Whole-genome sequencing and comparative genome analysis of multi-drug resistant *Acinetobacter baumannii* clinical strains isolated from Rawalpindi and Peshawar, Pakistan

Shah Fawad

Reg number: 00000329045

A thesis submitted in partial fulfillment of the requirements for the degree of

MS Industrial Biotechnology

Supervised by

Dr. Amjad Ali

Industrial Biotechnology

Atta ur Rahman School of Applied Biosciences (ASAB)

National University of Sciences and Technology (NUST), Islamabad, Pakistan

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THESIS ACCEPTANCE CERTIFICATE

Certified that the contents and form of thesis entitled "**Whole-genome sequencing and comparative genome analysis of multi-drug resistant *A. baumannii* clinical strains isolated from Rawalpindi and Peshawar, Pakistan.**" submitted by Shah Fawad, have been .found satisfactory for the requirement of the degree

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MS THESIS WORK

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be accepted in partial fulfillment of the requirements for the award of **MS Industrial Biotechnology** degree with (A grade).

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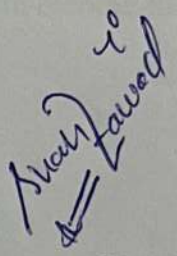

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DEDICATION

To

My wonderful father and my mother

Thanks for bearing the stressful moments I had, for the prayers and support to overcome them and for all the joy you have brought to me.

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Shah Fawad

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Abbreviations	Explanation
HAP	Hospital Acquired Pneumonia
VAP	Ventilator Associated Pneumonia
ESKAPE	Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Pseudomonas aeruginosa, and Enterobacter
WHO	World Health Organization
CDC	Centers for Disease Control and Prevention
ICU	Intensive Care Units
UTIs	Urinary Tract Infections
OMPs	Outer Membrane Proteins
OMVs	Outer Membrane Vesicles
LPS	Lipopolysaccharide
Bap	Biofilm-associated proteins
T2SS	Type 2 Secretion System
T6SS	Type 6 Secretion System
ESBLs	Extended Spectrum beta-lactamases
MBLs	metallo-beta-lactamases
ADC	Acinetobacter-derived Cephalosporinase
QRDRs	Quinolone Resistance-determining Regions
EPS	Extracellular Polymeric Substances
ENA	European Nucleotide Archive
CAB	Center for Algorithmic Biotechnology
RAST	Rapid Annotation Utilizing Subsystem Technology
MLST	Multi-Locus Sequence Typing
PubMLST	Public Multilocus Sequence Typing
CGE	Center of Genomic Epidemiology
iTOL	Interactive Tree of Life
CARD	Comprehensive Antibiotic Resistance Database
VFDB	Virulence Factor Database
COGs	Clusters of Orthologous Groups
PATRIC	Pathosystems Resource Integration Centre
RGI	Resistance genes identifier
VF	Virulence Factor
GI	Genomic Islands
AMR	Anti-Microbial Resistance
MDR	Multi-Drug Resistant
PHASTER	PHAge Search Tool Enhanced Release
QS	Quorum Sensing

ABSTRACT

Acinetobacter baumannii, a bacterium classified as gram-negative, has become a significant pathogen in hospital settings worldwide. It can cause a broad spectrum of illnesses and is of particular concern because of its resistance to many antibiotics. Nevertheless, the investigation of the genetic diversity and features of *A. baumannii* strains in specific geographical regions, such as those obtained from Pakistan, has been rather limited. The present investigation explores the genetic composition of *A. baumannii* strains by means of sequencing and analyzing four genomes obtained from local sources. The research endeavors to elucidate the genomic architecture by the utilization of comprehensive techniques such as genome sequencing, assembly, and annotation. The following comparative study offers useful insights into the genetic diversity that is inherent in the *A. baumannii* strains collected from the surrounding area. The focus of this work is to analyze the presence of virulence factors and antibiotic resistance determinants encoded within the genomes under study. The initial results indicate a significant genetic diversity among the indigenous strains, thereby enhancing our comprehension of the species' capacity to adapt and endure. The clinical significance of these strains in hospital settings is underscored by the presence of genes linked to virulence factors and antibiotic resistance mechanisms. Moreover, this research provides insight into the genetic correlation between the strains collected locally and their global counterparts. The preliminary phylogenetic analysis suggests the presence of genetically different lineages that are unique to the strains found in Pakistan. This finding offers valuable information regarding the population dynamics and evolutionary patterns of *A. baumannii* in this geographical area. In brief, this study reveals the genomic composition of *A. baumannii* strains obtained from local sources, revealing their genetic variability, possible virulence attributes, and patterns of antibiotic resistance. Through the clarification of these elements, the research enhances the overall comprehension of the clinical importance of *A. baumannii* and its consequences for healthcare initiatives at the local level.

Chapter 1

1. Introduction

Acinetobacter baumannii is a bacterium that is widely distributed and categorized as a Gram-negative coccobacillus, characterized by the absence of flagella. It is commonly seen in numerous environmental sources. This specific bacterium, characterized by its opportunistic nature, is responsible for infections acquired in both hospital and community settings within the realm of human medicine. A wide array of diseases, such as hospital-acquired pneumonia (HAP) and urinary tract infections, gastrointestinal infections, ventilator-associated pneumonia (VAP), bacteremia, meningitis, and skin/wound infections, serve as examples of the different types of infections that may occur (Harding, Hennon, & Feldman, 2018).

A. baumannii is categorized as one of the ESKAPE pathogens, a group that encompasses Klebsiella pneumoniae, Staphylococcus aureus, Pseudomonas aeruginosa, Enterococcus faecium, and Enterobacter spp. According to WHO *A. baumannii* has been recognized as one of the foremost ESKAPE species, possessing the capacity to elude the efficacy of antibacterial pharmaceuticals. These microorganisms collectively offer a substantial threat to human health globally and present a medical challenge due to their increasing and persistent resistance patterns. The bacterium's capacity to exhibit resistance against antibiotics that are seen as last-resort options, such as colistin, tigecycline, and carbapenems, classifies it within the group of nosocomial ESKAPE infections that pose significant challenges. The World Health Organization (WHO) has classed it as a bacterium that requires immediate study and development of novel antibiotics. At the same time, the Centers for Disease Control and Prevention (CDC) regards it as a major public health concern (Vrancianu et al., 2021).

The ability of *A. baumannii* to form biofilms and demonstrate resistance to desiccation and disinfectants is a matter of concern, since these characteristics facilitate its effective persistence

and propagation within healthcare settings. The genome of *A. baumannii* exhibits characteristics like as antibiotic resistance, environmental persistence, and the absence of recognized host-damaging toxins, suggesting that its virulence potential is likely attributed to a strategy centered around persistence and resistance. Moreover, these bacterial organisms possess the capacity to withstand complement-mediated death and oxidative stress. The existence of a malleable genome in *A. baumannii* at the genetic level results in notable diversity among isolates, hence bringing complexity to the study of *A. baumannii* as a separate organism. Therefore, the presence of *A. baumannii* is a current and worldwide challenge that requires continuous surveillance by the public health sector. The microbe demonstrates vigorous proliferation on widely employed laboratory culture media, like MacConkey agar, Blood agar and CHROM-agar. Following an incubation period lasting between 18 and 24 hours at a temperature of 37°C, the organism demonstrates the emergence of smooth, glossy, and mucoid colonies on blood agar. The observed colonies exhibit a lack of color, do not cause hemolysis, and possess a diameter that falls within the range of 1 to 2 millimeters. The bacterium demonstrates the attribute of generating colorless colonies on MacConkey agar, which have a smooth and slimy appearance and a tomb-shaped structure. This observation indicates that the bacterium does not possess the ability to metabolize lactose. When grown on Leeds Acinetobacter Medium enriched with certain nutrients, the Acinetobacter bacteria develop colonies that have a pink hue (Almasaudi SB. Acinetobacter spp. as nosocomial pathogens: epidemiology and resistance aspects (Vázquez-López et al., 2020).

A. baumannii strains are incredibly adaptable creatures that can thrive in both terrestrial and aquatic environments without being parasitic. What's even more impressive is their ability to quickly shift to an infectious state when necessary. Their survival strategies are resilient, which indicates that they have developed a broad genetic repertoire that enables them to adapt to changing environmental conditions with ease. It's important to be aware that in hospitals, there

are many surfaces that can harbor microorganisms. These surfaces can include everything from beds and curtains to walls and medical equipment. Even personal items belonging to healthcare providers can be potential sources of contamination. Additionally, items like tap water basins, telephones, door handles, and waste receptacles can all be breeding grounds for harmful bacteria. It's crucial to maintain cleanliness and hygiene in these environments to ensure the safety of patients and staff. They can persist for prolonged periods on inanimate objects. The persistence of bacteria in a hospital setting can be ascribed to various factors, such as their ability to withstand crucial antimicrobial drugs and disinfectants, as well as their capability to flourish in dry conditions (Al Atrouni, Joly-Guillou, Hamze, & Kempf, 2016).

1.1 Research Objectives

1. To isolate, characterize, and perform whole genome sequencing on *A. baumannii* isolates that exhibit resistance to local treatments.

2. To estimate the pangenome of all available *A. baumannii* strains worldwide.

3. To conduct a comparative genome analysis of multidrug-resistant *A. baumannii* strains to determine its genetic diversity, antibiotic resistance, and virulence profiles.

Chapter 2

Literature Review

2.1 Pathogenesis

A. baumannii is a concerning pathogen which has been found responsible for causing Hospital acquired infections, most commonly in ICUs around the world. The organism's tendency to contaminate hospital surfaces for longer period is associated with nosocomial outbreaks. The bacteria have acquired the capacity to contaminate individuals receiving medical care in hospitals and individuals within the broader population. Within medical facilities, the mortality rate is 26%, which escalates to 43%, specifically within intensive care units (ICUs). *A. baumannii* is a prominent causative agent of ventilator associated pneumonia (VAP), a type of infection that contributes to around 15% of all hospital-acquired illnesses. This infection exhibits the greatest morbidity and fatality rates in medical wards, particularly in intensive care units (ICUs). It comprises around 50% of the overall utilization of antibiotics inside intensive care units (ICUs) (Lee et al., 2017).

A. baumannii is typically not regarded as a pathogen that is commonly transmitted within the community. However, it has been observed to colonize tracheostomy sites in persons with weakened immune systems and children, leading to the development of bronchiolitis and tracheobronchitis that is acquired within the community. Studies indicate that this pathogen is linked to community-acquired pneumonia in sultry regions of Australia and Asia. Especially those with primary conditions like drinking, smoking and diabetes mellitus. Additionally, *A. baumannii* is responsible for approximately 10% to 15% of blood infections that are a result of invasive medical procedures. These procedures include the use of intravascular or respiratory catheters, tubes, or cannulas. The etiology of infection in a significant proportion of *A.*

baumannii infections, ranging from 20% to 70%, remains undetermined (Asif, Alvi, Rehman, & resistance, 2018).

A. baumannii poses a growing menace to those undergoing neurosurgical procedures. This pathogen is accountable for 4% of the total cases of meningitis and infections associated to shunt procedures, exhibiting a death rate of 70%. ICU-acquired wound infections are attributed to it, accounting for 2.1% of cases. Although it is not commonly associated with urinary tract infections (UTIs), this particular agent has been found to induce infections in older patients with weakened health and in patients with long-term catheter-related infections in intensive care units (ICUs). In these settings, it accounts for approximately 1.6% of all UTIs. The utilization of contact lenses and eye surgery has been associated with potential complications such as endocarditis, keratitis, and ophthalmitis. *A. baumannii* is generally regarded as a pathogen with low pathogenicity, except when it is found in patients who have underlying health conditions, like older individuals with persistent diseases like cancer and newborns with low birth weights. Significant predisposing factors that play a crucial role in the acquisition of *A. baumannii i*nfection encompass an extended duration of hospitalization, employment of mechanical ventilation, use of intravascular devices, advanced age, compromised immune system, prior administration of broad-spectrum antibiotic therapy, previous occurrence of sepsis, stay in the intensive care unit, and the use of enteral feedings (Whiteway, Breine, Philippe, & Van der Henst, 2022).

2.2 Clinical symptoms

2.2.1 Hospital-acquired pneumonia

The development of ventilator-associated pneumonia due to Acinetobacter is a severe ailment due to several risk factors. These include extended lengths of hospitalization, prolonged reliance on mechanical ventilation, and previous administration of antibiotics. Nosocomial

outbreaks have also been documented as a result of healthcare professionals who have colonized hands and exhibit inadequate personal cleanliness. These individuals can serve as opportunistic carriers of an epidemic strain. The initiation of an outbreak can be attributed to the presence of contaminated ventilators or respiratory care equipment, as well as the transfer of pathogens inside the hospital setting (Lim, Abidin, Liew, Roberts, & Sime, 2019).

2.2.2 Community-acquired pneumonia

The occurrence of community_acquired pneumonia caused by Acinetobacter has been observed in Australia and Asia. Throat carriage has been identified as a potential route of infection, particularly among community residents who engage in excessive alcohol intake, affecting approximately 10% of this population. The numerical value provided is 57. The condition is distinguished by an abrupt and intense initiation accompanied by subsequent bloodstream infection and exhibits a fatality rate ranging from 40% to 60% (Dexter, Murray, Paulsen, & Peleg, 2015).

2.2.1 Meningitis

Nosocomial infections, specifically those occurring after neurosurgery procedures, the incidence of Acinetobacter meningitis is on the rise, along with the emergence of various other Gram-negative bacteria that pose challenges in post-operative treatment. The placement of an external ventricular drain can serve as a potential site for opportunistic infection. The death rate potentially reaches a significant level of 70%; yet, determining the precise reason of mortality remains elusive (Lim et al., 2019).

2.3 Virulence factors

A. baumannii employs multiple virulence factors in order to induce infection within the host organism (Figure 1). Despite previous understanding that *A. baumannii* lacks motility, recent research has revealed the presence of two distinct types of motilities in this nosocomial

pathogen. These motility mechanisms, namely twitching motility and surface-associated motility, have been found to contribute to the pathogen's ability to survive and spread on surfaces, ultimately leading to an increase in its pathogenicity (Harding et al., 2018).

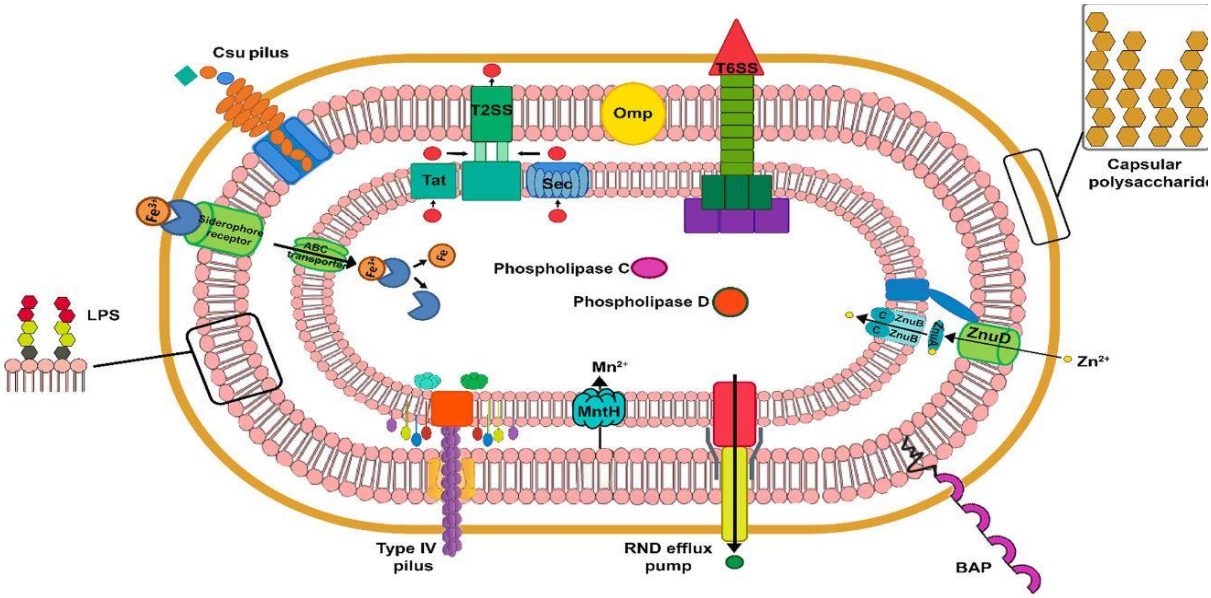


Figure 1. *A. baumannii* employs multiple virulence factors in order to induce infection within the host organism (Shadan et al., 2023).

2.3.1 Outer membrane proteins (Omps)

The outer membrane proteins (OMPs) of *A. baumannii* are pivotal in both pathogenicity and the organism's interaction with its environment. OmpA, the primary extensively studied outer membrane protein in A. baumannii, is implicated in virulence through its multifaceted functions. These include the triggering of apoptosis in host cells by the production of apoptosis-inducing proteins, attachment to epithelial cells through the utilization of host fibronectin, and participation in biofilm formation. The protein known as Outer membrane protein A (OmpA) has been found to be closely linked with the enhancement of adhesion, particularly to the epithelial cells located in the respiratory system. The compound localizes within the mitochondria and nucleus, leading to the activation of the proapoptotic molecule cytochrome c and subsequent initiation of cellular apoptosis. *A. baumannii* employs a strategy to dodge the

death mechanism of the complementary pathways by effectively nullifying factor H, a key regulator of this pathway via the assistance of OmpA. The observed occurrence is commonly referred to as the serum resistance of *A. baumannii*. OmpA has been seen to stimulate the differentiation of CD4+ cells, as well as drive the maturation and activation of dendritic cells. Additionally, it has been found to trigger premature apoptosis in dendritic cells (Uppalapati, Sett, & Pathania, 2020).

2.3.2 Outer membrane vesicles (Omvs)

The release of extracellular vesicles originating from the outer membrane, which carry several proteins associated with virulence (such as phospholipases, catalase, superoxide dismutase, and proteases) enhances the activation of the inborn immune response in the immediate vicinity of the infection site, ultimately resulting in tissue injury. The outer membrane vesicles additionally enhance the process of biofilm formation on non-living surfaces. The polysaccharide capsule seen in Gram-negative bacteria is widely recognized as a significant virulent factor. The function of this mechanism is crucial in providing protection for microbes against phagocytosis by the host's intrinsic immune system (Dehbanipour, Ghalavand, & Therapeutics, 2022).

2.3.3 Lipopolysaccharide (LPS)

The lipopolysaccharide is an important factor for bacterial survival as it may affect colonization and production of proinflammatory responses. Chin et al. found evidence that modifying the lipopolysaccharide (LPS) can lead to reduced antimicrobial susceptibility and the development of resistance mechanisms. The surface of bacteria is covered by a protective capsule that shields them from changes in the environment and certain antimicrobial substances, thereby aiding their survival, especially in serum. The lipopolysaccharides in *A. baumannii* comprised of three primary parts a carbohydrate core, O-antigen, and a lipid A moiety. lipopolysaccharides functions as a drawing inflammatory cell, chemotactic agent, and

encouraging them to secrete their cytotoxic materials (Skariyachan, Taskeen, Ganta, & Venkata Krishna, 2019).

2.3.4 Phospholipase

A further significant virulence factor exhibited by *A. baumannii* is the production of phospholipase enzymes, specifically phospholipase C and phospholipase D. The lipolytic action of these entities is linked to phosphatidylcholine, a substrate for phospholipases found in eukaryotic membranes. Phospholipases exhibit the capacity to hydrolyze human erythrocytes, hence assuming a significant role in the acquisition of iron. Moreover, these enzymes play a crucial role in the invasion of epithelial cells and the development of resistance to serum (Dehbanipour et al., 2022).

2.3.5 Biofilm-associated proteins (Bap)

Biofilm production is well recognized as the most potent virulence mechanism contributing to multidrug resistance in *A. baumannii*. The aforementioned pathogen possesses the capacity to utilize biofilm-associated proteins (Bap) in order to facilitate the formation of biofilm architecture as a reaction to adverse environmental circumstances. Similar to other characteristics that enhance the ability of bacteria to cause disease, the creation of biofilms plays a role in the colonization and survival of bacteria. Biofilms allow bacteria to attach to both living and non-living surfaces, which can lead to infections associated with medical devices in healthcare settings. Previous studies have provided evidence to support the involvement of quorum sensing (QS) in the development of biofilm, wherein autoinducers serve as signaling molecules. Furthermore, the significance of type IV pili and Csu pili lies in their role in facilitating the production of biofilms. The capacity of *A. baumannii* to generate biofilms on both living and non-living surfaces has been extensively investigated as a recognized mechanism of resistance. In order to endure adverse conditions, it undergoes metabolic inactivity within the deeper strata of biofilms. The limited ability of antibiotics to

effectively target metabolically inactive bacteria and their poor penetration into bacterial cells contribute to the increased pathogenicity of these germs. *A. baumannii* strains implicated in epidemics exhibit a notable ability to withstand desiccation and form biofilms on biotic surfaces. The power of *A. baumannii* to produce pellicle through the presence of polysaccharide, for example csuA/B usher protein and poly-N-acetyl glucosamine, which serves as a mechanism to evade the effects of antibiotics. Additional elements contributing to the evolution of biofilms encompass many virulence determinants, such as biofilm_associated protein (BAP), outer membrane protein A (OmpA), BAP like protein-1 (BLP_1), and BAP like protein_2 (BLP_2). Additionally, the acquisition of nutrients from the host aids in the long-term survival of *A. baumannii* allowing it to evade the immune system and facilitate the dissemination of infection. *A. baumannii* utilizes many mechanisms to acquire iron, zinc, and manganese to accomplish this objective. These processes include the production of siderophores, the utilization of a zinc-scavenging system, and the utilization of transporters that belong to the natural resistance-associated macrophage protein (NRAMP) family for each respective element (Gedefie et al., 2021).

2.3.6 Type II secretion system (T2SS)

The presence of protein secretion systems in *A. baumannii* is noteworthy, as it allows this microbe to interact with both its surrounding environment and its host organism. The Type II secretion system (T2SS) is essential, as it uses a biphasic mechanism to facilitate the extracellular transportation of virulence-associated effector proteins, such as CpaA, LipA, and LipH. These proteins play a crucial role as enzymatic factors. Another important system is Type VI secretion system (T6SS), used by *A. baumannii* to selectively target various bacteria by delivering toxins, including nucleases, peptidoglycan hydrolases, and toxins that specifically target the cell membrane. This mechanism is particularly significant in the

development and progression of polymicrobial infections (Weber, Kinsella, Harding, & Feldman, 2017).

2.3.7 Quorum sensing

Bacteria have the ability to communicate with each other through a process called quorum sensing. This allows them to respond collectively to changes in their environment. Autoinducers, which are small hormone-like molecules, are synthesized by bacteria. These molecules help the bacteria monitor their population density and make necessary adjustments to their environment. Similar to other Gram-negative rod shape bacteria, Acinetobacter produces certain signaling molecules such as acyl homoserine lactones for both inter-species and intra-species communication. Additionally, it also generates lesser-explored signaling molecules like 2-heptyl_3-hydroxy-4_quinolone. retention factor 1, and diketopiperazines. (Saipriya, Swathi, Ratnakar, & Sritharan, 2020).

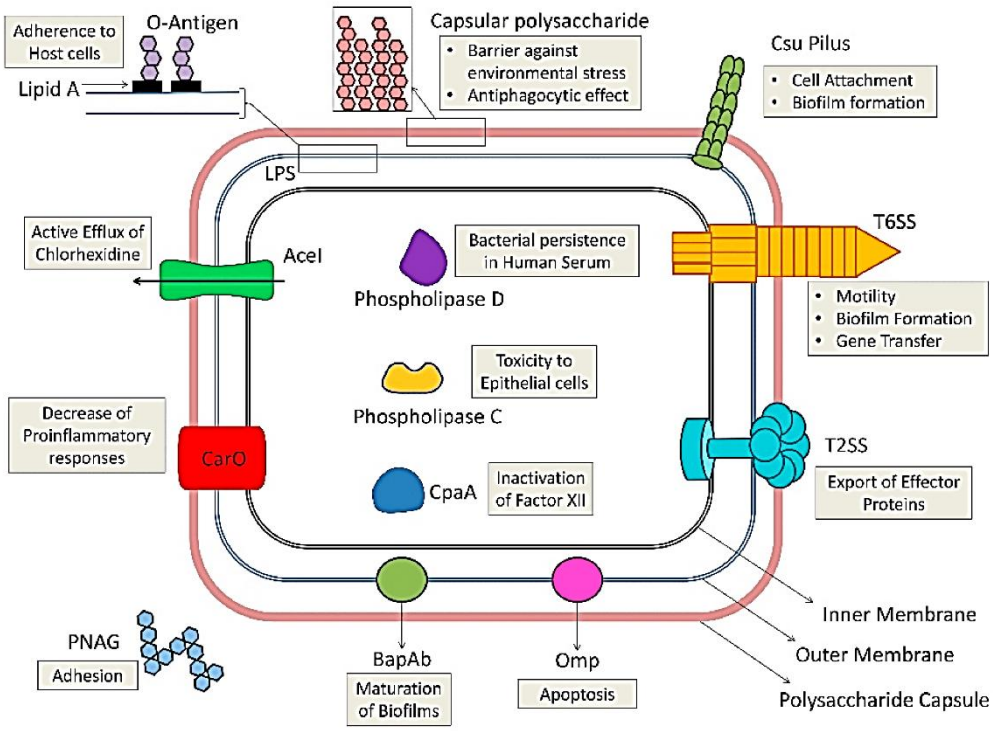


Figure 2. Illustrates the virulence Factors possessed by *A. baumannii (Ayoub Moubareck & Hammoudi, 2020).*

Table 1. shows the *A. baumannii* virulence factors and their role in disease progression and resistance.

Sr#	Virulence Factors (Genes)	Potential Contribution to the Pathogenesis
1	Outer membrane protein A (OmpA)	The cellular processes encompassed by the phenomenon include epithelial cell adhesion and invasion, biofilm formation, resistance to serum, surface motility, and the ability to trigger apoptosis in host cells or elicit cytotoxicity (Beceiro et al., 2014).
2	Biofilm-associated protein (Bap)	The process of biofilm development and the following binding between cells within the fully formed biofilm (Goh et al., 2013).
3	Lipopolysaccharide (LPS)	The evasion of the host immunological response, resistance to cationic antimicrobial peptides, initiation of the host inflammatory response, attenuation of TLR4 signaling, and enhancement of desiccation survival (Pelletier et al., 2013).
4	Penicillin-binding protein 7/8 (pbpG)	The process of peptidoglycan biosynthesis, cellular stability, and proliferation in serum (Geisinger et al., 2020).
5	Outer membrane vesicles (OMV)	The transportation of virulence genes into the cytoplasm of host cells and the transfer of genetic material between bacterial cells (Jun et al., 2013).
6	Phospholipase	The in vivo survival of bacteria, their ability to withstand serum, and their dispersion (Jun et al., 2013).
7	*Acinetobacter* or Siderophore mediated iron acquisition mechanism	The bacterium acquires iron from the host in order to ensure its existence and also triggers cell death (Pulido, García-Quintanilla, Pachón, & McConnell, 2020).
8	Capsule	The protein under consideration plays a role in mediating resistance to cationic antimicrobial peptides, as well as resistance to serum and survival inside a living organism (Kim et al., 2019).
9	Phospholipase C	Demonstrating hemolytic activity towards erythrocytes and facilitating iron absorption (Stahl, Bergmann, Göttig, Ebersberger, & Averhoff, 2015).
10	Poly-β-1-6-N-Acetylglucosamine (PNAG)	The biofilm formation process involves the adherence of cells to each other and the development of mechanisms to guard against the host's natural defense mechanisms (Jha, Ghosh, Gautam, Malhotra, & Ray, 2017).
11	Two-component regulatory system, BfmRS	The expression of the chaperone-usher assembly system in Csu, as well as its impact on biofilm development and cellular morphology (Pakharukova et al., 2018).
12	AbaI autoinducer synthase	normal biofilm formation (Pakharukova et al., 2018).
13	CsuA/BABCDE chaperone usher pili assembly system	The process of pilus assembly and the production of biofilms on non-living surfaces (Jha et al., 2017).

2.4 Environmental persistence

The concept of environmental persistence refers to the ability of a substance or organism to remain in the environment for an extended period of time.

The general opinion is that the ability of *A. baumannii* to thrive in the nosocomial setting can be largely attributed to two key characteristics: resistance to drugs and environmental persistence. In the following discourse, we examine the characteristics that facilitate the persistence of *A. baumannii* in habitats that are unfavorable for numerous bacterial pathogens,

hence establishing the conditions for human colonization and subsequent infection**.** Health-care situations sometimes involve extended periods of dryness and regular cleaning procedures. Nevertheless, *A. baumannii* has developed mechanisms to withstand these challenges (Whiteway et al., 2022).

2.4.1 Desiccation resistance

The desiccation resistance of clinical isolates of *A. baumannii* referring to their capacity to sustain viability in dry environments, exhibits variability, as certain isolates can retain viability for about 100 days. The multifactorial nature of desiccation resistance in *A. baumannii* remains incompletely characterized. Nonetheless, it is evident that the capacity of *A. baumannii* to sustain viability in situations characterized by limited water availability is a crucial determinant of its desiccation resistance. Undoubtedly, the presence of capsular polysaccharides in *Acinetobacter baylyi*, a nonpathogenic counterpart of *A. baumannii* plays a crucial role in enhancing its ability to endure and thrive under conditions of desiccation. The polysaccharides in question consist of recurring carbohydrate units and serve as a glycan shield, enveloping the bacteria as a whole and providing protection from external hazards. According to a recent study, the overall bacterial counts and culture counts of *A. baumannii* remained constant over extended periods of dryness. This suggests that *A. baumannii* does not primarily rely on transitioning to a dormant state, characterized by a significant portion of the bacterial population entering a viable but nonculturable state, as a survival strategy in arid environments (Whiteway et al., 2022).

2.4.2 Oxidative stress resistance

Oxidative stress can also be triggered during episodes of desiccation. Consequently, *A. baumannii* significantly increases the expression of proteins that are linked to the process of detoxification of reactive oxygen species. Certain species of Acinetobacter are thought to possess the greatest capacity for tolerating hydrogen peroxide, comparable to that of spore-

forming Gram-positive bacteria. The strain of *Acinetobacter gyllenbergii* exhibits the capacity to endure 100mM hydrogen peroxide without experiencing any decrease in its viability. Furthermore, it is able to sustain its viability even when exposed to a higher concentration of hydrogen peroxide, specifically 320 mM. Given that *A. gyllenbergii* has been obtained from human samples, it is plausible to anticipate that other medically significant Acinetobacter species, such as A. baumannii, may exhibit heightened resistance to oxidative stresses due to the genetic adaptability shown in members of the Acb complex. Indeed, it has been observed that *A. baumannii* strains have developed a response to oxidative stress by acquiring the insertion sequence element ISAba1 upstream of the catalase gene, katG. This genetic alteration leads to increased production of katG and subsequently boosts the bacterium's resistance to elevated levels of hydrogen peroxide. However, further empirical evidence is necessary (Vázquez-López et al., 2020).

2.4.3 Disinfectants resistance

Disinfectants, such as chlorhexidine, are widely utilized within medical facilities and several other healthcare environments. Chlorhexidine, an antiseptic agent known for its efficacy against both Gram-positive bacteria and Gram-negative bacteria, exerts its antimicrobic activity by disrupting cellular membranes. The research findings indicate that *A. baumannii* has an active mechanism wherein it utilizes the Acinetobacter chlorhexidine (AceI) efflux protein to expel chlorhexidine from its cellular environment. This process potentially facilitates the bacteria's ability to endure and thrive in challenging circumstances. Ethanol, an additional stressor, has been demonstrated to enhance the proliferation and pathogenicity of A. baumannii, albeit when present in low quantities. In addition, it has been observed that the presence of ethanol at physiological levels in the bloodstream of individuals with a past alcohol use disorder significantly hampers the process of phagocytosis, hence impeding the clearance of *A. baumannii* 38. Consistent with this assertion, prolonged alcohol intake is recognized as a key

contributing factor linked to the development of community-acquired *A. baumannii* infections (Whiteway et al., 2022).

2.5 Antibiotic resistance

After the discovery of antibiotics, a significant breakthrough has been observed in the annals of contemporary medicine. The discovery of penicillin, which was accompanied by aminoglycosides and sulfonamides, encouraged scientists to hypothesize the discovery of a potent remedy capable of eradicating infectious infections had been achieved. However, regrettably, the circumstance does not align with previous assumptions. In his Nobel talk, Fleming expressed his belief that antibiotics will become readily accessible to the general population in the future. However, he also cautioned about the potential negative consequences of unqualified individuals, referred to as "quacks," who may inadvertently contribute to the development of antibiotic resistance by subjecting microorganisms to prolonged and suboptimal treatment regimens. The initial administration of penicillin occurred in 1941, with the subsequent identification of penicillin-resistant isolates in 1942. In a similar vein, the introduction of methicillin occurred in 1960, followed by the reporting of methicillin-resistant organisms in 1961 and subsequent years. Presently, there have been documented instances of *A. baumannii* isolates exhibiting resistance to all antimicrobial agents currently in existence. The diligent efforts of the scientific community have led to the discovery of numerous antibiotics; nevertheless, their improper utilization has resulted in a significant level of resistance. One could argue that we are entering a period reminiscent of the pre-antibiotic age, characterized by the resurgence of bacteria possessing heightened lethality. Acinetobacter possesses the genetic capacity for expedited acquisition of antibiotic resistance, thereby earning the designation of a natural transformant. The scientific literature contains numerous findings characterizing it as one of the most resilient bacteria. At the start of 1970s, Acinetobacter infections were well managed with the administration of carbenicillin, ampicillin, nalidixic

acid, and gentamicin either through standalone treatments or using combination therapy. However, a significant increase in resistance rates was observed subsequent to 1975. Currently, a number of important pharmaceuticals, including extended_spectrum cephalosporin, narrow_spectrum and aminopenicillins. ureidopenicillin, chloramphenicol, tetracycline, cephamycin's like cefoxitin, and the majority of aminoglycosides, have experienced a decline in their effectiveness against *Acinetobacter (Kyriakidis, Vasileiou, Pana, & Tragiannidis, 2021)*.

2.5.1 Resistance to Beta-Lactams

The class of beta-lactam antibiotics encompasses penicillin, cephalosporins, carbapenems, monobactams, and beta-lactamase inhibitors. Beta-lactam antibiotics possess a structural resemblance to d-Ala-d-Ala moiety, which is found in peptidoglycan, to which penicillin-binding proteins are attached. Consequently, these antibiotics impede the transpeptidation process, which represents the final stage in the manufacture of peptidoglycan. *A. baumannii* currently has inherent resistance to cephalosporins and penicillin. Resistance against beta-lactam antibiotics can be acquired via the processes already discussed, namely hydrolytic inactivation, enhanced and reduced influx, and safeguarding of the target for antibiotic. (Akhtar, Fatima, Khan, & Challenges, 2022).

2.5.1.1 Beta-Lactamases

Beta_lactamases are a class of enzymes which facilitate the hydrolysis of beta_lactam antibiotics. These enzymes are categorized into four different classes, which are determined by their sequence motifs and variations in their hydrolytic mechanisms.

2.5.1.2 Class A

Class A beta_lactamases are responsible for showing resistance to a range of antibiotics, including carbapenems, cephalosporins, penicillin, and monobactams. Lactamases possess the

potential to exhibit a limited range of action, or they can obtain an expanded range of antibiotic effectiveness through point mutations. Besides this Narrow range lactamases exhibit activity primarily against penicillin which can be effectively suppressed by clavulanic acid. Extended_spectrum Beta_lactamases (ESBLs) are enzymes that can break down certain antibiotics, including ceftazidime, ceftriaxone, cefotaxime, and aztreonam. These enzymes are found in many types of Gram-negative bacteria and can spread easily through plasmids and other genetic materials. It's important to regularly monitor for ESBL-producing bacteria and identify specific genetic markers (like blaGES-11, blaTEM-92, blaVEB-1, blaPER-1, blaPER-7, and blaGES-14,) to help with clinical decisions. Some other important members of this group include CTX-M and KPC (Philippon, Jacquier, Ruppé, & Labia, 2019).

2.5.1.2 Class B

Metallo_beta-lactamases (MBLs) also known as class B enzymes are enzymes that are produced by bacteria through the expression of mobile genetic elements such as plasmids and integrons. These enzymes facilitate the breakdown of a wide range of beta-lactam antibiotics, including carbapenems, hence leading to the development of multidrug resistance. It is important to note that MBLs do not possess the ability to hydrolyze monobactams. These enzymes necessitate the presence of any heavy metal like zinc etc. to facilitate the catalytic process. Additionally, they can be categorized further into three subclasses like (B1, B2, and B3) which is based on variations in sequence and disparities in configuration of their respective active sites. Furthermore, four distinct classes of metallo_beta_lactamases have also been identified in *A. baumannii* specifically, SIM, NDM, IMP and VIM. The identification of bacteria that produce metallo-beta-lactamase enzymes has shown to be difficult using phenotypical methods, such as MBL E-test, the double disc synergy test, and combination disk test. However, the application of molecular methods, particularly next generation sequencing, is expected to provide valuable insights into the detection of these organisms. Phenotypic

techniques exhibit limited sensitivity, hence failing to identify all bacteria that produce Metallo-β-lactamases (MBLs). The utilization of polymerase chain reaction (PCR) facilitated the identification of blaVIM-1 in around 14.3% of *A. baumannii* isolates that were classified as metallo-β-lactamase negative using the E-test. This finding underscores the significance of incorporating molecular techniques into routine clinical procedures to effectively identify concealed MBLs. In a recent study, López et al. provided evidence which indicates the presence of NDM lactamases do not have role in hindering the development of bacteria. Furthermore, they found that NDM lactamases are more advantageous compared to other metallo-beta-lactamases (MBLs) due to their lack of fitness cost. This favorable characteristic has contributed to the global spread of NDM lactamases among Gram-negative bacteria (Kyriakidis et al., 2021).

2.5.1.3 Class C

Class C beta-lactamases, namely Acinetobacter-derived cephalosporinase (ADC), are cephalosporinases that are encoded in the chromosome and are naturally present in all strains of A. baumannii. The induction of lactamase overexpression can be achieved through the incorporation of ISAba125 and ISAba1 sequences upstream to the encoding gene blaADC (previously known as blaAmpC). These inserted sequences are observed to possess greater promoter activity compared to the intrinsic promoter. Insertion sequences (IS) are a class of small transposable elements that exhibit many repetitions throughout the genome, rendering their detection challenging. A recently established open-source bioinformatics pipeline, known as panISa, has been designed to address this issue which utilizes the whole genome sequencing (WGS) data as its primary input. Numerous variations of ADC have been documented, a significant proportion of which demonstrate an expanded range of antibiotic resistance. As an illustration, ADC_30 shows resistance not only towards Cephalosporins, but also shows resistance towards carbapenems and sulbactam. The findings from the phosphoproteomic

analysis indicate that the dephosphorylation of ADC could potentially result in the development of imipenem resistance in clinical isolates (Kyriakidis et al., 2021).

2.5.1.4 Class D

Class D beta_lactamases, known as Oxacillinases (OXA) or carbapenem_hydrolyzing class D β-lactamases (CHDLs), has the ability to render all beta-lactams (mostly those belonging to the OXA-10 family) ineffective, therefore serving as the primary mechanism for carbapenem resistance. The enzymes in question exhibit serine dependency, similar to Class A and C beta-lactamases. Furthermore, it is worth noting that class D beta-lactamases typically exhibit resistance to inhibition by tazobactam, sulbactam and clavulanic acid. Multiple blaOXA genes have been identified, such as blaOXA_143, blaOXA_23 , blaOXA_24 , blaOXA_58, blaOXA_235 and blaOXA_51. Enzyme_encoding genetic material is found on chromosomes and plasmids. Carbapenem resistance in *A. baumannii* is due to upregulation of OXA-23 or OXA-51. Molecular tools like PCR and sequencing have identified carbapenemase-producing *A. baumannii* in companion animals and pigeons, emphasizing the need for global surveillance (Kyriakidis et al., 2021).

2.5.2 Mechanism of Resistance to aminoglycosides

According to the findings of MicroBIGG-E, it has been discovered that *A. baumannii* can develop resistance to aminoglycosides (AG) through three different mechanisms. These include the presence of aminoglycoside-modifying enzymes (AMEs) that reduce the binding capability of AG, alteration of the active site by 16S_rRNA Methyltransferases, and reduced AG uptake due to decreased permeability or increased activity of efflux pumps. Acyl-modifying enzymes (AMEs) can be classified into three main categories based on the specific location of modification on the AG molecule. Recent studies have shown that there are several categories of transferases that modify AG, including acetyl_transferases, adenyl-transferases, and phospho-transferases. These modifications can occur through N-acetylation,

O_phosphorylation and O_nucleotidylation. Unfortunately, *A. baumannii* has developed resistance to aminoglycosides in a significant number of cases, ranging from 19% to 31%. This inherent resistance adds to the challenge of treating infections caused by *A. baumannii* (Kyriakidis et al., 2021*)*. Aminoglycosides (AGs) are a group of substances that inhibit protein synthesis. They do this by penetrating the bacterial cell wall and interfering with the extension of peptides at the 30S ribosomal subunit. Resistance genes may be transmitted through various methods, such as gene cassettes, integrons, conjugative elements and transposons. At the cellular level, the transfer of AG resistance genes can occur through many mechanisms, including mobilizable or conjugative plasmids, natural transformation, or transduction. These processes enable the transmission of AG resistance genes beyond the molecular level (Karakonstantis, Kritsotakis, & Gikas, 2020).

2.5.3 Mechanism of Resistance Colistin

Colistin, also known as polymyxin E, is a bactericidal pharmaceutical agent that functions by disrupting the cell membrane in a manner like to that of a detergent. The cationic area, which has a positive charge, forms a binding interaction with the hydrophilic component of lipopolysaccharides (LPSs) that is negatively charged. Cell death occurs as a consequence of the subsequent loss of integrity. The present state of alarm over antibiotic resistance in Acinetobacter infections has resulted in the utilization of colistin, a medication that was previously disregarded. Various retrospective and prospective investigations have demonstrated a significant incidence of nephrotoxicity associated with the administration of Colistin, with reported rates ranging from 11% to 76%. Consequently, the utilization of this technology was promptly terminated shortly after its initial identification in the late 1950s. Nevertheless, contemporary research does not support the notion of a significantly increased occurrence of nephrotoxicity when compared to earlier studies, provided that associated risk variables are taken into consideration. These risk factors include characteristics such as dosage,

patient age, treatment duration, and the presence of comorbidities including hypertension, obesity, and hypoalbuminemia (Da Silva & Domingues, 2017). The precise mechanisms underlying colistin-induced nephropathy remain incompletely elucidated. Nevertheless, extant research in this domain has posited that the buildup of colistin within the proximal renal tubules may give rise to oxidative injury. Endothelial nitric oxide synthase, inducible nitric oxide synthase, and Caspase_mediated apoptosis have also been implicated in the pathophysiology of nephrotoxicity (Qureshi et al., 2015). The occurrence of neurotoxicity induced by colistin is rare, and it manifests as cough, chest tightness and bronchoconstriction. when delivered through the rout of respiratory tract, ultimately leads towards chemical meningitis. Additional reversible adverse effects that are dependent on the dosage include apnea, psychosis, ataxia, neuromuscular defects visual problems, seizures, paresthesias, and vertigo. In recent years, there has been significant focus directed on Colistin as a final option for treating Multi-Drug Resistant (MDR) *A. baumannii* infections. Regrettably, widespread emergence of colistin resistance has been documented globally. Asia had the greatest reported resistance rate, with Europe following closely behind. The prevalence of hetero resistance to colistin in *A. baumannii* was observed to be typically higher compared to the prevalence of resistance. The precise mechanism underlying resistance remains uncertain; however, several studies have provided evidence suggesting a potential association with the depletion of lipopolysaccharide (LPS) and/or the PmrAB two-component system (Novović & Jovčić, 2023).

2.5.4 Mechanism of resistance to Quinolones

Quinolones are a class of bactericidal compounds that exhibit a wide range of activity against various bacteria. They are distinguished by their structural features, which consists of a bicyclic core that shares similarities with 4-quinolone. Quinolone antibiotics, particularly fluoroquinolones, are known for their effectiveness against a wide range of infections, including both Gram-negative and Gram-positive bacteria. Quinolone antibiotics exert their

method of action by impeding DNA replication through the prevention of bacterial DNA relaxation and subsequent cloning. The mechanism of action of quinolones involves the inhibition of the ligase activity of type II topoisomerases, including topoisomerase IV , and DNA gyrase. The phrase "quinolone resistance-determining regions" (QRDRs) primarily denotes modifications in the target sites of gyrase (specifically, mutations Ser83Leu, Gly81Asp, and Ser81Leu) that impede the binding of quinolones to its alpha-subunit. Additionally, mutations in subunit C of topoisomerase IV (namely, Ser80Leu, Glu84Lys, Gly78Cys, and Ser84Leu) also contribute to quinolone resistance. While a single point mutation in DNA gyrases is typically insufficient to confer resistance to fluoroquinolones in *A. baumannii* (except possibly against levofloxacin, as single parC mutations are associated with ciprofloxacin resistance), the presence of simultaneous mutations within the QRDR region of the parC and gyrA genes is strongly correlated with a substantially elevated level of quinolone resistance. The observed modifications in the gyrB and parE genes exhibit limited importance. Quinolones are a class of bactericidal compounds that exhibit a wide range of activity against various bacteria. They are distinguished by their structural feature, which consists of a bicyclic core that shares similarities with 4-quinolone. Quinolone antibiotics, particularly fluoroquinolones, are known for their effectiveness against a wide range of infections, including both Gram-negative and Gram-positive bacteria. Quinolone antibiotics exert their method of action by impeding DNA replication through the prevention of bacterial DNA relaxation and subsequent cloning. The mechanism of action of quinolones involves the inhibition of the ligase activity of type II topoisomerases, including topoisomerase IV and DNA gyrase. These enzymes often work in conjunction with DNA nucleases to produce supercoiling. By inhibiting the activity of ligase, bacteria experience the persistence of double_stranded DNA breaks, ultimately resulting into cellular demise. It is worth mentioning that the main impact of quinolones is on the activity of gyrase, with any toxicity towards

topoisomerase IV being of secondary importance. In other words, there is no evidence to support the occurrence of parC mutations alone without simultaneous changes in gyrA. Quinolone resistance manifests through three distinct mechanisms. (i) resistance facilitated by Qnr proteins, AMEs AAC(6′)_Ib-cr and plasmid-encoded efflux pumps. (ii) mutations in gyrase and topoisomerase IV targets, leading to diminished interactions between quinolones and these enzymes and (iii) resistance arising from chromosomal factors, such as reduced porin expression or increased expression of efflux pumps encoded within the chromosome (Hooper & Jacoby, 2015).

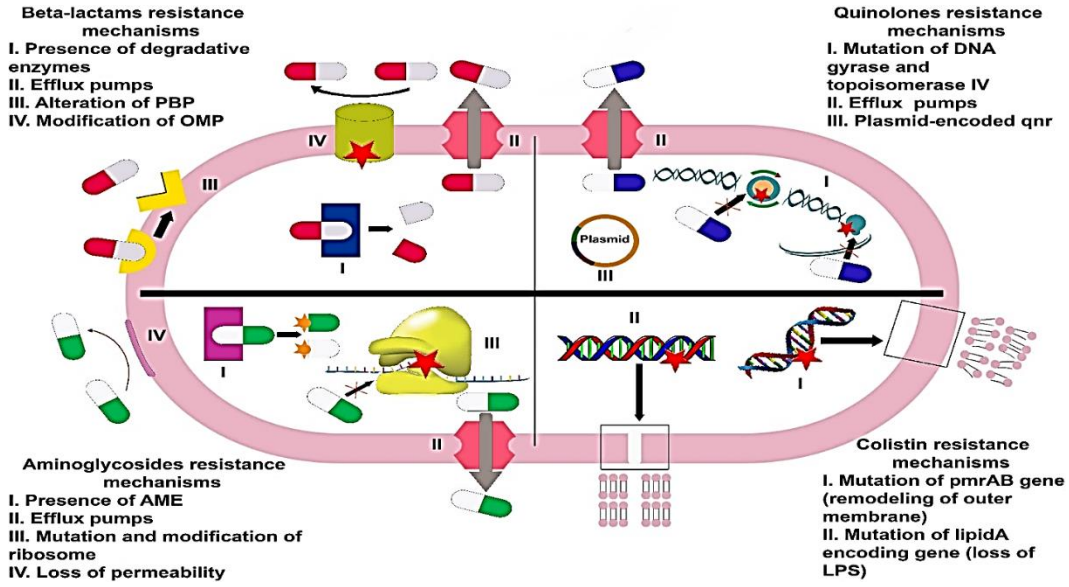


Figure 3. Illustrates the pathways of antibiotic resistance observed in *A. baumannii* (Dehbanipour, Ghalavand, & Therapeutics, 2022).

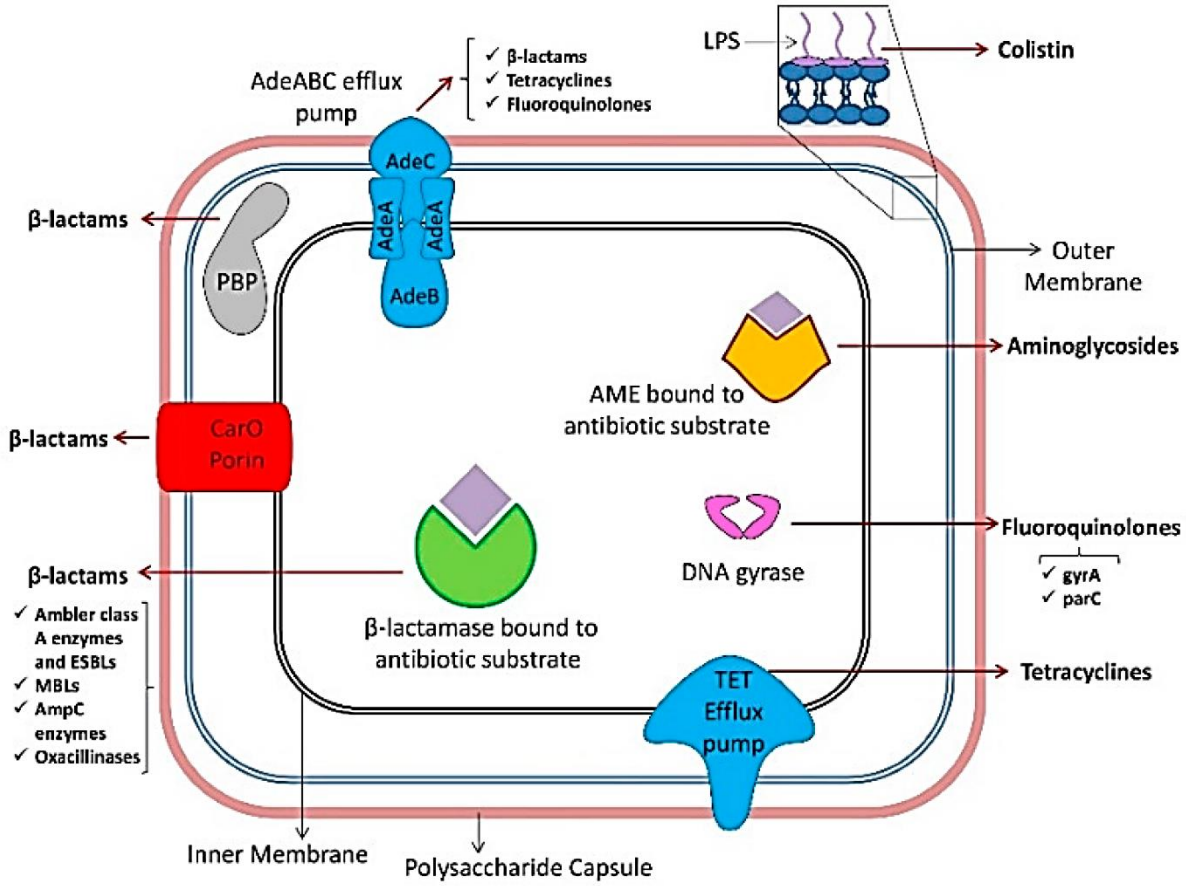


Figure 4. Shows different resistance mechanisms of *A. baumannii* to antimicrobial agents (Dehbanipour et al., 2022).

2.6 Biofilm development

A biofilm is a complex, three-dimensional arrangement that arises when microbial cells cling to either living or non-living surfaces due to a combination of physiological and environmental factors, some of which remain undiscovered. Moreover, these biological entities undergo continual proliferation and generate extracellular polymeric substances (EPS), resulting in the formation of a matrix that envelops these microorganisms. The process of biofilm generation and development encompasses five primary stages. Figure 1 (Reena, Subramaniyan, & Kanungo, 2017).

Biofilm-associated infections can arise from the introduction of pathogens via medical devices that have been contaminated, including intravascular catheters, cardiac devices, prosthetic joints and shunts, and prosthetic vascular grafts. These infections can develop independently

through open wounds, dental plaques, or native valve endocarditis. *A. baumannii* possesses the capacity to generate biofilm on medically significant surfaces, enabling its persistence within the hospital milieu. This organism serves as the primary etiological agent for a variety of infections, such as pneumonia, bacteremia, meningitis, urinary tract infection (UTI), and other ailments observed among critically ill patients in intensive care units (ICUs) within hospital settings (Kyriakidis et al., 2021). These infections are correlated with the formation of biofilms and exhibit a significantly higher resistance to antibiotics compared to individual planktonic cells. The biofilm matrix serves as a protective barrier for bacterial cells, shielding them from the effects of antibiotics and bacteriophages, and enabling their survival in challenging environments, including desiccation. Numerous research has been conducted to examine the correlation between drug resistance patterns and the capacity for biofilm formation in clinical isolates of A. baumannii. A prior study has demonstrated that *A. baumannii* isolates with a high level of resistance exhibit a diminished capacity for biofilm formation, while *A. baumannii* isolates with lower resistance tend to generate more robust biofilms. Thereafter, research conducted by Al-Shamiri et al. revealed that strains exhibiting resistance possess the capacity to generate biofilms of moderate to high intensity. Conversely, susceptible isolates initially demonstrated the ability to develop robust biofilms during a 24-hour timeframe, but thereafter saw a reduction in their biofilm-forming capability, resulting in the formation of weaker biofilms. According to data from the National Institutes of Health and the Center for Disease and Prevention, it is estimated that around 65-80% of bacterial illnesses in people are connected with biofilms (Vázquez-López et al., 2020). Additionally, these circumstances have resulted in significant economic difficulties as a result of equipment impairment, product adulteration, energy wastage, and the spread of illnesses. In addition to treatment difficulties, biofilm-associated infections in tissues provide diagnostic challenges due to the inability to identify the causal microorganism by non-invasive imaging techniques. Moreover, biofilms

have the ability to induce polymicrobial behavior by exerting influence on the surrounding bacteria, thereby promoting their association and colonization. In the final analysis, the course of treatment entails the surgical extraction of implants or grafts afflicted with biofilm-associated infections (Mea, Yong, & Wong, 2021) (Reena et al., 2017).

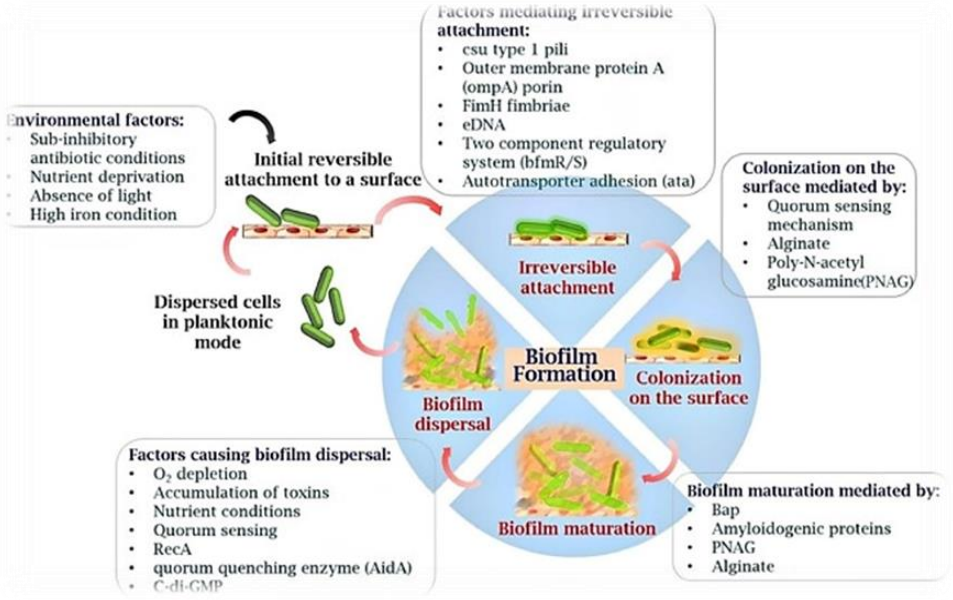


Figure 5. shows the biofilm formation of *A. baumannii* (Upmanyu, Haq, & Singh, 2022)

Chapter 3 Materials and Methods

3.1 Study Approval

Dr. Amjad Ali approved and directed the formulation of the study, which was then approved by the Department of Industrial Biotechnology (IBT), Atta-ur-Rahman School of Applied Biosciences (ASAB), National University of Sciences and Technology (NUST).

3.2 Sample Collection

Samples were obtained from Fuji Foundation Hospital in Rawalpindi and Khyber Teaching Hospital in Peshawar. The specimens were obtained from the blood and Urine of individuals across various age groups. The isolates were sent to the Lab at the corresponding hospital for initial identification, which involved assessing their colony/cell morphology, doing biochemical and molecular characterization, as well as performing an antibiotic susceptibility assay.

3.3 Culturing the Isolates

The Isolate was inoculated onto MacConkey agar which were used as nutrient agar to enhance bacterial growth uniformly then colonies were picked and inoculated on CHROM Agar plates, which serve as a selective medium for A. baumannii species. The plates were then incubated at a temperature of 37 ˚C. Following a 24-hour incubation period, observations were made on the form, colony size, texture, and margin. In order to achieve optimal growth, colonies of *A. baumannii* were subjected to re-culturing on nutrient agar plates. The colonies were chosen from the growth plates based on their size in order to harvest DNA and carry out subsequent processing. Colonies were picked on coal swabs which were sealed properly and stored at 4 °C.

Phenotypic Resistance Profile

The antibiotic resistance assay provides the phenotypic resistance profile of the A. baumannii isolate. The isolates were tested against eighteen antibiotics according to CLSI guidelines. Isolates showed resistance to 10 antibiotics whereas it was susceptible to 7 antibiotics. The *A. baumannii* isolates were resistant against Amikacin (30µg), Meropenem (10µg), Cefotaxime (30µg), Chloramphenicol (30µg), Ciprofloxacin (5µg), Imipenem (10µg), Ceftriaxone (30µg), Gentamycin (10µg), Ceftazidime (30µg), Levofloxacin (5µg) and Ceftriaxone (30µg). Whereas the isolate was susceptible to Doripenem (10µg), Doxycycline (30µg), Colistin (30µg). The zone of diameter is given in the table below.

Table. 2 Shows Phenotypic resistance profile of A. baumannii isolates.

Antibiotics (CLSI guidelines)	**Isolates**			
	AB_1	AB_2	AB_3	AB_4
Amikacin (30µg)	R (13mm)	R (11mm)	R (14mm)	R (13mm)
Meropenem (10µg)	R (10mm)		R (14mm)	
Cefotaxime (30µg)	R (11mm)		R (13mm)	
Chloramphenicol (30µg)		R (11mm)	R (13mm)	
Ciprofloxacin (5µg)			R (15mm)	R (12mm)
Imipenem (10µg)		R (17mm)	R (18mm)	R (17mm)
Ceftriaxone (30µg)			R (10mm)	R (13mm)
Gentamycin (10µg)	R (9mm)	R (12mm)	R (12mm)	R (10mm)
Ceftazidime (30µg)			R (13mm)	
Levofloxacin (5µg)			R (11mm)	

3.4 DNA Extraction, Quantification, and sequencing

The Qiagen DNA purification kit was employed for the purpose of DNA extraction. The quantity of DNA extracted from the isolate was determined through Qubit 2.0 fluorometer. The purity and quality were assessed via the utilization of Agarose Gel Electrophoresis. The DNA was diluted, and libraries were prepared following standard protocol. Samples were then sent for sequencing and the DNA was sequenced on Illumina Next generation sequencing 500 platform.

3.5 Sequence submission

The sequence data was then submitted to the online database European Nucleotide Archive (ENA) with project Accession_PRJEB56918.

3.6 Sequence retrieval

The genomes of the isolated strains were downloaded from ENA database and global complete genomes were retrieved from PATRIC database.

3.7 Assembly and QC check

The initial step in the genome assembly process involved the assessment of the raw sequencing reads' quality using the FastQC software. The genome assembler SPAdes (Galaxy version 3.15.4) were utilized to assemble the raw reads. SPAdes is a bioinformatic tool that has garnered considerable attention and widespread usage owing to its notable efficacy in the assembly of DNA sequences, particularly in the context of intricate genomes. SPAdes offer a precise approach for building short readings by employing a combination of de Bruijn graph and graph-based algorithms. This facilitates the ability of researchers to reconstruct genomic sequences and interpret genetic information with increased precision and enhanced continuity. The acquired data encompasses the aggregate number of contigs, genome magnitude, GC content, and comprehensive coverage. The following parameters were determined: the

total count of contigs, the size of genome, GC content, and the overall coverage. Assembled genomes Were then subjected for quality check through the quality evaluation tools QUAST, developed by the Center for Algorithmic Biotechnology (CAB). QUAST (Quality Assessment) utility for Genome Assemblies a bioinformatics is widely utilized for the purpose of evaluating the quality of genome assemblies. The tool offers comprehensive metrics and visualisations for evaluating the precision, comprehensiveness, and structural soundness of assembled genomes. This empowers researchers and bioinformaticians to make well-informed judgements regarding the dependability of their genomic data. The QUAST tool is particularly valuable for the purpose of comparing different assembly approaches and refining pipelines for the process of genome assembly. The minimum contig length option was set to zero, while the default values were utilized for the remaining parameters.

3.8 Specie Finder

After assembly and quality check genomes were run by SpeciesFinder 2.0 of CGE to confirm the species.

3.9 Genome Annotation

The genomes that were compiled were subjected to structural annotation using the Prokka pipeline with the default settings. Prokka is a bioinformatics tool developed to provide precise and efficient annotation of bacterial genomes. The process is streamlined through the use of gene prediction, coding region identification, and functional annotation assignment to various genomic components. Prokka is considered an essential tool for researchers engaged in the study of microbial genomes owing to its notable efficiency and automated capabilities. This process resulted in the generation of numerous output files, including a Protein FASTA file, a Nucleotide FASTA file, and an Annotation file in GFF3 format. The Rapid Annotation utilizing Subsystem Technology (RAST) server was utilized for the purpose of functional annotation. .

The RAST (Rapid Annotation utilising Subsystem Technology) bioinformatics tool is a comprehensive and accessible platform utilized for genome annotation and analysis. The tool facilitates the annotation of microbial genomes, aiding researchers in the discovery of genes, functional elements, and metabolic pathways present in DNA sequences. The utilization of the automated and curated methods of RAST expedites the understanding of microbial genetics and biology. The output files were acquired in several file formats, including GenBank and EMBL.

3.10 Genome Analysis

3.10.1 MLST

The contig sequences of four isolates were analyzed using the MLST Software version 2.0.9. The MLST Software version 2.0.9 is a state-of-the-art bioinformatics tool widely employed for the purpose of conducting Multi-Locus Sequence Typing (MLST) analysis. This updated edition provides enhanced precision and effectiveness in the characterization of genetic diversity among microorganisms, rendering it an essential instrument for researchers and physicians aiming to comprehend microbial evolution and epidemiology. The study employed the Institute Pasteur scheme (MLST-IP) which utilized a set of seven housekeeping genes (cpn60, fusA, gltA, pyrG, recA, rplB, and rpoB) to ascertain the Allelic profiles or STs of each isolate. The validation of these profiles was conducted by PubMLST. PubMLST, (Public Multilocus Sequence Typing), is a robust bioinformatics tool that is employed for the purpose of microbial strain typing and epidemiological study. This technology enables researchers to evaluate genetic differences in numerous genes, thereby facilitating the monitoring and understanding of the transmission of infectious diseases. PubMLST enables scientific community collaboration and data exchange through the provision of standardized and publicly accessible databases.

3.10.2 Phylogenetic Analysis

To comprehend the genetic variants and evolutionary links better Using the Center of Genomic Epidemiology's CSI Phylogeny 1.4 program, CSI Phylogeny 1.4 is a bioinformatics tool widely utilized in the field of evolutionary research. The software exhibits remarkable expertise in the creation of phylogenetic trees and the exploration of genetic relationships among organisms. The software possesses a user-friendly interface and employs advanced algorithms, making it an essential tool for scientists involved in the study of evolutionary biology and genetics. The phylogenetic relationships between the four genomes were created together with fifteen other genomes from global strains and one reference genome. The Interactive Tree of Life (iTOL) web program (version 6.8) was used to visualize the output Newick file. iTOL (Interactive Tree Of Life) version 6.8 is a state-of-the-art bioinformatics application commonly used for visualizing and annotating phylogenetic trees and other hierarchical data. Its accessible online, extensive customization options, and interactive features make it an indispensable tool for researchers and scientists seeking to explore and communicate complex evolutionary relationships and taxonomy. The tree was rendered and then saved in PNG format.

3.10.3 Pangenome Analysis

The pan_genome refers to the complete repertoire of genes within a given species. which includes both the core genome and the dispensable genome. The core genome consists of genes which are universally present in all genomes of a certain species and which are crucial for the growth of bacteria. On the other hand, dispensable sequences are not necessarily found in all genomes and may be responsible for strain-specific functions like pathogenicity, stress response, and tolerance to various factors. This study conducted a pangenome analysis on four genomes obtained from isolated strains, as well as twenty-nine draft genomes sourced from Pakistan and four hundred and sixty-six complete global genomes retrieved from PATRIC. The Pathosystems Resource Integration Centre (PATRIC) is a comprehensive bioinformatics

framework specifically developed for the purpose of analyzing microbial genomes and the accompanying data. This software enables scientists to engage in the exploration, annotation, and comparison of genomes, as well as the conduct of comprehensive analyses pertaining to microbial communities, metabolic pathways, and functional annotations. The utilization of PATRIC significantly enhances understanding of diverse diseases and microbiomes, rendering it a crucial tool for doing research on microorganisms. The pangenome study was conducted using the pangenome pipeline, namely Roary version 3.13.0. The Pathosystems Resource Integration Centre (PATRIC) is a comprehensive bioinformatics framework specifically developed for the purpose of analyzing microbial genomes and the accompanying data. This software enables scientists to engage in the exploration, annotation, and comparison of genomes, as well as the conduct of comprehensive analyses pertaining to microbial communities, metabolic pathways, and functional annotations. The utilization of PATRIC significantly enhances understanding of diverse diseases and microbiomes, rendering it a crucial tool for doing research on microorganisms. The GFF3 format was utilized as the input file. The selection of output files was based on the desired results, while the remaining parameters were left in their default settings.

3.10.4 Identification of virulence genes

The identification of virulence factors involved an analysis of the genomes of four isolated strains using VFDB, a comprehensive database dedicated to cataloging virulence factors. . VFDB (Virulence Factor Database) is a computational database tool developed to conduct exhaustive analysis of bacterial virulence factors. It provides an archive of organized data on pathogenicity-related genes, proteins, and functional annotations. Researchers can utilize VFDB to further comprehend molecular processes underlying bacterial infections and to devise preventative and therapeutic strategies. The input file utilized for the analysis was in the

Nucleotide FASTA format, and a reference genome was chosen for the sake of comparison. The outcomes were acquired and recorded in an Excel spreadsheet as the final output.

3.10.5 Identification of Resistance genes

The antibiotic resistance determinants included in the sequenced genomes of isolates were identified through the utilization of the CARD database. The Comprehensive Antibiotic Resistance Database (CARD) is a bioinformatics tool that has been specifically developed to enhance the investigation and analysis of antibiotic resistance. The platform offers an extensive compilation of meticulously selected data pertaining to antibiotic resistance genes, their corresponding proteins, and the underlying molecular pathways that contribute to antibiotic resistance. Scientists can employ the Comprehensive Antibiotic Resistance Database (CARD) as a valuable tool for the examination and interpretation of genetic information pertaining to antibiotic resistance. This resource plays a crucial role in enhancing comprehension and facilitating the mitigation of this pressing worldwide health issue. The Resistance Gene Identifier version 2 was utilized to access the CARD database (http://arpcard.mcmaster.ca/).

3.10.6 Identification of Genomic Islands

The analysis of isolated isolates' genomes was conducted using IslandViewer 4 in order to identify Genomic Islands (GIs). IslandViewer 4 is a well utilized computational tool renowned for its efficacy in the prediction and study of genomic islands inside bacterial genomes. This technology provides precise identification of these genetic components, facilitating the comprehension of their involvement in horizontal gene transfer and the evolutionary processes of bacteria. The user-friendly interface and comprehensive capabilities of IslandViewer 4 render it an indispensable tool for investigating genetic diversity and pathogenicity across diverse bacterial species. The input file utilized in this study was in the GenBank file format. IslandViewer 4 incorporates four distinct algorithms, namely IslandPath-DIMOB, SIGI-HMM, Island-Pick, and Islander. The selection of an algorithm is contingent upon the characteristics

of the input file. The preferred tools for the draft genome analysis are IslandPath-DIMOB and SIGI-HMM. IslandViewer 4 has maintained its adaptable plus dynamic user interface for the purpose of visualizing genomic islands (GIs) and their corresponding gene content. This is achieved through the utilization of Genome3DPlot, which was previously referred to as IslandPlot. The analysis of genomes yielded results that were visually represented as circular rings, wherein genomic islands were depicted in distinct colors corresponding to the specific algorithm employed. The downloaded files were obtained in the formats of CSV and PNG.

3.10.7 Identification of Prophages

The prophages present in the isolated strains were identified through the analysis of their genomes using PHASTER (PHAge Search Tool Enhanced Release). The PHASTER (PHAge Search Tool Enhanced Release) bioinformatics tool is widely recognized for its efficacy in the identification and characterization of bacteriophages, which are viral entities that specifically target and infect bacteria, inside the genetic makeup of bacterial organisms. By utilizing sophisticated algorithms, PHASTER effectively identifies phage-associated sequences, gene annotations, and offers valuable insights into the genomic arrangement of phages. PHASTER is a valuable tool utilized by researchers to elucidate the viral constituents present in bacterial genomes, hence facilitating the investigation of phage-bacteria interactions and their prospective implications across diverse disciplines. The input file utilized for this study was in the format of Nucleotide FASTA. The phage sequence was subjected to a search process, during which the identified areas were categorized as intact, questionable, or incomplete, based on their respective scores. The score is determined based on the geographical region, the length of the region, and the degree of completeness of the sequence.

3.10.8 COG Analysis

The COG Bioinformatics tool is a highly robust and adaptable software utilized in the field of biological research. The utilization of this tool facilitates the effective examination of Clusters of Orthologous Groups (COGs), hence assisting researchers in the identification of common functional attributes across genes across diverse organisms. The COG Bioinformatics tool plays a significant role in providing useful insights into evolutionary links and gene functions through the facilitation of comparative genomics. COG analysis was performed through Bacterial Pangenome Pipeline B-Pan. B-Pan is a Bacterial pangenome pipeline which is used for pangenome, phylogenetic and COG analysis. In COG analysis Core genes and unique genes involved in different pathways were determined.

Chapter 4 Results

4.1 Sequence Retrieval

A total of 466 complete genome sequences of *A. baumannii* were retrieved from PATRIC reported from around the world on 26th of May 2023. Also 29 draft genomes of *A. baumannii* from Pakistan were retrieved from PATRIC database on the same date. The *A. baumannii* strains included in the study were all reported as MDR and causing infections in human host.

4.2 Pangenome of Multi Drug Resistant *A. baumannii*

Roary estimated a total of 33,300 genes in 499 *A. baumannii* genomes, out of which 1394 were core genes that were conserved among 90-100% strains and which is 4.18% of total genes. 683 soft core genes were present among 68-72% strains, 2345 were shell or accessory genes that were present among 10-68% of the strains, and 28878 cloud or unique genes were present among 10% of the strains (Fig 6). Figure 7 shows the tree compared to a matrix with the presence and absence of core and accessory genes among *A. baumannii* genomes. Pangenome analysis reveals that there is an increase in the number of unique genes with an increase in number of genomes (Fig. 8). It was an open pangenome showing an increase in the number of total genes with an increase in the number of *A. baumannii* genomes.

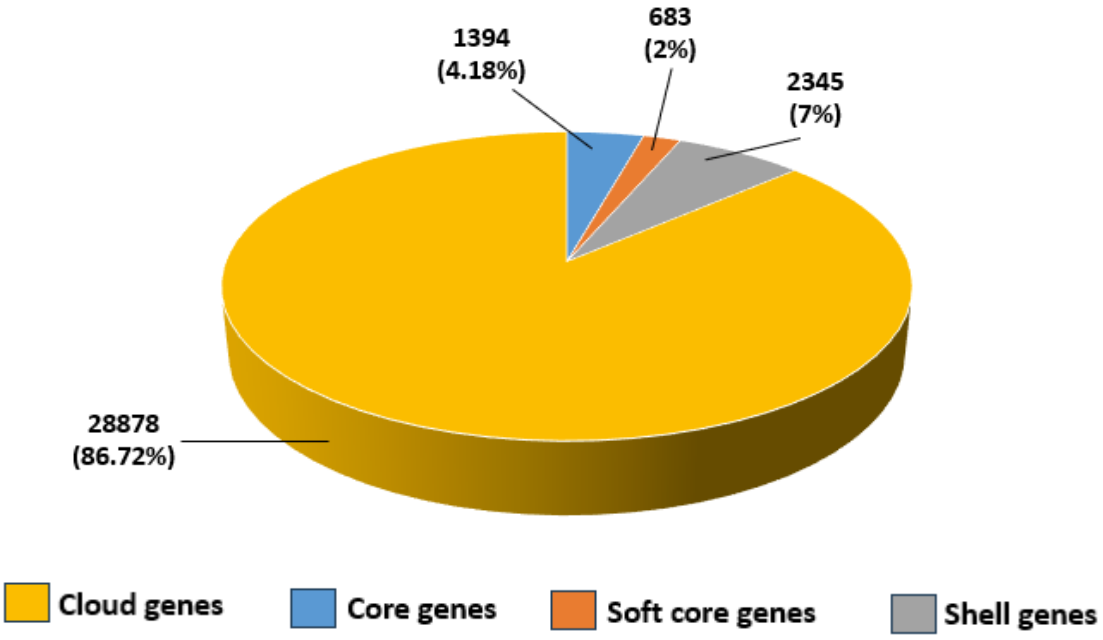


Figure 6. Figure 6. Pie chart of gene clusters in *A. baumannii* pangenome shows Core genes, Soft core genes and Accessory genes.

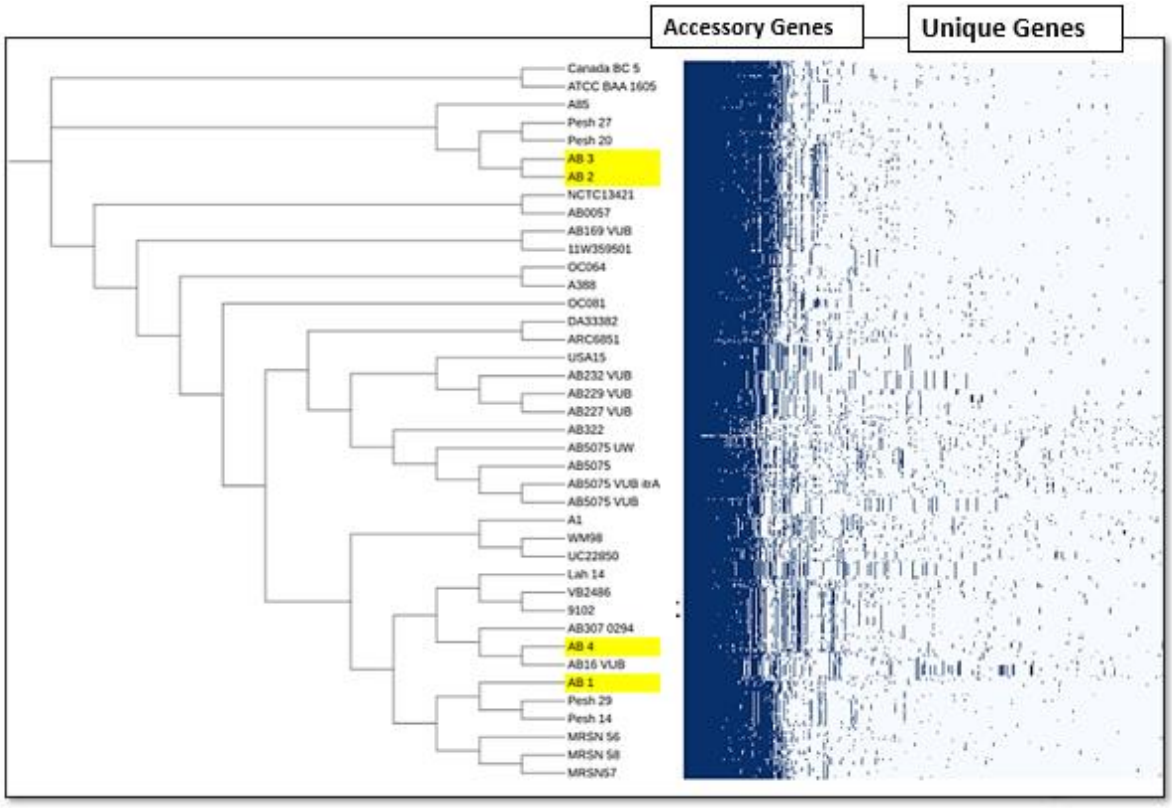


Figure 7. Pangenome tree compared to the matrix with presence/absence of core and accessory genes among *A. baumannii.*

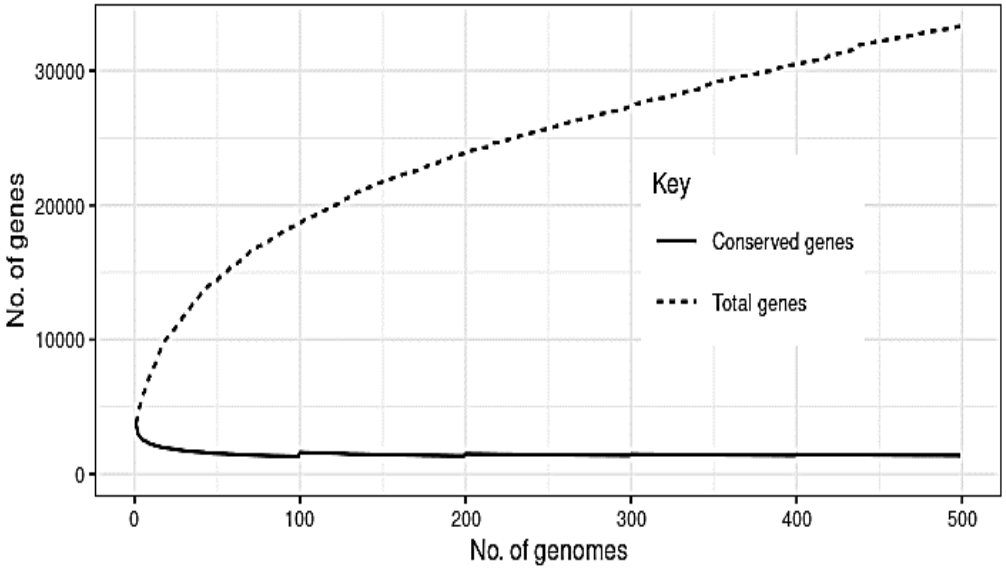


Figure 8. Graph showing increase in number of total genes with increase in number of *A. baumannii* Genomes.

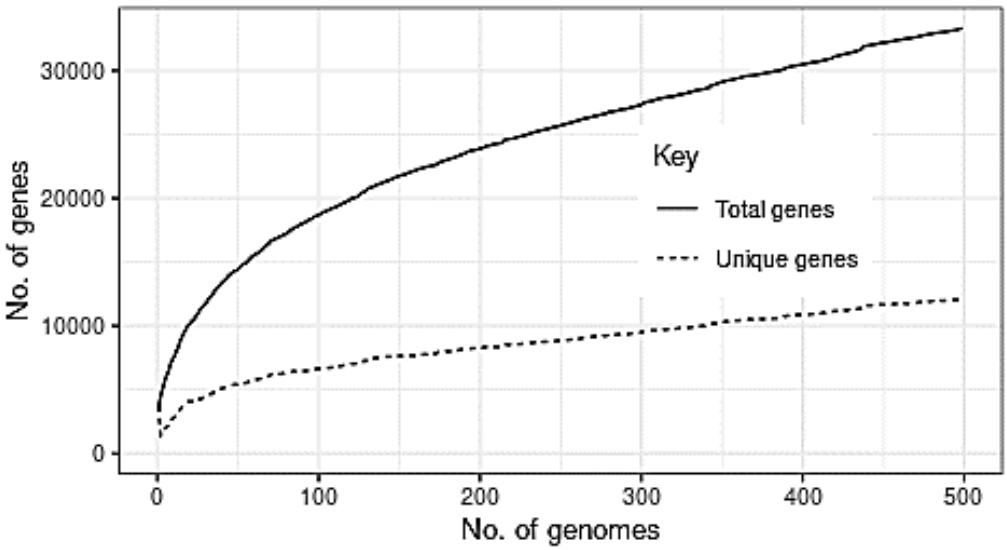


Figure 9. Graph shows increase in number of unique genes with increase in number of *A. baumannii* Genomes which indicates diversity and adoptability of isolates.

Table 2. Shows genomic characteristics of *A. baumannii* isolates.

Genomic Characteristics				
	AB_1	AB_2	AB_3	AB_4
Size	3,919,810	4,039,027	4,006,632	3,700,547
Number of Contigs	343	81	302	249
GC content	39.3	39.0	39.1	39.0
N50	22027	126706	28157	29155
L50	58	12	49	40
Number of coding sequences	3810	3953	3941	3513
Longest contig size	70210	249736	94386	109202
Smallest contig size	512	507	501	549
Mean sequence size				
Number of subsystems	310	313	313	302
tRNA	55	55	60	61
rRNA	3	5	4	3
tmRNA	1	1	1	1

4.3 Multi Locus Sequence Typing of *A. baumannii* Isolates.

The MLST analysis of all the *A. baumannii* (n=499) was conducted using the Pasteur scheme that comprises of seven housekeeping genes cpn60, fusA, gltA, pyrG, recA, rplB and rpoB. For each strain the allelic profiles were determined and new STs were assigned to the strains with unknown STs. MLST analysis revealed 88 different sequence types among 499 strains that show high genetic diversity between *A. baumannii* genomes. The most frequent sequence type was ST2 (n=240) but none of the isolated strains belong to sequence type 2. Among other

STs shared by *A. baumannii* isolates, the most frequently encountered were ST1 (35 isolates), ST553 (21 isolates), ST636 (12 isolates), ST437 (11 isolates), ST (78,422,622) have (10 isolates) and 52 STs has only one isolate.

Table 3. Multi Locus Sequence Type of selected *A. baumannii* strains

House-keeping genes	Sequence types (STs)	No. of Isolates
genes cpn60, fusA, gltA, pyrG, recA, rplB and rpoB	ST2	240
	ST1	35
	ST553	21
	ST636	12
	ST437	11
	ST78, ST422, ST622	10 each
	ST10, ST25	9 each
	ST499	7
	ST79, ST85	6 each
	ST86	5
	ST23, ST158	4 each
	ST16, ST40, AT52, ST63, ST570, ST103, ST126,	3 each
	ST45, ST46, ST64, ST138, ST162, ST164, ST203, ST215, ST374, ST544, ST548, ST554, ST1106	2
	ST15, ST20, ST32, ST33, ST49, ST54, ST58, ST77, ST81, ST90, ST98, ST107, ST108, ST109, ST111, ST142, ST172, ST187, ST193, ST205, ST218, ST221, ST224, ST239, ST248, ST256, ST268, ST279, ST312, ST318, ST322, ST345, ST396, ST398, ST400, ST433, ST464, ST549, ST551, ST533, ST575, ST604, ST623, ST635, ST638, ST655, ST750, ST752, ST801, ST922, ST1039, ST1076	1 each

4.4 COG Analysis

The functional annotation of isolated strains shows the involvement of the Cluster of Orthologous Genes (COGs) in various functions. For strain AB_1, 148 (17.51%) Cluster of Orthologous Genes (COGs) are involved in translation; 89 (10.53%) in amino acid metabolism and transport; 86 (10.17%) in coenzyme metabolism; 62 (7.33%) in transcription, and 54 (6.39) are found to be associated with energy production and conversion. The same pattern goes for strain AB_2 and AB_3 but there is a slight difference when it comes to Strain AB_4. In strain AB_4 the involvement of COGs in coenzyme metabolism is higher than that of the transcription. Graphical representation of the COGs involvement in various metabolic pathways are given below using the following key:

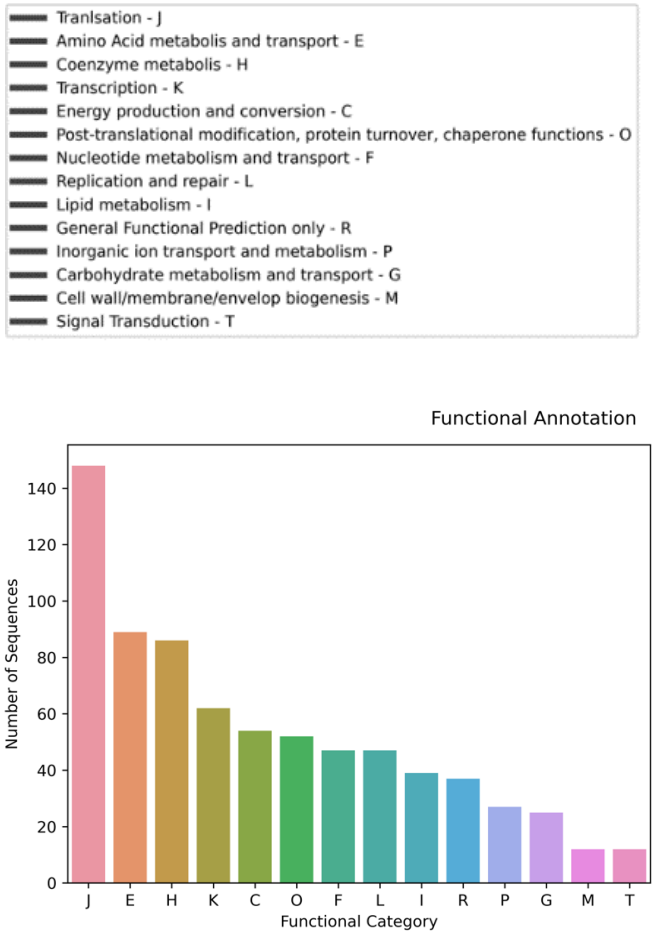


Figure 10. Graph shows distribution of Cluster of orthologous genes in *A. baumannii* isolate AB_1

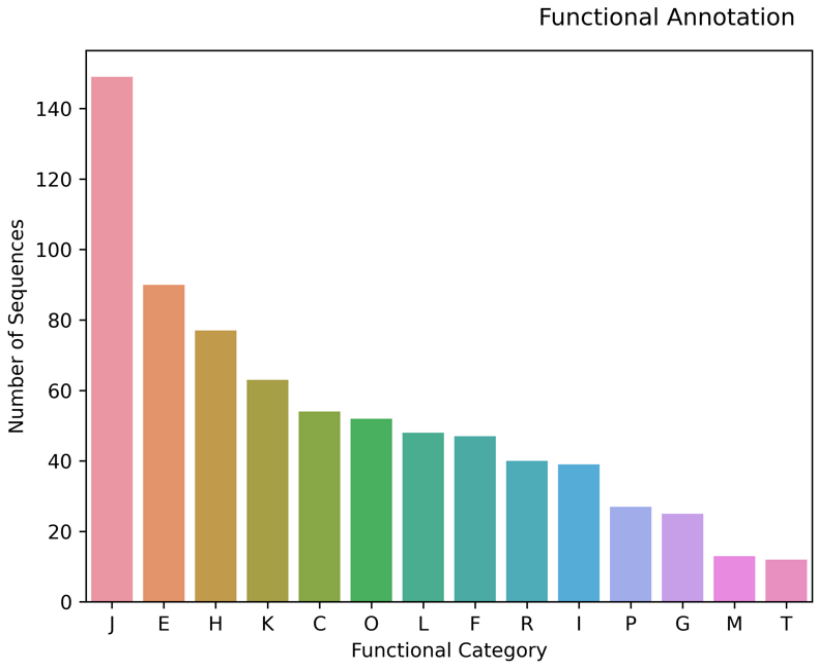


Figure 11. Graph shows distribution of Cluster of orthologous genes in *A. baumannii* isolate AB_2

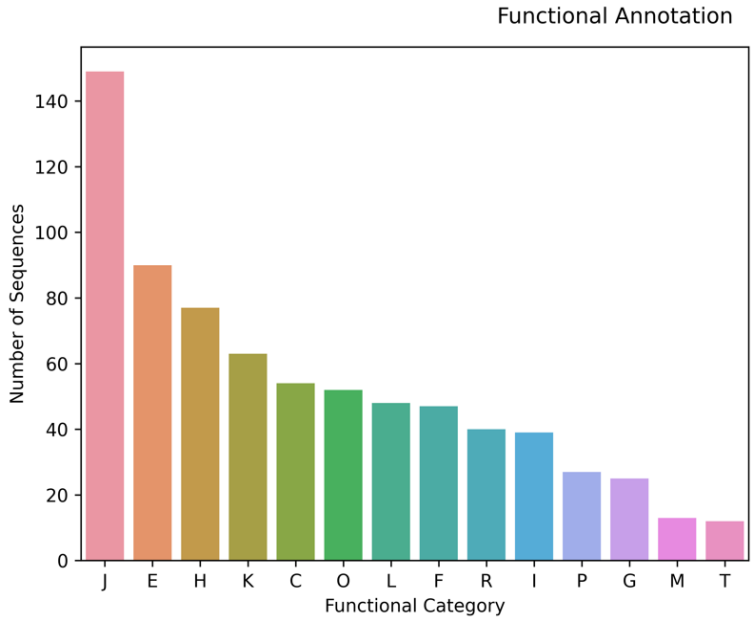


Figure 12. Graph shows distribution of Cluster of orthologous genes in *A. baumannii* isolate AB_3

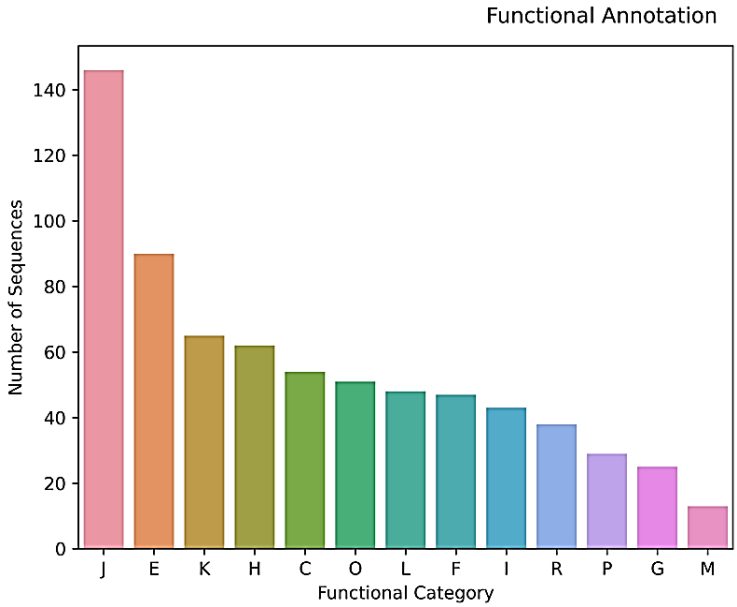


Figure 13. Graph shows distribution of Cluster of orthologous genes in *A. baumannii* isolate AB_4

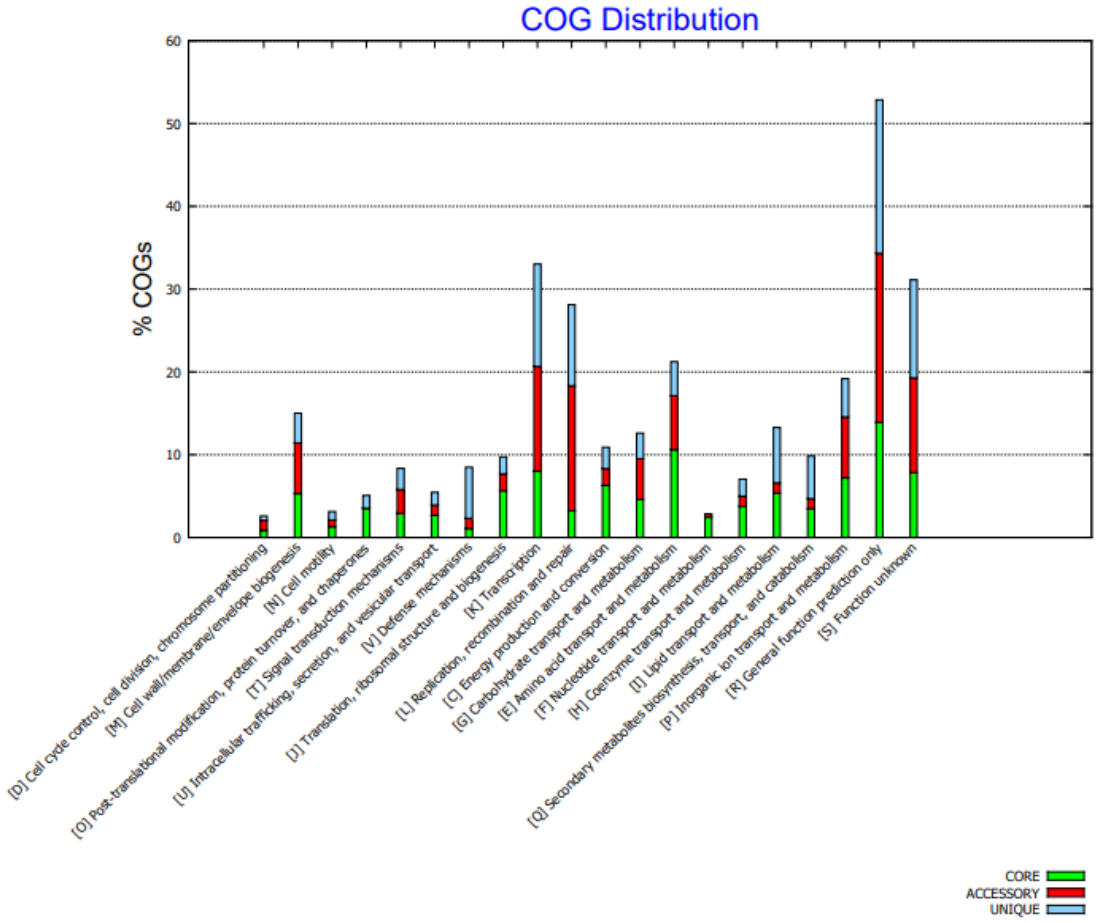


Figure 14. Graph shows distribution of Cluster of orthologous genes (COGs) in *A. baumannii* isolates.

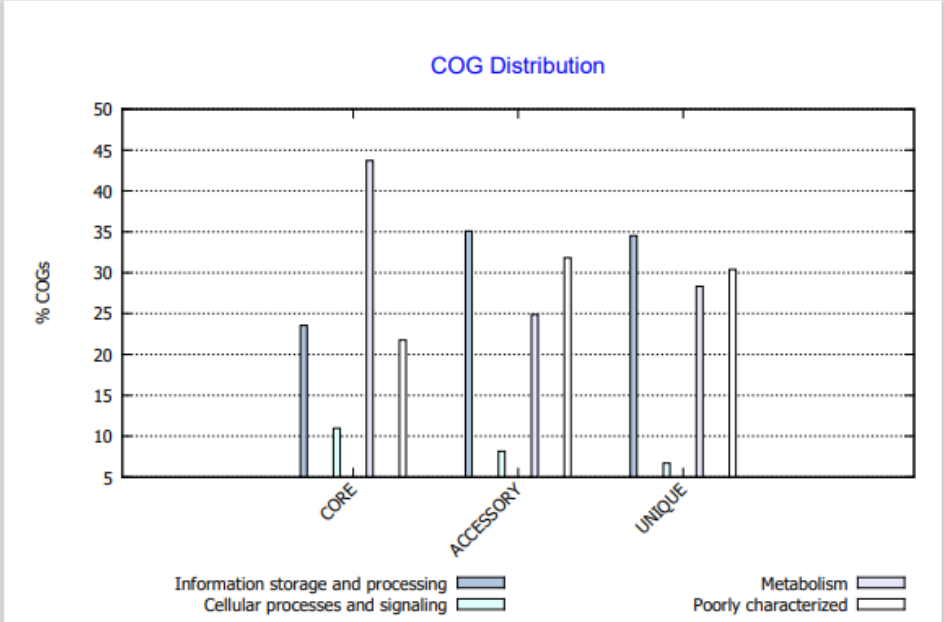


Figure 15. Graph shows distribution of Cluster of orthologous genes (COGs) based on Core, Unique and Accessory genes in *A. baumannii* isolate.

4.5 Identification and Comparative Analysis of Antibiotic Resistance Genes

Several antibiotic Resistance Genes were identified through RGI of Comprehensive Antimicrobial Resistance Database (CARD) in four isolated strains of *A. baumannii*. Which cause resistance to several class of drugs. 36,31,30 and 16 resistance genes were predicted in AB_1, AB-_2, AB_3 and AB_4 respectively. Which show resistance towards multiple class of antibiotics like fluoroquinolone (adeI, adeK, adeG, adeF, adeJ, adeK, *A. baumannii* parC, *A .baumannii* gyrA, *A. baumannii* AbaQ), carbapenem (adeI, OXA-69, NDM-1, OXA-23, adeJ, adeN_), aminoglycoside antibiotic (AAC(3)-Ia, aadA, ANT(3')-IIc, APH(3)-Ib, APH(3')_VIa), tetracycline antibiotic (adeR, tet(B), adeJ, adeC, tetR,), cephalosporin (OXA-23, adeN, NDM-1, ADC-191, OXA-69, ADC-10,), macrolide antibiotic (*A. baumannii* AmvA, abeS, msrE, mphE, adeJ), phenicol antibiotic (catI, adeK, adeN,). Several antimicrobial gene families encoding for MFS, RND and SMR antibiotic efflux pump are also identified providing resistance against macrolide, fluoroquinolone, cephalosporin, cephamycin, penam and tetracycline antibiotics. Resistance mechanism adopted by *A. baumannii* against antibiotics

includes antibiotic efflux, antibiotic target replacement, antibiotic inactivation and reduced permeability to antibiotics. The resistance profile of the above four strains have been compared with global publicly available strains which was selected on the base of sequence typing ST profile. The most common resistance conferring genes in all strains are (AbaQ, AbaF, abeS, adeL, adeI, adeK, ANT(3")-IIc, LpSB, AmvH, adeN, adeR, adeF, adeJ, parC, gyrA). dfrA1 gene was identified in locally isolated strain AB_1 which confer resistance to diaminopyrimidine antibiotic, nucleoside antibiotic (SAT-2), NDM-1 (cephamycin), BRP(MBL) (glycopeptide antibiotic).

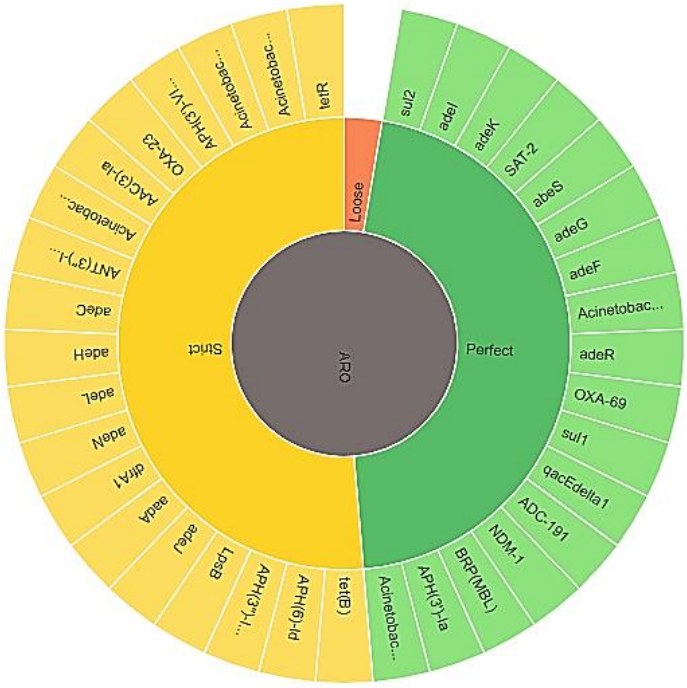


Figure 16. Shows resistance genes identified in *A. baumannii* isolates.

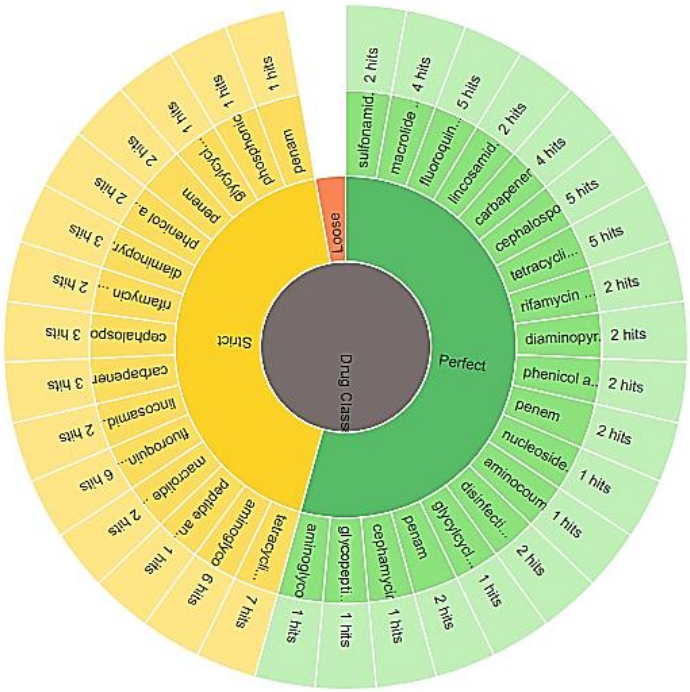


Figure 17. Drugs class against which *A. baumannii* isolates are resistant.

Table 4. Shows resistance genes, drug class and mechanism of resistance in *A. baumannii* isolates.

ARO Term	AMR Gene Family	Drug Class	Resistance Mechanism
AbaQ	major facilitator superfamily (MFS) antibiotic efflux pump	fluoroquinolone antibiotic	antibiotic efflux
AbaF	major facilitator superfamily (MFS) antibiotic efflux pump	phosphonic acid antibiotic	antibiotic efflux
abeS	small multidrug resistance (SMR) antibiotic efflux pump	macrolide antibiotic, aminocoumarin antibiotic	antibiotic efflux
adeI	resistance-nodulation-cell division (RND) antibiotic efflux pump	macrolide antibiotic, fluoroquinolone antibiotic, carbapenem, cephalosporin	antibiotic efflux
ANT(3'')-IIc	ANT(3'')	aminoglycoside antibiotic	antibiotic inactivation
LpSB	Intrinsic peptide antibiotic resistant Lps	peptide antibiotic	reduced permeability to antibiotic

parC	fluoroquinolone resistant parC	fluoroquinolone antibiotic	antibiotic target alteration
gyrA	fluoroquinolone resistant gyrA	fluoroquinolone antibiotic	antibiotic target alteration
AmvH	major facilitator superfamily (MFS) antibiotic efflux pump	macrolide antibiotic, disinfecting agents and antiseptics	antibiotic efflux
adeN	resistance-nodulation-cell division (RND) antibiotic efflux pump	macrolide antibiotic, fluoroquinolone antibiotic, carbapenem, cephalosporin, tetracycline antibiotic,	antibiotic efflux
adeJ	resistance-nodulation-cell division (RND) antibiotic efflux pump	carbapenem, cephalosporin, tetracycline antibiotic, rifamycin antibiotic, phenicol antibiotic,	antibiotic efflux
adeL	resistance-nodulation-cell division (RND) antibiotic efflux pump	fluoroquinolone antibiotic, tetracycline antibiotic	antibiotic efflux

*SMR; Small multidrug resistance antibiotic efflux pump

*MFS; Major facilitator superfamily antibiotic efflux pump

*RND; Resistance-nodulation-cell division antibiotic efflux pump

4.6 Identification and Comparative Analysis of Virulence genes

Genomes of isolated strains were checked for both structural and secreted virulence factors and it was determined that all of the strains carry different number of virulence genes i.e. strains AB_1, AB_2, AB_3 and AB_4 carry 68, 74, 74 and 75 genes respectively. This includes genes clusters encoding for Adherence, Biofilm formation, Enzyme, Immune evasion, Iron uptake, Regulation and Serum resistance etc. These virulence factors were further compared with global isolates. The predicted virulence factors in A. *baumannii* isolates were common between global strains and local isolates. A comparison of virulence factors of several *A. baumannii* given in figure.

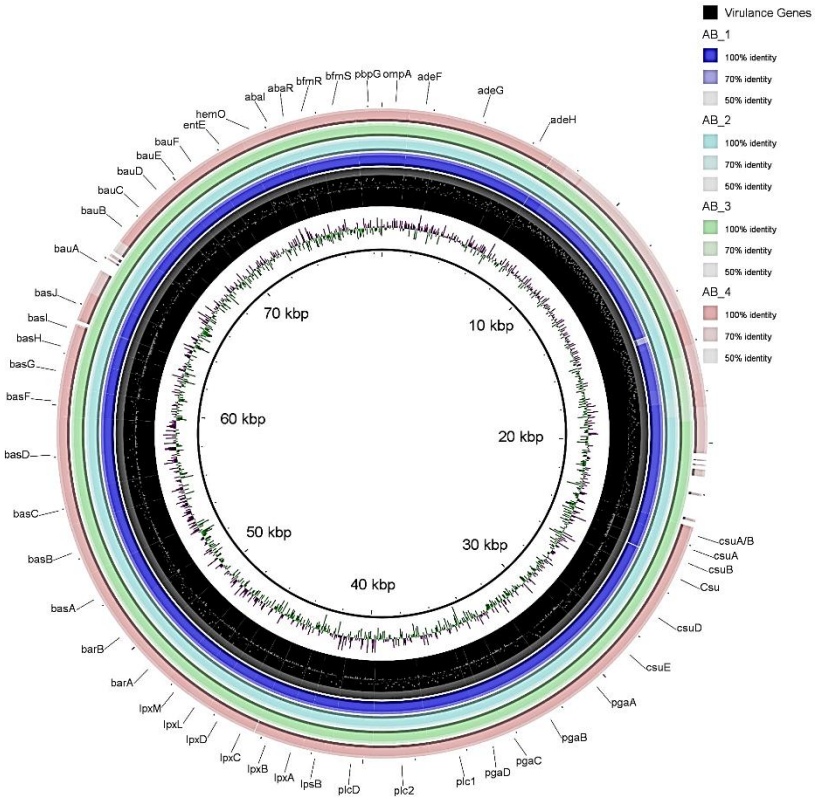


Figure 18. Shows virulence factors identified in A. baumannii isolates.

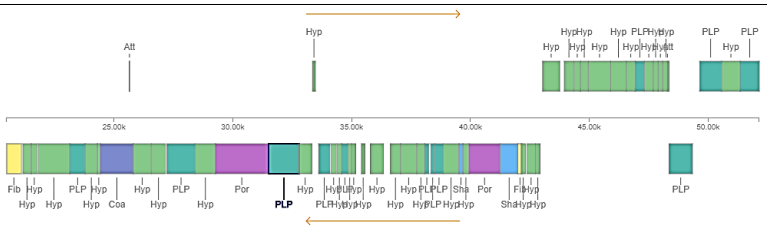
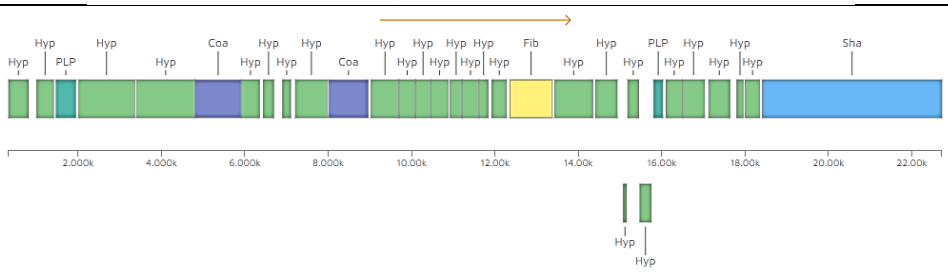
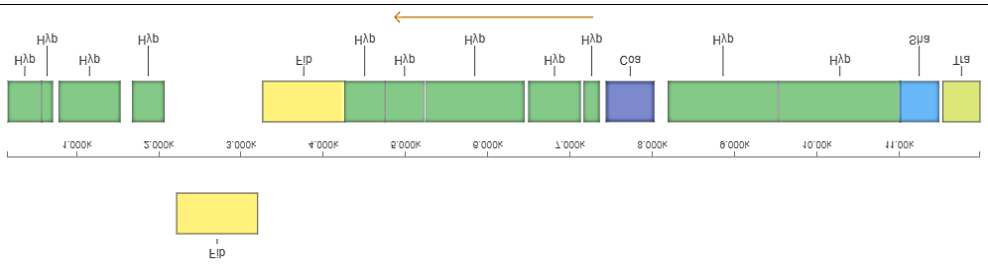
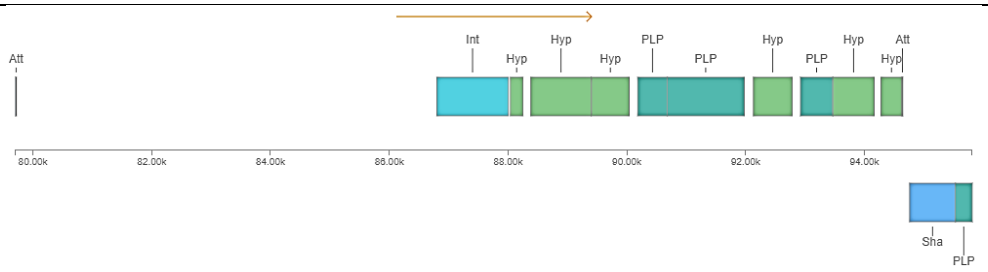
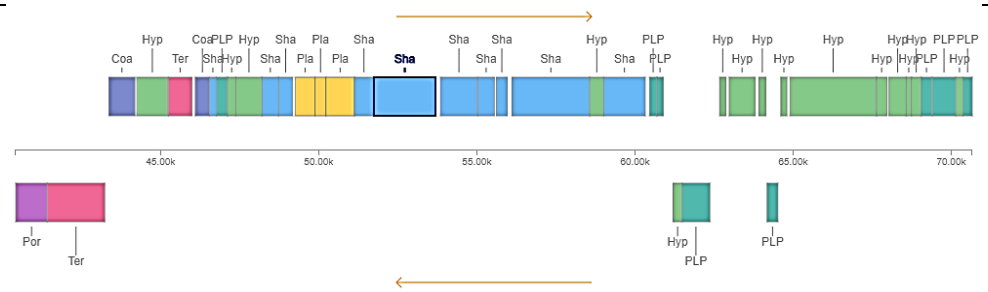
4.7 Identification and Comparative Analysis of Prophage Sequences

PHASTER identified a total of 25 regions in four genomes of isolated strains in which there is 4 regions in AB_1 (2 intact, 2 incomplete), 10 regions in AB_2 (9 incomplete, 1 questionable), 9 regions in AB_3 (incomplete) and two regions in AB_4 (incomplete) strain. No complete region was found in all four strains. Two out of eight regions were intact, and six regions were incomplete. Sequences of the phages were further screened for resistance and virulence factors and no resistance or virulence factors were found. Characteristics of the identified regions are given in the table below.

Table 5. Shows characteristics of phage sequences identified in A. baumannii isolates.

A. baumannii Strains		Prophage	Length (kb)	Total Proteins	Phage Hit Proteins	GC%	Annotations	Most common Phages	Virulence Factors
AB_1 Total regions (4)	1	Intact (150)	41.3	63	42	40.59%	lysin, tail, coat, terminase, portal, head, capsid	PHAGE_Acinet_LZ35 NC_031117	None
	2	Incomplete (50)	22.3	32	27	39.70%	head, capsid, tail	PHAGE_Klebsi_JD001 NC_020204	None
AB_2 Total regions (10)	1	Incomplete (60)	11.8	16	15	40.35%	tail, head, transposase	PHAGE_Psychr_pOW20_A	None
	2	incomplete (30)	16.1	12	7	37.19%	integrase, plate	PHAGE_Salmon_vB_SosS_Oslo	None
AB_3 Total regions (9)	1	intact (150)	30.2	41	32	39.13%	portal, terminase, capsid, tail, plate	PHAGE_Pseudo_Dobby	None
	2	incomplete (10)	17.6	8	6	37.57%	NA	PHAGE_Escher_RCS47	None
	3	incomplete (20)	8.2	10	6	39.84%	Tail	PHAGE_Salmon_SJ46	None
AB_4 Total regions (2)	1	Incomplete (40)	16.4	26	21	39.54%	head, coat, tail	PHAGE_Acinet_YMC11/11/R3177	None
	2	Incomplete (30)	7.2	13	10	38.22%	tail, integrase	PHAGE_Acinet_Bphi_B1251	None

Table 6. Detailed linear genome display of prophage sequences found in A. baumannii isolate. Color key represents various regions encoding various proteins.

Region1 (AB_1)	
Region 2 (AB_1)	
Region 1 (AB_2)	
Region 2 (AB_2)	
Region 1 (AB_3)	

4.10 Identification and comparative analysis of Genomic Island

Genomes of the isolated strains were searched for Genomic Islands (GIs) by Island viewer 4. Multiple GIs were identified using different algorithms. Genomes of isolated strains were compared with various reference genomes of publicly available A.baumannii strains (AB181-VUB, AB181-VUB, AS51, BDT201, OCU Ac18) by default. Customized reference genomes can also be selected for comparison. The genomes were also annotated for Virulence Factors (VF) and Antimicrobial Resistance (AMR) but no resistance or virulence gene were found. Other genes encoding for various proteins were present in the predicted GIs. Compared view of the genomic island in isolated strain with publicly available strain is shown in Figure.

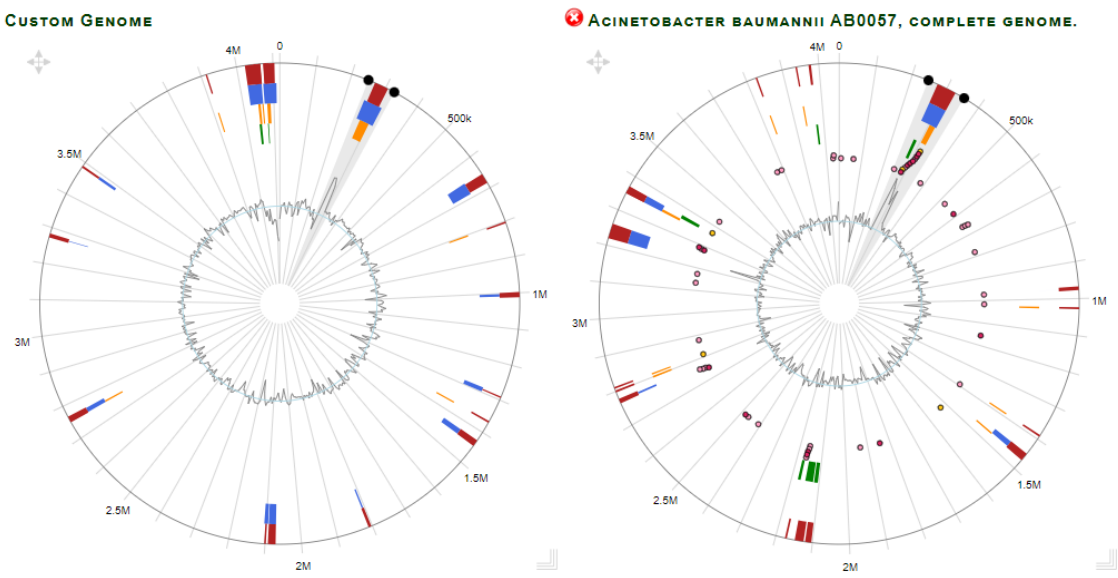


Figure 19 . Various Genomic Islands (IGs) identified in A. baumannii isolates. Different colors show prediction method. Key: IslandPick = Green, IslandPath-DIMOB = Blue, SIGI-HMM = Orange, Islander = Dark red

Figure 20. Horizontal view of GIs found in A. baumannii isolates.

Chapter 5 Discussion

Discussion

This research on Multi-Drug Resistant (MDR) *A. baumannii* provides an in-depth examination of the pathogen's genetic makeup, mechanisms of antibiotic resistance, and virulence factors. *A. baumannii* is a type of bacteria that is shaped like a coccobacillus and has a negative reaction to the Gram stain. It is known for its ability to take advantage of opportunities and cause infections in different places, particularly in healthcare settings (Huang et al., 2018). *A. baumannii* is categorized as one of the ESKAPE diseases because it is resistant to antibiotics. This makes it a significant global health problem recognized by the World Health Organization (WHO) and the Centre for Disease Control and Prevention (CDC). By analyzing 499 genomes of A. baumannii, this study reveals important information on the genetic variations and mechanisms of resistance in this bacterium. The pangenome analysis offers a concise representation of the genomic terrain. Out of a total of 33,300 genes, there are 629 core genes that are conserved in 90-100% of the strains, and 1447 soft core genes that are present in 68-72% of the strains. In addition, there are 2345 accessory genes that are present in 10-68% of strains, and a remarkable 38,878 distinct genes that are detected in 10% of strains (Jafari et al., 2013). The open pangenome of *A. baumannii* demonstrates its potential to continuously acquire genes, highlighting its genomic flexibility. The correlation between genome counts and the increase of unique genes emphasizes the adaptability of organisms. This highlights the need for ongoing research to monitor and comprehend the changing genomic environment. Utilizing the Pasteur system for Multi-Locus Sequence Typing (MLST), 499 strains exhibit 88 unique sequence types (STs), indicating a significant degree of genetic diversity (Ibrahim, Al-Saryi, Al-Kadmy, & Aziz, 2021). The ST2 strain of *A. baumannii*

is the most prevalent, with a total of 240 occurrences, highlighting the extensive evolutionary diversity of this bacterium. The MLST analysis highlights genetic diversity, underscoring the importance of focused interventions that take into account the various genetic backgrounds that contribute to the pathogen's ability to withstand challenges. Functional annotation by the Cluster of Orthologous Genes (COGs), provides insights on the strains' participation in diverse metabolic processes (Chen et al., 2015). The presence of strain-specific changes in COG involvement underscores the genetic diversity observed in functional characteristics. Strain AB_4 demonstrates a greater degree of COG participation in coenzyme metabolism as compared to transcription. The variations in COG profiles offer crucial insights into the metabolic preferences of the strains, which can possibly impact their adaptation and survival strategies. *A. baumannii's* resistance profile is far more serious than previously thought, as revealed by the identification and comparative analysis of antibiotic resistance genes. The isolated strains (AB_1, AB_2, AB_3, AB_4) have predicted resistance genes that span several drug classes, such as aminoglycosides, tetracyclines, cephalosporins, carbapenems, phenicols, and tetracyclines. The analysis of worldwide strains demonstrates the presence of both common and distinct resistance genes, indicating regional variations. The high occurrence of resistance-associated genes such as AbaQ, AbaF, abeS, adeL, and adeI underscores the necessity for antibiotic stewardship programs tailored to specific regions (Antunes, Visca, Towner, & disease, 2014). Analysis of virulence genes revealed a wide range of factors that contribute to the pathogenicity of *A. baumannii*. The presence of genes linked to immune evasion, biofilm formation, adhesion, and iron uptake improves our comprehension of the processes by which *A. baumannii* causes infections. Prophage sequences and genomic islands, despite lacking identified resistance or virulence genes, enhance the pathogen's ability to adapt and change its genetic makeup. These factors can contribute to the processes of evolution, adaptation,

or interactions with the host environment. The study's importance rests in its potential to influence infection control tactics and therapeutic procedures (Kyriakidis et al., 2021). The genomic variety, resistance mechanisms, and virulence variables that have been uncovered provide a foundation for precise and focused therapies. Further investigation could focus on the creation of antibiotic guidelines tailored to individual regions, taking into account the distinct resistance profiles. In addition, investigating the found prophage sequences and genomic islands may reveal concealed aspects of *A. baumannii* evolution and adaptation. The study's results highlight the pressing necessity for ongoing monitoring and investigation to proactively address *A. baumannii's* adaptive tactics. The open pangenome nature of a genome implies a constantly changing genomic environment, which requires continuous genomic surveillance. Effective infection control strategies and antibiotic stewardship programs require collaborative efforts among researchers, doctors, and policymakers. Ultimately, this study offers a thorough comprehension of MDR *A. baumannii*, by integrating genetic studies with functional observations. The genetic diversity, antibiotic resistance, and virulence variables that have been identified provide a basis for future research and intervention efforts. This work provides valuable guidance for researchers and practitioners in tackling the growing problem of multidrug-resistant infections caused by *A. baumannii* in hospital settings (Kyriakidis et al., 2021).

Chapter 6

Conclusion and Prospects

Whole genome sequencing and comparative genome analysis of local isolates of A. baumannii reveals that the isolates are virulent and multi drug resistant. Out of four isolates three isolates belong to ST1 which is second most prevalent sequence type after ST2 while one isolate belongs to ST1509 which is a unique ST. It was also concluded that the isolates AB_1 AB_2 and AB_3 was closely related to local strains from Peshawar Pakistan while one isolate AB_4 was closely related to strain AB16-VUB from Belgium. From comparative genome analysis it was revealed that high genomic diversity was found among A. baumannii genomes which contribute to the adoptability and increasing antibiotic resistance.

As the antibiotic resistance is increasing day by day because of the excessive use of antibiotics. Therefore, alternative strategies need to be adopted to overcome the challenge. Vaccine development and combination therapy can play their role in combating the challenge. For better understanding of A. baumannii more genomes would be made available to study genetic diversity and population structure. Also, appropriate surveillance of A. baumannii will be needed to determine its prevalence in the region, routes of infection and local antibiotics resistance trends.

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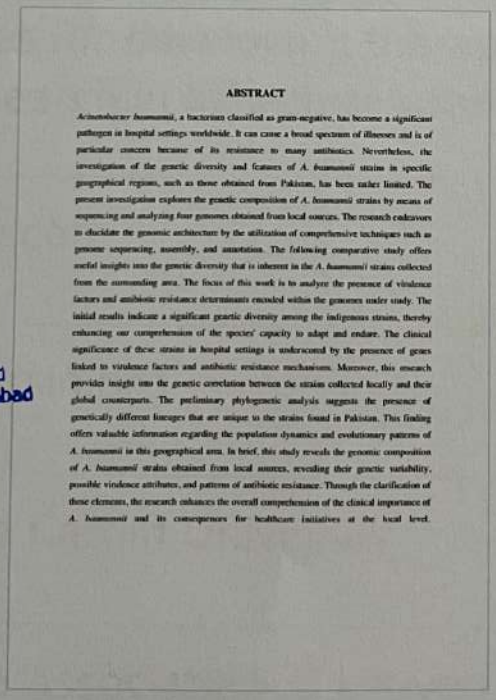

ABSTRACT

Acinetobacter baumannii, a bacterium classified as gram-negative, has become a significant pathogen in hospital settings worldwide. It can cause a broad spectrum of illnesses and is of particular concern because of its resistance to many antibiotics. Nevertheless, the investigation of the genetic diversity and features of *A. baumannii* strains in specific geographical regions, such as those obtained from Pakistan, has been rather limited. The present investigation explores the genetic composition of *A. baumannii* strains by means of sequencing and analyzing four genomes obtained from local sources. The research endeavors to elucidate the genomic architecture by the utilization of comprehensive techniques such as genome sequencing, assembly, and annotation. The following comparative study offers useful insights into the genetic diversity that is inherent in the *A. baumannii* strains collected from the surrounding area. The focus of this work is to analyze the presence of virulence factors and antibiotic resistance determinants encoded within the genomes under study. The initial results indicate a significant genetic diversity among the indigenous strains, thereby enhancing our comprehension of the species' capacity to adapt and endure. The clinical significance of these strains in hospital settings is underscored by the presence of genes linked to virulence factors and antibiotic resistance mechanisms. Moreover, this research provides insight into the genetic correlation between the strains collected locally and their global counterparts. The preliminary phylogenetic analysis suggests the presence of genetically different lineages that are unique to the strains found in Pakistan. This finding offers valuable information regarding the population dynamics and evolutionary patterns of *A. baumannii* in this geographical area. In brief, this study reveals the genomic composition of *A. baumannii* strains obtained from local sources, revealing their genetic variability, possible virulence attributes, and patterns of antibiotic resistance. Through the clarification of these elements, the research enhances the overall comprehension of the clinical importance of *A. baumannii* and its consequences for healthcare initiatives at the local level.

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