring resistance to antibiotics and other antimicrobial agents. Additionally, LOS may contribute to the bacterium's ability to survive in dry environments, such as those encountered on surfaces or medical equipment (Boll et al., 2015). The capsular polysaccharide of *A. baumannii* serves a protective function by preventing complement-mediated killing of cells. Complement is part of the innate immune system and plays a role in recognizing and destroying pathogens. By shielding the bacterial cell surface, the capsular polysaccharide helps *A. baumannii* evade detection and destruction by the host immune system (Russo et al., 2010). Glycoproteins are also involved in *A. baumannii* virulence by enhancing the formation and stability of biofilms. Glycoproteins within the biofilm matrix contribute to its structural integrity, promoting bacterial survival and persistence in the host environment. Furthermore, glycosylated type IV pili have been suggested to aid in immune evasion by shielding antigenic proteins from detection by antibodies. Type IV pili are filamentous structures on the bacterial surface that play roles in adherence, motility, and biofilm formation. By glycosylating these pili, *A. baumannii* may mask antigenic epitopes, making them less recognizable to the host immune system (Piepenbrink et al., 2016).

2.3.8 Phospholipase

Acinetobacter baumannii contains a set of phospholipase enzymes, including phospholipase C (PLC) and phospholipase D (PLD), each with distinct hydrolytic properties that target phosphatidylcholine, a key component of eukaryotic cell membranes (Fiester et al., 2016). Each of the enzymes contributes uniquely to the bacterium's pathogenicity. PLD enzymes are particularly crucial for several aspects of *A. baumannii* virulence. They play a pivotal role in the penetration of epithelial cells, facilitating the bacterium's ability to invade host tissues. Additionally, PLD enzymes contribute to the bacterium's resistance against human serum, a component of the innate immune system, and are implicated in its overall pathogenesis in vivo, suggesting their importance in causing disease (Stahl et al., 2015).

Conversely, phospholipase C (PLC) enzymes play a significant role in the hemolytic and cytolytic activities displayed by *A. baumannii* toward host cells. These enzymes contribute to the bacterium's ability to disrupt host cell membranes, leading to cell lysis and tissue damage. This activity eventually leads to evading the host immune defenses and establishing infection (Fiester et al., 2016).

2.3.9 Iron Acquisition System

Acinetobacter baumannii has evolved a sophisticated iron acquisition system to thrive in the iron-limited environment encountered within the host, where iron is predominantly bound to heme, the iron-containing component of hemoglobin and other proteins (Sheldon & Skaar, 2020). This system comprises eight gene clusters, each with specific functions related to iron acquisition. One cluster is involved in direct iron acquisition, enabling the bacterium to scavenge iron from the host environment. Two clusters facilitate the uptake of heme, a rich source of iron found within host tissues and blood. Heme uptake systems allow *A. baumannii* to extract iron from heme molecules for its metabolic needs. Additionally, five clusters are dedicated to the biosynthesis and utilization of siderophores, specialized molecules that bind iron with high affinity and participate in iron acquisition from host cells (Sheldon & Skaar, 2020). Siderophores play a crucial role in the competition for iron between bacteria and host cells. *A. baumannii* produces ten distinct types of siderophores, including acinetobactin, baumannoferrins, and fimsbactins. Acinetobactin has been extensively studied, and its structural characteristics have been well-documented among the others

(Eijkelkamp et al., 2011). The efficient iron acquisition system of *A. baumannii* reflects its adaptation to the host environment and its ability to compete for essential nutrients such as iron.

2.3.10 Efflux Pumps

Efflux systems of bacteria exhibit a crucial role for the survival and virulence of gram-negative pathogens, including *Acinetobacter baumannii*. These systems span the bacterial membrane and function to expel various potentially harmful compounds from the periplasm to the extracellular environment. They contribute significantly to bacterial resistance against antimicrobial agents and host immune defenses. Efflux pumps play diverse roles in gram-negative bacteria, including the expulsion of bile salts, antimicrobial fatty acids, peptides, and toxins. Additionally, some efflux pumps actively secrete virulence factors such as siderophores, which are molecules involved in iron acquisition and pathogenesis (Morris et al., 2019).

There are six families of bacterial efflux pumps identified to date, each with its unique substrate specificity, functional characteristics, and mechanisms of action: 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 wide range of efflux systems, allowing bacteria to expel various antimicrobial agents and toxins, thereby promoting survival and virulence in different environmental niches. In *A. baumannii*, efflux pumps, particularly those belonging to the RND family, play a critical role in multidrug resistance, contributing to the bacterium's ability to survive in diverse environments, including clinical settings (Morris et al., 2019).

2.4 Host- Pathogen Interactions

During disease progression, the interactions between hosts and pathogens usually involve factors such as strain virulence, host characteristics and bacterial load, profoundly affecting the infected individual's health. Interestingly, according to a study by Fournier (2006), several genes responsible for resisting antibiotics, heavy metals, and antiseptics in *A. baumannii* has likely originated from other highly pathogenic organism (Fournier et al., 2006). When comparing the

genomes of *A. baumannii* strain 17978 and the non-pathogenic *A. baylyi*, researchers identified 28 gene clusters specific to *A. baumannii*, with 16 strains potentially linked to virulence with the five gene csu polycistronic operon cluster being the most notable one (Rastogi et al., 2007). Some of these genes share similarities to proteins in other Gram-negative bacteria involved in chaperon functions and pilus assembly (Tomaras et al., 2003). Studies have shown that the pathogenicity of *A. baumannii* isolates is due to their ability to form biofilms, by the formation of exopolysaccharides and pili. These factors promote biofilm development and stability, where it can adhere to surfaces and cells. Additionally, in many Gram-negative organism, quorum sensing plays a major role in controlling various virulence mechanisms.

In *Acinetobacter,* the presence of N-acyl homoserine lactones (AHSL) as quorum sensing molecules indicates their role in self-inducing virulence factors (Joly-Guillou, 2005). Various types of AHSL molecules have been identified, including 3-oxo substituted variants with acyl chains of different lengths, 3-unsubstituted AHSL (excluding C4), and 3-hydroxyl AHSL (C6, C8, and C10) (Gonzalez et al., 2001). The production of exopolysaccharides is also believed to provide protection against host defenses. Exopolysaccharides act as a physical barrier, enhancing bacterial survival and persistence within the host environment, by shielding the bacteria from immune system components. Toll-like receptors (TLR) signaling also plays a crucial role in the innate immune response to *A. baumannii* (Joly-Guillou, 2005). Animal mouse model studies have shown that mice lacking Toll-like receptor 4 (TLR4) exhibits increases bacterial counts, higher bacteremia levels, impaired cytokine/chemokine responses, and delayed lung inflammation compared to wild-type mice. This underscores the importance of TLR4 in recognizing and responding to bacterial infections and the impact of TLR4 deficiency on immune response and host defense mechanisms. *A. baumannii* lipopolysaccharide (LPS) is a significant stimulant triggering immune responses. CD14, a receptor protein, binds to *A. baumannii* LPS and Toll-like receptor 4 (TLR4), facilitating immune system detection of *A. baumannii* LPS and initiating immune responses against bacterium. Importantly, TLR2 in human cells also play a significant role in signaling pathways for *A. baumannii* LPS, inducing strong inflammatory responses akin 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 shown bactericidal and opsonizing effects by inhibiting iron uptake.

Conducting experimental infections in controlled animal models has offered significant insights into disease mechanisms. Nonetheless it is essential to approach the interpretation of these findings carefully since they might not capture all the intricacies of natural infection scenarios (Clutterbuck et al., 2007). The results indicates that *A. baumannii* endotoxin can provoke a robust inflammatory response during infection. Furthermore, monoclonal antibodies derived from mice, specifically targeting the outer membrane proteins expressed in *A. baumannii* under low iron conditions, have shown both bactericidal and opsonizing effects by impeding iron uptake (Joly-Guillou, 2005).

2.5 Epidemiology of *A. baumannii*

In the early years, *Acinetobacter* spp. was viewed as an opportunistic pathogen with limited clinical importance. However, from the 1980a, there has been a noticeable rise in the occurrence and severity of *A. baumannii* infections, particularly patients in the intensive care units (ICUs) (Zhao et al., 2019). This surge has been observed globally, with significant prevalence in regions like Europe (including the UK, Germany, Italy, Spain) and the United States, often involving strains resistant to multiple drugs (Akrami & Namvar, 2019). In European ICUs, *A. baumannii* infections account for 2% -10% of all Gram-negative bacterial infections, and approximately 2.5% in the United States (Fournier et al., 2006). Factors including person-to-person contact and development of antimicrobial resistance also contribute to the spread of *A. baumannii* infections (Akrami & Namvar, 2019; Munoz-Price & Weinstein, 2008). A study conducted in the US have indicated that there is a high prevalence of nosocomial infections caused by *Acinetobacter* spp. during periods of high temperatures (Eber et al., 2011). Asian countries like Thailand, Malaysia, and India have reported high rates of multidrug-resistant (MDR) and extensively drug-resistant (XDR) *Acinetobacter* spp., with particularly elevated resistance rates to imipenem (Chung et al., 2011). In Bosnia, cases of nosocomial infections caused by Acinetobacter is substantial, with a significant proportion involving respiratory infections. Community-acquired *A. baumannii* infections are more commonly found in tropical and subtropical regions such as Singapore, Hong Kong, and Taiwan, often coinciding with rainy seasons (Custovic et al., 2014). Carbapenem-resistant *A. baumannii* (CRAB) poses a significant challenge, constituting around 65% of *A. baumannii* pneumonia cases in the United States and Europe. On the other hand, the highest levels

of resistance among *Acinetobacter* spp. have been reported in Baltic and southern/southeastern European countries (Zhao et al., 2019).

2.6 Biofilm Formation among *A. baumannii*

Biofilm is a complex substance comprising microorganisms adhered to surfaces and enclosed within a hydrated polymeric matrix, consisting of polysaccharides, proteins, and nucleic acids, forming three-dimensional structures (Sauer et al., 2007). *Acinetobacter baumannii*, commonly isolated from healthcare settings, forms biofilms facilitating its persistence and protection from disinfectants, thus serving as a continuous infection source. Additionally, the ability of *A. baumannii* isolates to form biofilms contributes to antibiotic resistance. Various factors influence biofilm synthesis in *A. baumannii*, including nutrient availability, surface structures like pili and outer membrane proteins, quorum sensing, and the presence of DNA and polysaccharides (Cerqueira & Peleg, 2011). Pili, encoded by the csu/BABCDE chaperone-usher assembly system and regulated by the *bfmS* and *bfmR* genes in a two-component regulatory system, play a crucial role in biofilm formation and regulation in *A. baumannii*, impacting its persistence and antibiotic resistance (Luo et al., 2015). Studies employing transposon mutagenesis have shown that csuA and csuE are vital for bacterial attachment, biofilm formation, and pili synthesis (Tomaras et al., 2003). Another study revealed that inactivation of the bfmR response regulator led to the absence of csu gene expression, resulting in complete loss of pili production and biofilm formation in *A. baumannii*. In contrast, a mutation in the *bfmS* gene only partially hindered biofilm synthesis, indicating a less significant role in the process.

Another protein, known as biofilm-associated protein (Bap), is also linked to biofilm production in *A. baumannii*. Mutation of the Bap gene resulted in a substantial decrease in biofilm thickness, reducing it by over 50% compared to the wild-type strain (Loehfelm et al., 2008). Additionally, *ompA* is crucial for the formation of robust biofilms, particularly on polystyrene surfaces (Gaddy & Actis, 2009). Exopolysaccharides, specifically poly-β-(1-6)-N-acetylglucosamine (PNAG), were identified as essential components for the structural integrity of *A. baumannii* biofilms formed under stressful conditions, maintaining stability and cohesion (Choi et al., 2009). PNAG also aids in cell-to-cell adhesion (Cramton et al., 1999). Besides the aforementioned factors,

quorum sensing, a well-known bacterial cell-to-cell communication mechanism, regulates biofilm formation in *A. baumannii*. The quorum sensing molecule 3-hydroxy-C12-HSL, produced by the autoinducer synthase encoded by the abaI gene, is crucial for later stages of biofilm production, especially on non-living surfaces. Quorum sensing allows *A. baumannii* cells to synchronize their behavior, facilitating the formation and maturation of biofilm structures (Niu et al., 2008).

Many researchers have observed that clinical strains of *A. baumannii* are proficient in forming strong biofilms (Gurung et al., 2013). Besides its role in spreading nosocomial infections, biofilm formation by *A. baumannii* significantly impacts antibiotic resistance and virulence. Clinical samples of multidrug-resistant (MDR) *A. baumannii* have demonstrated strong biofilm formation, leading to increased adherence to human bronchial epithelial cells. This enhanced biofilm production enhances the organism's ability to persist and thrive within the host, contributing to its antibiotic resistance (Gordon & Wareham, 2010). Thus, it is crucial to target and inhibit biofilm formation in *A. baumannii* to effectively control its transmission in healthcare settings, while also addressing its antibiotic resistance and virulence.

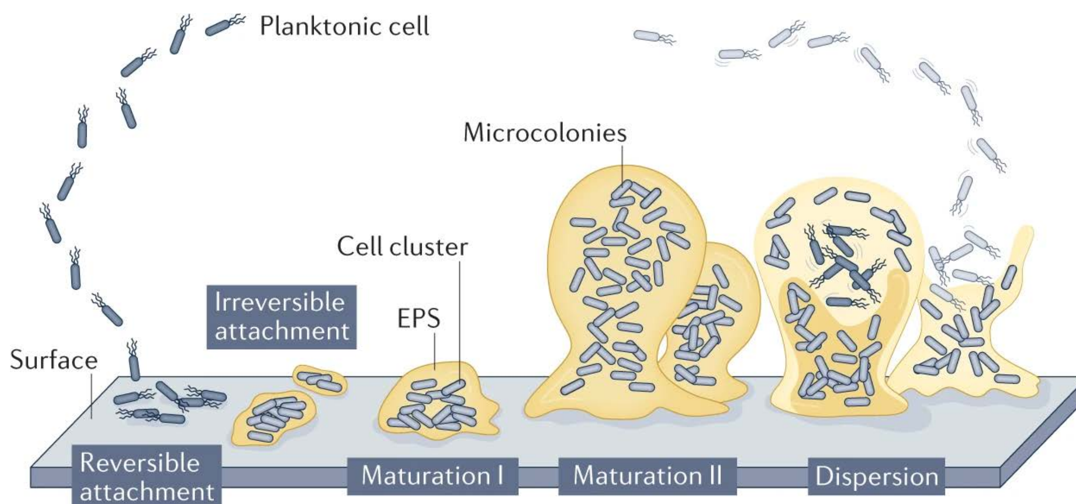


Figure 2.3: The original five-step model of biofilm development (Sauer et al., 2022)

2.7 Biofilm and Increased Antibiotic Resistance

Escherichia coli is widely recognized as the primary causative agent for urinary tract infections (UTIs), with significant cases of *Acinetobacter baumannii* contributing to UTI cases. Many *A. baumannii* strains exhibit resistance to commonly used antibiotics such as ampicillin, amoxicillin-clavulanic acid, norfloxacin, cefuroxime, ceftriaxone, and co-trimoxazole. Diabetes and kidney

diseases, along with the use of intrauterine devices are few of the risk factors for UTIs, 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 formation complicates UTIs by creating a protective environment for bacterial cells, rendering them more resistant to antibiotics compared to free-floating planktonic cells. Biofilms commonly develop on medical devices such as catheters, ventilators, and contact lenses, posing a significant challenges for treatment.

However, there are studies showing that cells growing in planktonic state after being disrupted from a biofilm can regain antibiotic susceptibility (Zuroff et al., 2010). Extensive studies on the association between biofilms and antibiotic resistance has shown that bacteria within biofilms exhibit a higher resistance to antibiotics such as ampicillin, cefotaxime, norfloxacin, and nalidixic acid compared to no-biofilm forming bacteria. Additionally, the effectiveness of antibiotics in preventing biofilm formation is also impacted by the presence of biofilm. For example, while ampicillin can inhibit attachment and early development of biofilms, it does not prevent mature biofilm formation within 72 hours after treatment cessation. This underscores the importance of considering both multidrug-resistant mechanisms, including efflux pumps, and the biofilm matrix itself in understanding penicillin resistance and presents challenges for effective treatment (Ito et al., 2009). Addressing biofilm formation and its impact on antibiotic response are crucial in combating UTIs caused by bacteria like A. baumannii.

2.8 Antibiotics

Since the launch of the first antibiotic, salvarsan, in 1910, the field of antibiotics has seen significant progress, transforming modern medicine and substantially extending human life expectancy by approximately 23 years. The discovery of penicillin in 1928 marked the onset of a golden era in exploring natural product antibiotics, which peaked in the mid-1950s. The integration of antibiotics into medical practice is widely acknowledged as one of the most extraordinary medical breakthroughs of the 20th century. Antibiotics have not only been vital in treating infectious diseases but have also paved the way for numerous contemporary medical procedures such as cancer treatment, organ transplants, and open-heart surgery. Their impact on medicine has been profound, shaping healthcare and enhancing the lives of millions globally.

The five major mechanisms of antibiotics are described below:

2.8.1 Interference with Cell Wall Synthesis

Penicillin and cephalosporin are categorized as β-lactam antibiotics, and their antimicrobial effectiveness stems from their capacity to target enzymes vital for synthesizing the peptidoglycan layer of bacterial cell walls. These antibiotics function by inhibiting the activity of penicillin-binding proteins (PBPs), which are responsible for linking peptide chains within the peptidoglycan structure. By impeding these enzymes, penicillin and cephalosporin disrupt the construction and integrity of bacterial cell walls, leading to cell rupture and eventual bacterial demise. Their ability to interfere with peptidoglycan synthesis renders them potent against a broad spectrum of bacterial infections, contributing to their extensive use in clinical settings (Leach et al., 2007).

2.8.2 Inhibition of Protein Synthesis

The oxazolidinone family of antibiotics, a recent addition to the antibiotic arsenal, operates by interacting with the A site of the bacterial ribosome. This interaction blocks the binding of aminoacyl-tRNA molecules to the site, thus halting the initiation of protein synthesis. Tetracyclines, however, disrupt protein synthesis by binding to the 30S component of the bacterial ribosome. This binding weakens the connection between the ribosome and tRNA molecules, hindering the elongation phase of protein synthesis. Macrolide antibiotics bind to the 50S ribosomal subunit, impeding the elongation of nascent polypeptide chains during protein synthesis. Chloramphenicol also binds to the 50S ribosomal subunit but operates differently by inhibiting the peptidyl transferase process, which forms peptide bonds during protein synthesis. Aminoglycosides attach to the 30S ribosomal subunit, preventing protein synthesis initiation and leading to the production of defective proteins. Their binding disrupts the accurate reading of the genetic code, potentially resulting in the synthesis of non-functional or harmful proteins. Each antibiotic class targets a specific ribosomal component and disrupts distinct stages of protein synthesis, ultimately halting bacterial growth and promoting bacterial cell death (Leach et al., 2007).

2.8.3 Interference in Nucleic Acid Synthesis

Rifampicin functions as an antibiotic by inhibiting DNA-directed RNA polymerase, a crucial enzyme involved in transcription. This inhibition disrupts the synthesis of RNA from DNA templates, thereby halting bacterial gene expression (Strohl, 1997).

2.8.4 Inhibition of a Metabolic Pathway

Sulfonamides, like sulfamethoxazole, and trimethoprim are antibiotics that target various stages in the folate synthesis pathway, essential for nucleotide production in DNA and RNA synthesis. Sulfonamides mimic a component of folic acid, a precursor molecule in this pathway, competitively inhibiting the enzyme dihydropteroate synthase. This enzyme is responsible for integrating the precursor molecule into the folate synthesis pathway. By inhibiting it, sulfonamides disrupt folate production necessary for nucleotide synthesis, ultimately interfering with bacterial DNA and RNA synthesis, leading to inhibited bacterial growth (Strohl, 1997).

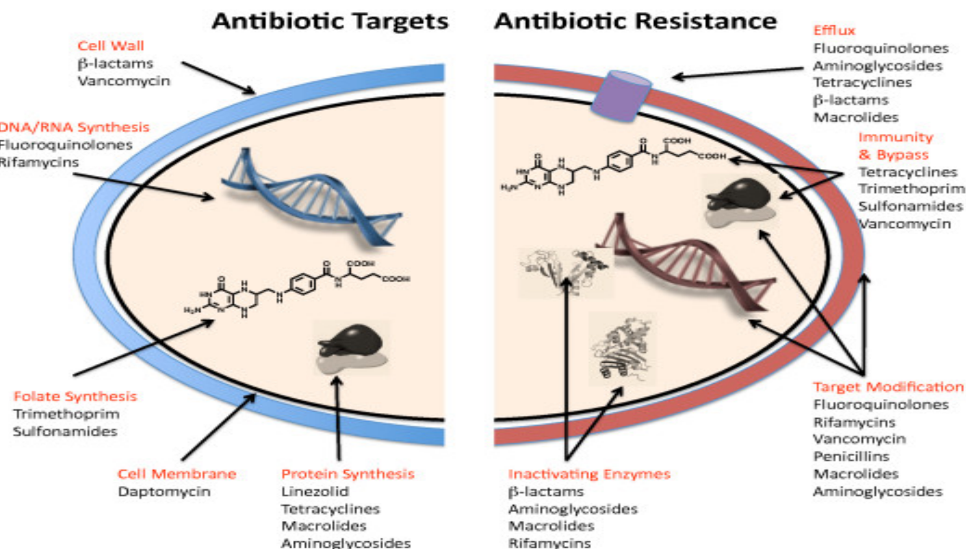


Figure 2.4: Antibiotic target vs resistance mechanism (Wright, 2010).

2.8.5 Disorganization of the Cell Membrane

Gram positive bacteria possesses a cytoplasmic membrane, while Gram-negative bacteria have an inner membrane, both being primary targets for specific antibiotics. Polymyxins are believed to function by increasing bacterial membrane permeability, causing the release of cellular contents

and subsequent cell death. Conversely, daptomycin, a cyclic lipopeptide, rapidly eradicates bacteria by binding to the cytoplasmic membrane in a calcium-dependent manner. This binding triggers membrane complex formation, leading to potassium ion efflux from the bacterial cell and eventual cell death (Straus & Hancock, 2006).

2.9 Antibiotic Resistance

Antibiotic resistance, as defined by the World Health Organization (WHO), occurs when bacteria, viruses, fungi, or parasites no longer respond to treatments that were previously effective against them. Specifically, antibiotic resistance refers to bacteria becoming unaffected by antibiotics that once treated their infections effectively.

2.9.1 Categories of Antibiotic Resistance Pattern

The European Centre for Disease Prevention and Control (ECDC) and the Centers for Disease Control and Prevention (CDC) have established three categories to classify resistance patterns:

1. Multidrug-resistant (MDR): Acquired non-susceptibility to at least one agent in three or more antimicrobial categories.
2. Extensively drug-resistant (XDR): Non-susceptibility to at least one agent in all except two or fewer antimicrobial categories, leaving bacterial isolates susceptible to only one or two categories.
3. Pan drug-resistant (PDR): Non-susceptibility to all agents in all antimicrobial categories.

2.9.2 Mechanisms of Antibiotic Resistance

Antimicrobial resistance denotes the diminishing efficacy of medications, encompassing antibiotics and antineoplastics, in combating diseases or conditions. It shares similarities with dose failure or drug tolerance, where the desired antibacterial outcome is not attained. This term is frequently employed in discussions concerning pathogen resistance, particularly when it arises from acquired mechanisms. Multidrug-resistant organisms are those that have evolved resistance to multiple medications (Fisher & Mobashery, 2014). Bacterial strains exhibit diverse resistance mechanisms, as outlined below.

2.9.3 Biochemical aspects of Antibiotic Resistance

2.9.3.1 Through Hydrolysis

Enzymes produced by resistant bacteria have the ability to hydrolyze specific chemical bonds found in many antibiotics, such as amides and esters, resulting in the inactivation of these antibiotics. Extended-spectrum β-lactamases (ESBLs) are enzymes that specifically target and cleave these vulnerable bonds, affecting antibiotics like penicillin, third-generation cephalosporins (e.g., ceftazidime, cefotaxime, and ceftriaxone), and aztreonam. However, cephamycin (cefoxitin and cefotetan) and carbapenems are not susceptible to inactivation by ESBLs (Bonnet, 2004).

2.9.3.2 Through Redox Process

While the oxidation or reduction of antibiotics is not commonly utilized by pathogenic bacteria, there are instances where this strategy has been observed. For example, the TetX enzyme oxidizes tetracycline antibiotics. Additionally, the bacterium *Streptomyces virginiae* produces the streptogramin antibiotic virginiamycin M1, and it has evolved a mechanism to protect itself from the antibiotic's action by modifying its structure, rendering it less effective against the producing bacterium (W. Yang et al., 2004).

2.9.3.3 Antibiotic Inactivation through Group Transfer

Transferases, an extensive group of enzymes, play a significant role in antibiotic resistance by chemically modifying antibiotics, such as aminoglycosides, chloramphenicol, streptogramin, macrolides, or rifampicin, through the addition of various groups to the antibiotic molecule. This modification alters the antibiotic's structure, making it unable to bind to its target effectively. Various chemical reactions, including O-acetylation, N-acetylation, and phosphorylation, are involved in this resistance process. Importantly, these covalent modification reactions typically require specific co-substrates and occur primarily in the cytoplasmic environment (Shaikh et al., 2015).

2.9.3.4 Antibiotic Inactivation through Target Modification

Another significant mechanism of resistance involves altering the antibiotic's target site, reducing the antibiotic's ability to bind and inhibit the target. This alteration can occur through genetic mutations, resulting in reduced susceptibility to the antibiotic's inhibitory effects while allowing normal cellular function to continue. This mechanism enables bacteria to evade the antibiotic's intended action and survive in its presence (Spratt, 1994).

2.9.4 Genetics of Antibiotic Resistance

2.9.4.1 Antibiotic Resistance via Mutations

Antibiotic resistance can arise through various biochemical pathways, with a significant portion of them resulting from mutational events. Mutations in genes encoding the targets of antibiotics play a crucial role in conferring resistance. For instance, mutations in genes like RpoB and DNA-topoisomerases can lead to resistance against rifampicin and fluoroquinolones, respectively (Ruiz, 2003). Besides target alterations, mutations can also impact the expression of proteins involved in antibiotic absorption or efflux systems. For example, in Pseudomonas aeruginosa, mutations can reduce the expression or absence of the OprD porin, limiting the permeability of the cell wall to carbapenem antibiotics (Wolter et al., 2004).

2.9.4.2 Antibiotic Resistance via Horizontal Gene Transfer

Horizontal gene transfer is a significant mechanism facilitating the spread of antibiotic resistance. Genes responsible for antibiotic resistance can be transferred between bacteria through processes such as conjugation, transformation, and transduction. In recent years, there has been a notable emergence of β-lactamase enzymes called extended-spectrum β-lactamases (ESBLs), capable of resisting a wide range of β-lactam antibiotics, including cephalosporins (excluding carbapenems). One specific ESBL variant, CTX-M-15, initially discovered in *E. coli*, has spread to other bacteria within the Enterobacteriaceae family. CTX-M-15 is often associated with a particular lineage called the uropathogenic clone ST131. It is frequently found on highly mobile plasmids of the IncFII type, often linked to a mobile genetic element called IS26. Individuals with prolonged renal

or liver dysfunction and those traveling to high-risk areas face an increased risk of infection by antibiotic-resistant bacteria carrying CTX-M-15 (Nordmann et al., 2011).

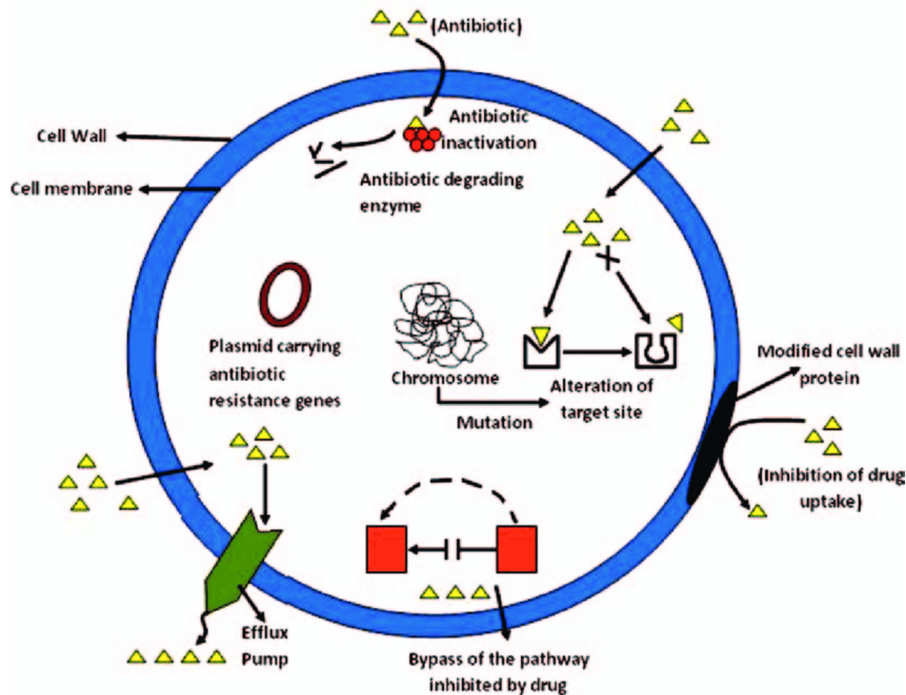


Figure 2.5: Antibiotic resistance mechanisms (Singh et al., 2014)

2.10 Antibiotic Resistance in *A. baumannii*

Before 1970s, a variety of antibiotics, including β-lactams, aminoglycosides, and tetracyclines, were effective against *Acinetobacter* infections. However, the emergence of *A. baumannii* with both extensive scale of intrinsic and acquired resistance determinants has rendered it resistant to all established antibiotics, posing significant challenges in treatment (Peleg et al., 2008). *A. baumannii* is the most resistant species within the *Acinetobacter* genus, exhibiting variations in antimicrobial susceptibility (Van Looveren et al., 2004). The acronym ESKAPE is used to categorize high frequency multidrug-resistant (MDR) bacteria, including *Enterococcus faecium*, *Staphylococcus aureus, Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa,* and *Enterobacter* spp., with *A. baumannii* being included. The ESKAPE pathogens are major contributors to healthcare-associated infections and have developed resistance to antimicrobial agents (Navidinia, 2016). In 2017, the World Health Organization (WHO) identified a list of pathogens requiring immediate global attention. Carbapenem-resistant *A. baumannii*

(CRAB) was designated 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).

2.11 β-lactamases

Resistance to β-lactam antibiotics has been a common challenge in bacterial infections, often mediated by enzymes called β-lactamases which are produced by bacteria (Kumarasamy et al., 2010). These enzymes possess the ability to hydrolyze or break down β-lactam antibiotics, rendering them ineffective in combating bacterial infections. β-lactamases can be classified into four main classes, namely class A, B, C, and D, based on their sequence motifs and hydrolytic mechanisms. Classes A, C, and D are categorized as serine β-lactamases (SBLs) and rely on a serine residue in their active site to catalyze the hydrolysis of β-lactam antibiotics. Class B β-lactamases, on the other hand, are zinc-dependent enzymes known as metallo-β-lactamases (MBLs) (Massova & Mobashery, 1998). Class D β-lactamases, also referred to as oxacillinases or carbapenem-hydrolyzing class D β-lactamases (CHDLs), are particularly significant in conferring resistance to carbapenem antibiotics, which are considered as last-resort treatments for many bacterial infections. CHDLs have the ability to inactivate a broad range of β-lactam antibiotics, including carbapenems, rendering them ineffective against bacterial pathogens (N. T. Antunes & Fisher, 2014).

2.11.1 Extended-spectrum β-lactamases (ESBLs)

Extended spectrum β-lactamases (ESBLs) are enzymes produced by many pathogenic bacteria that confer resistance to extended spectrum cephalosporins, rendering antibiotics such as ceftazidime, ceftriaxone, cefotaxime, and oxyimino-monobactams ineffective against these bacteria. ESBL-producing strains typically remain susceptible to carbapenems and cephamycin. The activity of ESBLs can be inhibited by β-lactamase inhibitors like clavulanic acid and tazobactam (Paterson et al., 2000). Gram-negative bacteria, including *Enterobacteriaceae* and *Pseudomonas aeruginosa*, are common hosts of ESBLs, with the TEM-1 β-lactamase being the most prevalent (Bradford, 2001). TEM-1 can also hydrolyze penicillin and first-generation cephalosporins. TEM-2 is a variant of TEM-1 with a single amino acid substitution. Different β-lactamase enzymes exhibit

diverse phenotypes due to amino acid changes that alter their structure and function (Bradford, 2001). TEM, SHV, and OXA enzymes often have substitutions at specific amino acid positions, resulting in varied abilities to hydrolyze third generation cephalosporins and increased resistance to β-lactamase inhibitors (Winokur et al., 2001). The emergence of ESBL-producing strains is associated with the overuse of certain β-lactam antibiotics, which has selected for strains with various phenotypes and structural alterations in porins such as Omp (Blazquez et al., 2000), leading to resistance against cephalosporins and other antimicrobials (Shakib et al., 2011). Plasmids carrying ESBL genes may confer resistance to aminoglycosides and cotrimoxazole (Villa et al., 2000).

Quinolone resistance is more common in ESBL-producing strains, although the mechanism of co-resistance is not fully understood. ESBL-producing strains pose significant challenges in clinical settings, as they exhibit resistance to multiple antibiotics, including aminoglycosides and fluoroquinolones (Paterson et al., 2000). The prevalence of ESBL-producing strains is increasing, leading to higher morbidity, mortality, and healthcare costs. ESBL-producing *Acinetobacter baumannii* strains can be transmitted between humans and animals and contaminate and infect aquatic environments, posing risks to exposed individuals. These strains have been found in vegetables in various parts of the world and have been reported in wild animals and environments on multiple continents.

There are more than 350 different ESBL variants that have been found and being studied to date, of which the prevalent and thoroughly studied ones are stated below.

2.11.1.1 TEM

There are two predominant prototypical examples of TEM-type β-lactamases (TEM-1 and TEM-2), which were initially discovered in *E. coli* strains isolated from a patient named Temoneira in Athens, Greece, in 1965 (Datta & Kontomichalou, 1965). TEM-1 exhibits enhanced hydrolysis of ampicillin compared to other β-lactam antibiotics such as carbenicillin, oxacillin, or cephalothin. However, it does not affect extended spectrum cephalosporins. The activity of TEM-1 can be inhibited by clavulanic acid. TEM-2 shares a similar hydrolytic profile with TEM-1 but differs in having a more active native promoter and a different isoelectric point. TEM-13 also shows an identical hydrolytic profile to TEM-1 and TEM-2. Notably, TEM-1, TEM-2, and TEM-13 are not

considered extended spectrum β-lactamases (ESBLs) (Jacoby, 1997). However, in 1984, TEM-3 was identified in *K. pneumoniae* in France and initially referred to as CTX-1 due to its activity against cefotaxime. TEM-3 differs from TEM-2 by two amino acid changes.

Currently, there are over 100 TEM-type β-lactamases, and apart from TEM-1 and TEM-2, all of them are classified as 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*. These enzymes pose significant challenges in clinical settings due to their ability to confer resistance to multiple β-lactam antibiotics, limiting treatment options for infections caused by bacteria carrying them (Brun-Buisson et al., 1987).

2.11.1.2 SHV

SHV-type extended spectrum β-lactamases (ESBLs) are frequently found in clinical isolates and are more prevalent than other types of ESBLs (Jacoby, 1997). The acronym SHV stands for sulfhydryl variable, reflecting the initial observation that the activity of SHV enzymes could be influenced by p-chloromercuribenzoate in a substrate-dependent manner, with variations depending on the specific substrate being tested (SYKES & BUSH, 1982). In contrast to TEM-type β-lactamases, SHV-1 produces a smaller number of derivatives within the SHV family of β-lactamases. This gene is commonly carried on plasmids in most strains and typically contains 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 critical 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, demonstrating the diversity within this family of β-lactamases. SHV-type ESBLs are found in a wide range of bacteria, including *Enterobacteriaceae*, *Pseudomonas aeruginosa*, and *Acinetobacter* spp. These enzymes contribute significantly to antibiotic resistance in clinical settings and present challenges for the treatment of infections caused by bacteria carrying them (Huang et al., 2004).

2.11.1.3 CTX-M

CTX-M-type β-lactamases were first described by Tzouvelekis and colleagues in 2000 (Tzouvelekis et al., 2000). The term "CTX-M" refers to the enzyme's ability to metabolize cefotaxime effectively (Bonnet, 2004). These β-lactamases exhibit varying hydrolytic activity towards different antibiotics. They are particularly efficient in hydrolyzing cephalothin compared to benzylpenicillin, and they demonstrate a higher affinity for cefotaxime than ceftazidime. While minimum inhibitory concentration (MIC) analysis is commonly used to test bacterial resistance to ceftazidime, certain CTX-M-type β-lactamases have been found to confer resistance to this antibiotic despite the testing results (Poirel et al., 2002). Additionally, resistance to aztreonam can vary among CTX-M-producing strains. However, cefepime can still be effective against these strains when combined with sulbactam or clavulanate (Bush et al., 1993). Unlike TEM and SHV enzymes, CTX-M β-lactamases do not typically exhibit point mutations. It is believed that CTX-M enzymes were initially found in the chromosomal DNA of *Kluyvera* spp. before being transferred to plasmids (Radice et al., 2002).

There have been reported 128 different variants of CTX-M so far, which are categorized into five main groups: CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, and CTX-M-25. These enzymes are commonly found in *Salmonella* spp. and other members of the *Enterobacteriaceae* family (Bradford et al., 1998).

2.11.1.4 OXA

The discovery of OXA-type extended spectrum β-lactamases (ESBLs) originated from *Pseudomonas aeruginosa* isolates in a hospital in Ankara, Turkey. This family of β-lactamases includes various variants such as OXA-1, OXA-10, OXA-13, OXA-18, OXA-19, and OXA-28, among others. They are named "OXA-type" due to their ability to hydrolyze oxacillin and exhibit significant hydrolysis rates for cloxacillin and oxacillin compared to benzylpenicillin (Bush et al., 1993). OXA-type β-lactamases are predominantly found in *Pseudomonas aeruginosa*, although they have also been identified in other gram-negative bacteria (Weldhagen et al., 2003). While many OXA-type enzymes lack the ability to efficiently hydrolyze extended-spectrum cephalosporins, some variants like OXA-10, OXA-18, and OXA-19 exhibit extended-spectrum activity, conferring resistance to antibiotics such as cefotaxime, ceftazidime, and aztreonam

(Toleman et al., 2003). The identification of novel variants like OXA-28, OXA-18, and OXA-19 underscores the ongoing evolution and diversity of β-lactamase enzymes, contributing to the challenge of antimicrobial resistance. Their ability to confer resistance to multiple β-lactam antibiotics highlights the importance of surveillance and infection control measures to combat the spread of multidrug-resistant organisms in healthcare settings. Additionally, the co-production of carbapenem-hydrolyzing metalloenzymes and aztreonam-hydrolyzing OXA enzymes poses a significant threat, leading to resistance against a broad spectrum of β-lactam antibiotics.

2.11.1.5 Toho β-lactamase

Toho-1 and Toho-2 are β-lactamases that structurally resemble CTX-M β-lactamases. They were named after Tokyo's Toho University School of Medicine Omori Hospital, where a child was hospitalized with an infection caused by *Escherichia coli* producing Toho-1 β-lactamase. Like other CTX-M-type β-lactamases, Toho-1 and Toho-2 demonstrate higher hydrolytic activity against cefotaxime compared to ceftazidime. This preference for cefotaxime is a characteristic feature shared by many CTX-M-type β-lactamases and contributes to their clinical significance (Ma et al., 1998).

2.11.1.6 PER

The PER-type extended spectrum β-lactamases (ESBLs) exhibit relatively low homology, approximately 25% to 27%, with known TEM- and SHV-type ESBLs (Bauernfeind et al., 1996). Despite this difference, PER-type ESBLs are significant in their ability to confer resistance to β-lactam antibiotics (Neuhauser et al., 2003). PER-1 β-lactamase efficiently hydrolyzes penicillin and cephalosporins, but it is susceptible to inhibition by clavulanic acid. Initially discovered in *Pseudomonas aeruginosa*, PER-1 has also been identified in *Salmonella enterica* serovar *Typhimurium* and *Acinetobacter* isolates (Vahaboglu et al., 2001).

In Turkey, PER-1 was detected in a significant proportion of nosocomial *Acinetobacter* spp. isolates and a smaller proportion of *Pseudomonas aeruginosa* isolates. This highlights the clinical relevance of PER-1-mediated resistance in these pathogens (Vahaboglu et al., 2001). PER-2, another variant of PER-type ESBL, has been identified in various bacterial species, including

Salmonella enterica serovar *Typhimurium*, *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, and *Vibrio cholerae* O1 El Tor. It shares a relatively high homology of 86% with PER-1, indicating a close evolutionary relationship between these variants (Petroni et al., 2002).

2.11.1.7 GES -type β – lactamases

The discovery of GES-1, initially isolated from a *K. pneumoniae* strain obtained from a newborn kid relocated from French Guiana to France, marked an important milestone in understanding the diversity of extended spectrum β-lactamases (ESBLs) (Poirel et al., 2002). GES-1 represents a unique member of the ESBL family with distinct characteristics. GES-1 exhibits sensitivity to inhibition by β-lactamase inhibitors and demonstrates the ability to hydrolyze penicillin and extended-spectrum cephalosporins. However, unlike some other ESBLs, GES-1 does not hydrolyze cephamycin or carbapenems. Despite these differences, GES-1 was designated as an ESBL due to its enzymatic properties being comparable to those of other class A ESBLs.

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

In addition to well-known ESBLs like TEM, SHV, and CTX-M, there are several other less common enzymes that exhibit extended spectrum β-lactamase activity. BES-1, CME-1, VE-B-1, PER, SFO-1, and GES-1 are among these unusual ESBLs (Bradford, 2001). These enzymes may have unique characteristics or substrate specificities compared to more widely studied ESBLs. While they may not be as prevalent as TEM, SHV, or CTX-M enzymes, they still play a role in antibiotic resistance and can pose challenges in clinical settings (Naas et al., 2008)..

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

Carbapenemases, which are also known as metallo-β-lactamases (MBLs), refer to enzymes that utilize zinc ions to hydrolyze β-lactam antibiotics. Among these enzymes, New Delhi metallo-β-lactamase (NDM-1) stands out as a broad-spectrum β-lactamase, belonging to class B carbapenems. NDM-1 is capable of inactivating almost all β-lactams, except aztreonam. NDM-1 is particularly notable because most NDM-1-producing bacteria also possess other β-lactamases,

such as CTX-M and CMY-type extended spectrum β-lactamases (ESBLs), which hydrolyze aztreonam. This renders these bacteria resistant to all β-lactam antibiotics (Yong et al., 2009).

The name "NDM-1" originated from New Delhi, India, where *Klebsiella pneumoniae* and *Escherichia coli* strains carrying this gene were discovered in a Swedish patient who had received medical treatment there (Yong et al., 2009). NDM-1 producers typically exhibit resistance to several antibiotics, including imipenem, meropenem, ertapenem, gentamicin, amikacin, tobramycin, and ciprofloxacin. However, they are often susceptible to colistin and tigecycline (Kumarasamy et al., 2010).

2.11.2 ESBLs in Bangladesh

A study conducted in Bangladesh revealed that around 3.5% of Gram-negative clinical isolates had the ability to produce NDM-1, a carbapenemase associated with broad-spectrum antibiotic resistance (Islam et al., 2012). Another hospital study revealed that ESBL genes from the $bla_{CTX-M-1}$ groups were present in all clinical isolates. Additionally, bla_{TEM} type and bla_{OXA-1} type genes were detected in 82.5% and 47.5% of the isolates respectively (Talukdar et al., 2013). In a separate study focusing on waste-water sources, it was discovered that 51% of the isolates tested positive for bla_{NDM-1} gene, with *Klebsiella pneumoniae* being the most common species harboring this gene, followed by *Escherichia coli, Acinetobacter* spp., and *Enterobacter* spp.. These bacteria also showed resistance to other antibiotics, including $bla_{CTX-M-1}$, $bla_{CTX-M-15}$, bla_{TEM}, bla_{SHV}, bla_{CMY-2}, bla_{OXA-48}, bla_{OXA-1}, and bla_{OXA-47} like genes (Ali et al., 2021). These findings underscore the widespread presence of antibiotic resistance genes among bacterial isolates in Bangladesh, posing significant challenges for infection treatment in the area.

CHAPTER 3: METHODOLOGY

3.1 Sampling Site and Sample Collection:

With the approval of the Institutional Review Board (IRB) of icddr,b sampling was conducted from the largest Goranchatbari sub-catchment of western Dhaka city (Figure 3.1).

Within the whole sub-catchment area, seven distinct severed waterbody sites including canals, local drains and retention ponds or wetland were selected for sample collection. The latitudes and longitude of sampling sites are mentioned in Table 3.1.

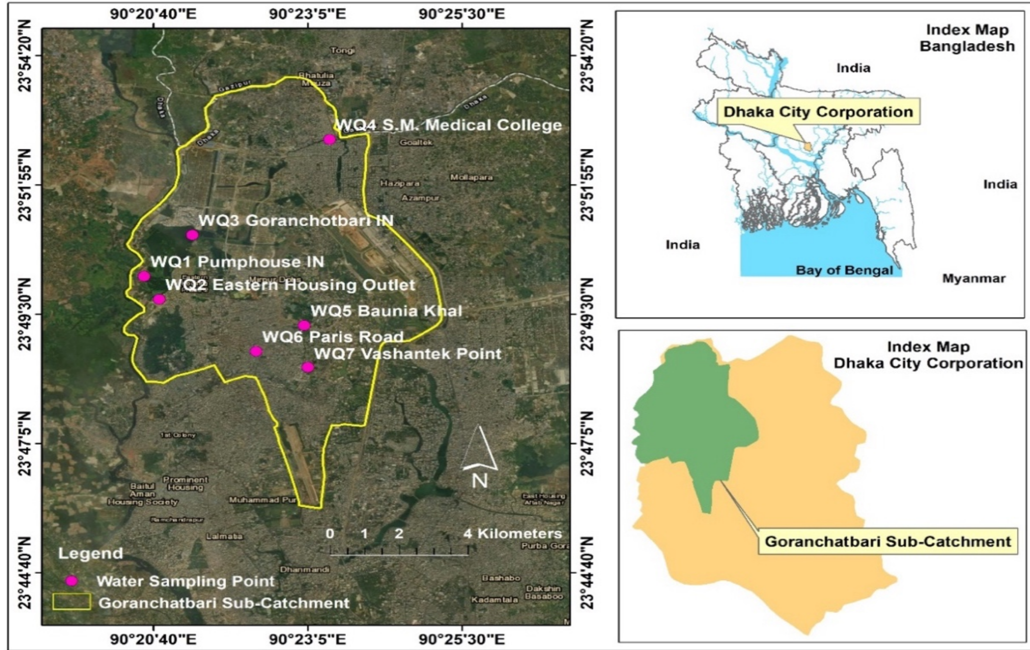


Figure 3.1: Locations of sampling points in Goranchatbari sub-catchment.

Table 3.1: Geographical details of sampling sites.

Location ID	Latitude	Longitude	Area
WQ1	23.837534	90.339649	Goranchatbari Pumphouse Inlet
WQ2	23.829787	90.345469	Eastern Housing Khal
WQ3	23.850702	90.358800	Goranchatbari Detention Pond Inlet
WQ4	23.879618	90.392134	Drain near Shaheed Monsur Ali Medical College Hospital
WQ5	23.822027	90.383494	Baunia Khal near Steel bridge
WQ6	23.813129	90.371583	Paris Road Khal, Mirpur
WQ7	23.808490	90.384767	Vashantek Khal, Mirpur

The samples were collected in four rounds during the months of April, September, December in 2022 and February in 2023. In climatic perspective of Bangladesh, the months April and September represented the wet season and December and February representing the dry season. A total of 28 samples were collected over these 4 rounds, each round consisting to 7 samples in total. Approximately 500ml sample was collected in sterile 500ml plastic bottle (NALGENE, NY, USA) with proper label. 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 constant temperature between 4-10°C. The samples were then processed within 8 hours of collection.

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). 100µl of the diluted samples was then spread on CHROMagar™ Acinetobacter (CAB) agar (CHROMagar, Paris, France) plates and incubated at 37 ± 0.5°C between 18-24 hrs.

3.3. Isolation and Identification of ESBL Positive *Acinetobacter* spp. on selective media:

Following incubation, colonies with a red color were considered as presumptive *Acinetobacter* spp. Colonies from a countable dilution were counted and noted as CFU/100ml. For the subsequent screening and isolation of ESBL producing *Acinetobacter* spp., the patch inoculation method was employed. Single random colonies were picked and maximum 10 colonies were patched on CHROMagar™ ESBL (CHROMagar, Paris, France) media, one colony in one slot at a time, and incubated at 37 ± 0.5°C between 18-24 hrs. Growth of cream, opaque colonies on CHROMagar™ ESBL media confirmed the production of extended-spectrum β-lactamases (ESBLs) by the *Acinetobacter* isolates. Following the presence of growth, single colonies were then streaked onto CHROMagar™ Acinetobacter agar plates and incubated at 37 ± 0.5°C between 18-24 hrs. to further obtain discreate colonies. Growth of unique red colonies on CHROMagar™ Acinetobacter agar plates were further confirmation of *Acinetobacter* spp.

3.4. Preparation of stock culture for further analysis:

After confirmation, single colonies from CHROMagar™ Acinetobacter agar plates were picked and cultured into 3ml of Luria-Bertani (LB) broth for enrichment at 37 ± 0.5°C between 18-24 hrs. A stock culture supplemented with 30% (v/v) glycerol (700µl enriched culture + 300µl glycerol) was prepared from enrichment culture of respective isolates and stored at -80° C and -20°C for further analysis. For bacterial revival, one loopful from the stock culture was taken, streaked on CHROMagar™ Acinetobacter agar and incubated at 37 ± 0.5°C between 16-18 hrs.

3.5. Bacterial Cell Lysate Preparation:

The DNA from the samples were isolated using the boiling lysis method (Hossain et al., 2021). For this purpose, one to two discrete colonies were taken from CHROMagar™ Acinetobacter agar plates of pure culture and inoculated into 3ml of LB broth, and incubated overnight at 37 ± 0.5°C. Following incubation, 1.5ml of fresh culture was taken and centrifuged at 13000rpm for 5 min. The supernatant was discarded, and the pellet was resuspended into 600µl of autoclaved distilled water and mixed well by pipetting. The solution was then subjected to boiling at 100°C for 10 minutes on Stuart® block heater (Cole-Parmer, Stone, UK), and then immediately cooled on ice for 10 minutes. Following the heat and cold treatment, the solution was centrifuged at 13000rpm for 7-8 minutes. Finally, 100µl of the supernatant was transferred to a new microcentrifuge tube and stored at -20°C for further use.

3.6. Molecular Analysis:

3.6.1. Identification of *Acinetobacter* spp. and *A. baumannii* by PCR:

For the identification of *A. baumannii* and non-*baumannii* spp., the putative ESBL positive isolates were subjected to molecular detection following previously published protocol (Chen et al., 2007). *A. baumannii* NCTC 12156 was kept as a reference for positive control in reactions where available. Using sequence specific primers, a multiplex PCR was performed for the genes 16S-23S rRNA ITS and the recA gene. The recA gene is highly specific for the genus *Acinetobacter* while the ITS region between the 16S and 23S rRNA is a good candidate for the identification of bacterial species (Chen et al., 2007). The reaction mixture was prepared by mixing components

given in Table 3.2. To provide for a contamination free environment, the PCR reaction mixture was prepared inside a laminar flow cabinet. Following preparation, the PCR tubes containing the reaction mixture and template DNA were capped and briefly centrifuged to spin down the contents. The PCR tubes were placed in BIORAD T100™ Thermal cycler (BIORAD, USA). PCR conditions for this reaction is given in Table 3.3. After completion of the PCR, the tubes were stored at -20°C until further analysis.

Table 3.2: PCR reaction mixture for *recA*, *16S-23S rRNA* gene

PCR reaction mixture for 1 sample of total 25µl volume		
Serial no.	**Reagent**	**Volume**
1	Thermo Scientific™ DreamTaq™ Green PCR Master Mix (2X)	12.5 µl
2	Primer Ab-ITS Forward (Concentration-0.2µm)	1 µl
3	Primer Ab-ITS Reverse (Concentration-0.2µm)	1 µl
4	Primer rA1 (Concentration-0.2µm)	1 µl
5	Primer rA2 (Concentration-0.2µm)	1 µl
10	Template DNA	2 µl
11	Nuclease free water	6.5 µl

Table 3.3: PCR conditions for *recA, 16S-23S rRNA* gene

Segment	Process	Temperature	Time
Segment 1	Initial denaturation	94°C	5 minutes
Segment 2 30 Cycles	Denaturation	95°C	30 seconds
	Annealing	54°C	30 seconds
	Extension	72°C	30 seconds
Segment 3	Final Extension	72°C	7 minutes

3.6.2. Detection of antibiotic resistance genes by PCR:

The total DNA content of the *A. baumannii* isolates was used to detect the presence of *bla* gene group, bla_{SHV}, bla_{TEM}, $bla_{CTX\text{-}M}$, bla_{OXA} according to previously published protocols (Fang et al., 2008). For available reactions, *E.coli* isolates acting as positive controls were used. A multiplex PCR was designed and performed with sequence specific primer for the *bla* gene group. The PCR reaction mixture was prepared in a contamination free environment inside the laminar flow cabinet. The PCR reaction mixture contained 25µl of reaction mixture containing 2µl of template DNA, 12.5µl of Thermo Scientific™ DreamTaq™ Green PCR Master Mix (2X), 0.5µl each of shv, tem, ctx-m, oxa primers and 6.5µl of nuclease free water (Fang et al., 2008). The PCR conditions for the following reactions were composed of initial denaturation at 95°C for 15 mins, subsequently 30 cycles of denaturation for 30 secs at 94°C, annealing for 90 secs at 62°C and extension for 1 min at 72°C followed by 10 mins elongated extension at 72°C. Reaction mixture composition is given is Table 3.4 and condition is listed in Table 3.5.

Table 3.4: PCR reaction mixture for bla_{SHV}, bla_{TEM}, $bla_{CTX\text{-}M}$, bla_{OXA} gene

PCR reaction mixture for 1 sample of total 25µl volume		
Serial no.	**Reagent**	**Volume**
1	Thermo Scientific™ DreamTaq™ 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 $bla_{CTX\text{-}M}$ Forward (Concentration-0.2µm)	0.5 µl
7	Primer $bla_{CTX\text{-}M}$ 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 DNA	2 µl
11	Nuclease free water	6.5 µl

Table 3.5: PCR conditions for bla_{SHV}, bla_{TEM}, $bla_{CTX\text{-}M}$, bla_{OXA}

Segment	Process	Temperature	Time
Segment 1	Initial denaturation	95°C	5 minutes
Segment 2 30 Cycles	Denaturation	94°C	30 seconds
	Annealing	62°C	90 seconds
	Extension	72°C	1 minute
Segment 3	Final Extension	72°C	7 minutes

3.6.3. Detection of Virulence genes:

All the *A. baumannii* isolates were tested for the presence of ten virulence genes. Those pathogenic markers were- *espA*, *ompA* (Toledo-Arana et al., 2001), csuE (Braun & Vidotto, 2004), *bfmS*, *bap* (H.-W. Lee et al., 2008), *fimH*, *kpsMII* (Bahador et al., 2013), $bla_{PER\text{-}1}$ (Tayabali et al., 2012), *ptk* and *pgaB* (Liou et al., 2014). For the identification of pathogenic gene presence, three separate multiplex PCR was carried out according to previously published protocols. The primers required for the multiplex reactions are listed in Table 3.12 and the PCR conditions for each multiplex reaction are given in Table 3.7, Table 3.9, and Table 3.11. The composition of the reaction Mixture for the multiplex reactions are given in Table 3.6, Table 3.8, and Table 3.10.

Table 3.6: PCR reaction mixture for virulent gene detection (1st multiplex)

PCR reaction mixture for 1 sample of total 25µl volume		
Serial no.	**Reagent**	**Volume**
1	Thermo Scientific™ DreamTaq™ Green PCR Master Mix (2X)	12.5 µl
2	Primer *espA* Forward (Concentration-0.2µm)	0.5 µl
3	Primer *espA* 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 DNA	3 µl
11	Nuclease free water	5.5 µl

Table 3.7: PCR conditions for virulent gene detection (1st multiplex)

Segment	Process	Temperature	Time
Segment 1	Initial denaturation	94°C	5 minutes
Segment 2 30 Cycles	Denaturation	94°C	1 minute
	Annealing	60°C	1 minute
	Extension	72°C	1 minute
Segment 3	Final Extension	72°C	10 minutes

Table 3.8: PCR reaction mixture for virulent gene detection (2nd multiplex)

PCR reaction mixture for 1 sample of total 25µl volume		
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 DNA	3 µl

11	Nuclease free water	5.5 µl

Table 3.9: PCR conditions for virulent gene detection (2nd multiplex)

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

Table 3.10: PCR reaction mixture for virulent gene detection (3rd multiplex)

PCR reaction mixture for 1 sample of total 25µl volume		
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 DNA	2 µl
7	Nuclease free water	7.5 µl

Table 3.11: PCR conditions for virulent gene detection (3rd multiplex)

Segment	Process	Temperature	Time
Segment 1	Initial denaturation	94°C	5 minutes
Segment 2 30 Cycles	Denaturation	94°C	1 minute
	Annealing	58°C	1 minute
	Extension	72°C	1 minute

Segment 3	Final Extension	72°C	10 minutes

Table 3.12: List of primers used in this study.

	Target Gene	Primer Name	Sequence (5' → 3')	Product Size (bp)	Reference
Species Specific	*16S-23S-rRNA ITS*	Ab-ITS-F	CATTATCACGGTAATTAGTG	208	(Tsai et al., 2018)
		Ab-ITS-B	AGAGCACTGTGCACTTAAG		
	recA	P-rA1	CCTGAATCTTCTGGTAAAAC	425	(Chen et al., 2007)
		P-rA2	GTTTCTGGGCTGCCAAACATTAC		
Antibiotic Resistant Gene 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		
	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	blaPER-1-F	ATGAATGTCATTATAAAAGC	927	

Virulence Gene Primers					
		bla_{PER-1}-R	AATTTGGGCTTAGGGCAAGAAA		(Tayabali et al., 2012)
	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 & Vidotto, 2004)
		csuE-R	CGACTTGTACCGTGACCGTATCTTGATAAG		

3.6.4. Post PCR detection of amplified DNA through agarose gel electrophoresis:

The presence and subsequent amplification of the targeted gene is determined 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°C, 0.1µl/ml of Invitrogen SYBR™ Safe DNA Gel Stain was added and thoroughly mixed and 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 was 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 5-6cm from the wells (after around 1.5 – 2.0 hrs.). The gel was then observed, and a photograph was taken on GelDoc Go Imaging System (BIORAD, USA).

3.7. Antibiotic Susceptibility Assay:

3.7.1. Determination of Antimicrobial Resistance Profiles:

The patterns of antibiotic susceptibility for the virulent gene harboring *A. baumannii* isolates were obtained by following the standard Kirby-Bauer disk diffusion method as per the suggestion of the Clinical and Laboratory Standards Institute (CLSI) guidelines (PA, 2019). 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 antibiotic disks used were Imipenem (IMP, 10µg), Meropenem (MEM, 10µg), Cefepime (FEP, 30µg), Cefotaxime (CTX, 30µg), Ceftriaxone (CRO, 30µg), Ciprofloxacin (CIP, 5µg), Gentamicin (CN, 10µg), Amikacin (AK, 30µg), Tetracycline (TE, 30µg), Cotrimoxazole (SXT, 25µg), and Piperacillin/Tazobactam (TPZ, 110µg).

3.7.2. Procedure:

For inoculation, isolated *A. baumannii* colonies 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 in the suspension and swirled several times. The swab stick was then removed from the solution while pressing 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 (Difco, MD, USA) following a repetitive streaking pattern to ensure even distribution. Within 3-5minutes, the antibiotic disks were firmly placed on the streaked agar plates and were then incubated at 37°C for 18 (±2hrs). The

plates were then investigated for the presence of clear zones due to growth inhibition and the diameter of the zone of inhibition was recorded in millimeter along with a 6mm disc diameter.

3.7.3. Interpretation:

According to the diameter of the zone of inhibition, the isolates were categorized as susceptible or resistant as per CLSI guidelines (Table 3.13).

Table 3.13: Zone of diameter interpretation for *A. baumannii*

Groups	Antibiotics	Susceptible	Intermediate	Resistant
Carbapenem	Imipenem	≥ 22	19-21	≤ 18
Carbapenem	Meropenem	≥ 18	15-17	≤ 14
3G Cephalosporin	Cefepime	≥ 18	15-17	≤ 14
3G Cephalosporin	Cefotaxime	≥ 23	15-22	≤ 14
3G Cephalosporin	Ceftriaxone	≥ 21	14-20	≤ 13
2G Cephalosporin	Ciprofloxacin	≥ 21	16-20	≤ 15
Aminoglycoside	Gentamicin	≥ 15	13-14	≤ 12
Aminoglycoside	Amikacin	≥ 15	13-14	≤ 12
Tetracycline	Tetracycline	≥ 15	12-14	≤ 11
Sulfonamide	Cotrimoxazole	≥ 16	11-15	≤ 10
Penicillin	Piperacillin/Tazobactam	≥ 21	18-20	≤ 17

3.8. Quantitative Adherence Assay:

3.8.1. Biofilm Formation Assay:

The biofilm formation capability of the isolates was detected using the quantitative adherence assay (Ali et al., 2021; Nirwati et al., 2019). Fresh inoculum culture was prepared for individual

isolates, inoculating single colonies in Lurie Bertani broth, and incubating overnight at 37°C. Following overnight incubation, a sterile 96 well flat bottom polystyrene microtiter plate (Costar, USA) containing 198µl of fresh LB was prepared to which 2µl of the suspension was inoculated. For each run, uninoculated LB was used as negative controls, and the plates were incubated at 25°C and 37°C for 48 hours. Following incubation, the plates were gently washed with PBS and dried in an inverted position. The biofilm mass was then stained with 200µl of 0.1% crystal violet solution for 15 minutes. To remove the unbound crystal violet stain, the wells were further washed with distilled water and dried in an inverted position. Lastly, the stained biofilm mass was 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 590nm wavelength.

3.8.2. Interpretation:

Following published protocols, the biofilm forming capacity of the isolates were categorized as strong, moderate, weak, or non-biofilm former (Nirwati et al., 2019). The optical density cut-off value (ODc) was established from the negative values. The ODc value is three standard deviations (SD) above the mean OD of the negative control, that is ODc = average OD of negative control-3xStandard deviation (SD) of negative controls. The isolates with OD≤ODc are termed as non-biofilm producer. On the contrary, the isolates with ODc<OD≤2xODc are categorized as weak biofilm producer, whereas 2xODc<OD≤4xODc and OD>4xODc are categorized as moderate and strong biofilm producer respectively.

3.9. DNA Fingerprinting by ERIC-PCR:

For all the *A. baumannii* isolates carrying 4 or more pathogenic marker, the genetic relatedness was investigated using the Enterobacterial Repetitive Intergenic Consensus (ERIC) sequences and the PCR performed using the ERIC-2 primer (5'-AAGTAAGTGACTGGGGTGAGCG-3'). The PCR was performed following previous published protocol (Meacham et al., 2003). The amplified PCR products were separated using 2% agarose gel, with the gel being run at 90 volts for approximately 3 hours. The first and last lanes of the gel were filled with 1kb plus ladder. After

completion, the gel was removed carefully and placed in a staining (0.5 mg/ml EtBr) tray and stained for 15 minutes. Then the gel was de-stained for about 10 minutes in deionized water. The gel image analysis was performed using the GelJ v.2.0 software (Mahmud et al., 2022) and gaussian regression method was used to normalize the image. The clusters of ERIC-PCR patterns were generated using the dice coefficient and the unweighted pair group method using arithmetic averages (UPGMA) with 1.0% tolerance value. The UPGMA algorithm computes genetic distances among isolates and then constructs a hierarchical tree using these distances.

3.10: Statistical Analysis

The statistical analyses were conducted using Microsoft Excel (2019) and R (Version 4.3.3). In order to conduct statistical analysis, the summaries of antibiotic resistance genes and the presence or absence of both resistance and virulence genes were transformed into binary code, where 1 represented the presence of a specific gene and 0 indicated its absence. The 'cor' function was utilized to compute correlations among the variables. Significant correlations between the variables were visualized using the 'corrplot' function (Murtagh & Legendre, 2014; Okanda et al., 2021).

CHAPTER 4: RESULTS

The present study was conducted to isolate *Acinetobacter baumannii* carrying major antibiotic resistant genes from the environmental samples. The samples were collected in 4 rounds over the two seasonal variations across the Goranchatbari sub-catchment area, and their molecular characterization was conducted through various methods.

4.1 Sample Isolation and Identification:

Upon spreading 100µl of sample on CHROMagar™ Acinetobacter Base (CAB), the results were recorded for unique, red-colored colonies indicating *Acinetobacter* spp. (Figure 4.1). Out of the 28 samples, 27 samples were presumed to show the occurrence of *Acinetobacter* spp. The abundance of *Acinetobacter* spp. from different sampling points, over the 2 seasonal variation are presented in Figure 4.2.

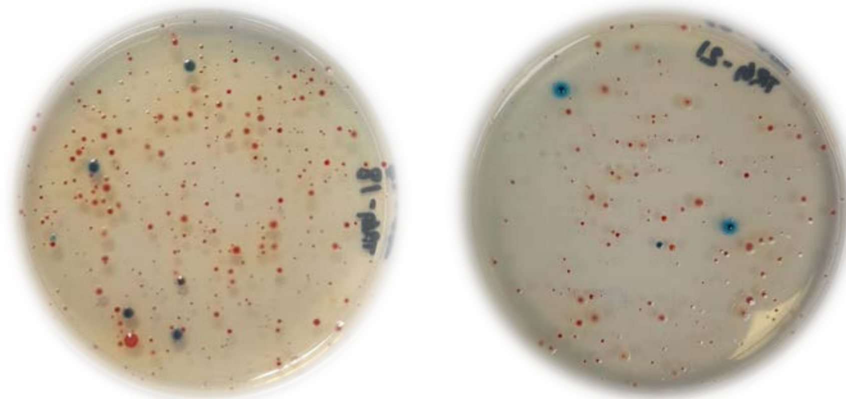


Figure 4.1: Red colonies on CAB indicative of *Acinetobacter* spp.

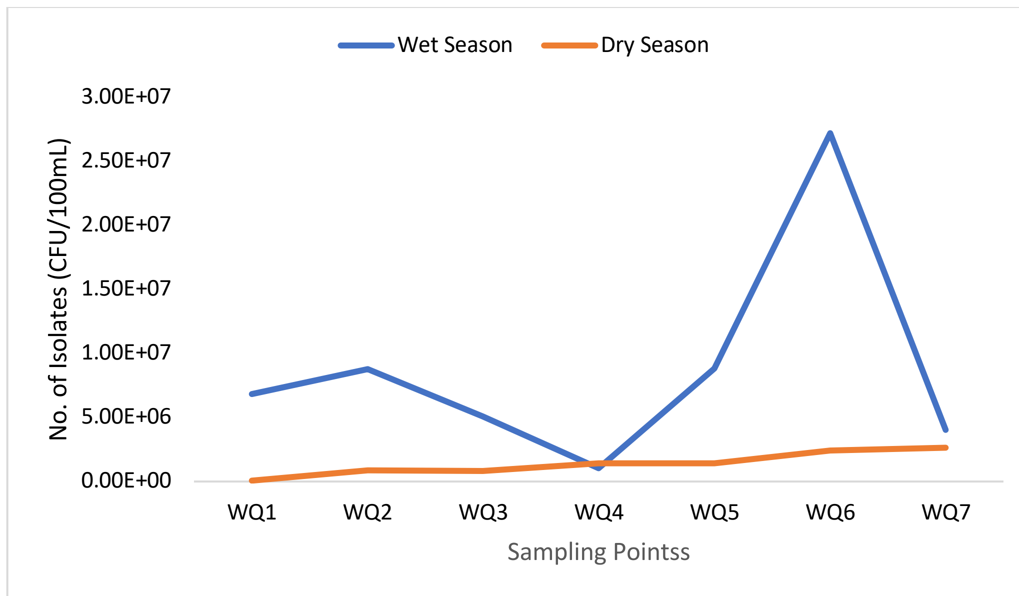
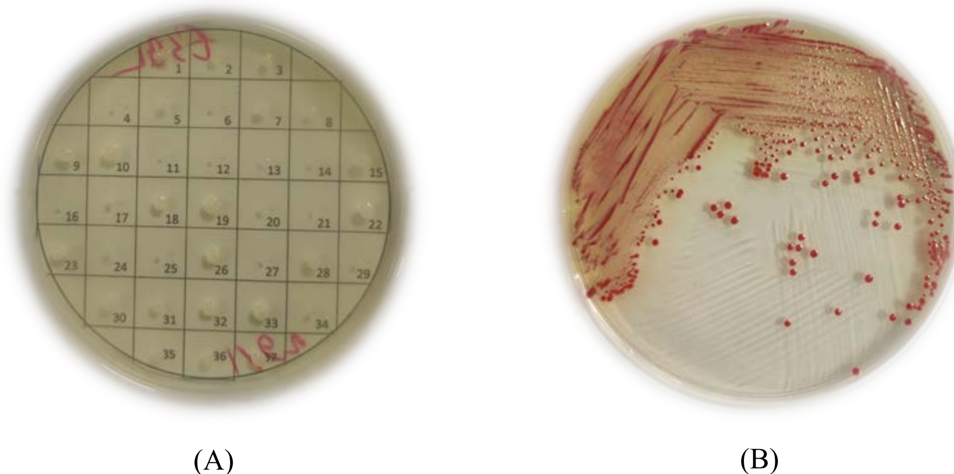


Figure 4.2: Distribution of presumptive *Acinetobacter* spp. across the seasonal variation.

4.2 Identification of ESBL producing *Acinetobacter* spp.*:*

The suspected colonies of *Acinetobacter* spp. from CAB media, were inoculated into CHROMagar ESBL and CHROMagar CAB through patch inoculation method to phenotypically identify ESBL producing *Acinetobacter*. Only colonies that gave cream, opaque color on CHROMagar ESBL were representative of ESBL producing *Acinetobacter* isolates, which were further assigned with numerical IDs and the data was stored in a log sheet. Out of 249 isolates, a total of 106 (42.6%) *Acinetobacter* colonies were found to be ESBL producers. Representative plates of presumptive *Acinetobacter* producing ESBL are given in Figure 4.3(A). All 106 ESBL producing isolates were streaked onto CHROMagar CAB plates to obtain single discrete colonies as shown in Figure 4.3(B). A single colony was then used for enrichment followed by preparation of stock culture.

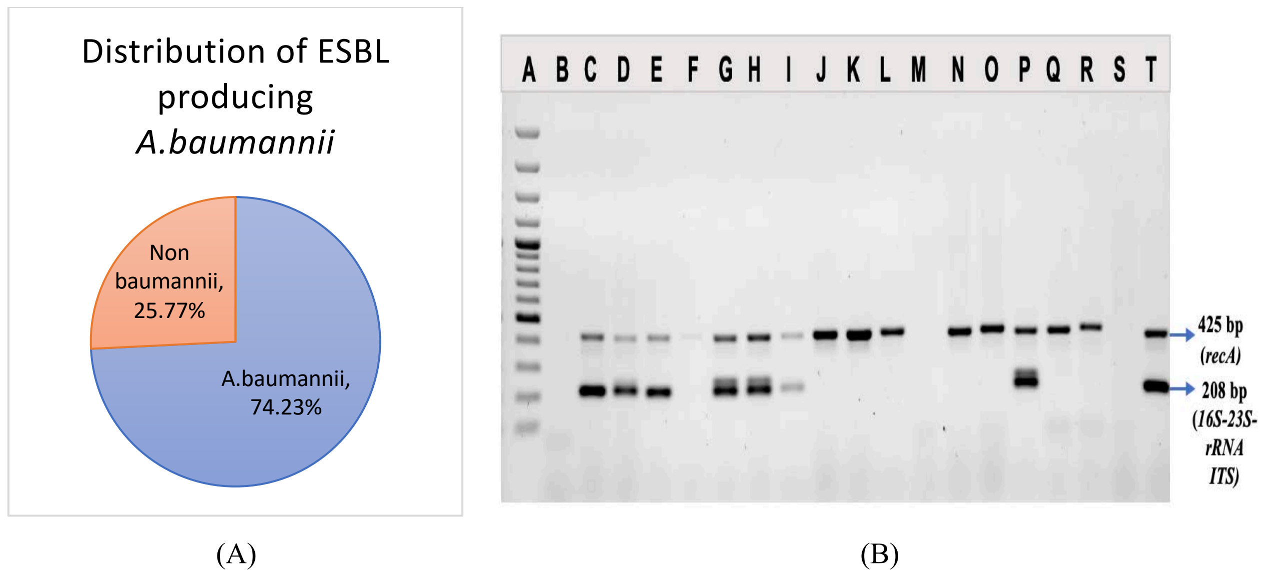


(A) (B)

Figure: 4.3: (A) ESBL producing *Acinetobacter* spp. on CHROMagar^{TM} ESBL plate identified in slots 9, 10, 18, 19, 26, 32 and 33 due to their growth and distinctive color. (B) *Acinetobacter* spp. on CHROMagar^{TM} Acinetobacter Base plate

4.3.1 Molecular detection of *Acinetobacter* spp. and *A. baumannii*:

Upon screening the ESBL producing isolates, they were subjected to PCR (Polymerase Chain Reaction) for the presence of *recA* gene (T.-L. Chen et al., 2007) and *16S-23S-rRNA ITS* gene (Tsai et al., 2018). Among the isolates, 97 of them harbored the *recA* gene affirming them to be *Acinetobacter* spp. Out of the 97 *Acinetobacter* isolates, 72 (74.2%) of them tested positive for *A. baumannii* based on the presence of *16S-23S-rRNA ITS* gene and the rest 25 (25.8%) were identified as non-*baumannii*. The distribution of *A. baumannii* and non-*baumanni*i is shown in Figure 4.4(A) and the gel image for the PCR reaction is given in Figure 4.4(B).



(A) (B)

Figure 4.4: (A) Schematic distibution of *Acinetobacter baumannii* and non-*baumannii* isolates. (B) Agarose gel image for the molecular detection of *Acinetobacter* spp. and *A. baumannii*. Lane-A contains 100bp DNA ladder, whereas Lane-B is the negative control and Lane-C represent positive controls. Lane-E to T represent samples being tested.

4.3.2 Climatic influence on the prevalence of *A. baumannii*:

The influence of season on the occurrence of *A. baumannii* in the environment was studied upon in this study. It was observed that the prevalence of *A. baumannii* in the wet season, was 38/51 (74.51%) and in the dry season, it was 34/46 (73.91%). The prevalence ratio of *A. baumannii* to non-baumannii under different climatic circumstances are shown in Figure 4.5.

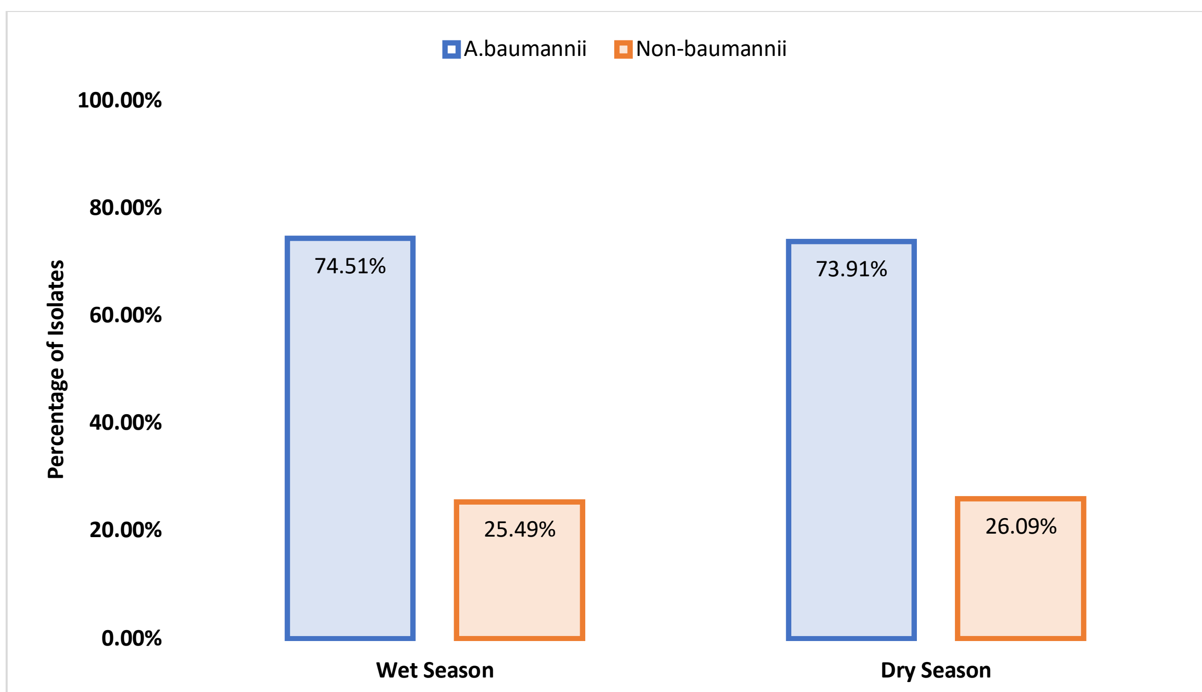


Figure 4.5: Prevalence of *A. baumannii* influenced by seasons.

4.4 Detection of antibiotic resistant genes by PCR:

All the *A. baumannii* isolates (n= 72) were screened for the *bla* gene group responsible for antibiotic resistance. Among the isolates, 40 (55.6%) tested positive for bla_{TEM} , 3 (4.2%) tested positive for bla_{SHV} and none of the isolates tested positive for $bla_{CTX\text{-}M}$ or bla_{OXA}. Additionally, among the four genes tested, bla_{TEM} and bla_{SHV} co-existed in 2 (2.8%) of the isolates. The counts for the individual genes are given in Figure 4.7.

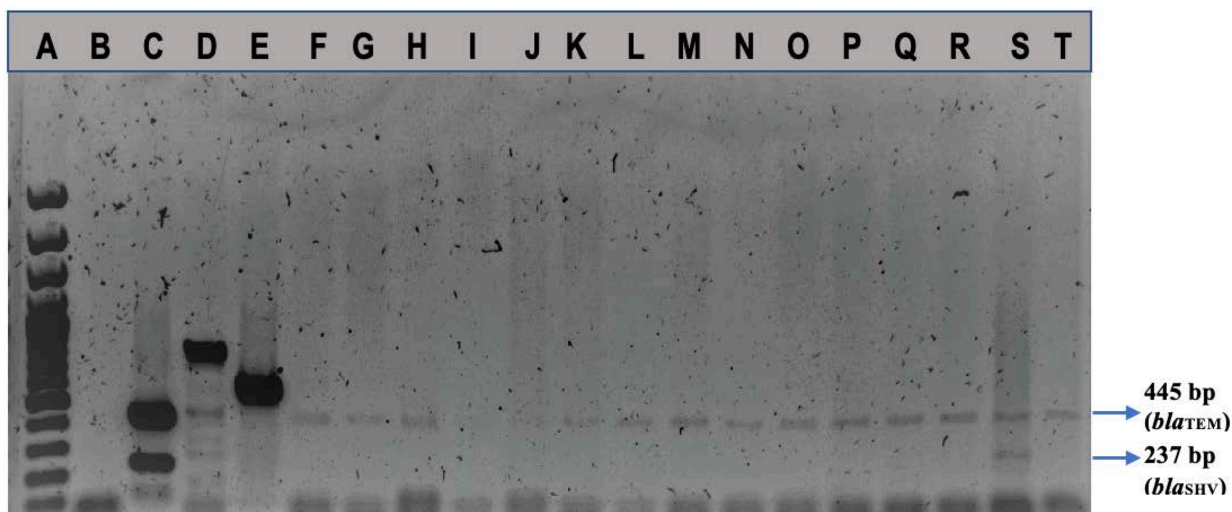


Figure 4.6: Bands obtained from agarose gel electrophoresis of PCR amplicon from ESBL Multiplex Reaction. Here, the presence of *bla_{TEM}* and *bla_{SHV}* is confirmed as the amplicon size for the genes are 445 bp and 237 bp respectively. Lane-A contains 100 bp DNA ladder, whereas Lane-B is the negative control and Lane-C, Lane-D and Lane-E represent positive controls. Lane-F to T represent samples of *Acinetobacter* spp. being tested.

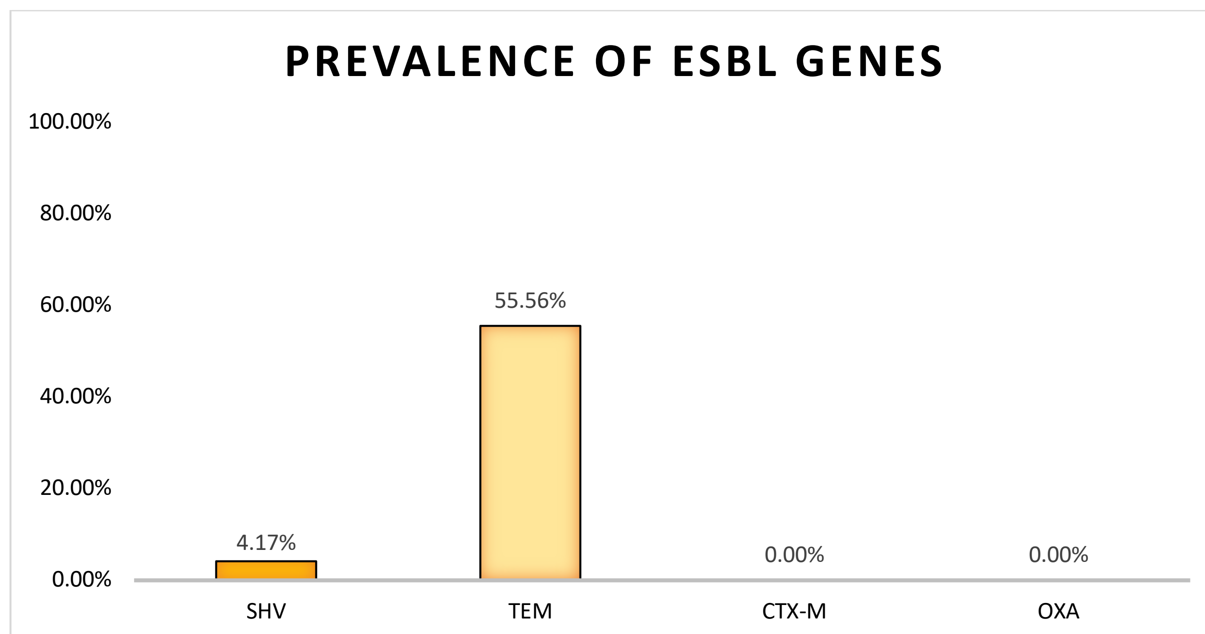
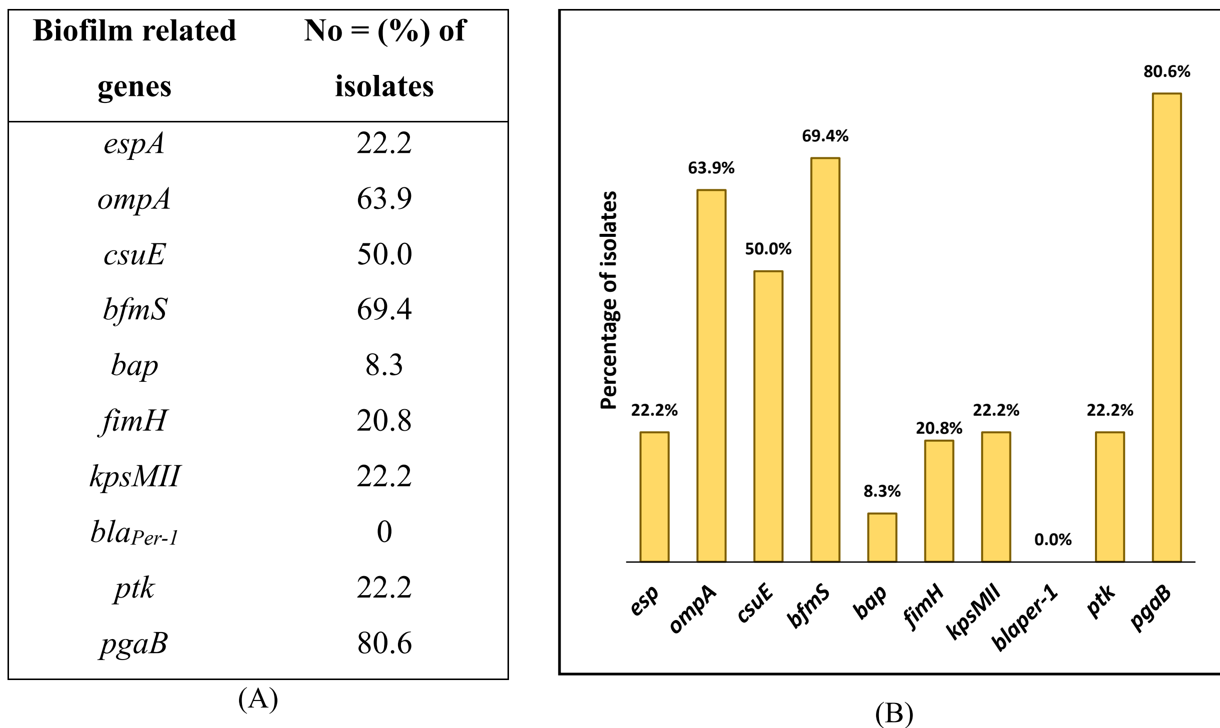


Figure 4.7: Prevalence of *bla* genes in ESBL producing *A. baumannii.*

4.5 Confirmation of Pathogenic Isolates:

For the detection of virulence genes, nine out of ten genes were detected using the multiplex reactions. Among the isolates, 16 (22.2%) harbor the *espA* and *ptk* genes, 46 (63.9%) harbors the

ompA gene, 36 (50%) harbors the csuE gene, 50 (69.4%) harbors the *bfmS* gene, 6 (8.3%) harbors the *bap* gene, 15 (20.8%) harbors the *fimH* gene, 16 (22.2%) harbors the *kpsMII* gene, 58 (80.6%) harbors the *pgaB* gene and none of the isolate contained the *bla$_{Per-1}$* gene. Additionally, among the genes tested, only 8 (11.1%) isolates carried only one gene, 9 (12.5%) isolates carried a combination of two genes, 17 (23.6%) isolates carried three genes, 16 (22.2%) isolates carried four genes, 15 (20.8%) isolates carried five genes, 6 (8.3%) isolates carried six genes and 1 (1.4%) isolate carried seven genes. Information regarding the gene presence is given in Figure 4.8 (A) and Figure 4.8 (B).



Biofilm related genes	No = (%) of isolates
espA	22.2
ompA	63.9
csuE	50.0
bfmS	69.4
bap	8.3
fimH	20.8
kpsMII	22.2
bla$_{Per-1}$	0
ptk	22.2
pgaB	80.6

(A)

(B)

Figure 4.8: (A) Frequency of biofilm related genes among isolates; (B) Prevalence of virulence genes. Predominantly, *pgaB* gene was present among the isolates, followed by *bfmS* and *ompA* genes, respectively. None of the isolates carried *bla$_{Per-1}$* gene.

Table 4.1: Patterns of biofilm related genes among isolates

No of Biofilm Related Genes	Patterns of Biofilm Related Genes	Percent (%) of *A. baumannii*
1 gene	*ompA*	2.8
(n=8)	*pgaB*	2.8

	bfmS	5.6
2 Genes (n=9)	*fimH-pgaB*	1.4
	bfms-pgaB	6.9
	bfmS-csuE	2.8
	bfmS-ompA	1.4
3 Genes (n=17)	*bfms-csuE-pgaB*	9.7
	fimH-bap-ompA	2.8
	espA-ptk-pgaB	1.4
	fimH-pgaB-ompA	4.2
	bfms-pgaB-ompA	4.2
	espA-fimH-pgaB	1.4
4 Genes (n=16)	*bfms-csuE-pgaB-ompA*	5.6
	csuE-pgaB-ompA-kpsMII	2.8
	bfms-csuE-ptk-pgaB	1.4
	csuE-ptk-ompA-kpsMII	1.4
	bfms-pgaB-ompA-kpsMII	1.4
	espA-fimH-pgaB-ompA	2.8
	bfmS-fimH-pgaB-ompA	2.8
	fimH-bap-pgaB-ompA	1.4
	fimH-ptk-pgaB-ompA	1.4
	espA-bfms-pgaB-ompA	1.4
5 Genes (n=15)	*bfms-csuE-pgaB-ompA-kpsMII*	5.6
	bfms-csuE-ptk-pgaB-ompA	4.2
	espA-bfms-csuE-ptk-pgaB	2.8
	espA-csuE-pgaB-ompA-kpsMII	1.4
	espA-csuE-bap-ompA-kpsMII	1.4
	bfms-ptk-pgaB-ompA-kpsMII	1.4
	bfmS-fimH-bap-pgaB-ompA	2.8
	espA-bfms-csuE-ptk-ompA	1.4
6 Genes (n=6)	*espA-bfms-csuE-pgaB-ompA-kpsMII*	2.8
	espA-bfms-csuE-ptk-pgaB-ompA	2.8

	bfms-csuE-ptk-pgaB-ompA-kpsMII	1.4
	espA-csuE-ptk-pgaB-ompA-kpsMII	1.4
7 Genes (n=1)	*epsA-bfms-csuE-ptk-pgaB-ompA-kpsMII*	1.4

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

The antimicrobial susceptibility testing was to determine the drug resistance characteristics of the ESBL forming *A. baumannii*. Antibiotic susceptibility pattern was conducted for 72 ESBL producing *A. baumannii*. Among the isolates, all of them were sensitive to Imipenem, Meropenem, Gentamicin, Amikacin and Ciprofloxacin. Around 97% (70/72) were susceptible to Cefepime, 94% (68/72) were susceptible to Cotrimoxazole and Tetracycline and 44% (32/72) were susceptible to Piperacillin-Tazobactam and only 10% (7/72) were susceptible to Ceftriaxone. Interestingly, none of the isolates were sensitive to Cefotaxime. According to the CLSI guidelines, the isolates were intermediately resistant, with 7% (5/72) showing resistance against Cefotaxime. The information regarding susceptibility pattern is given in Figure 4.9.

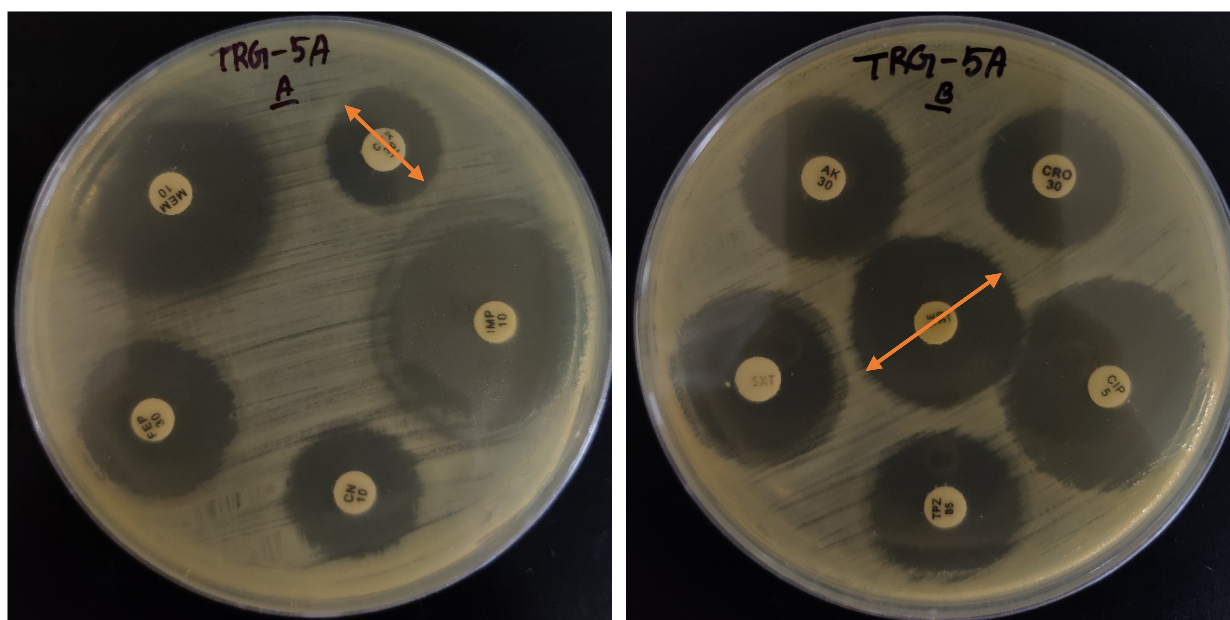


Figure 4.9: Two representative MHA plate of antibiotic susceptibility testing for one of the isolates. Arrow indicates measuring of zone of inhibition.

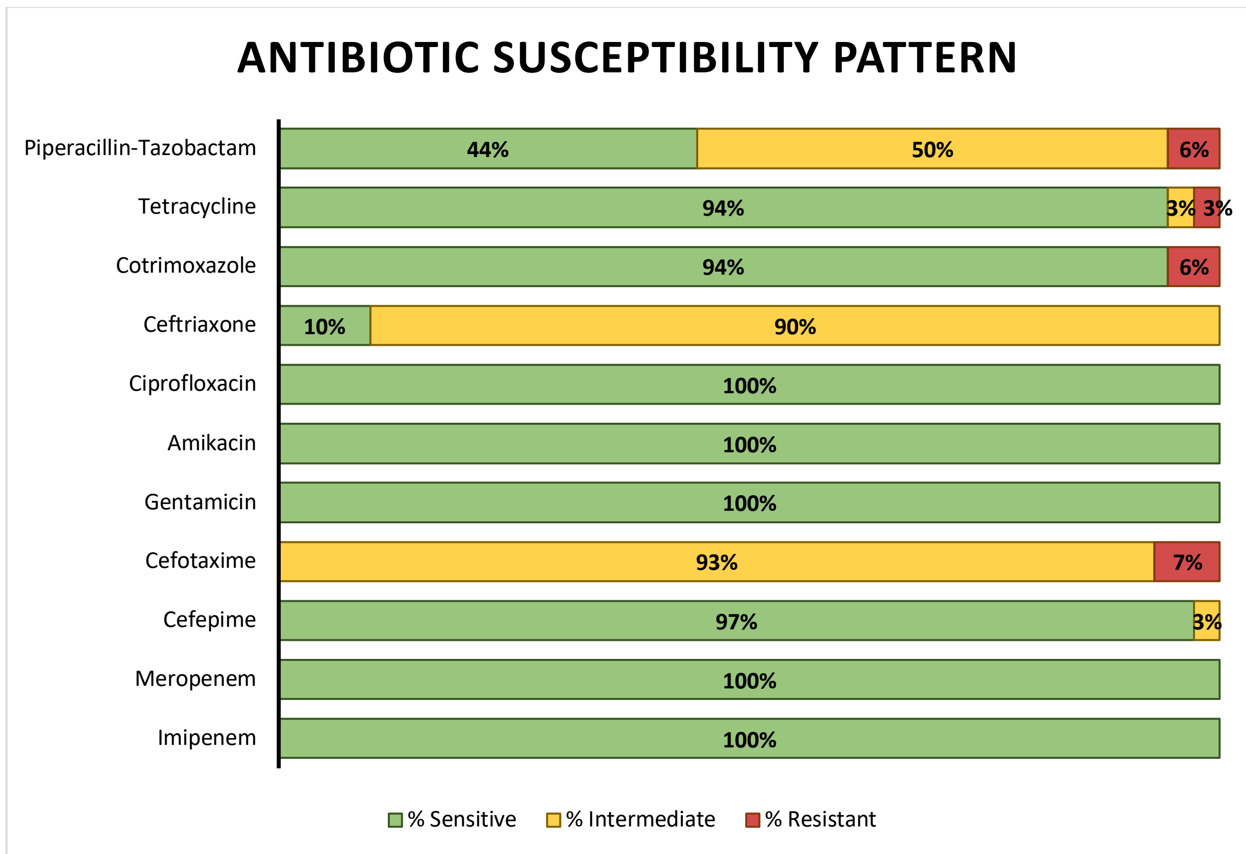


Figure 4.10: Antibiotic resistant patterns of ESBL producing *A. baumannii*.

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

The biofilm formation assay was performed for all ESBL producing *A. baumannii*. Among the isolates tested, temperature variation was observed with incubation temperatures of 25°C and 37°C. Regarding 25°C, a total of 32 (44.4%) isolates were strong biofilm producer, 19 (26.4%) isolates were moderate biofilm producer, 17 (23.6%) isolates were weak biofilm producer and 4 (5.6%) were no biofilm producers. Regarding 37°C, a total of 35 (48.6%) isolates were strong biofilm producer, 18 (25%) were moderate and weak biofilm producers and only 1 (1.4%) isolate did not produce any biofilm. The biofilm formation capability according to temperature are illustrated in Figure 4.11.

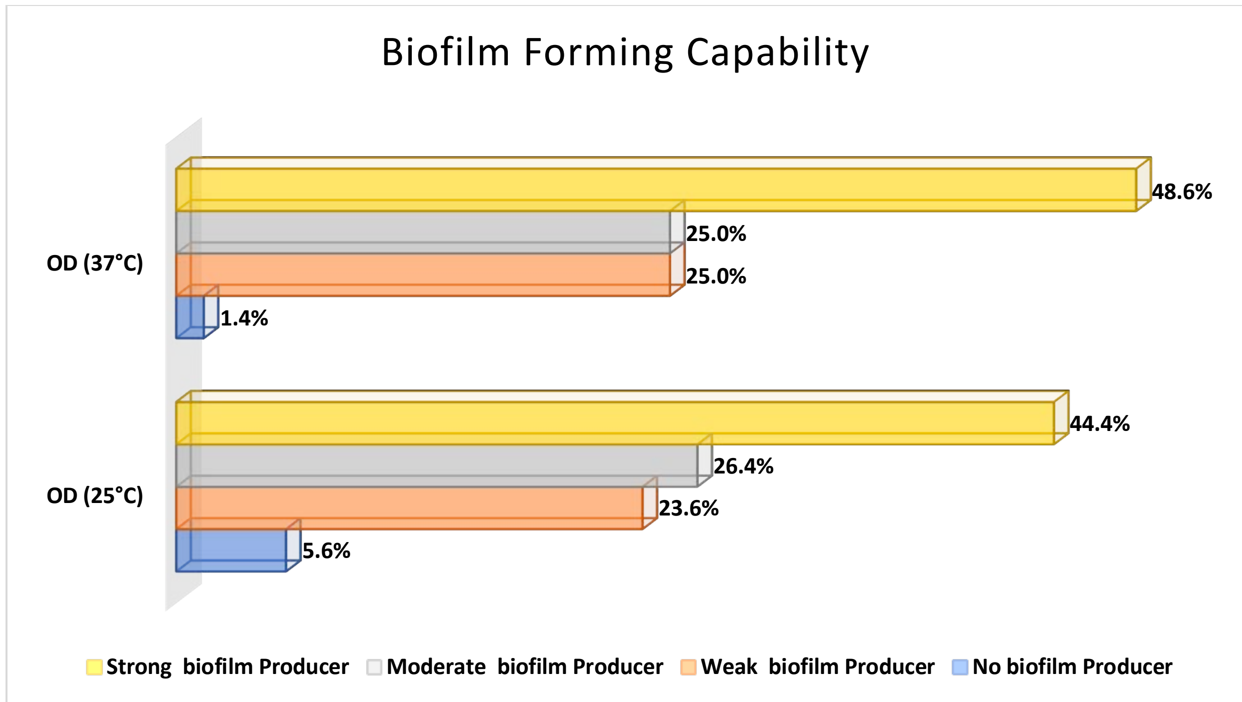


Figure 4.11: Biofilm formation capability of *A. baumannii* isolates at 37°C and 25°C.

4.8 Correlation Matrix

The associations between the phenotypic and genotypic trains were explored through the use of a correlation matrix. The results of the analysis revealed positive associations between the presence of pathogenic genes and phenotypic resistance to antibiotics (Figure: 4.12). For example, the presence of the *bla*$_{TEM}$ was positively correlated with existence *csuE* gene. Furthermore, the presence of *bla*$_{TEM}$ was positive correlated with presence of other pathogenic genes such as *ptk, pgaB* and *kpsMII*. Similarly, the formation of biofilm was positively associated with the presence of *bfmS*, *csuE*. Likewise, if the isolates formed biofilm at 25°C, they were also likely to form biofilm at 37°C.

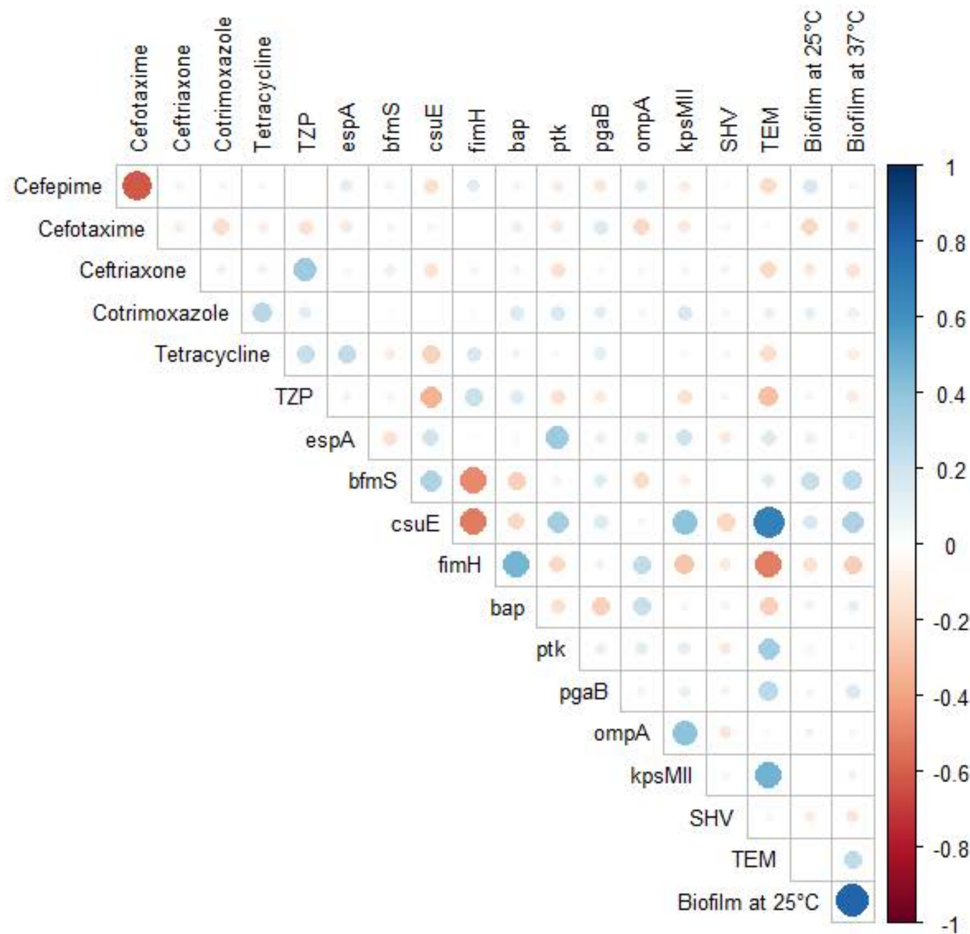


Figure 4.12: Correlation matrix of phenotypic (antibiotic resistance profiles and biofilm formation) and genotypic (resistance and virulence genes) traits of *A. baumannii* portrays correlation among the variables. Blue circles indicate a positive correlation, and red circles are indicative of negative correlation. The size and strength of color represents the numerical value of the correlation coefficient.

4.9 ERIC PCR Analysis:

The genetic relatedness of the ESBL producing *A. baumannii* carrying four or more pathogenic genes was performed using ERIC-PCR. Using the ERIC-PCR band, patterns were calculated using the visibility and placements of gels according to molecular weights and molecular markers. The genetic profiles of 34 ESBL producing virulent isolates were confirmed. Following dendrogram analysis (Figure: 4.13), the isolates were grouped into 10 clusters with a 60% similarity index. The isolates produced amplicons ranging from 4-19 per isolate, and among them 400 and 500 bp were most common. The largest cluster-J contained a total of 13 isolates. Notably, several isolates obtained from multiple rounds of sampling were grouped under the same cluster. For example,

AB-6B and AB-23D were grouped under the same cluster although being isolated during different seasons of the year, suggesting that they likely extend from the same clonal lineage and contamination within the sampling points. Similarly, the isolates AB-13F and AB-5C were also grouped under the same cluster. Furthermore, several *A. baumannii* isolates obtained during different sampling seasons were also grouped under same cluster.

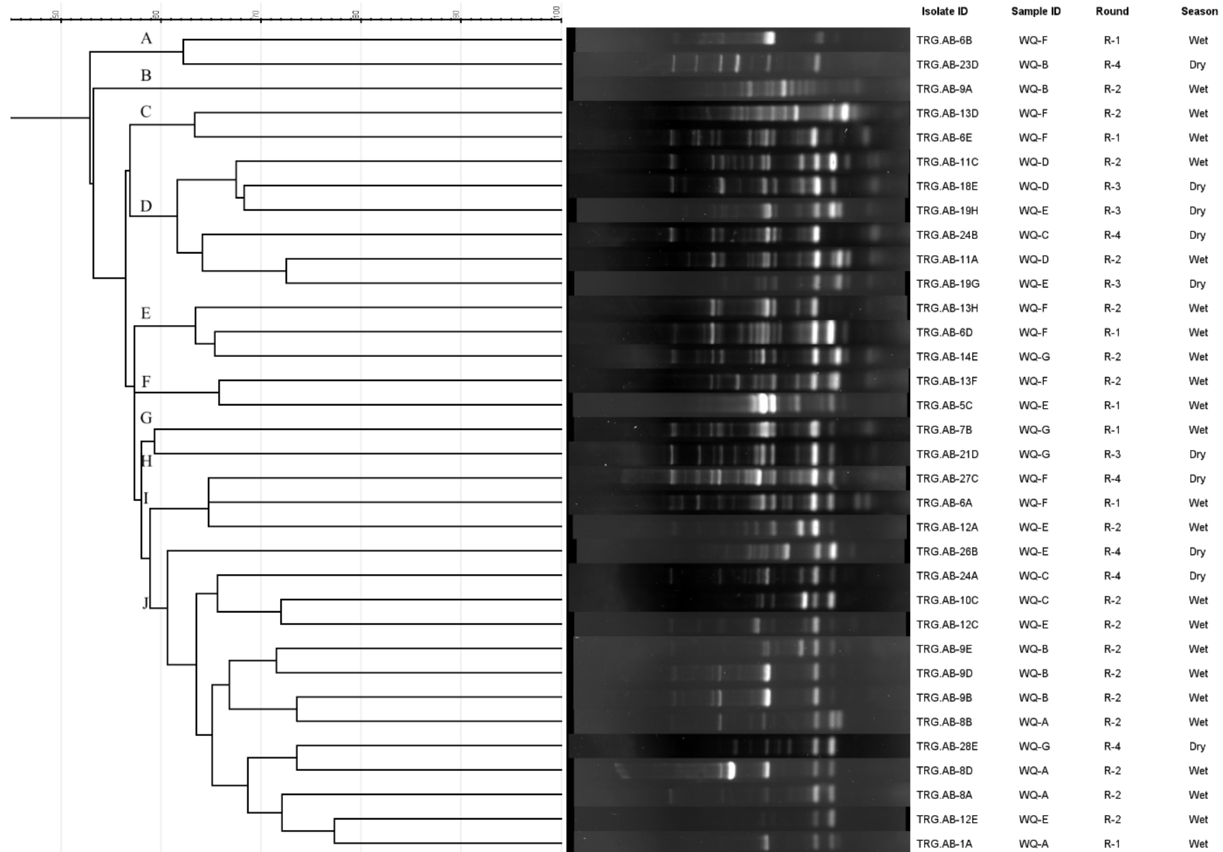


Figure 4.13: DNA fingerprinting pattern obtained using ERIC PCR.

CHAPTER 5:
DISCUSSION

Acinetobacter spp. are widespread and can be detected across various environments (Adewoyin & Okoh, 2018; Doughari et al., 2011), however, unlike other species of the genus, *A. baumannii* is primarily linked to healthcare acquired infections and hospital outbreaks (Fournier et al., 2006; Peleg et al., 2008). In the recent years, there has been an alarming emergence of multidrug resistant strains possessing serious threats to public health (L. C. S. Antunes et al., 2014; Gedefie et al., 2021). The biofilm forming capacity of *A. baumannii* on living and non-living surfaces facilitates persistent infections, antibiotic resistance, and survival in hospital environments (C.-H. Yang et al., 2019). Consequently, available medications are ineffective in treating *A. baumannii* infections associated with biofilm, exhibiting resistance to multiple drugs (Gedefie et al., 2021; Roy et al., 2018). The prevalence of antibiotic resistance and virulence traits among *Acinetobacter* spp., particularly *A. baumannii*, in hospital settings in Bangladesh have been extensively studied, but their presence in the natural environment remains inadequately explored. This study therefore aimed to examine the phenotypic and molecular characteristics of antibiotic resistance and virulence factors in ESBL-producing *A. baumannii* isolated from the largest sub-catchment area of Dhaka City.

Out of the 28 environmental samples processed over the four rounds for this study, 27 samples showed the occurrence of *Acinetobacter* spp. indicating a significant burden of the organism in the environment. Besides, the prevalence of the organism was higher in the wet season compared to the dry season as observed phenotypically according to the growth on the selective media. The findings of our study are in accordance with a previous study (Sabour, 2023), which raises concerns regarding increased measures to contain this isolate during the wet seasons. In addition, 106 *Acinetobacter* colonies were ESBL producers, of which, 46.2% (49/106) were isolated during the wet season and 53.8% (57/106) were isolated from the dry season, contrary to the general population.

Acinetobacter spp. was highly prevalent (91.5%) within the ESBL isolates and a majority of the isolates were *A. baumannii* (74.2%), as per molecular confirmation. In a study by Chen et al. (2007), the confirmation of *Acinetobacter* spp. was conducted utilizing the primers P-rA1 and P-rA2, targeting a highly conservative region of the *recA* gene (Chen et al., 2007). Previously conducted research by Safari et al. (2015) detected *A. baumannii* by targeting the bla_{OXA51}-like carbapenamase gene, intrinsic to this species (43,44). However, Lee et al. (2009) reported the

presence of this gene in a clinical isolate of *Acinetobacter* genomic species 13TU, deviating from the initial understanding that it was exclusive to *A. baumannii* (Y.-T. Lee et al., 2009). Of the samples analyzed, 91.5% (97/106) were identified to belong to the *Acinetobacter* genus based on the presence of the *recA* gene. The molecular detection also gave insight that during the wet season, the prevalence of *A. baumannii* was 74.5%, compared to 73.9% during the dry season. Contrary to previous studies (Murray et al., 2022), stating the pathogen is more commonly prevalent in the wet season, this study implied that throughout the wet and dry season, the rate of appearance of the pathogen is almost similar.

A. baumannii has several mechanisms rendering antibiotics ineffective, comprising the synthesis of extended spectrum β lactamases (ESBLs), carbapenemases and metallo β-lactamases (Jamal et al., 2018; Pfeifer et al., 2010). Within the ESBL category, the primary groups responsible for causing significant infections include bla_{SHV} and bla_{TEM}, however, recent investigations indicated a rise in the prevalence of hospital acquired infections carrying the $bla_{CTX\text{-}M}$ gene (Ibrahim et al., 2021; Kittinger et al., 2018; Tawfik et al., 2011). In this study, bla_{TEM} was identified as the predominant gene present in 55.7% (40/72) isolates, followed by bla_{SHV} identified in 4.2% (3/72) isolates. None of the isolates were found to harbor $bla_{CTX\text{-}M}$ or bla_{OXA} genes. Although limited research has been conducted on environmental ESBL-producing *A. baumannii*, this study result aligns with previous findings on clinical samples, which have shown bla_{TEM} to be the most prevalent β-lactamase gene in *A. baumannii*, followed by bla_{SHV} (Turton et al., 2006).

It is well-established that virulence genes such as *bap*, *espA*, *bfmS*, *ompA*, and *csuE* are involved in the biofilm formation of multidrug-resistant clinical *A. baumannii* isolates (Ghasemi et al., 2018; Sung, 2018; Thummeepak et al., 2016). PCR analysis revealed that more than 85% of the isolates carried at least two virulence genes. The *pgaB* gene (80.6%) was most frequently expressed, which was explained considering the necessity of the gene for poly-β-1,6-Nacetylglucosamine (PNAG) operon production and biofilm formation (Choi et al., 2009). The *bfmS* gene was the second predominant (69.4%) followed by the *csuE* gene (50%). The *bfmS* gene participates in a two-component system regulating the csuA/BABCDE pilus usher-chaperon assembly system (Tomaras et al., 2008), whereas *csuE* is a member of the usher-chaperon assembly system involved in attachment and biofilm formation (C.-H. Yang et al., 2019), which explains the higher persistence of the genes. Notably, *ompA* gene is associated with drug resistance in *A. baumannii* (Smani et al.,

2014), which was highly prevalent in our isolates (63.9%) even though the isolates were not multi-drug resistance. High prevalence of *ompA* was reported in studies conducted in Thailand, Korea, and China (Liu et al., 2016; Sung, 2018; Thummeepak et al., 2016). Notably, none of the isolates harbored the $bla_{PER\text{-}1}$ gene. Other studies also documented a similar absence of $bla_{PER\text{-}1}$ gene among *A. baumannii* isolates (Ghasemi et al., 2018; Liu et al., 2016; Sung, 2018; Zeighami et al., 2019).

The emergence and extensive spread of bacterial infections resistant to β-lactams, particularly third-generation cephalosporins and carbapenems, have emerged as a significant global concern (Pfeifer et al., 2010). Antimicrobial susceptibility testing conducted on the *A. baumannii* isolates indicated that all isolates were susceptible to imipenem, meropenem, gentamicin, amikacin, and ciprofloxacin. Another high proportion showed susceptibility to cefepime, cotrimoxazole and tetracycline. Only one isolate exhibited resistance to 3 antimicrobial categories (cefotaxime, cotrimoxazole, and tetracycline), thus meeting the criteria for classifications as multi-drug resistant (MDR). Despite all the 72 isolates being ESBL producers phenotypically, not all the resistance could be explained by the disc diffusion assay. Given that the CHROMagar™ ESBL plates detect resistance to a few third-generation antibiotics, it can be speculated that the remaining isolates might be resistant to other antibiotics not covered in the study. This finding complies with a previous study conducted in Europe where only 2% *Acinetobacter* spp. isolates showed resistance to carbapenems (Kittinger et al., 2018). On the contrary, a study in South Africa reported a higher resistance against carbapenems, cephalosporins, and cotrimoxazole (Anane A et al., 2020).

Biofilm formation in *A. baumannii* infections has been significantly associated with the severity of the infections as well as enhanced persistence of the isolates. This finding is concerning considering reports suggest that *A. baumannii* isolates have higher rates of biofilm forming capacity compared to other *Acinetobacter species* (Sung, 2018). In our study, the isolates demonstrated different degrees of biofilm formation with a comparatively higher percentage of isolates forming biofilm at 37°C than 25°C. Previous evidence suggests that the biofilm formation of clinical *A. baumannii* isolates is stronger than environmental isolates (Gedefie et al., 2021), however, our study demonstrates that the environmental *A. baumannii* isolates can also form a stronger degree of biofilm. This necessitates further analysis of the extent of *A. baumannii* biofilm formation irrespective of the temperature or the climatic condition they were isolated from.

In addition, the study also assessed the correlation between biofilm formation capability and antimicrobial resistance of the isolates. A negative correlation exists between isolates that were resistant to the tested antibiotics and strong biofilm forming tendency. The results of this research are consistent with a previous study, where the non-MDR *baumannii* strains produced strong biofilm compared to their MDR strains (Li et al., 2021; Qi et al., 2016). However, there are certain contradictions centered around the relationship between the degree of biofilm formation and antimicrobial resistant patterns (Li et al., 2021; C.-H. Yang et al., 2019). Moreover, the coexistence of virulence and antimicrobial resistance is concerning. The correlation matrix revealed positive correlations between the existence of virulence determinants and antibiotic resistance genes. These findings are in accordance with previous studies which have shown similar associations (Fasciana et al., 2019; Tasneem et al., 2022). The positive associations also correlate with an ability to form biofilms, which exhibit the ability of *A. baumannii* to persist despite seasonal and environmental variations.

The genetic fingerprint patterns for the *A. baumannii* isolates revealed 10 clusters at a 60% similarity index. The largest cluster-J contained a total of 23 isolates obtained during different sampling periods and seasons. The grouping of isolates bearing different phenotypic and genotypic traits under the same cluster demonstrates the genetic relatedness among the isolates. These findings are in coherence with previous studies which have shown relatedness between *A. baumannii* isolates obtained from distinct sampling points (Havenga et al., 2022). Furthermore, isolates obtained during different sampling seasons were grouped under the same cluster, and this provides evidence of the continued persistence of *A. baumannii* within these environmental reservoirs despite seasonal fluctuations in environmental parameters. The isolates screened during this study demonstrated a wide genetic variation, and ERIC-PCR provides a cost-efficient method to track the persistence of infectious organisms within large environmental reservoirs over a long period of time.

There are a few limitations of this study. Firstly, this study finding cannot be broadly generalized considering only one municipal waste area was targeted for the analysis. A broader sampling area across Dhaka city could have augmented statistical significance. Secondly, we conducted PCR for four major ESBL genes, so there might be a chance of under-detecting the genes. Thirdly, this study did not analyze the presence of plasmids, hence, plasmid-mediated resistance was left

unchecked. Moreover, a comparative analysis of the environmental isolates with the clinical isolates or hospital surrounding environments could have established an association among the spread of these organisms from one environment to another.

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CHAPTER 6: CONCLUSION

This study aimed to characterize ESBL-producing *Acinetobacter baumannii* isolates obtained from environmental sources. The most commonly identified ESBL genes among the isolates were bla_{TEM} and bla_{SHV}. Additionally, a notable proportion of ESBL-producing isolates tested positive for various virulence genes, with *pgaB*, *bfmS*, *ompA* and csuE being particularly prevalent. Antibiotic susceptibility testing revealed that approximately 90% of the isolates showed intermediate resistance to cefotaxime and ceftriaxone. Biofilm formation capability was assessed for all isolates, revealing rates of biofilm formation among the environmental isolates. The study also gave an insight into the correlation between the phenotypic and genotypic characteristics of the ESBL-producing isolates.

The findings of the study highlight the widespread presence of ESBL-producing *A. baumannii* in the environment, emphasizing the urgent need to intervene to prevent their transmission and combat resistance transmission. Subsequent research should focus on monitoring the prevalence of ESBL *A. baumannii* in diverse environmental sources and investigating plasmid profiles and conjugation experiments to understand resistance and virulence factor dissemination, ultimately aiding in reducing antimicrobial resistance.

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