Evaluation of Biofilm Formation in Multi Drug Resistant Isolates of *Acinetobacter baumannii*

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DECLARATION

The material and information contained in this thesis is my original work that was carried out at the Department of Microbiology, Quaid-i-Azam University, Islamabad. I have not previously presented any part of this work elsewhere for any other degree.

Ayesha Azher

DEDICATION

This thesis is dedicated to my beloved parents late **Muhammad Azher** and **Tasneem Kousar**, and my grandfather late **Jalal Din**, who have been my source of inspiration and gave me strength when I thought of giving up. Though their demise made a wide gap in my life but the values they taught me prepared me for a human and meaningful life. Their memories gave me the nourishment to fulfill my long-cherished dream. Special thanks to my mother for her moral, spiritual, emotional and financial support. This moment fills me with gratitude to them...

CERTIFICATE

This thesis submitted by **Ayesha Azher** is accepted in its present form by the Department of Microbiology, Quaid-i-Azam University, Islamabad, in partial fulfillment of the requirements for the Degree of Master of Philosophy in Microbiology.

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LIST OF ABBREVIATIONS

ESBL	Extended spectrum beta lactamase
MDR	Multidrug resistant
NNIS	National nosocomial infections surveillance
HAI	Hospital acquired infections
OMPs	Outer membrane proteins
PBPs	Penicillin binding proteins
ABC	ATP-binding cassette
EPS	Extracellular polymeric substance
VAP	Ventilator associated pneumonia
PLA	Phospholipase A
T1SS	Type 1 secretion system
AHL	Acyl homoserine lactone
MFS	Major facilitator superfamily
MATE	Multidrug and toxic compound extrusion
RND	Resistance nodulation division
OMV	Outer membrane vesicles

CarO	Carbapenem-associated outer membrane protein
AMC	Amoxicillin-Clavulanate
CTX	Cefotaxime
CAZ	Ceftazidime
EDTA	Ethylenediamine tetra acetic acid
FEP	Cefepime
CRO	Ceftriaxone
TSB	Tryptic soy broth
MHB	Muller Hinton broth
MIC-b	Minimum inhibitory concentration of biofilm
MRC	Minimum regrowth concentration
MBEC	Minimum biofilm eradication concentration

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ABSTRACT

Acinetobacter baumannii is an important troublesome nosocomial pathogen and predominant cause of catheter and ventilator associated infections along with skin and bloodstream infections. The capability of multidrug resistant (MDR) *A. baumannii* to adopt various mechanisms of antimicrobial resistance against multiple classes of antibiotics enables its long-term survival in clinical settings and promotes the dissemination of MDR strains over the world. Biofilm forming ability of *A. baumannii* on both biotic and abiotic substrates is liable for high prevalence of hospital acquired infections, plays a key role in its pathogenesis and making alternative treatment options more challenging. The first objective of this research was to check the extended spectrum beta lactamase (ESBL) enzyme production in 100 clinical strains of *A. baumannii* and out of 100 only 28% strains were ESBL producers. ESBL producing strains were further screened for *TEM-1*, *SHV-1* and *CTXM-15* and their occurrence was 89%, 32% and 57% respectively. Biofilm formation potential of 100 clinical strains of *Acinetobacter baumannii* was also determined by microtiter plate and crystal violet staining method. Out of 100 only 35% strains were strong biofilm formers, 60% strains were moderate biofilm formers and only 5% strains were weak biofilm formers. Previous data showed that all these isolates were 100% sensitive to colistin. So, the present study also checked the effect of colistin on strong biofilm formers and compared its MIC-b with MIC-p of *A. baumannii*. MIC-p and MIC-b of isolate no 71, 72, 58 and 92 were performed by test tube method and microtiter plate method respectively and the values of MIC-p and MIC-b of two isolates were same and two isolates have MIC-b values two-fold more than MIC-p. Last objective of this research was to check the minimum regrowth concentration (MRC) and minimum biofilm eradication concentration (MBEC) of colistin on strong biofilm formers of *A. baumannii*. The MRC values of strong biofilm formers are 8 folds more than MIC-p and MBEC values are 128 folds more than MIC-p of strong biofilm formers. So, our study indicated that higher concentration of colistin can completely eradicate the strong biofilm formers of MDR *A. baumannii.*

INTRODUCTION

Acinetobacter baumannii is a Gram-negative, non-fermentative, aerobic, non-motile coccobacilli that can cause healthcare-related infections and can stay in the environmental settings and on healthcare workers' hands for longer durations (Sunenshine *et al.*, 2007). It is commonly present in clinical settings, soil, water, skin and mucosal membrane of intestine, respiratory tract and urinary tract (Wendt *et al.*, 1997). The survival of *A. baumannii* in the living arrangements even after being dried for many weeks facilitates its proliferation in clinics by contamination. Due to its broad spectrum of antimicrobial resistance *A. baumannii* has been declared as a "high alert" pathogenic bacterium in past few years, causing concern between many medical professionals (Cerqueira et al., 2011).

Several infections were caused by *A. baumannii* in military people injured in war during the 2003–2004 epidemic of MDR *Acinetobacter baumannii*. This led to dissemination of MDR strains of *A. baumannii* from military hospitals towards other places, as well as an increase in the rate of antibiotic resistance gene distribution (Scott *et al.*, 2007). According to the WHO, A. baumannii is among the most dangerous ESKAPE pathogen. ESKAPE is basically group of six pathogenic bacteria that includes *Enterococcus faecium*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *A. baumannii* and Enterobacter species. These pathogenic bacteria have successfully evaded themselves from antibacterial effects of different antibiotics (Boucher *et al.*, 2009).

The genus Acinetobacter comprises a diverse variety of bacteria that doctors and scientists have labelled as "the most troublesome." This genus has experienced significant taxonomic changes. Traditional phenotypic approaches, such as use of biochemical testing, have created many challenges in the identification of Acinetobacter species. The genus Acinetobacter now contains 59 different species, each with its own name. There have been reports of 11 properly named but not officially published species and roughly 15 tentative species with designation (Vaneechoutte *et al.*, 1995). On the other hand, Acinetobacter species identification is difficult. using biochemical tests and automated processes, resulting in incorrect identification (Jain *et al.*, 2006). Even though many

molecular approaches are accessible, but they are rarely used for identification in diagnostic labs (Wisplinghoff *et al.*, 2012).

Most of species of Acinetobacter are mainly spread to patients by colonization of environmental settings and through contaminated hands of health care personnel (Spellberg and Bonomo 2013). Transmission of *A. baumannii* through inhalation has also been reported. A case report revealed that during endotracheal suctioning of a patient on ventilator a healthcare professional inhaled airborne droplets of *A. baumannii* and acquired severe pneumonia (Whitman *et al.*, 2008). Another investigation revealed that carbapenem-resistant *Acinetobacter baumannii* (CRAB) was detected in about fourth half of air samples collected from patient's rooms and all those patients were suffering from various infections caused by carbapenem resistant *A. baumannii*. This study also confirmed that only infected patients were source of transmission of *A. baumannii* in air because this pathogen was not detected in air ducts of contaminated rooms.

According to the National Nosocomial Infections Surveillance (NNIS) System, the prevalence of infections caused by *A. baumannii* in the U.S jumped by 54% between 1987 and 1996. (McDonald *et al.*, 1999). The existing pipeline of new therapies, especially for *A. baumannii*, is woefully insufficient. The inadequacy of approved animal models for the preclinical investigation of *A. baumannii* infections is one major hurdle that continues to impede antibiotic development efforts (Boucher *et al.*, 2009).

Acinetobacter baumannii is linked to both community and hospital-acquired infections. Community acquired infections are associated with a high death rate and are frequently marked by a serious onset of pneumonia with secondary septicemia. Because *A. baumannii* primarily affects the mucus membrane in the pharynx, trachea, and bronchi, the respiratory system is the major route of infection (Howard *et al.*, 2012). Shamsizadeh et al did air sampling in four different wards in four different hospitals and observed that 11% of them tested positive for *A. baumannii*, with the highest prevalence in intensive care units. The study also revealed that the tap water could be a source of *A. baumannii* transmission, which is worrying because it could indicate contamination of the water

supply. Kotay *et al* on the other hand, discovered that this pathogen might be spread through handwashing basins.

Hospital Acquired pneumonia and bacteremia are the two most prominent clinical presentations of *Acinetobacter baumannii*. Air borne droplets containing *A. baumannii* are the cause of nosocomial pneumonia (Raad *et al.*, 2011). The presence of an indwelling catheters is an excellent source for the environmental dissemination of Acinetobacter. Acinetobacter easily attaches to plastic tubes and can form biofilms on it. Skin and soft tissue infections, endocarditis, meningitis, and urinary tract infections particularly linked with indwelling catheters and lithotripsy ducts are all well-known clinical symptoms of *A. baumannii* (Gil *et al.*, 2012).

Because of the rise in antibiotic resistance and the emergence of new strains conferring resistance to almost all currently available antibiotics, *Acinetobacter baumannii* is getting a lot of popularity (Seifert *et al.*, 1993). *A. baumannii* is innately resistant to so many drugs that mainly involves aminopenicillins, 1st- and 2nd generation cephalosporins and chloramphenicol. It also has a greater potential for acquiring antibiotic resistance mechanisms to broad-spectrum beta-lactams, fluoroquinolones, tetracyclines and aminoglycosides (Vila *et al.*, 1993).

Various investigations have revealed that the number of strains of *A. baumannii* resistant to above mentioned drugs is increasing. The ability to upregulate intrinsic mechanisms of resistance and acquiring novel determinants has earned *A. baumannii* a lot of attention (Bacher *et al.*, 2006). All of the basic mechanisms of antimicrobial resistance such as alteration in target site, enzymatic degradation of drug, active efflux and decreased influx of drug confer antibiotic resistance in *Acinetobacter baumannii*.

Enzymatic degradation of many antibiotics with beta-lactam rings by beta-lactamases is most commonly present antibiotic resistance mechanism in *A. baumannii*. Beta - lactamases are the most diversified class of enzymes linked to antimicrobial resistance, with more than fifty different enzymes, or their allelic variants, discovered in *A. baumannii* up till now. Beta lactamases are categorized into four main classes that includes Ambler class A, Ambler class B, class C and Ambler class D. Both class A and

class D are dependent on serine while class B or metallo beta-lactamses are Zinc dependent because they have zinc at their active site (Seward *et al.*, 1998). Nonenzymatic resistance mechanisms such as alteration in outer membrane proteins (OMPs), multi drug efflux pumps, and modifications in expression of penicillin-binding proteins (PBPs) and their binding affinity towards various antibiotics also contribute to beta-lactam resistance in *A. baumannii*.

At least 9 different enzymes have been linked to resistance against aminoglycoside, and some isolates have multiple combinations of them. Within class 1 integrons, MDR strains of *A. baumannii* have a high prevalence of genes that encode for aminoglycoside-modifying enzymes. Binding affinity of aminoglycosides with bacterial ribosomes is reduced by 16S rRNA methylation, which imparts resistance to all potentially effective aminoglycosides such as neomycin, tobramycin and amikacin. Aminoglycoside resistance is also caused by different types of efflux pumps that mainly includes AdeABC and AbeM efflux pump (Doi *et al.*, 2007).

The binding affinity of fluoroquinolones towards topoisomerase IV and DNA gyrase is disrupted in *A. baumannii* when *gyrA* and *parC* genes undergo point mutation. Multidrug efflux pumps including resistance nodulation division type efflux pumps and multidrug and toxic compound extrusion (MATE) transporters are substrates for several quinolones. *A. baumannii* has yet to be found with plasmid-based quinolone resistance conferred by *qnr* genes (Higgins *et al.*, 2004).

Despite recent publications revealing increased polymyxin resistance in *A. baumannii* but the exact polymyxin resistance mechanism is unknown. Modifications in outer membrane proteins (OMPs) have also been reported for decreased colistin susceptibility in *P. aeruginosa*. Phosphoethanolamine transferase, which is regulated by *pmrC* gene, adds phosphoethanolamine to the lipid A in *A. baumannii*, causing colistin resistance (Dortet *et al.*, 2018).

Ribbera and colleagues reported that resistance against tetracycline is associated with the *tet(A)* gene. According to Foong and his colleagues overexpression of the *tet(A)*, *tet(K)*, *tet(E)* and *tet(G)* genes provides antibiotic resistance to minocycline, and tetracycline.

Tet(A) also contributes in tigecycline resistance by interacting with efflux pumps with the help of resistance nodulation division system. This class of antibacterial drugs is vulnerable to efflux through multidrug efflux pumps, like the AdeABC transporters.

Biofilm formation also confers antibiotic resistance in *A. baumannii*. A microbial biofilm is a cluster of microorganisms that attach to one other on a biotic or abiotic substrates. These adhering cells are frequently enclosed in a matrix composed of extracellular polymeric substances (EPS) (Gonzalez *et al.*, 2001). Biofilm production may be caused by colonization of *A. baumannii* on mucosal lining or contaminated medical equipment such as intravenous or urinary catheters and endotracheal tubes. Colonization of *A. baumannii* on such medical devices is raising the risk of bacteremia and other respiratory infections (Cabral *et al.*, 2011).

Highly regulated systems, such as those dependent on the expression of antibiotic resistance genes, environmental factors (such as surface porosity, fluid movement and accessibility to food) or cell density, control mechanisms involved in biofilm development in *A. baumannii* (Jamal *et al.*, 2015). A study was conducted in 2013 on *A. baumannii* ATCC 17978 to check the expression of genes in planktonic and biofilm forming strains. The transcriptome profiles of planktonic and biofilm forming bacteria revealed that 1,621 genes were upregulated and overly expressed in biofilm forming bacterial cells relative to planktonic bacterial cells, as well as 55 genes that were only expressed in planktonic bacteria (Rumbo *et al.*, 2013).

Many virulence factors confer the development of biofilm in *Acinetobacter baumannii* such as biofilm associated proteins, EPS matrix, poly-β-(1,6)-N-acetyl glucosamine, chaperon-usher pilus, outer membrane proteins and quorum sensing (Tomaras *et al.*, 2003). Outer membranes proteins and pili help in attachment and colonization of *A. baumannii* on plastic objects and epithelial cell layer of alveoli and bronchi (Gaddy *et al.*, 2009). Biofilm formation is a complex mechanism in which the growth style of motile planktonic bacterial cells is changed into sessile mode and cells get attached to each other.

In the first step of biofilm formation bacterial cells attach with living and non-living substrates. The attachment of bacterial cells with non-polar and hydrophobic substrates such as plastic materials is stronger than attachment with polar and hydrophilic substrates such as stainless steel. After the adherence with substrate extra cellular polysaccharide matrix is secreted that maintains bacterial cells' attachment with their substrate (Jamal at al., 2018). Then microbial cell proliferation begins, resulting in the formation of multiple microcolonies and biofilm becomes mature. In biofilm bacterial cells communicate with each other in the form of signals through quorum sensing (Stewart *et al.*, 2008).

The transfer of beneficial mutations, enhanced accessibility of bacterial cell towards nutrients, and increased antibiotic tolerance level are also facilitated by quorum sensing (Hannan *et al.*, 2010). Eventually, the sessile mode of growth transforms into a planktonic mode, resulting in biofilm dispersion in surroundings. Biofilm dissemination in surroundings is triggered by a lack of oxygen or food, as well as the decomposition of extra cellular polysaccharide matrix by saccharolytic enzymes (Jamal at al., 2018).

Due to increased antibiotic resistance towards broad spectrum antibiotics and strong biofilm forming capacity, proper treatment of *A. baumannii* infections may be more challenging. Different antimicrobial peptides, bacteriophage therapies and natural compounds such as derivatives of microbes, animals and plants may be more beneficial for treating *A. baumannii* infections (Eze *et al.*, 2018).

AIM AND OBJECTIVES

Aim

Aim of this research was evaluation of biofilm formation in multi drug resistant (MDR) isolates of *Acinetobacter baumannii*.

Objectives

1. To detect the extended spectrum beta lactamases production by ESBL test in *A. baumannii*.
2. To detect the occurrence of ESBL genes *TEM-1*, *SHV-1* and *CTXM-15* in *A. baumannii*.
3. To evaluate the biofilm formation in clinical strains of *A. baumannii*.
4. To determine the MIC-p of colistin on strong biofilm formers of *A. baumannii*.
5. To determine the MIC-b, MRC/MBIC and MBEC of colistin on strong biofilm formers of *A. baumannii*.

LITERATURE REVIEW

Acinetobacter baumannii is a Gram-negative opportunistic nosocomial pathogen responsible for about 2-10 % of all Gram-negative nosocomial infections. The Infectious Disease Society of America stated that *A. baumannii* is among the six most common multidrug-resistant bacteria found in clinical settings around the world (Antunes *et al.*, 2014). It is aerobic, oxidase negative, non-fastidious, catalase positive, Gram-negative coccobacilli that is currently classified as belonging to the genus Acinetobacter. It is non-motile, non-fermentative, non-spore former bacteria that has GC content of 39% to 47% mol (Howard *et al.*, 2012).

It produces smooth, domed shaped yellow to greyish color colonies on agar medium with entire edges at 37°C (Joshi, 2013). During rapid growth phase size of this bacteria ranges from 1.0-1.5μm by 1.5-2.5μm. It produces white or creamy mucoid colonies with diameter of 1-2mm on Blood agar whereas on Leeds Acinetobacter medium it produces pink color colonies with purple color background. On Eosin Methylene Blue agar colonies are bluish grey color while on Herellea agar Acinetobacter colonies appear in pale lavender color. Acinetobacter is non-lactose fermenter and displays light lavender color colonies on MacConkey agar. Most of the strains of *Acinetobacter baumannii* are not able to produce nitrites from nitrates. The optimum temperature of *A. baumannii* is 35°C to 37°C. De-staining of *Acinetobacter baumannii* is not easy as it retains purple color of principal stain, crystal violet which can cause false identification as Gram positive coccobacilli (Almasaudi, 2018).

2.1 Taxonomy

Due to complex classification schemes devised by taxonomist earlier with over 40 identified species belonging to the genus, the exact phylogeny and taxonomic classification of Acinetobacter genus has been muddled. In 1911 Beijerinck a Dutch scientist first ever isolated Acinetobacter from environmental samples on calcium acetate containing minimal media (Howard *et al.*, 2012). Genus Acinetobacter was originally known as Micrococcus calco-aceticus and after 43years Acinetobacter genus was

presented by Brisou and Prevot to distinguish it from non-motile species of Achromobacter genus. The name Acinetobacter comes from the Greek word "akinetos" which means "non-motile" (Brisou and Prevot 1954). Baumann presented a comprehensive study on microorganisms like *Alcaligenes hemolysans, Herellea vaginicola, Micrococcus calco-aceticus, Mima polymorpha, Bacterium anitratum* and *Moraxella lwoffi* and after that the genus Acinetobacter had gained widespread acceptance. He concluded that all these species are belonged to only one genus and could not be further subdivided into subspecies on the basis of their phenotype (Baumann *et al.*, 1968). On the basis of DNA-DNA hybridization almost 25 different genomic species have been found and the second genomic species is *Acinetobacter baumannii* (Schreckenberger *et al.*, 2003).

2.2 Genome

Acinetobacter baumannii XH386 has a genome of 4,199,500 nucleotides with G+C content of 39.1%. Chromosome of *A. baumannii* XH386 is circular, and its size is 4,087,343 bp. *A. baumannii* XH386 also contains a circular plasmid with a size of 112,157bp. A study from China reported 3968 protein-coding genes and 94 RNAs among the 4062 genes. This study also reported 26 pseudogenes (Fang *et al.*, 2016).

Korean research investigated that *Acinetobacter baumannii* 1656-2 has genome of 3,940,614 bp with coding region of 88.0 %. This study also revealed that *A. baumannii* 1656-2 has 3,715 open reading frames along with 18 genes encoding rRNA and 71 genes encoding tRNA. 1656-2 strain is composed of two plasmids named as p1 and p2. The size of p1 plasmid is74,451 base pairs and size of p2 is 8,041base pairs. p1 plasmid is composed of 101 open reading frames while p2 plasmid has 8 open reading frames.

Thirty-nine genes have been identified during the genotypic analysis of MDR A. *baumannii* 1656-2. *blaPER-1*, *blaOXA-109*, and the tellulite resistance genes have also been reported. 17 *ISAba1* copies have been detected in genomic analysis with 15 copies of chromosome and 2 copies of plasmid. These copies are mainly linked to antibiotic resistance in Acinetobacter species. 13 genes have been identified for the synthesis of polyglutamic acid (PGA) along with thirty-four genes for cell adhesion. This study

reported more genes encoding vesicular transport, cell traffic and cell motility in the genome of *A. baumannii* 1656-2 than in other strains of *A. baumannii* (Park *et al.*, 2011).

2.3 Habitat

Most of the species of genus Acinetobacter are frequently thought to be present everywhere in environment and they can survive in variety of settings including both living and non-living surfaces. Many species of Acinetobacter are commonly present in soil and water. *A. baumannii* is most commonly present in hospital settings especially in ventilators, indwelling catheters and intensive care units (Fournier *et al.*, 2006). Pathogenic species of Acinetobacter mostly target exposed skin and moist tissues like mucosal lining of respiratory tract, urinary tract and gastrointestinal tract. Infected skin and soft tissues have a "peau d'orange" (like the skin of an orange) appearance at first then a sandpaper-like appearance that gradually forms small vesicles over the skin (Sebeny *et al.*, 2008). It is yet unknown if *A. baumannii* isolated from other sources like animals, plants, water or soil, may be due to environmental contamination from the hospital settings or indicate another natural reservoir of this species (Antunes *et al.*, 2014) Although *A. baumannii* causes skin infections but a study indicated that this bacterium colonizes only 3% of the population. Acinetobacter was also found in 22% of body lice samples collected from homeless people, indicating that the pathogen may have another important reservoir (Scola and Raoult 2004).

2.4 Pathogenesis

In order to assert infection, a pathogen must adhere to cells; however, *Acinetobacter baumannii* has a limited ability to adhere to cells/mucosal cells in contrast to other pathogens such as *P. aeruginosa*, *Neisseria meningitides*, Campylobacter and Helicobacter (Tibor *et al.*, 2013). The decreased virulence of *A. baumannii* is due to its limited adherence and penetration; yet its hydrophobic propensity allows it to adhere to foreign substrates such as plastics used within intravascular catheters. Surface hydrophobicity has been shown to be strongly exhibited in isolates obtained from patients in comparison to normal skin microflora (Peleg *et al.*, 2008). So far, five primary pathogenic mechanisms have been identified:

Biofilm formation: Attachment through pili and the consequent development of biofilms increase colonization of abiotic surfaces and objects. Biofilm development and maturation require the biofilm-associated protein (Bap). *Bap* is also helpful for colonization because it promotes cell adhesion (Richards *et al.*, 2015). Biofilm formation appears to be necessary for Acinetobacter's capability to withstand harsh circumstances, as strong biofilm formers are less vulnerable to dryness than weak biofilm formers (Greene *et al* 2016).

Outer membrane protein A (ompA): It has been linked to improved adherence, particularly to epithelial cells of trachea and bronchi. It attaches to mitochondria and nuclei and enables the apoptosis - inducing protein cytochrome c to be expressed, leading to apoptosis (Schweppe *et al* 2015). *Acinetobacter baumannii* avoids being killed via alternative complement pathway through neutralizing factor H, a fundamental modulator of the process, with the support of outer membrane protein A. OmpA stimulates differentiation of CD4+ lymphocytes, dendritic cell activation and maturation, and premature cell death (Lee *et al.*, 2010).

K1 capsule: About a third of isolates of *A. baumannii* form a polysaccharide capsule that interacts with the liposaccharide of bacterial cell wall to block activation of complement system (Kaplan *et al.*, 1985). Phagocytosis may also be inhibited by the bacterial capsule. Siderophore-mediated iron-acquisition system: Acinetobacter may withstand iron deficiency for longer durations. This is due to a catechol siderophore called "acinetobactin" which can sequester iron from the host cells (Goel and Kapil 2001).

Fimbriae: As previously stated, fimbriae assist in the attachment of the microorganism to its surrounding substrates. They also aid in the colonization of living surfaces like bronchial and tracheal epithelial cells (Seifert *et al.*, 1993).

2.5 Clinical significance

The most frequently known clinical symptoms of infection caused by *A. baumannii* include bloodstream infections and ventilator-associated fulminant pneumonia. Acinetobacter can also invade skin, wounds, and the gastrointestinal and respiratory

tracts (Albrecht *et al.*, 2006). It might be hard to tell the difference between colonization and actual infection, especially when so many infections emerge during colonization.

Infections caused by *A. baumannii* have been linked to a wide range of body sites, with different levels of severity and clinical outcomes (Gordon *et al.*, 2010). The actual sign and symptoms of infection and its correlation with patient's death are hotly debated topics. While a few investigations have found that infection caused by *Acinetobacter baumannii* has a negative impact on patient outcomes (Lee *et al.*, 2007) other investigations have found that infection has little or no impact on patient outcomes (Jang *et al.*, 2009). Most common infections caused by *A. baumannii* are elaborated below.

2.5.1 Ventilator associated pneumonia (VAP)

The well-known risk factors that enhance the risk of ventilator associated pneumonia caused by *A. baumannii* include prolonged hospital stays, advanced age, prolonged ventilator exposure and immune suppression are all recognized to increase. Hospital acquired infections are also caused by contaminated hands and inadequate hygiene of health care practitioners. Such persons may behave as opportunist carriers of an infectious agent (Peleg *et al.*, 2008).

2.5.2 Community acquired pneumonia

Pneumonia caused by Acinetobacter that is caught outside of the hospital environment has been reported in Australia and Southeast Asia. A history of alcohol consumption, diabetes, or bronchopulmonary illness is common in patients with acute pneumonia. It has a mortality rate of about 40 to 60% and is marked by a severe and abrupt onset, as well as subsequent bloodstream infection (Anstey *et al.*, 2002).

2.5.3 Meningitis

Acinetobacter is also an important cause of hospital acquired meningitis. Neurosurgical operations, cerebrospinal fluid leaks, long - term antibiotic medication, cerebral bleeding, contaminated methotrexate and suctioning equipment are all risk factors of meningitis. The mortality rate due to meningitis is about 70% (Korinek *et al.*, 2006).

2.5.4 Bloodstream infections

Acinetobacter baumanii is most common cause of intensive care unit associated bloodstream infection. Ventilators, catheters, immune suppression, prolonged stay at hospital settings and antibiotic use for a longer period are most common risk factors for septic shocks and bacteremia (Glew *et al.*, 1977).

2.5.5 Skin, wound and soft tissues infections

Acinetobacter baumanii is most frequent cause of cellulitis, folliculitis skin abscesses, necrotizing fasciitis and osteomyelitis. Cellulitis is indicated by a demarcated patch with erythema at injured site with orange skin like appearance. After that it changes into sandpaper like lesion with multiple vesicles and finally a hemorrhagic bullae is formed (Glew *et al.*, 1977).

2.6 Virulence factors

In recent years, different virulence factors of *A. baumannii* have been discovered by using various genotypic and phenotypic approaches and infection model analyses. Most common virulence factors of MDR *Acinetobacter baumannii* involves capsular polysaccharides, porins or OMPs, lipopolysaccharides, MDR efflux pumps, phospholipase enzymes, biofilm formation and protein secretion systems.

2.6.1 Outer membrane proteins (OMPs)

Outer membrane proteins (OMPs) are also known as porins, and they are present on the outer surface of cell along with controlled cellular permeability. The OMPs of Gram-negative bacteria perform a key role in environmental interaction and adaptability, as well as virulence.

In *Acinetobacter baumannii* the well-known and well characterized outer membrane is OmpA. It is a b-barrel shaped protein with a size of 38KDa. It induces apoptosis and cell invasion. Apoptosis is induced with the help of apoptosis inducing factors and cytochrome C. It binds to the surface of the host cell, localizes both in mitochondria and nuclei, and causes cell death. These OmpA are very important for small solute

penetration. OmpA has 70-fold lower pore forming capacity. With the help of host fibronectin OmpA attaches and captures epithelial cells. These proteins are also responsible for pathogen's biofilm formation, surface motility and dissemination into blood stream. Regulation of biogenesis of outer membrane vesicles is usually mediated by OmpA (Lee *et al.*, 2017). Therefore, these proteins are promising target for development of antibacterial drug (Iyer *et al.*, 2018). Choi and his colleagues investigated the invasion of epithelial cells by *Acinetobacter baumannii* in a mice model and they reported that significant histopathological changes of lungs were only found in wild-type infected mice but not in OmpA-mutant infected mice. Resistance against carbapenem drugs has been linked to the overexpression of OmpA in *A. baumannii* (Choi *et al.*, 2008).

A study investigated that *OMP* 33-36 was secreted both in immune and connective cells, where it blocked autophagy and triggered apoptosis, allowing intracellular persistence and the development of cytotoxicity (Rumbo *et al.*, 2014). Moreover, strains of *A. baumannii* in which outer membrane protein genes (*Omp33* and *Omp36*) were knocked out had abnormal growth rates as well as drastically reduced attachment, cytotoxicity and invasion showing that both these genes act as key elements to maintain fitness and virulence in *A. baumannii* (Smani *et al.*, 2013).

2.6.2 Lipopolysaccharides (LPS)

LPS is one of the most important components of outer membrane of *A. baumannii*. It stimulates macrophages to release tumor necrosis factor and interleukin 8, also responsible for survival of bacteria (Rossi *et al.*, 2016). Bacterial sensitivity to different antimicrobial agent can be affected due to modifications in structure of lipopolysaccharides. In laboratory animals, it can also trigger a proinflammatory response. One study investigated that lipopolysaccharide modulates multiple inflammatory cytokines and chemo attractants in 3T3-L1 adipocytes at the mRNA level. In 3T3-L1 adipocytes, the primary role of lipopolysaccharides is to lower leptin and adiponectin levels (Sinosh *et al.*, 2019). During the first step of colonization pili and the antigenic O of lipopolysaccharide stimulate the attachment between pathogen and host cells (Haseley *et al.*, 1997).

2.6.3 Capsular polysaccharides

Antimicrobial resistance is linked to capsular polysaccharides, which have been identified as a target for vaccination against *A. baumannii*. Antimicrobial resistance of *A. baumannii* is mediated by capsular polysaccharides, according to a latest report. A report indicated that mutants lacking capsular polysaccharides have less resistance to antibacterial peptides and produce excessive capsular polysaccharides. In a mouse model, it was found that over production of capsular polysaccharide can elicit the virulence (Geisinger and Isberg 2015).

Overexpression of capsular polysaccharides was linked with an increase in transcription in the K-locus gene, which is predominantly regulated by a two-component regulatory system, such as AdeRS (Liou *et al.*, 2014). Bacterial capsule is repeated, tightly packed sugar units and protect bacterial cells from harsh conditions such as desiccation and disinfection, as well as immune response such as phagocytosis and some antimicrobial agents. Even though *A. baumannii* has over 100 different types of capsular polysaccharides, the capsule is always responsible for the pathogen's survival in duration of infection and increases the capability of replication in serum (Kenyon and Hall 2013).

2.6.4 Enzymes

Different enzymes act as virulence factors for *A. baumannii*. Phospholipases are most important lipolytic enzymes that play a key role in the phospholipid metabolism. They are hydrolytic enzymes that cause hydrolysis of phospholipids present in plasma membrane of host cells. Phosholipase A (PLA), phospholipase C (PLC), and phospholipase D (PLD) are the three most common types of phospholipase enzymes.

Phospholipase A hydrolyzes fatty acid from glycerol and Phospholipase C cleaves the phospholipid and releases a phosphorylated head group. Phospholipase D cleaves the head group and known as the transphosphatidylase (Flores *et al.*, 2016). The integrity of the host cell membrane is altered when phosphorylated and polar head groups are released. *A. baumannii* has two Phspholipase C genes, *A1S-2055* and *A1S-0043,* and the silencing of these genes cause the epithelial cells to lose their cytotoxic activity (Fiester *et*

al., 2016). An in vivo study investigated that PLD also facilitates the long-term stay of *Acinetobacter baumannii* in human blood serum. CpaA, a glycan-specific adamalysin-like protease, was recently discovered as a virulence factor that prevents blood clotting by inactivating Hageman factor or factor XII. As a result, this protease inhibits the thrombi formation at intravascular regions, allowing *A. baumannii* to spread more widely (Waack *et al.*, 2018).

2.6.5 Protein secretion system

Many cell surface structures secrete different kind of proteins that allow *A. baumannii* to interact with its environment and hosts, making it an excellent target for treatment. The first protein secretion system discovered in *Acinetobacter baumannii* was trimeric autotransporter (Ata). This trimeric autotransporter is involved in biofilm formation, pathogenesis, and adhesion to human matrix components, mainly collagen.

The most common secretion system in *A. baumannii* is Type 1 Secretion System (T1SS). The components of cell membrane, transmembrane fusion proteins and ATP binding transporter are the three most important components that make up Type 1 Secretion System. Substrates of Type 1 Secretion System have a C-terminal domain along with a secretion signal, and secretion is carried out in a single step through lipid membranes. Substrate gene clusters and exporter proteins are encoded by type 1 Secretion System. A study investigated the correlation between biofilm formation and *bap* gene expression, and it was increased by low availability of iron. Lower concentrations of iron were found to be important in early biofilm formation in studies (Bielaszewska *et al.*, 2014). *A. baumannii* have 460 genes and most of them acts as virulent factors. Bap is widely expressed and found in multi drug resistant *A. baumannii* which is linked to increased resistance to desiccation (Bardbari *et al.*, 2017).

Multiple factor proteins are also exported by Type 2 Secretion System (T2SS). The Twin-arginine (Tat) system or the general secretory pathway (Sec) transport different proteins through the inner membrane of *A. baumannii*. N-terminal secretion signal transports protein in this two-step secretion mechanism. After protein transportation this secretion signal is released and finally a folded protein is translocated across the outer membrane

of cell. CpaA, LipA, and LipH are the virulence factors of Type 2 Secretion System. LipH and LipA are most common lipases that are required for exogenous lipid uptake while metallo-endopeptidase CpaA is involved in zinc-dependent degradation of factor V and fibrinogen (Tilley *et al.*, 2014). 12-16 genes encode this secretion system.

The autotransporter secretion system is also known as Type 5 Secretion System and it secretes multiple proteins without the use of any additional component or other sources of energy. In comparison to other transporter systems, this autotransporter secretion system transports proteins by using the simplest way of transportation. The signal sequence, the passenger domain, and the autotransporter domain are the three key protein-targeting domains in Type 5 Secretion System. The signal sequence is present in the protein's N-terminal domain and contains a recognition sequence. this sequence targets protein to the periplasm and supports secondary pathway secretion. The C-terminal proteins have autotransporter domain at their end, which form pores in outer membrane and then secrete nascent proteins into the external environment (Noha *et al.*, 2018).

Like many Gram-negative bacteria, some strains of *A. baumannii* also carry genetic locus for Type 6 Secretion System. This secretion is encoded by 13 core structural proteins and additional regulatory proteins (Lopez *et al.*, 2020). The Type 6 Secretion System allows *A. baumannii* to inject different protein toxins into other bacteria, such as peptidoglycan hydrolases, nucleases, or cell membrane effectors, in order to outcompete nearby bacteria and causing polymicrobial infections. Type 6 Secretion System demands high energy cost, so it is only triggered in response to stressful stimuli such as nutrient deprivation, cell injury, and competing microorganisms. The Type 6 Secretion system contracts, allowing the effector protein to be released, which normally targets neighboring bacteria without causing self-intoxication (Weber *et al.*, 2017).

2.6.6 Motility

Pathogenicity and virulence of bacteria can be increased by bacterial motility. *A. baumannii* do not possess flagella. It has long been considered a non-motile pathogen for a long time. However, investigations revealed that by employing twitching motility, this

pathogen can thrive in whole duration of infection, and it can also disseminate in surroundings throughout hospital stay. Type IV pili helps *A. baumannii* to move in media by extension and retraction movements. Surface-associated motility is a type of motility in which some strains of *A. baumannii* do not use any flagella to move on both biotic and abiotic substrates. Quorum sensing mechanism (QS), type IV pili and lipo-oligosaccharides are needed for surface-associated motility (Skiebe *et al.*, 2012).

2.6.7 Micronutrient acquisition system

Growth and survival of bacteria highly depends upon availability of micronutrients. *Acinetobacter baumannii* has different metal uptake modes for scavenging host nutrients such as iron, manganese, and zinc. *A. baumannii* uptakes iron primarily by the usage of siderophores. Siderophores are low molecular weight iron chelating agents with a size of 400-1000 kDa and acinetobactin is most common siderophore in *A. baumannii* (Sarshar, *et al.*, 2021). Furthermore, it also scavenges iron directly with the help of transporters and receptors like FecA and FecI.

Acinetobacter baumannii uses ZigA GTPase and ABC transporters such as ZnuABC transporter for zinc uptake. ZigA is a Zn metallochaperone and involves in zinc metabolism while ZnuABC transporters maintain intracellular zinc uptake. *A. baumannii* escapes from immune system protein calprotectin, which prevents bacterial growth by intrinsically forming a complex of divalent metal ions with manganese and zinc. A study investigated that after infection by *A. baumannii* with zigA mutant and depleted zinc availability, there will be less chances of systemic dissemination of pathogen (Ayoub and Hammoudi 2020).

Manganese acts as a cofactor for many enzymes like ribonucleotide reductase and superoxide dismutase and protects bacteria from reactive oxygen species (ROS). MumT is a high affinity manganese transmembrane transporter that utilizes the proton motive force (PMF) as a source of energy. *A. baumannii* uses these MumT transporters for the uptake of manganese present outside of the cell (Sarshar, *et al.*, 2021).

2.6.8 Biofilm formation and quorum Sensing

Biofilm formation is the most important virulence factor for making *A. baumannii*, a multidrug resistant pathogen. Biofilms are micro-communities of microorganisms that are encased in an extracellular polymeric substance (EPS), making them more resistant to stresses such as desiccation, acid exposure, immune system clearance, and antimicrobial agents. The biofilm formation involves multiple steps. The ability of pathogen to attach to non-living surfaces is determined by the hydrophobicity of the cell surface, accompanied by the intercellular aggregation of exopolysaccharide proteins and macromolecules (Zhang *et al.*, 2018).

Many reports suggested that quorum sensing is very important to form biofilm of *A. baumannii*. Quorum sensing is basically a way of cell-to-cell communication among bacteria that involves the formation of signaling molecules called as autoinducers to maintain population density (Whiteley *et al.*, 2017). These auto-inducers modulate motility, biofilm formation, conjugation and interaction with eukaryotic cells. The most common autoinducers are acyl homoserine lactones (AHLs). In *A. baumannii*, the quorum sensing cycle includes the *AbaI* inducer and its corresponding *AbaR* receptor. *AbaI* inducer is a sensor protein that is produced by the *abaI* gene, and it acts as an autoinducer synthase enzyme to produce acyl homoserine lactone (AHL) signal. Whereas *AbaR* acts as a receptor protein that triggers a series of reactions when it binds to acyl homoserine lactones. *A. baumannii* has obtained these genes from *Halothiobacillus neapolitanus* (Saipriya *et al.*, 2019).

2.6.8 Efflux pumps

The efflux pump mechanisms may also be linked to the ability of *A. baumannii* to survive in natural habitats and in the hospital environment. Efflux mechanisms are present in all cells and perform physiological functions. The basic function of efflux pumps in a prokaryote cell includes evading naturally produced compounds and removing metabolic waste and toxins. Early stages of infection like attachment of pathogen with host cells and its colonization can complicate efflux pumps. Antimicrobial agents that are used to

treat infections caused by bacteria are also extruded out from bacterial cell by using efflux pumps (Abdi *et al.*, 2020).

Antibiotic efflux is not the main function of efflux pumps so, they are also involved in release of different toxins out of the cell, balancing of nutrients and heavy metals and alleviation of stress on cell pathogenesis. So far, there are 6 main classes of bacterial efflux pumps and all these classes are present in *Acinetobacter baumannii*. These multi drug efflux pumps contribute in resistance to a broad range of antimicrobial agents in *A. baumannii* (Kornelsen and Kumar 2021).

2.6.9.1 Major facilitator superfamily (MFS)

It is the largest and most complex superfamily of multidrug efflux pumps that has been reported up till now. Members of Major Facilitator Superfamily are carried from bacteria to human. Based on sequence homology this family can be further divided into 70 families. Based on transport mode this family is divided into uniporters, symporters and antiporters. Uniporters do not need any extra energy for transportation of their substrates along their concentration gradient. Symporters uses stored energy to transport their two types of substrates in same direction. Antiporters also uses stored energy to transport their substrates in opposite directions (Kornelsen and Kumar 2021).

There are three Major Facilitator Superfamily transporters related to multidrug resistance in *A. baumannii* that are found in all known strains. It includes CraA, AmvA and AbaQ. Chloramphenicol resistance Acinetobacter (CraA) demonstrated to increase in chloramphenicol susceptibility in *A. baumannii* by up to 128-fold when removed. It is still unknown whether craA expression is persistent or not. AmvA mostly effluxes dyes and disinfectants and its effect on susceptibility of *A. baumannii* to erythromycin has also been reported. AmvA expression is modulated by a TetR-like regulators also known as AmvR. AbaQ is the third transporter and responsible for quinolones transportation.

Tet type efflux pumps and AbaF are also present in this super family. Tet(A) is responsible for tetracycline resistance while Tet(B) is responsible for minocycline and tetracycline resistance. AbaF effluxes out fosfomycin and increases virulence and biofilm formation potential of MDR *A. baumannii* (Kornelsen and Kumar 2021).

2.6.9.2 Small multidrug resistance (SMR) Family

These transporters are formed by small proteins with four transmembrane α-helical domains. Small Multidrug Resistance proteins are important inner membrane proteins, and some members of this family act as oligomers. Many lipophilic molecules are transported outside the cytosol by utilizing proton motive force and several members of the Small Multidrug Resistance family are also involved in the efflux of quaternary ammonium compounds, which are frequently used as disinfectants. A1S-0710, AbeS and QacE transporters are commonly present in *A. baumannii* and responsible for resistance against different antibiotics, dyes and detergents (Bay *et al.*, 2008).

2.6.9.3 ATP-binding cassette family (ABC)

Transporters belonging to the ABC family are ATP dependent and use free energy provided by ATP hydrolysis to enable export and import of solutes through a lipid membrane. There are four protein domains in all ATP-binding cassette (ABC) transporters: 2 ATP-hydrolyzing domains and 2 membrane-spanning domains that might be located on a single lengthy protein or spread over several proteins (Davidson and Chen 2004). Though little is known about MacAB-TolC in Acinetobacter genus it involves in antibiotic resistance against macrolides in other species (Greene *et al.*, 2018).

2.6.9.4 Multidrug and toxic compound extrusion (MATE) family

Transporters belonging to MATE family are reported to extrude many cationic antimicrobial agents in exchange for H or Na ions. These transporters can also efflux out ciprofloxacin, norfloxacin and acriflavine. There are three MATE family efflux pumps in *A. baumannii*, but only AbeM has been linked with antibiotic resistance; so, there is still needed to study more about these efflux pumps in *A. baumannii* (Kornelsen and Kumar 2021).

2.6.9.5 Resistance nodulation division (RND) Family

RND family is present in all domains of life. The AdeABC transporter in *Acinetobacter baumannii BM4454* confers antimicrobial resistance against variety of antibacterial

drugs. It is made up of three proteins: AdeA, AdeB, and AdeC. AdeB belongs to the RND superfamily. In gram-negative bacteria, these efflux pumps are found inside the plasma membrane and form a complex with outer membrane factors and transmembrane fusion proteins. These transporters have also been linked with reduced sensitivity to a wide range of antibiotics including fluoroquinolones, aminoglycosides, chloramphenicol, tetracycline and certain β -lactam drugs (Magnet *et al.*, 2001).

Table 2.1: Overview of *A. baumannii* virulence factors, Adapted from (Sarshar *et al.*, 2021).

Virulence Factors	Functions	Reference
Lipopolysaccharides (LPS)	Induces release of tumor necrosis factors and evasion from the host immune response	Chin *et al.*, 2015
Capsule	Persistence	Ayoub and Hammoudi 2020
Porin (*OmpA*, *Omp33-36*)	Induces apoptosis, biofilm formation, persistence, and regulation of biogenesis	Novovic *et al.*, 2018
Protein secretion system T1SS T2SS T5SS	ATP binding membrane transporters Helps in in vivo survival Acts as an autotransporter	Noha *et al.*, 2018
ABC efflux pump	Oxidative stress response	Srinivasan *et al.*, 2015
RDN efflux pump	Resistance to osmotic stress	Magnet *et al.*, 2001

MFS efflux pump	Resistance to disinfectants and osmotic stress	Lin and Lan 2017
AbaI and *AbaR*	Motility, virulence, conjugation, biofilm formation and host-pathogen interactions	Niu *et al.*, 2008
CarO	Resistance against carbapenem antibiotics	Labrador *et al.*, 2020
Bap	Biofilm formation	Loehfelm *et al* 2008
Acinetobactin	Acts as an Iron chelator	Sheldon *et al* 2020
Phospholipase	Helps in invasion and reduces cytotoxic effects	Fiester *et al.*, 2016

2.6.9.6 Antimicrobial resistance in *Acinetobacter baumannii*

Gram-negative *A. baumannii* is a ESKAPE pathogen that causes severe and invasive mainly hospital acquired infections that are associated with high mortality rates. Acinetobacter has developed multidrug resistance (MDR) in recent years, owing to widespread antibiotic overuse and inadequate management (Kyriakidis *et al.*, 2021). The capability of *Acinetobacter baumannii* to acquire resistance against wide range of antimicrobial agents may be attributed due to impermeability of plasma membrane and its exposure to a huge pool of antimicrobial resistance genes in the environment (Bonomo and Szabo 2006). The susceptibility of *A. baumannii* towards numerous antimicrobial classes has been significantly reduced by different antibiotic resistance mechanisms (Lee *et al.*, 2017).

Antibiotic resistance in *A. baumannii* against all antimicrobial classes is increasing rapidly since 1970. From 2007 to 20 up to 70% strains of *Acinetobacter baumannii* were Multi Drug Resistant, particularly resistance to carbapenem drug which was originally

thought to be most effective against infections of MDR *A. baumannii*. Carbapenem resistance has been detected in strains isolated from both military and civilian settings, which is not unusual (Keen *et al.*, 2010). Colistin tends to be the most reliable antibiotic against Multi Drug Resistant *A. baumannii* now, although it has a lot of complications and it is not ideal to treat all forms of bacterial infections (Rafailidis *et al.*, 2009). Colistin resistance has now been reported worldwide leading to the emergence of novel strains conferring resistance against all known antibiotics in different areas of world (Cai *et al.*, 2012).

The three principal causes that induce antibiotic resistance includes poor hospital hygiene, selective pressure owing to overuse of antimicrobial drugs and transposable elements encoding different mechanisms of antibiotic resistance (Joshi, 2013).

Multidrug-resistant *Acinetobacter baumannii* has no standard definition, however it usually means resistance to at least three of the five classes of antibiotics that would typically be selected for treatment (i.e., fluoroquinolones, amino-glycosides, ampicillin-sulbactam, cephalosporins and carbapenems). Pan-drug-resistance relates to resistance to all beta lactams, fluroquinolones, and aminoglycosides, but not polymyxins or tigecycline (Alsan and Klompas 2010).

The susceptibility of *Acinetobacter baumannii* to antibiotics varies greatly between regions, clinics, and even wards within a same hospital. These variations could be due to diverse antibiotic usage patterns and epidemiological conditions, along with antibiotic control strategies. The disparities in resistance patterns among strains highlight the necessity of local awareness in identifying the best treatment for *Acinetobacter baumannii* (Cisneros *et al.*, 2002).

2.7 Mechanisms of antimicrobial resistance

Antibiotic resistance mechanisms of *Acinetobacter baumannii* can be divided into five categories. An elaboration of these mechanisms is given below.

2.7.1 Antimicrobial inactivating enzymes

The most prevailing antibiotic resistance mechanism in Acinetobacter towards beta-lactam drugs is enzymatic inactivation by beta-lactamase enzymes. Beta-lactamase enzymes are classified into four main classes: class A, class B or metallo enzymes, class C and class D or oxicillinases.

Ambler Class A or serine-dependent beta-lactamases can be inactivated by Clavulanate or tazobactam. Except for cephamycins, these serine dependent beta-lactamases can cause hydrolysis of all cephalosporins and penicillins. Class A beta-lactamases such as *TEM*, *CTX-M* and *SHV* have also been reported in many strains of MDR *Acinetobacter baumannii*. Many Extended Spectrum Beta Lactamases have been found in *A. baumannii* strains, particularly *TEM-92* from Italy and *TEM-116* from Netherland, as well as *SHV-12* from Netherland and China (Perez *et al.*, 2007).

CTX-M-43 and *CTX-M-2* have also been detected in MDR strains of *Acinetobacter baumannii* from Bolivia and Japan. *VEB-1*, which disseminated among hospitals in France and was recently discovered in Belgium and Argentina, *PER-1*, which originated in France, Turkey, Belgium, Romania, Korea, and the United States, and *PER-2*, which originated in Argentina. Some narrow spectrum β-lactamases such as *TEM-1* and *TEM-2* have also been identified in many strains of *A. baumannii* (Perez *et al.*, 2007).

Class B or metallo beta-lactamases strongly catalyze the hydrolysis of all known beta-lactam drugs including carbapenems except aztreonam. Ambler class B is present on chromosome or plasmids and can be inactivated by different metal chelators diplocolinic acid and EDTA. In the active site of class B, a unique metal ion, generally zinc is present so, they are also known as Zinc-dependent metallo β-lactamases. Zinc helps in catalysis and distinguishes metallo beta-lactamases from carbapenemases belonging to both class A and D. (Perez *et al.*, 2007).

A. baumannii has two main class B beta-lactamases: Imipenem hydrolyzing beta-lactamase (*IMP*) and Verona integron-encoded metallo beta-lactamase (*VIM*). Both these enzymes have been reported in Korea, Japan and Italy. Metallo-β-lactamases are considered a significant concern since they are frequently found on transposable elements

of bacteria. (Urban *et al.*, 2003). *NDM-1* also belongs to metallo-β-lactamases and has also been reported in different parts of world including China, Saudi Arabia, Lebanon, Iran and Tunisia.

In *A. baumannii*, the commonly acquired metallo-β-lactamase genes are located at class I integrons and they also carry aminoglycoside resistance genes. These strains of *A. baumannii* express more resistance than strains without class I integrons, which means that using one antibiotic can lead to overexpression of resistance towards other antibiotics, especially because integrons are genetically located on transposable elements such as plasmids or transposons, making them highly transferable (Jeon *et al.*, 2015). Acinetobacter-derived cephalosporinases or class C beta-lactamases are encoded on chromosomes, and they are present in all known MDR strains of *Acinetobacter baumannii*. They can hydrolyze different antibiotics including penicillins, cephamycins and cephalosporins.

When expressed at a low level, these enzymes have little effect on the efficacy of cephalosporins. The occurrence of an insertion sequence (IS) element called as ISAba1or *bla* gene coding for class C cephalosporinases in *A. baumannii* is the primary indicator of AmpC enzyme production (Manchanda *et al.*, 2010). Around 28 *bla ADC* genes have been identified and documented in GenBank. *AmpC-69*, *AmpC-70*, and *AmpC-71* have been detected with the help of whole-genome sequencing. The novel AmpC alleles encoded by *blaADC-196* have also been detected in MDR strains of *A. baumannii* from China (Ayoub and Hammoudi 2020).

Class D beta-lactamase or serine dependent OXAs (oxacillinases) cause hydrolysis of isoxazolyl penicillin more rapidly than benzylpenicillin (Jeon *et al.*, 2015). Around 400 OXA-type beta-lactamases have been discovered, with many of them having hydrolytic activity against carbapenem drugs. One of the key carbapenem resistance mechanisms in *Acinetobacter baumannii* is the occurrence of carbapenem-hydrolyzing oxacillinases, or metallo-β-lactamases. (Lin and Lan, 2014). Class D oxacillinase was first time isolated from a Scottish patient's blood culture infected with *A. baumannii*, named as *ARI-1* in 1985, which is now known as *OXA-23*. This enzyme is widely distributed and the *ISAba1*

insertion sequence in the *blaOXA-23* promoter sequence of *A. baumannii* has been linked with overexpression of *blaOXA-51*, *blaOXA-23* and *blaOXA-58*.

It's important to note that *OXA-23* enzymes have been found in clinical samples of *A. baumannii* from all around the world, along with other carbapenemases. Different studies from Kuwait and Lebanon investigated the coexistence of *OXA-2* gene with *GES-11*. A rare, concerning occurrence of *A. baumannii* was found to have *OXA-23*, *VIM-2*, and *NDM-1* gene, indicating the variety of carbapenemases that can be carried by this pathogen at the same time (Ayoub and Hammoudi 2020).

2.7.2 Changings in outer membrane proteins

Outer membrane proteins or OMPs have gotten a lot of attention as a part of major virulence factors in pathogenic bacteria. These proteins are a type of integral membrane proteins embedded in outer membrane with eight to twenty-six strands forming a β-barrel shape. On the extracellular side, there are long loops in middle of the strands, while on the periplasmic side, there are small loops. Due to these features outer membrane proteins have high membrane stability and can withstand highly severe environments.

Outer membrane proteins have diverse sequences and roles, but they all have the same structure and biological features. Bacterial outer membrane proteins have an even number of strands, their role and shear number are determined by strand sequences. OmpX in *Escherichia coli* and fibronectin and heparin-binding protein in *Yersinia pestis* have a close resemblance as virulence associated proteins but their sequence similarity is less than 45 percent. The N-terminal domain of outer membrane proteins has significantly more variability than the C-terminal domain, and the conserved β-signal directs folding and proper assembling of outer membrane proteins.

However, the types of outer membrane proteins in *Acinetobacter baumannii* have not been fully defined, and some dispersed reports have been found, with CarO, BamA, Omp33–36, OprD, LptD and OmpW being the most common. β-barrel shaped OmpA protein has been found to confer antibiotic resistance in many species of Acinetobacter by facilitating slow diffusion of anionic β-lactam drugs and virulence through its toxic effects towards host cells (Gribun *et al.*, 2003). Overexpression of OmpA proteins in

Acinetobacter baumannii have increased the prevalence and incidence of Acinetobacter infections (Sanchez *et al.*, 2017).

Because OmpA is the most prevalent outer membrane protein in *A. baumannii*, and its significant role in antimicrobial resistance by conferring mutant gene disruption, which are more susceptible to chloramphenicol, carbapenems, monobactam and quinolones. Apart from diffusion, many studies suggest that these outer membrane proteins bind to peptidoglycan of *Acinetobacter baumannii* through its C-terminal domain and drive many antibiotics out of the periplasmic space by interacting with efflux pumps (Tsai *et al.*, 2020). This binding regulates the synthesis of outer membrane vesicles (OMVs) and integrity of plasma membrane in *A. baumannii* (Moon *et al.*, 2012).

The expression of OmpA in *Acinetobacter baumannii* has recently been linked to resistance to colistin, a last expedient antibacterial drug. The loss of cell wall stability caused by a homozygous mutation of OmpA makes the bacterium twenty times more susceptible to colistin and 5.3 times more susceptible to trimethoprim than wild type *Acinetobacter baumannii*. Researchers are racing to find new antibacterial drugs that target OmpA because of its unique role in developing antibiotic resistance. For the eradication of MDR strains of *A. baumannii*, OmpA blockers can work in tandem with last expedient antibacterial drug like colistin (Parra *et al.*, 2018).

Carbapenem-associated outer membrane protein or CarO, was first time discovered in *Acinetobacter baumannii* strains sensitive to imipenem. CarO develops resistance against imipenem and meropenem after losing a 29 kDa protein. CarO is an outer membrane channel protein with eight stranded β-beta barrel structure which does not have an ongoing channel but regulates the inflow of β-lactams (mainly imipenem) into *Acinetobacter baumannii*. CarO is divided into two subgroups: CarOa and CarOb with 2-fold higher imipenem sensitivity. At least 6 polymorphic CarO variants have been found in *A. baumannii*, each with different imipenem specificities, underlining the significance of protein (Uppalapati *et al.*, 2020).

Many hospitals epidemiological investigations have confirmed the therapeutic importance of CarO. These investigations also demonstrated the high proportion of CarO

deficiency among carbapenem-resistant strains harboring the BlaOXA and *TEM-1* genes in hospital strains of *Acinetobacter baumannii*. Insertion sequences including ISAba1, ISAba125 and ISAba36 disrupted the CarO gene in a variety of carbapenem-resistant strains of *A. baumannii* (Uppalapati *et al.*, 2020).

OprD was discovered during research on plasma membrane of carbapenem-resistant strains of *A. baumannii*. OprD is a protein analogous to a porin that's associated in translocation of basic amino acids and imipenem drug. Studies reported that OprD is a β-barrel shaped monomeric protein with a very narrow porin constriction (Uppalapati *et al.*, 2020).

2.7.3 Efflux pumps

Efflux pumps significantly influence the antimicrobial resistance in *Acinetobacter baumannii*. Efflux pump has three parts. It includes pump, exit portal and a linker lipoprotein. Pump is normally found in the plasma membrane of bacteria. Exit portal acts as a channel that flows through the plasma membrane while linker lipoprotein presents in middle of pump and exit portal. *Acinetobacter baumannii* possesses a resistance-nodulation division type efflux transporters that confer antimicrobial resistance against a wide range of antibiotics including fluoroquinolones, carbapenems, aminoglycosides, tetracyclines, chloramphenicol and beta-lactam drugs (Nowak *et al.*, 2015).

AdeABC appears to be cryptic in wild-type *Acinetobacter baumannii* due to the AdeRS 2-component system's strict regulation, however, point mutations or addition of insertion sequence element ISAba1 into the adeRS gene causes upregulation of AdeABC transporters (Marchand *et al.*, 2004). Cell density and the two-component BaeSR regulatory system, which is implicated in response to stress stimulus, tend to alter tigecycline sensitivity through regulating transcription of the *adeA* gene. Certain RND-type transporters like AdeABC and AdeIJK, are linked to tigecycline resistance in a synergistic way. The expression of AdeIJK transporters is controlled by transcriptional regulator *AdeN* (Rosenfeld *et al.*, 2012)

MDR strains of *A. baumannii* have a strong potential for biofilm formation and low-dose antimicrobial therapy seems to dramatically increase biofilm formation at sub-MIC level.

A study reported that overexpression of RND-type transporters via low dose antibiotic therapy promotes the production and transportation of autoinducers which promote biofilm formation. These findings suggest a correlation between low-dose antibiotic therapy and an increased risk of *A. baumannii* biofilm infections (Rodriguez-Bano *et al.*, 2008).

AbeM transporters are a part of MATE family in *A. baumannii*. The antibiotic substrates of this efflux pump seem to be limited to fluoroquinolones (Perez *et al.*, 2007). Imipenem and cephalosporin resistance are also linked to AbeM efflux pump. When the abeS gene is deleted, pathogen becomes more susceptible to antibiotic such chloramphenicol and erythromycin (Srinivasan *et al.*, 2009).

Fournier identified CmlA and CraA transporters linked to chloramphenicol resistance, while TetA efflux pump is linked to tetracycline resistance (Fournier *et al.*, 2006). Resistance to disinfectants, surfactants, dyes and antimicrobial agents such as macrolide antibiotics, acriflavine, benzalkonium chloride and chlorhexidine is mediated by the unique efflux pump AmvA (Migliaccio *et al.*, 2020). AbaF is another novel efflux pump, and it is linked to fosfomycin resistance (Sharma *et al.*, 2016).

*A*cinetobacter *baumannii* also possesses EmrAB-TolC efflux pump, which provides antimicrobial resistance against netilmicin, tobramycin, and imipenem. Through multiplex phenotypic screening, researchers discovered three new transporters in *A. baumannii* (A1S 1535 transporter, A1S 2795 transporter, and ABAYE 0913 transporter). A1S 1535 transporter provides resistance against chloramphenicol, aminoglycoside and chloroxylenol. A1S 2795 is the first MFS transporter linked to sulfathiazole resistance, while ABAYE 0913 transporter is associated to chloramphenicol and fusidic acid resistance (Li L. *et al.*, 2016).

2.7.4 Alteration in target sites

Antimicrobial resistance in *Acinetobacter baumannii* can be acquired through changes in antibiotic target sites. Overexpression of modified PBPs with a low binding affinity for carbapenems produces carbapenem resistance. Peptidoglycan synthesis and its incorporation into bacterial cell wall is catalyzed by penicillin-binding proteins. Because

beta-lactams resemble with substrate of penicillin binding proteins, so they attach to it. B lockage of penicillin binding proteins by beta-lactams causes an imbalance in cell wall biosynthesis, which leads to cell death. Although the significance of this mechanism of antimicrobial resistance seems to be minimal, but it cannot be neglected (Kyriakidis *et al.*, 2021).

In *Acinetobacter baumannii*, seven distinct PBPs (1a, 1c, 2, 3, 4, 4b, and 5) were discovered. As observed in MDR strains of *A. baumannii* isolated from Seville and Spain, carbapenem resistance is linked with lower drug influx because of porin insufficiency and decreased binding affinity for the antibiotic due to alteration in penicillin Binding Proteins which is defined by a decreased expression of PBP-2 (Perez *et al.*, 2007).

Quinolone resistance is linked to changes in GyrA (a subunit of DNA gyrase) and ParC (a subunit of topoisomerase IV) in some strains of *A. baumannii*. TetM of *A. baumannii* has 100% similarity with TetM of *Staphylococcus aureus*. Ribosome defence has linked TetM with resistance against tetracycline. In multidrug resistant strains of *A. baumannii*, dihydrofolate reductases have been identified as the cause of trimethoprim resistance (Ribera *et al.*, 2003).

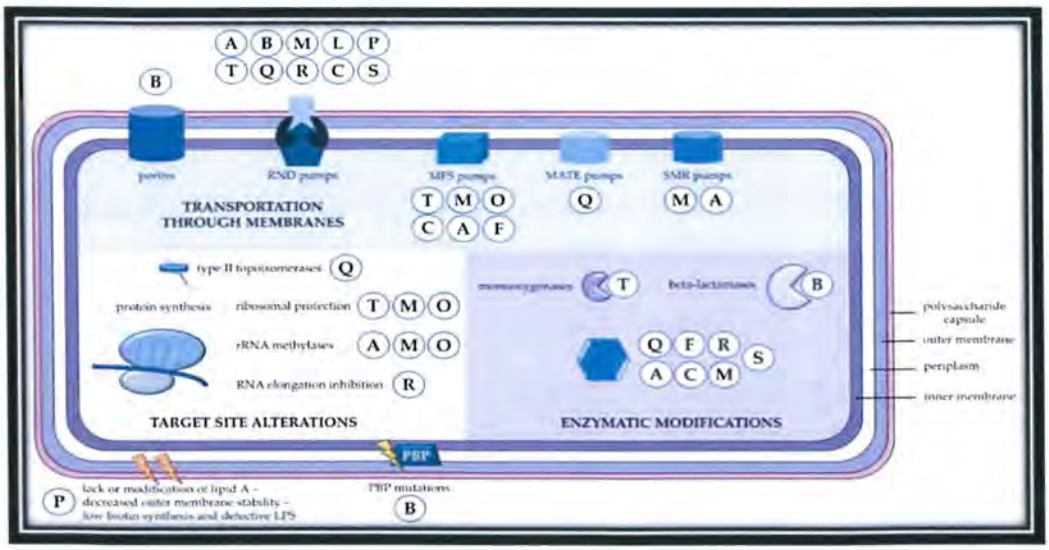

Figure 2.1: Mechanisms of antibiotic resistance in *A. baumannii*, Adapted from (Kyriakidis *et al.*, 2021).

2.8 Resistance to antibiotics

2.8.1 Resistance to aminoglycosides

The basic mechanism of aminoglycoside resistance in *Acinetobacter baumannii* is aminoglycoside-modifying enzymes. Acetyltransferases, adenyltransferases, and phosphotransferases are the three groups of aminoglycoside-modifying enzymes. Such enzymes are usually found on mobile genetic elements and spread between pathogenic bacteria horizontally (Lin and Lan, 2014). Many publications reported that most of MDR strains of *Acinetobacter baumannii* express a complex of aminoglycoside-modifying enzymes and the inclusion of genes encoding for aminoglycoside-modifying enzymes among class I integrons is quite common.

It was recently discovered that methylation of 16S rRNA is regulated by a newly identified set of 16S rRNA methylases-mediated process of increased resistance against currently used intravenously injected aminoglycosides. *Acinetobacter baumannii* isolates from Japan, Korea, and the U.S have been found to have 16S rRNA methylase enzyme. In Japan, a new form of aminoglycoside-modifying enzyme has been identified recently and proven to perform a significant role in resistance against amikacin in many species of Acinetobacter. Other resistance mechanisms involve modifications in the targeted ribosomal proteins, decreased aminoglycoside transportation to the microbial cell, and transporter-mediated clearance of aminoglycosides from inside of microbial cell (Jung and Park, 2015).

Changings in target site appear to perform a key role in aminoglycoside resistance, as seen by the rmA and rmtB1 genes found with the abundance of coexisting resistance genes. AdeABC and AbeM pumps eliminate gentamicin and netilmicin, while efflux is substantially weaker for more hydrophilic aminoglycoside such amikacin and kanamycin. Modifications in porins and lipids of cell membrane are some minor mechanisms of aminoglycoside resistance that are still being researched. Recent studies have demonstrated the capability of next-generation sequencing (NGS) in the early detection of resistance in *A. baumannii*, allowing for rapid diagnosis and treatment (Kyriakidis *et al.*, 2021).

2.8.2 Resistance to quinolones

Quinolones are broad-spectrum antibiotics with a bactericidal effect on both Gram-negative and Gram-positive bacteria. Quinolones have a bicyclic core structure like 4-quinolone. Quinolones inhibit the replication of DNA by preventing bacterial DNA from unwinding and being copied. Quinolones work by decreasing the ligase activity of type II topoisomerases, DNA gyrase, and topoisomerase IV, which usually work together with DNA nucleases to trigger supercoiling of DNA (Kyriakidis *et al.*, 2021).

The main cause of quinolone resistance in *A. baumannii* strains is modifications in topoisomerase IV and DNA gyrase developed by mutations in the quinolone resistance-determining sequences of *parC* and *gyrA* genes. Binding affinity of quinolones with enzyme-DNA complex is reduced because of these modifications. A single point mutation in amino acid of *gyrA* can develop resistance against fluoroquinolones and double amino acid alteration in both *gyrA* and *parC* genes induces enhanced resistance compared to single amino acid. There hasn't been any finding of *parC* mutations without a *gyrA* mutation, indicating that fluoroquinolones may have a complementary target in DNA topoisomerase IV (Kyriakidis *et al.*, 2021).

Efflux systems that limit intracellular accumulation of antibiotic, such as RND-type transporters AdeABC and MATE transporters AbeM, are also another mechanism of quinolone resistance. Quinolone resistance regulated by *qnr* genes and plasmids has not been described for *A. baumannii* till now (Potron *et al.*, 2015). Fluoroquinolones are the main target of the AbeM transporters, resulting in clinically substantial alterations in the minimum inhibitory concentrations of ciprofloxacin and norfloxacin.

Although plasmid-derived quinolone resistance has recently been found as a clinical issue in the treatment of *Acinetobacter baumannii* infections. It normally imparts very low-level resistance. Members of the pentapeptide-repeat protein family are encoded by qnrAI, qnrB, qnrB19, and qnrS genes. These members restrain gyrase activity by competing with DNA for binding, resulting in decreased DNA binding with topoisomerase. In this way the enzyme-DNA complex is prevented from effects of

quinolones. Chromosome mediated antibiotic resistance against quinolones has also been discovered in *A. baumanni* (Kyriakidis *et al.*, 2021).

2.8.3 Resistance to glycylcyclines and tetracyclines

Tetracycline attaches to the 30S subunit of ribosome, preventing the initiation of translation and thus inhibiting protein synthesis. Tetracycline resistance is caused by three primary mechanisms: (1) ATP-dependent efflux, (2) enzyme-mediated inactivation of tetracyclines, and (3) ribosomal protective proteins. Tetracycline efflux pumps in *Acinetobacter baumannii* can be divided into two types: Resistance Nodulation Division pumps and Tet efflux pumps (Kyriakidis *et al.*, 2021).

TetA and TetB are transposon-derived transporters. TetB regulates the efflux of tetracycline and minocycline and TetA only modulates tetracycline efflux. Through a non-covalent alteration of the ribosomes TetM/TetO/TetS efflux pumps eliminate the inhibitory effect of tetracycline on translation. Few studies have reported the link of *TetM* gene and protection of bacterial ribosome with minocycline resistance in *Acinetobacter baumannii*. The product of *TetS* gene uses a GTP-dependent mechanism to remove tetracycline from its binding site at bacterial ribosome, allowing protein synthesis to resume even if tetracycline is present (Ribera *et al.*, 2003).

According to gene disruption investigations resistance nodulation division efflux pumps can expel tetracyclines, increase the minimum inhibitory concentrations for minocycline, tigecyline and tetracycline. AdeIJK efflux pump has more impactful role in combination with AdeABC efflux pump. Tigecycline has a broader spectrum of activity than early tetracyclines. it can easily penetrate in tissues penetration and is resistant to a variety of tetracycline resistance mechanisms, particularly Tet efflux pumps and ribosomal protection protein mechanisms like Tet(O) and Tet(M). To avoid the development of resistance, tigecycline consumption should be limited (Kyriakidis *et al.*, 2021).

2.8.4 Resistance to polymyxin B and colistin

Plasma membrane of *A. baumannii* allows necessary molecules or ions to pass through it while blocking the passage of harmful substances. Lipid A of lipopolysaccharides is anionic in nature, and it forms bond with non-ribosomal cationic lipopeptides of polymyxins B and E. By compromising the integrity of both the inner and outer membranes, this bonding loses the stability of outer membrane of Gram-negative bacteria. It leads towards absorption of polymyxins into the periplasmic space. Although the exact mode of action is not known but the hydrophobic tail of polymyxin B damages the cell membrane just like detergents (Potron *et al.*, 2015).

Colistin resistance mechanisms in *Acinetobacter baumannii* are encoded on chromosomes these mechanisms include 1. Addition of phosphoethanolamine (PetN) into lipid A of the outer membrane modifying its structure 2. Mutation in genes required for synthesis of lipid A resulting in its complete loss 3. Low expression of proteins required for stability of outer membrane 4. Insufficient expression of cofactors required for lipopolysaccharide synthesis. Addition of cationic PetNt to lipopolysaccharide decreases the negative charge on the bacterial cell surface, limiting polymyxin-lipopolysaccharide interaction. Colistin resistance is linked to modifications in the *pmrCAB* operon in MDR strains of *Acinetobacter baumannii*(Lima *et al.*, 2018).

An in vitro study of different antibiotic combination therapies against colistin-resistant strains of *Acinetobacter baumannii* revealed that combination of colistin with teicoplanin and rifampin are the most effective. Similarly, combination of minocycline and colistin is more beneficial to treat minocycline-resistant *A. baumannii* infections and it also enhances the lifespan and decreases bacterial count in the lungs of mice (Yang *et al.*, 2016).

2.9 Biofilm formation

Biofilms are communities of microbial cells that are connected to surfaces by self-produced extracellular polymeric matrix and have a different phenotype than planktonic cells, particularly in gene transcription and interaction with one another. These microbial

colonies are recognized as a most prevalent cause of infection in human (Costerton *et al.*, 1999). Bacterial biofilms have been detected on the surfaces of endotracheal tubes, catheters, and mechanical heart valves, as well as water pipelines and cleaning equipment (Donlan and Costerton, 2002). Complex microbial communities frequently target surfaces.

Biofilm forming cells have low metabolic rate and are protected by the EPS, making them more resistant to antimicrobial agents and the host's innate defense mechanism. Biofilm associated infections such as catheter associated bacterial infections and ventilator related pneumonia are commonly caused by *Acinetobacter baumannii*. These infections can be highly resistant to antibiotic treatments, posing a significant clinical challenge in the treatment of *A. baumannii*-related biofilm infections. Due to the fast expansion of medical equipment-related infections and antimicrobial resistance, *A. baumannii* biofilms have become one of the most significant global concerns (Gedefie *et al.*, 2021).

During the last few decades, a variety of models for studying in vitro biofilm formation have been proposed (10). Biofilm biomass assays, viability assays and matrix quantification assays are mostly used for quantification of biofilm formers (Stepanovic *et al.*,2000). Environmental stressors such as Ultraviolet radiation, dehydration, food deprivation, extreme fluctuations in temperature, pH and pressure, high salt levels and antimicrobial drugs cause bacteria to develop biofilms. The events resulting in the formation of bacterial biofilms are much complicated (Galie *et al.*, 2018).

Biofilm formation is thought to begin through a reversible adherence of bacterial cells to a substrate, accompanied by an irreversible adhesion, which is frequently helped by adhesive bacterial properties and relatively small interactions. The synthesis of extracellular polysaccharide has enhanced reversible attachment of bacteria. They eventually change into a well-arranged form encased in an extracellular polysaccharide matrix. Finally, bacteria can release from the mature biofilm and occupy different places in the environment (Berne *et al.*, 2015). The steps involved in biofilm formation are described below.

2.9.1 Bacterial attachment

Bacterial adhesion is consisting of reversible and irreversible stages. The first phase is introducing bacteria to a surface, a procedure that is random in nature, powered by Brownian motion and gravitational forces, and affected by ambient hydrodynamic forces (Beloin *et al.* 2008). Depending on nutrient availability, pH, ion concentration, and temperature, bacteria face forces of attraction or repulsion within a habitat. The characteristics of the medium, as well as the structure of the bacterial cell surface, influence speed and orientation toward or away from the mating surfaces (Donlan 2002). Flagella help motile bacteria to withstand hydrodynamic and repelling forces, providing competitive benefits.

Adhesion is regulated by external sticky extensions and secretory surface proteins once they intercept the surface. Early attachment is transient and reversible. Bacterial detachment and returning into planktonic form can be seen if bacterial cells are agitated by hydrodynamic pressure, or nutrient accessibility and repulsive forces (Anderson *et al.* 2008).

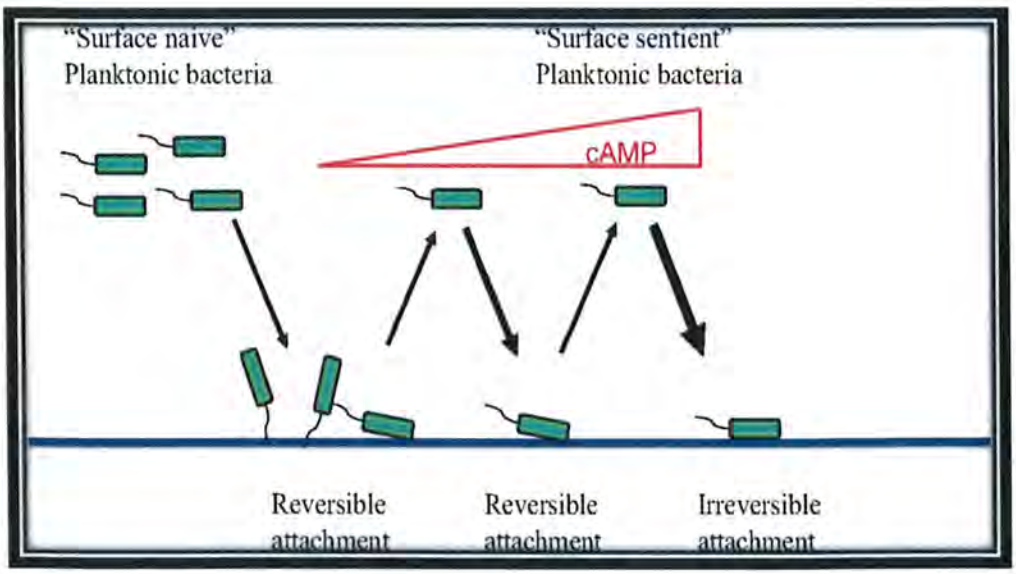

Figure 2.2: Reversible and irreversible attachment of planktonic cells to a substrate for biofilm formation, Adapted from (Armbruster and Parsek 2018).

Bacteria that can tolerate physical or chemical shear forces and hold a firm grip on the surfaces are able to achieve irreversible adhesion. Relatively short interactions with bacterial surface such as dipole-dipole interactions, hydrogen bonding, ionic bonding, and covalent bonding are used to achieve irreversible attachment (Bos *et al.*, 1999). Pathogenic bacteria also produce unique surface proteins that allow them to attach to receptors on the surface of eukaryotic cells.

Individual cells are also linked by a cell-to-cell communication system known as Quorum Sensing to trigger the development of microbial biofilms. Bacteria use this mechanism to produce and disseminate primary mediators such as signaling molecules (autoinducers) that allow cell-to-cell interaction within the bacterial community (Papenfort and Bassler 2016). Quorum sensing is used by both Gram-positive and Gram-negative bacteria to regulate biofilm formation.

2.9.2 EPS matrix production

The irreversible stage of bacterial attachment to a substrate is characterized by the production of extracellular polysaccharide. Because of the significance that *P. aeruginosa* biofilms have in the development of cystic fibrosis, the extracellular polysaccharide matrix of *P. aeruginosa* has been extensively investigated. Alginate is a key constituent of extracellular polysaccharide matrix of *P. aeruginosa* and it is synthesized in huge amounts by bacterial cells adhered to a specific substrate than by free-floating planktonic cells. Genes encoding flagella are downregulated by the σ factor *AlgT*, which is also requisite for alginate synthesis. Polysaccharides, proteins, DNAs, lipids, and other polymeric substances are among the main elements of extracellular polysaccharide matrix, which vary depending on the bacterial strains and environmental factors (Davies *et al.*, 1993).

2.9.3 Biofilm maturation

Bacteria with same or different species are driven towards the biofilm from the surrounding flow once the primary layer of the biofilm is formed. Biofilm forms from a thin coating to a mushroom or tower shape. Bacterial cells are placed in a thick biofilm (>100 layers) in accordance with their aerotolerance and metabolic rate. Anaerobic

microorganisms, for instance, choose to stay in deeper strata to avoid their exposure to oxygen. Bacteria present in communities of biofilm interact with each other and carry out important activities. Entrapped bacterial cells secrete extra biofilm scaffoldings, such as proteins, DNA and polysaccharides as the biofilm matures (Rabin *et al.*, 2015).

2.9.4 Dispersing mechanism

The biofilm dispersal process, also called as detachment. It is the last step of biofilm formation. It is thought to be a bacterial cell approach for leaving biofilms and starting a new biofilm life cycle. In other words, dissemination of attached bacterial cells from biofilms is a naturally occurring mechanism that permits bacteria to develop new microcolonies on new surfaces. Numerous factors, such as nutrient deprivation, population expansion and intense competition contribute to the spread of biofilms. Detachment can occur throughout the biofilm or only in some sections (Diaz-Salazar *et al.*, 2017).

Although detachment process varies among bacteria and even at species level, the entire mechanism can be classified into three phases: bacterial detachment from microcolonies, bacterial migration to a new surface, and bacterial attachment to the new substrate (Shen *et al.*, 2018). Dispersion of bacteria can also be an active behavior in which bacteria in biofilms undergo detachment in response to environmental fluctuations such as antibiotic stress, matrix-degrading enzymes, and nutrient deprivation, or passively regulated by external factors like shear stress (Lee and Yoon, 2017).

Genes associated in bacterial cell's motility, like genes for flagella synthesis and breakdown of extracellular polysaccharide matrix, are typically up-regulated during active detachment, whereas genes involved in production of extracellular polysaccharide matrix, adhesion, and fimbriae formulation are frequently down-regulated (Kostakioti *et al.*, 2013). Hindering the c-di-GMP signaling mechanism is another efficient strategy to detach biofilm because decreased intracellular c-di-GMP levels either restrict or enhance biofilm dispersion (Kaplan, 2010).

Different studies have reported the effect of colistin on biofilm of *A. baumannii*. Colistin acts on lipopolysaccharide layer and causes modifications in outer membrane of Gram-

negative bacteria. So, colistin reduces the attachment of bacterial cells on different biotic and abiotic surfaces. But some studies revealed that the sub minimal concentration of colistin may enhance the rate of biofilm formation in *A. baumannii*.

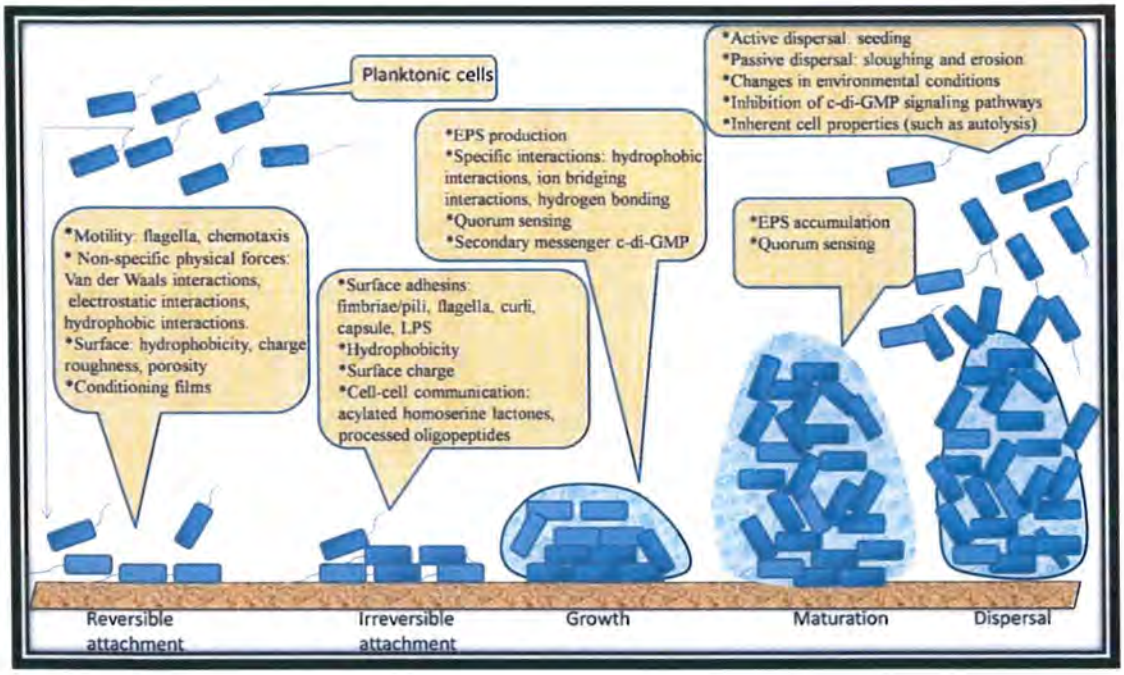

Figure 2.3: Five main stages leading to the development and dispersal of biofilm, Adapted from (Muhammad *et al.*, 2020).

2.10 Future treatment strategies

There is no new antibiotic in the pipeline that has a significant impact in control of multi drug resistant *A. baumannii*. Combination therapies can be effective, but it needs more in-depth understanding and research. Sato *et al* reported in vitro effect of colistin and tigecycline on biofilm of *A. baumannii*. Antibiotics can also be used in combination with bacteriophages for the treatment of infections caused by MDR *A. baumannii*. Wintachai *et al* reported the enhanced antibacterial activity of Friunavirus phage vWU2001 in combination with colistin to treat carbapenem resistant *A. baumannii* infections. Yang *et al* also reposted the antibacterial activity of AB1 bacteriophage against MDR *A. baumannii*. Nanoparticles can also be used in combination with multiple antimicrobial

agents. Hetta *et al* reported antibiofilm and anti-virulence activity of silver nanoparticles against MDR *A. baumannii*. Banoub *et al* reported the antibacterial activity of Chitosan nanoparticles in combination with tigecycline and meropenem. Zendegani and Dolatabadi Reported the antibacterial activity of meropenem conjugated with silver nanoparticles against clinical strains of *A. baumannii*.

MATERIALS AND METHODS

3.1 Sampling

This research was conducted on 100 strains of *Acinetobacter baumannii* collected from patients' samples at the Pakistan Institute of Medical Sciences (PIMS).

3.2 Sample source

100 clinical samples of *A. baumannii* were collected from multiple sources like sputum, blood, wounds, urine, endotracheal aspirates, and nasal swabs.

3.3 Study duration

This research was performed at Laboratory of Genomics and Molecular Epidemiology, Department of Microbiology, Quaid-I-Azam University Islamabad, and it was completed in 10 months.

3.4 Morphological identification of *Acinetobacter baumannii*

Isolates of *A. baumannii* were streaked on Luria Bertani agar and MacConkey agar. In morphological examination I observed coccobacilli shaped smooth, circular, mucoid, opaque, raised, creamy and pink color colonies.

3.4.1 Gram staining

Principal

Gram staining is most widely used procedure to differentiate among Gram positive and Gram-negative bacteria. Classification of bacteria into two groups is based on their ability to lose or retain the primary stain, crystal violet. Cell wall of Gram-positive bacteria have a thick layer of peptidoglycan as compared to Gram-negative bacteria. So, when smear of Gram-positive bacteria is stained with crystal violet, it deeply penetrates in multiple layers of peptidoglycan and when decolorizer is applied it dehydrates the cell wall and closes the pores. Primary stain does not come out and bacteria appear dark-blue

or purple. On the other hand, decolorizer degrades lipids of cell wall and allows crystal violet-iodine complex to come out from thin layer of Gram-negative bacteria. When cell is again stained with counter stain (safranin), it retains its color and appears pink in color.

Materials required

Glass slide, Platinum loop, Microscope, Gram's iodine solution, Crystal violet solution, Safranin solution and 95% ethanol.

a) Smear preparation

A drop of sterile distilled water was placed in the center of clean and grease free glass slide. Then a single bacterial colony from freshly streaked plate was mixed with distilled water and thin film was formed on glass slide. After that smear was air dried and heat fixed.

b) Staining

Air dried and heat fixed smear was stained with Primary stain (crystal violet) and kept it for about 1 minute. Then glass slide was washed with tap water and stained with mordant (Gram's iodine) for 1 minute. It was washed with water and decolorizer (95% ethanol) was added drop wise for ten to twenty seconds and again rinsed with tap water. After that (counter stain) safranin was added for 1minute and washed with water. Slide was air dried and observed under compound microscope at 100X.

c) Result interpretation

Purple or dark blue color indicates Gram positive bacteria while Gram-negative bacteria are indicted by pink color.

3.5 Biochemical identification of *A. baumannii*

3.5.1 Catalase test

a) Principal

Catalase is an enzyme that helps to break down hydrogen peroxide (H2O2) into oxygen and water. When bacterial colonies are mixed H2O2 the quick formation of oxygen bubbles shows that bacterial isolate is carrying catalase enzyme and bacteria is safe from the effect of H2O2 which is produced by host. No or inadequate bubble formation indicates the absence of catalase enzyme. It is recommended that bacterial culture should not be older than 24 hours.

Required materials

3% hydrogen peroxide, glass slide, platinum wire loop and bacterial culture

b) Procedure

A dry, clean and scratch free glass slide was taken and a loopful bacterial culture was transferred on glass slide with the help of wire loop. After that a drop of 3% hydrogen peroxide was placed on glass slide and bubble formation was observed.

c) Result interpretation

Rapid bubble formation indicates that bacteria is catalase positive and no or very few bubble formation after 2 minutes shows that bacteria is catalase negative.

3.5.2 Oxidase test

a) Principal

The oxidase test is used to check cytochrome oxidase system that catalyzes electron transfer among electron donors in the bacteria and a redox dye called tetramethyl-p-phenylene-diamine. Oxidase-positive microorganisms, that have cytochrome c as a component of their respiratory chain, change the dye into blue or purple. Organisms without cytochrome c cannot oxidize the dye, keeping it colorless and are therefore oxidase negative.

Required materials

Filter Paper, Kovacs oxidase reagent (1% tetra methyl-p-phenylenediamine dihydrochloride), Petri plate, bacterial Culture, wire loop

b) Procedure

A clean filter paper was placed on a petri plate and a loopful bacterial culture was transferred to the filter paper. Then 2 to 3 drops of 1% Kovacs oxidase reagent were added with the help of clean dropper and change in color was observed within 10 seconds.

c) Result interpretation

If color of reagent changes within 10 seconds, it means that microorganism is oxidase positive. If color of Kovacs oxidase reagent does not change even after 2 minutes, it means that microorganism is oxidase negative.

3.5.3 Indole test

a) Principal

Indole test is used to detect the presence of tryptophanase enzyme in microorganisms. In the presence of this enzyme tryptophan amino acid undergoes hydrolysis and deamination and indole and other byproducts such as pyruvic acid and ammonium are produced. When Kovacs reagent that is mixture of hydrochloric acid and p-dimethyl amino benzaldehyde in amyl alcohol is added indole combines with this reagent and color of solution changes from yellow to pink or cherry red color.

Material required

Kovacs reagent, bacterial culture, Test tube, tryptophan broth

b) Procedure

A sterilized test tube containing 4ml of tryptophan broth was taken and inoculated with 24 hours old bacterial culture under sterile conditions. After that test tube was incubated

overnight at 37°C. Next day 0.5ml of Kovacs reagent was added to the test tube with the help of clean dropper and results were observed within few minutes.

c) Result interpretation

If a pink or cherry red color ring is formed at the tip of medium it means that microorganism is positive for indole. If no ring is formed even after addition of appropriate Kovacs reagent it means that organism is negative for indole production.

3.5.4 Citrate utilization test

a) Principal

Citrate agar is being used to check whether the microorganism can use citrate as an energy source or not. Citrate is the sole source of carbon, and inorganic ammonium salts are the sole source of nitrogen in the citrate media. Bacteria that grow in this media produces pyruvate by conversion of citrate through citrate permease enzyme which is used to generate energy for bacterial metabolic cycle. The ammonium compounds are converted into ammonia and pH of media is increased. Due to increase in pH the color of bromothymol blue dye changes from green to blue.

Materials required

Test tube, Simmon's Citrate agar, bacterial culture and straight loop

b) Procedure

A slant of Simmon's Citrate agar was prepared under aseptic conditions. Then 2-3 colonies from overnight bacterial culture were picked with the help of straight loop and slant was streaked back and forth. After that test tube was incubated at 37°C for 24 to 48 hours and results were noted.

c) Result interpretation

If the color of slant changes from green to blue after 24 to 48 hours, it means that microorganism is citrate positive if color of slant remains green it means that microorganism is catalase negative.

3.5.5 Urease Test

a) Principal

Decarboxylation of amino acids produces urea which undergoes hydrolysis and produces dioxide and ammonia. Due to ammonia production the pH of media increases and color of phenol red indicator changes from orange to pink.

Materials Required

Christensen's Urea Agar, test tube, bacterial culture and straight loop

b) Procedure

Urea agar slant was prepared under sterile conditions. Then 2-3 colonies from overnight bacterial culture were picked with the help of straight loop and slant was streaked back and forth. After that test tube was incubated for 48 hours at 37°C and results were noted.

c) Result interpretation

If the color of media changes from orange to pink, it means that microorganism is urease positive. If color of media remains orange, it means that microorganism is urease negative.

3.5.6 Triple Sugar Iron (TSI) Test

a) Principal

As the name indicates TSI agar contains three sugars (lactose, sucrose and glucose) and iron. This test is used to check the fermentation of these three sugars and production of hydrogen sulfide and gas. When one of the sugars in the medium undergoes fermentation

the pH of media increases due to acid production. Bacteria reduces sodium thiosulfate into hydrogen sulfide that further reacts with iron and form iron sulfide.

Materials Required

TSI agar, test tube, straight loop and bacterial culture

b) Procedure

A slant of triple sugar iron agar was prepared under sterile conditions. Then 1-2 colonies from 24 hours old bacterial culture were picked with the help of straight loop and stabbed from medium to the bottom of test tube. Slant was also streaked back and forth. After that test tube was incubated for 18 to 24 hours at 37°C and results were observed.

c) Interpretation

If bacteria only ferment glucose, then the color of butt will be yellow and color of slant will be red. If bacteria ferment glucose, lactose or sucrose, then color of both butt and slant turns into yellow. If bacteria do not ferment any of three sugars, then color of both slant and butt remains red. Gas production is indicated by bubbles and cracks in the medium while production of hydrogen sulfide is indicated by blackening of the medium.

3.6 Extended Spectrum Beta Lactamase Test

a) Principal

Beta lactamase is an enzyme that is produced by bacteria and this beta lactamase enzyme inhibits the activity of Beta-lactam antibiotics. While clavulanic acid inactivates the beta lactamase enzyme, and the activity of beta lactam antibiotics is restored. When ESBL test is performed clavulanic acid from AMC disc inactivates beta lactamase enzyme, beta-lactam antibiotics become active and the diameter of zone of inhibition is increased.

Materials Required

Muller Hinton Agar, normal saline, 0.5 McFarland standard, 24 hours old bacterial culture Ceftazidime (CAZ 30µg), Cefotaxime (CTX 30µg), Ceftriaxone (CRO 30µg), Cefepime (FEP 30µg) and Amoxicillin-Clavulanate (AMC 20/10µg).

b) Procedure

Culture of *A. baumannii* was streaked on Luria Bertani agar and incubated for 24 hours at 37°C. After overnight incubation bacterial suspension was made by mixing 2-3 colonies from LB agar into 500µl normal saline. This suspension was compared with 0.5 McFarland standard and incubated for 10 minutes in shaker incubator at 37°C. After that a petri plate of Muller Hinton Agar (MHA) was prepared and lawn culture of bacteria was made on it with the help of swab. Then Amoxicillin-Clavulanate (AMC 20/10µg) disc was placed in the center of petri plate and discs of Ceftazidime (CAZ 30µg), Cefotaxime (CTX 30µg), Ceftriaxone CRO30µg were placed at 15mm distance and Cefepime (FEP 30µg) disc was placed at 20mm distance from AMC disc.

c) Result Interpretation

The synergism of zone of inhibition of any of 3rd generation Cephalosporins (Ceftazidime, Cefotaxime and Ceftriaxone) and 4th generation Cephalosporins (Cefepime) with zone of AMC disc indicated that bacteria is ESBL producer.

3.7 Genotypic Detection of Extended Spectrum Beta Lactamase Genes

3.7.1 Plasmid DNA Extraction by Alkaline Lysis Method

a) Principal

Solution 1 removes the cell wall of bacteria and transforms it into spheroplast form. Glucose in Solution 1 helps to prevent degradation of bacterial DNA by inhibiting early osmotic lysis of the bacteria. Ethylenediamine tetra acetic acid (EDTA) degrades the outer membrane of bacteria while Tris-HCL of Solution 1 works as a buffer for Solution 2 and maintains the pH up to 12. The spheroplast is lysed by NaOH of Solution

2, which partially degrades nucleases. SDS in Solution 2 causes the cells lysis and nuclease denaturation. The glacial acetic acid of Solution 3 involves in pH neutralization, which allows the DNA strands to renature while potassium acetate precipitates out cell debris and SDS.

Materials required

24 hours old bacterial culture, 99.9% ethyl alcohol, 70% ethyl alcohol, centrifuge tubes, RNA free tubes and T.E buffer

Solution 1: Glucose, Tris-HCL and EDTA (pH=8)

Solution 2: NaOH and 20% SDS

Solution 3: Potassium acetate and glacial acetic acid (pH=5.5)

b) Procedure

For plasmid DNA extraction cultures of *Acinetobacter baumannii* were freshed in 1.5 ml of Tryptic Soy Broth (TSB) overnight at 37°C. Next day Eppendorf tubes containing 1.5 ml of bacterial inoculum were vortexed and centrifuged at 14000rpm for one minute. Supernatant was discarded and pellet was resuspended in in 200µl of ice-cold Solution 1 and mixed as much as possible with the help of pipet. After that 200µl of ice-cold Solution 2 was added, tubes were inverted to mix it properly and placed them at room temperature for about five minutes. Then 200µl of ice-cold solution 3 was added to the tubes, mixed it by inverting the tubes and incubated them on ice for ten minutes. Then tubes were centrifuged at 14000rpm for ten minutes and supernatant was transferred into new tubes. 900µl of 99.9% ethyl alcohol was added to supernatant and mixed it gently. Tubes were again centrifuged at 14000rpm for twenty minutes and supernatant was discarded properly without disrupting the pellet. After that 100µl of 70% of chilled ethyl alcohol was added to the pellet and centrifuged the tubes at 14000rpm for 30 seconds. Supernatant was discarded and tubes were air dried in biosafety cabinet for 20 minutes. At the end pellet was resuspended in 50µl of TE buffer and stored it at -20°C.

3.7.2 Visualization of Plasmid DNA

The extracted plasmid DNA of *A. baumannii* was visualized by using agarose gel electrophoresis.

a) Principal

Agarose gel electrophoresis is a technique that separates DNA fragments of different sizes on agarose gel. when DNA sample is loaded into the wells of agarose gel and an electric current is applied then negatively charged DNA starts move towards the positively charged anode. The distance travelled by DNA fragment is inversely proportional to the size of the DNA fragment: smaller fragments migrate faster and end up towards the bottom of agarose gel. Ethidium bromide dye is used to visualize DNA fragments in the gel. As DNA fragments travel through the agarose gel, they pick up Ethidium bromide and fluoresces when subjected to UV light.

Materials Required

Electrophoresis chamber (Gel tank), power supply, gel casting tray, comb, Ethidium bromide, DNA loading dye, transilluminator and loading buffer (TBE).

b) Procedure

0.4g of agarose powder was measured and added it into a beaker. 36ml of distilled water and 4ml of 1x TBE buffer was added into the same beaker and heated it on a hot plate until a clear transparent solution is formed. Then beaker was removed from hot plate and allowed it cool down up to 50°C. 4µl of Ethidium bromide was added into gel and mixed properly. Then combs were fixed, gel was poured into the gel casting tray and allowed it to get solidify at room temperature. After solidification combs were removed and gel along with casting tray was transferred to the Electrophoresis chamber containing 1x TBE buffer. Then 3µl of each extracted plasmid DNA of *A. baumannii* was mixed with 1.5µl of DNA loading dye and loaded it into wells conscientiously. Lid was placed on gel tank; electrodes were connected to the apparatus and 70 volts with 400mA current was

applied for 40 minutes. After that gel was transferred to transilluminator and visualized under UV-light.

3.7.3 Polymerase Chain Reaction (PCR)

PCR was used to amplify Extended Spectrum Beta-Lactamase (ESBL) genes in *A. baumannii*.

3.7.3.1 Primers Dilutions

The preserved stock solution of primers was used to make a working solution of forward primers and reverse primers for polymerase chain reaction (PCR). 40µl of PCR water and 10µl of both forward and reverse primers from stock solution were added into autoclaved and labelled Eppendorf tubes.

Table 3.1: Oligonucleotide primers, annealing temperature and product size of *TEM-1*, *CTXM-15* and *SHV-1*.

Target Gene	Primer	Oligonucleotide sequence (5'-3')	Annealing Temperature (°C)	Amplicon size(bp)
TEM-1	Forward	GATCTCAACAGCGGTAAG	57	500
	Reverse	CAGTGAGGCACCTATCTC		
SHV-1	Forward	ACTATCGCCAGCAGGATC	55	600
	Reverse	ATCGTCCACCATCCACTG		
CTXM-15	Forward	GTGATACCACTTCACCTC	55	255
	Reverse	AGTAAGTGACCAGAATCAG		

3.7.3.2 Polymerase Chain Reaction (PCR) for screening of ESBL genes (*TEM-1*, *SHV-1* and *CTXM-15*)

a) Principal

Polymerase Chain Reaction (PCR) is a technique that is used to amplify the DNA by using enzyme DNA Polymerase. DNA Polymerase adds nucleotides at 3' OH group and forms new strands that are complementary to the template strands. PCR is divided into three main cyclic reactions based on temperature variations in thermos cycler.

1). Denaturation: It is the first step of PCR and temperature is usually up to 94°C. During this step hydrogen bonds between double stranded DNA are broken down to form single stranded DNA that are used as a template. **2) Annealing:** During this step temperature is decreased and primers bind with complementary sequences on DNA template strands. **3) Elongation:** Temperature increases and Taq polymerase enzyme adds nucleotides into 3' end of primers and DNA elongation starts from 5'-3' direction. These steps are continuously repeated for 25 to 40 times for making exact copies of DNA.

b) Procedure

Reaction mixtures of *TEM-1*, *SHV-1* and *CTXM-15* were prepared in PCR tubes according to recipe given in table no 3.3 and reaction was carried out in thermos cycler machine.

Table 3.2: PCR reaction mixture ingredients and volume for five DNA samples

Reaction Mixture Ingredients for 5 DNA samples	Volume
Diluted DNA (0.7µl DNA+0.8µl PCR H2O)	1.5µl
PCR water	31.5µl

Taq Buffer	5µl
MgCl2	3µl
DNTPs	1µl
Forward Primer	2µl
Reverse Primer	2µl
Taq Polymerase	0.5µl

Table 3.3: Cycles of polymerase chain reaction.

Sr. No	PCR steps	Temperature	Time	Number of Cycles
1	Initial Denaturation	94°C	5 min	1
2	Denaturation	94°C	30 sec	35
3	Annealing	*TEM-1*(57) *SHV-1*(55) *CTXM-15* (55)	30 sec	35
4	Initial Extension	72°C	30 sec	35
5	Final Extension	72°C	5 min	1

3.7.2 Agarose Gel Electrophoresis

a) Procedure

1.5% (0.6g agarose) of agarose gel was prepared in a beaker and allowed it to cool down up to 50-55°C. 4µl of Ethidium bromide was added and mixed with the help of pipette. Sample combs were placed in gel casting tray and gel was poured in it. After solidification for about 25-30 minutes gel casting tray was placed in electrophoresis chamber. 3µl of each PCR product of *TEM-1*, *SHV-1* and *CTXM-15* was mixed with 1.5µl of DNA loading dye and pipetted into sample wells. Chamber was covered by lid and both electrodes were connected to the apparatus. 70 volts with 400mA current was provided for 35 minutes.

b) Gel Visualization under UV-light

After 40 minutes gel was removed from electrophoresis chamber, transferred to transilluminator and visualized under UV-light.

3.8 Biofilm Formation and Characterization of *A. baumannii* Isolates

Required Materials

96-well microtiter plate, TSA, TSB. Pipette, Eppendorf tubes blue and yellow tips

20-25µl of bacterial samples were refreshed in 1ml of TSB and incubated overnightat 37°C. Next day 3-5µl of bacterial suspension was taken from TSB and streaked on petri plate containing TSA (Tryptic Soy Agar). Petri plates were incubated overnight at 37°C.

Microtiter Plate Inoculation

2-3 colonies of each sample were suspended into 1ml Muller Hinton Broth (MHB) in labeled Eppendorf tubes and incubated for 15-20 minutes at 37°C. 96-well microtiter plate was sterilized under UV-light of laminar air flow and labelled properly. 200µl of each bacterial sample was added into wells in triplicate form. 200µl of media and 200µl

of bacterial suspension were added into media and growth controls respectively and incubated the microtiter plate overnight at 37°C.

Biofilm Washing

Materials Required

Phosphate Buffer Saline (PBS), 70% methanol, 2% crystal violet and 95% ethanol

Procedure

After overnight incubation media was discarded and wells were washed 3 times with 200µl of Phosphate Buffer Saline (PBS) to remove planktonic microorganisms. After that biofilm was fixed with 150µl of 70 methanol for 15 minutes. Methanol was discarded and microtiter plate was air dried in laminar air flow for 5 minutes. Then biofilm was stained by 150µl of 2% crystal violet for 15 minutes. Crystal Violet was removed, and microtiter plate was again washed 3 times with 200µl of Phosphate Buffer Saline. After that Microtiter plate was air dried and 200µl of 95% ethanol was added to solubilize the stained biofilm. Optical Density (OD) was taken at 492nm and 630nm in ELISA reader.

Result Interpretation

Isolates are classified into weak, moderate, strong and non-biofilm formers based on following criteria:

Non-biofilm formers: $OD \leq ODc$

Weak biofilm formers: $ODc < OD \leq 2ODc$

Moderate biofilm formers: $2ODc < OD \leq 4ODc$

Strong biofilm formers: $OD > 4ODc$

3.9 Minimum Inhibitory Concentration (MIC-p)

MIC- p is the minimum amount of antibiotic required to inhibit the growth of planktonic microorganisms.

Stock Solution Preparation

The stock solution of colistin was prepared according to the CLSI guideline 2019 by using following formula.

$$W=CxVx100/P$$

Where,

W= Weight of powder antibiotic

C= Final concentration of antibiotic

V= Required volume of antibiotic

P= Potency of antibiotic

Stock solution of Colistin was prepared by dissolving 0.1g of powdered colistin into 10ml autoclaved distilled water and mixed it until drug dissolved completely. Stock solution was stored at -20°C.

Table 3.4: Solvent and diluent of colistin antibiotic.

Antibiotic	Solvent	Diluent
Colistin	Water	Water

Working Solution Preparation

For working solution preparation 4.5ml of Muller Hinton Broth (MHB) was added into a test tube and 500µl of stock solution was pipetted into the same test tube and mixed properly.

Serial Dilution of Antibiotic

11 autoclaved test tubes were placed in test tube rack and labelled from 1024µg/ml-1µg/ml. 1ml of MHB was pipetted into all test tubes except first test tube(1024µg). After

that 2ml of working solution was added into first test tube (1024µg/ml) and from it 1ml of MHB was transferred into second test tube. Then 1ml from second test tubes was transferred into third test tube. In this way drug was serially diluted up to 11th test tube from which 1ml was discarded.

Primary Suspension Preparation

With the reference of CLSI guideline 1-2 colonies of *A. baumannii* were picked from freshly streaked LB agar, missed it into 1ml of MHB and compared it with 0.5 McFarland standards. After that Eppendorf was incubated for 20 minutes in incubator.

Secondary Suspension Preparation

14ml and 850µl of MHB was added into test tube and 150µl from primary suspension was added and mixed properly.

1ml from secondary suspension was added into all test tubes and incubated them overnight at 37°C.

Result Interpretation

After incubation of 24 hours test tubes were checked for turbidity. The last test tube with no visible growth was considered as minimum inhibitory concentration (MIC-p).

3.10 Minimum Inhibitory Concentration of Biofilm (MIC-b)

The lowest concentration of a drug that inhibits the biofilm formation ability of planktonic bacteria is known as minimum inhibitory concentration of biofilm (MIC-b). All the isolates of *A. baumannii* were sensitive to colistin and MIC-b was performed only on two isolates.

Biofilm Formation

Tested isolates of *A. baumannii* were streaked on Luria Bertani agar petri plates and incubated overnight at 37°C. Next day 2-3 colonies were picked with the help of autoclaved toothpick and mixed in 1ml of Muller Hinton Broth and incubated for 15

minutes. After that 96-well microtiter plate was sterilized under UV-light of laminar air flow and labelled properly. 75µl of bacterial suspension was added into labelled wells. 75µl of MHB was added into last column as a media control while 75µl of bacterial suspension was added into last row as a growth control and incubated the microtiter overnight at 37°C.

Stock Solution Preparation

Stock solution of Colistin was prepared by dissolving 0.1g of powdered colistin into 10ml autoclaved distilled water and mixed it until drug dissolved completely. Stock solution was stored at -20°C.

Working Solution Preparation

For working solution preparation 4.5ml of Muller Hinton Broth (MHB) was added into a test tube and 500µl of stock solution was pipetted into the same test tube and mixed properly.

Serial Dilution of Antibiotic

11 autoclaved test tubes were placed in test tube rack and labelled from 1024µg/ml-1µg/ml. 1ml of MHB was pipetted into all test tubes except first test tube(1024µg). After that 2ml of working solution was added into first test tube (1024µg/ml) and from it 1ml of MHB was transferred into second test tube. Then 1ml from second test tubes was transferred into third test tube. In this way drug was serially diluted up to 11^{th} test tube from which 1ml was discarded.

Adding Antibiotic Dilutions into Microtiter plate

After overnight incubation planktonic suspension was removed from microtiter plate carefully without disrupting biofilm at the bottom. Then wells were washed 3 times with 100µl of Phosphate Buffer Saline (PBS). After that 100µl of diluted drug was added into wells carefully according to the labelling. Microtiter plate was again incubated overnight.

Result Interpretation

The lowest antibiotic concentration that has inhibited the biofilm formation of bacteria was considered as MIC-b. Clear and turbid wells checked carefully.

3.11 Minimum Regrowth Concentration of Biofilm (MRC/MBIC)

Minimum Regrowth Concentration (MRC) is defined as the lowest concentration of antibiotic that prevents regrowth of bacterial cells. Following the result interpretation of MIC-b in the previous stage, the transparent wells were washed 3 times with 100µl of phosphate buffer saline (PBS). After that 200µl of recovery media (Muller Hinton Broth) was added in clear wells and microtiter plate was again incubated overnight at 37°C. After overnight incubation the transparent wells were considered for MRC (Minimum Regrowth Concentration of antibiotic).

3.12 Minimum Biofilm Eradication Concentration (MBEC)

Minimum Biofilm Eradication Concentration (MBEC) is defined as the minimal concentration of antibiotic that completely eradicates regrowth of bacterial cells after antibiotic exposure or the lowest concentration of antibiotic that completely eradicates the biofilm. Microtiter plate was inoculated with 75µl of bacterial suspension and incubated the plate overnight for biofilm formation. Next day all the wells were washed 3 times with 100µl of phosphate buffer saline. Two-fold dilutions of antibiotics were made from 1024µg/ml to 1µg/ml and added in respective wells carefully and incubated it overnight. After 24 hours of incubation solution was discarded and wells were again washed three time with 100µl of phosphate buffer saline. Then biofilm from the bottom of wells was scraped with the help of scraper and mixed in to 1ml of phosphate buffer saline in an Eppendorf tube and vortex it to disrupt the biofilm. After that this material as streaked on Tryptic Soy Agar (TSA) and incubated the petri dish overnight at 37°C.

Result Interpretation

After 24 hours plates with no bacterial growth were considered for Minimum Biofilm Eradication Concentration (MBEC).

RESULTS

4.1 Morphological Identification of *Acinetobacter baumannii*

Isolates of *Acinetobacter baumannii* were confirmed by Gram staining and colony morphology on different growth media.

4.1.1 Colony Morphology

Isolates of *A. baumannii* were streaked on MacConkey agar, Luria Bertani agar, Tryptic Soy agar and Nutrient agar. Smooth, mucoid, shiny colonies with entire edges and raised elevation were observed.

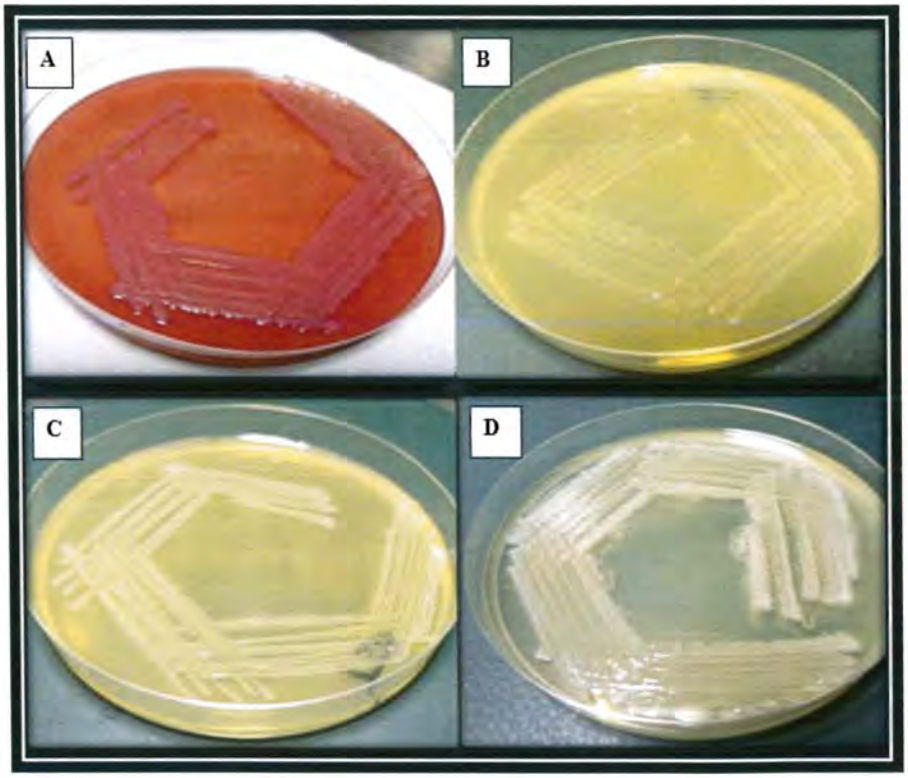

Figure 4.1: Colony Morphology of *A. baumannii*. (A) shows colony morphology of *A. baumannii* on MacConkey Agar. (B) shows colony morphology of *A. baumannii* on Luria Bertani Agar. (C) shows colony morphology of *A. baumannii* on Tryptic Soy Agar. (D) shows colony morphology of *A. baumannii* on Nutrient Agar.

4.1.2 Gram Staining

Isolates of *A. bumannii* were further confirmed by Gram staining. At 100X Gram negative rods were observed.

Figure 4.2: It indicates pink color rod shaped *A. baumannii*.

4.2 Biochemical Identification of *A. baumannii*

Table 4.1: Biochemical identification of *A. baumannii*

Biochemical Tests	Results
Catalase test	Positive
Oxidase Test	Negative
Indole Test	Negative
Citrate Utilization Test	Positive
Urease Test	Negative
Triple Sugar Iron (TSI) Test	No gas and H2S production with Alkaline slant and no change in color at bottom

4.2.1 Catalase Test

Catalase test was performed on *A. baumannii* and negative control. When 1-2 drops of hydrogen peroxide were added bubble formation was observed which showed that *A. baumannii* was catalase positive.

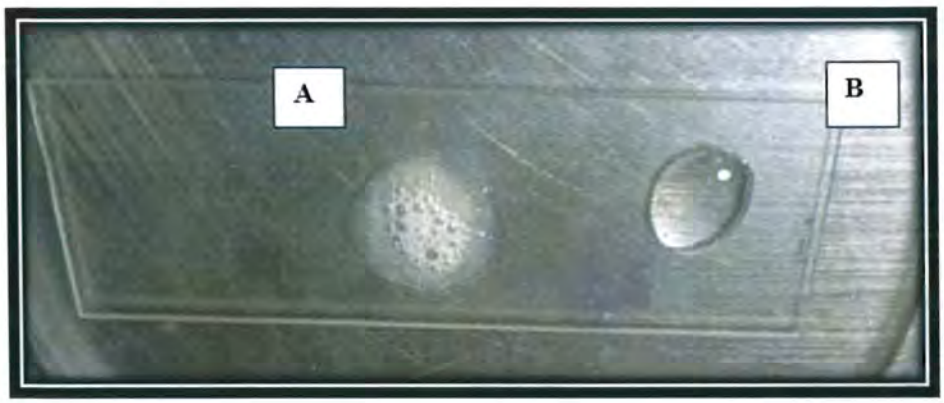

Figure 4.3: (A) shows catalase positive *A. baumannii* while (B) shows a negative control.

4.2.2 Oxidase Test

Oxidase test was also performed for confirmation of *A. baumannii*. When 1-2 drops of Kovacs oxidase reagent were added no color change was observed which indicated that *A. baumannii* is oxidase negative.

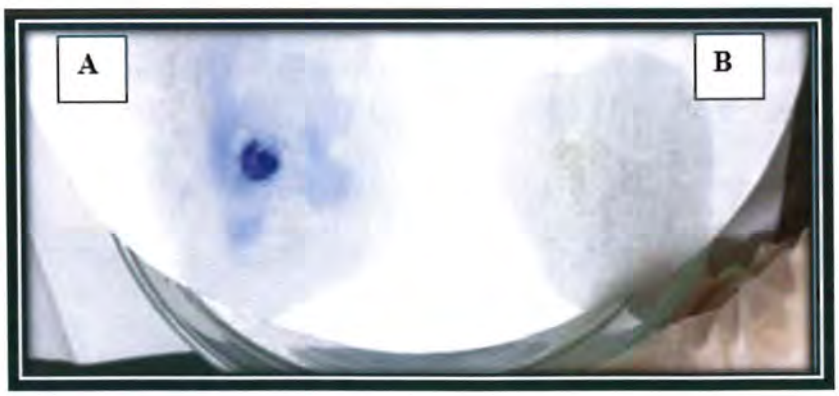

Figure 4.4: (A) indicates oxidase positive *P. aeruginosa*, used as a positive control while (B) indicates oxidase negative *A. baumannii*.

4.2.3 Indole test

Indole test was performed for confirmation of *A. baumannii*. No ring formation was observed.

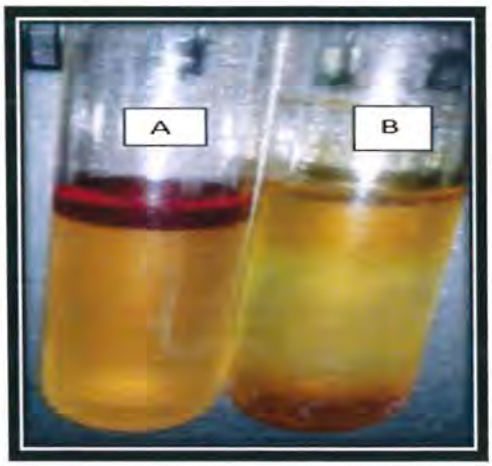

Figure 4.5: (A) indicates indole positive *Escherichia coli* with ring formation and (B) indicates indole negative *A. baumannii* with no ring formation.

4.2.4 Citrate Utilization Test

Citrate utilization test was performed to check the ability of *A. baumannii* to utilize citrate as a sole source of carbon. Color of slant was changed from green to blue which shows that *A. baumannii* is citrate positive.

Figure 4.6: (A) indicates citrate negative *E. coli* and (B) indicates citrate positive *A. baumannii*.

4.2.5 Urease Test

Isolates of *Acinetobacter baumannii* were also confirmed by urease test. No change in color was observed which indicates that *Acinetobacter baumannii* is urease negative.

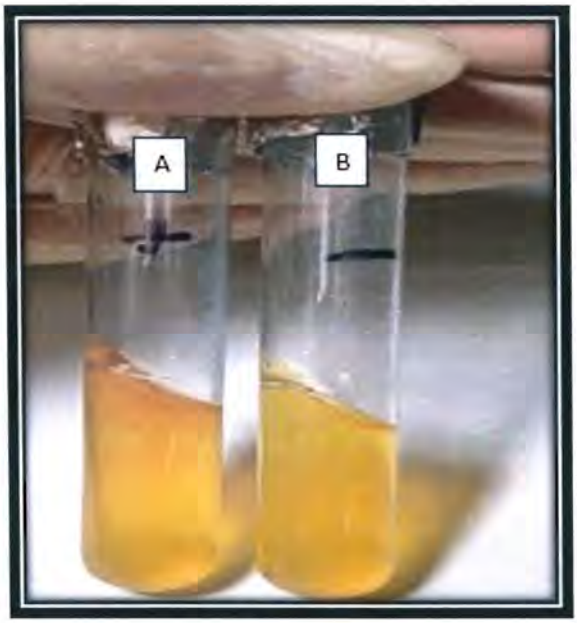

Figure 4.7: (A) shows urease positive *Escherichia coli* while (B) shows urease negative *Acinetobacter baumannii.*

4.2.6 Triple Sugar Iron Test

Triple Sugar Iron (TSI) was also performed for confirmation. *Acinetobacter baumannii* did not ferment any of three sugars. So, the color of slant and bottom remained red with no gas and H_2S production. TSI test was also performed on *Escherichia coli* for comparison. *E. coli* fermented all of three sugars, so the color of slant and bottom remained red and yellow respectively with gas production.

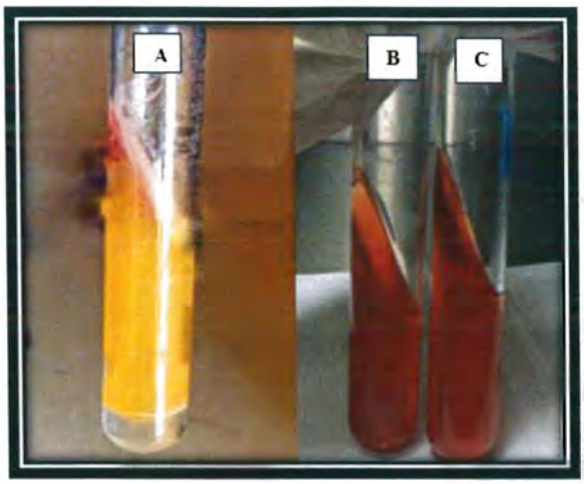

Figure 4.8: (A) indicates red slant and yellow bottom of *E. coli* with gas production at bottom while (B) and (C) indicates *A. baumannii* with red slant and bottom.

4.3 Extended Spectrum Beta Lactamase (ESBL) Test

Synergism of zone of inhibition of 3rd generation and 4th generation Cephalosporins with zone of Amoxicillin-Clavulanate (AMC) disc was observed which indicated that bacteria is ESBL producer. Out of 100 isolates of *A. baumannii* only 28 isolates were ESBL producers.

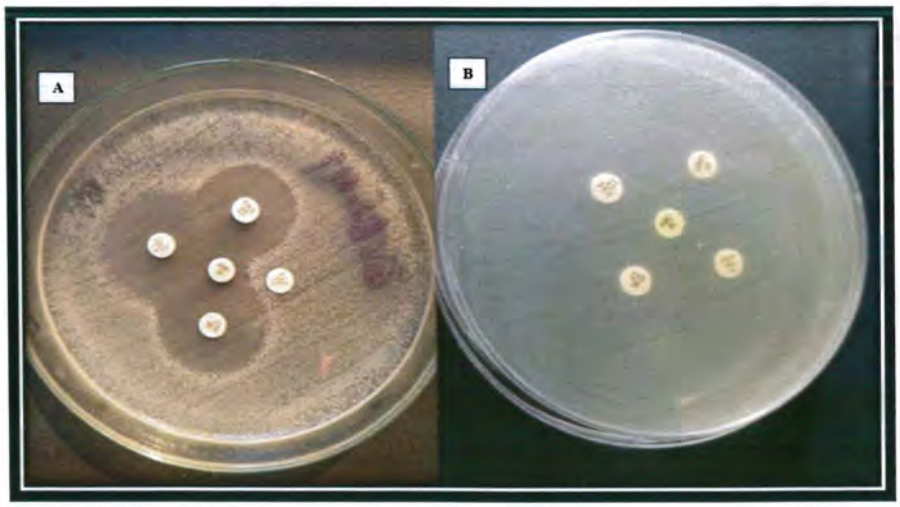

Figure 4.9: (A) indicates ESBL positive *A. baumannii* while (B) indicates ESBL negative *A. baumannii*.

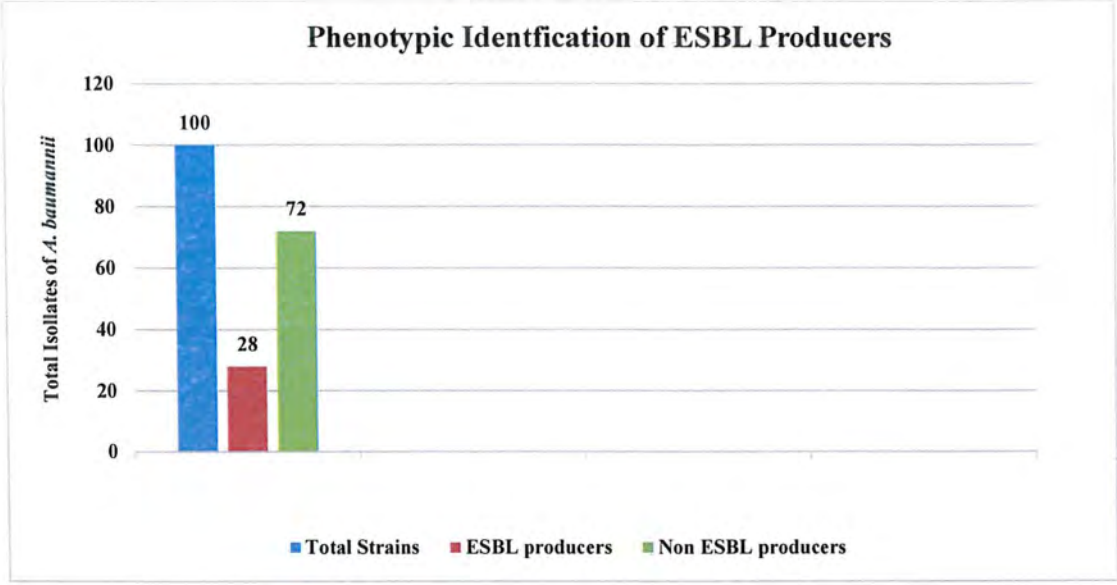

Figure 4.10: Bar graph indicating the 28 ESBL producers and 72 non-ESBL producers out of 100 isolates of *A. baumannii*.

4.4 Visualization of Plasmid DNA Extracted by Alkaline Lysis Method

Plasmid DNA of 28 isolates of *A. baumannii* was extracted by Alkaline Lysis Method and bands of plasmid DNA were visualized on agarose gel.

Figure 4.11: Bands of plasmid DNA on agarose gel.

4.5 Genotypic Detection of Extended Spectrum Beta Lactamase Genes

28 strains of *A. baumannii* were phenotypically positive for ESBL test and these isolates were further screened for *TEM-1*, *SHV-1* and *CTXM-15* genes. Out of 28 ESBL producers, 25 isolates have *TEM-1* gene, 16 isolates have *CTXM-15* gene and 9 isolates have *SHV-1* gene.

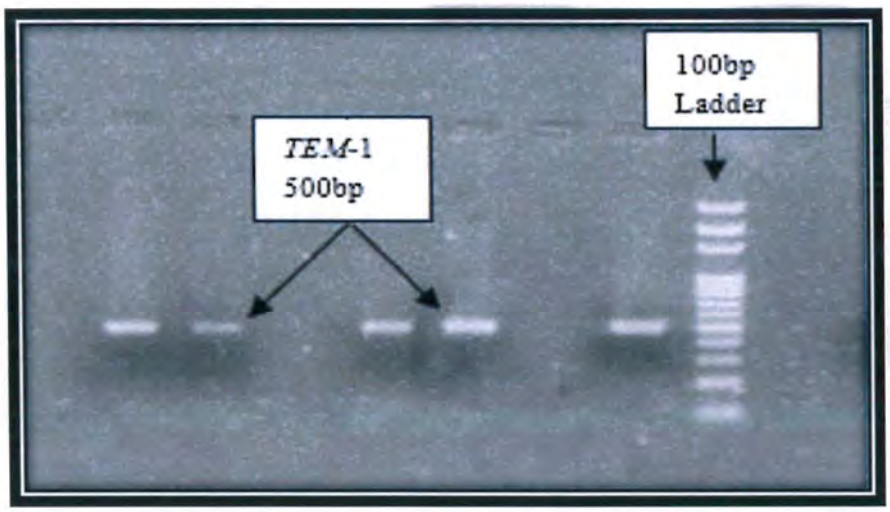

Figure 4.12: Shows *TEM-1* gene on agarose gel. Size of *TEM-1* is 500bp and 100bp ladder was also used for comparison.

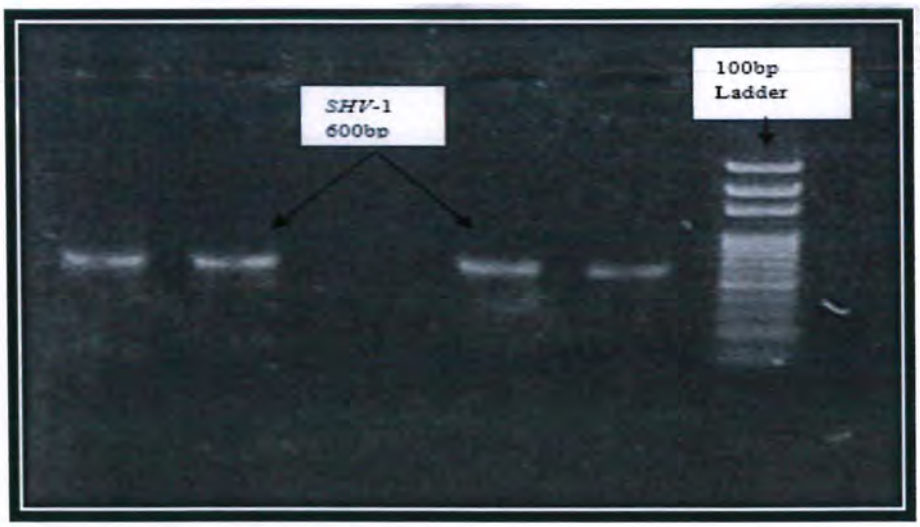

Figure 4.13: Shows *SHV-1* gene on agarose gel. Size of *SHV-1* is 600bp and 100bp ladder was also used for comparison.

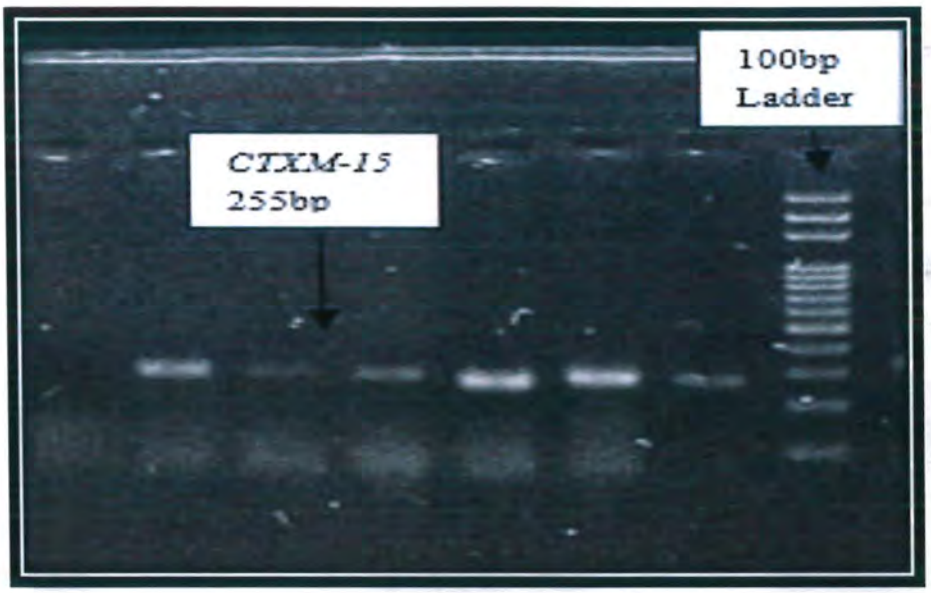

Figure 4.14: Shows *CTXM-15* on agarose gel. Size of *CTXM-15* is 255bp and 100bp ladder was also used for comparison.

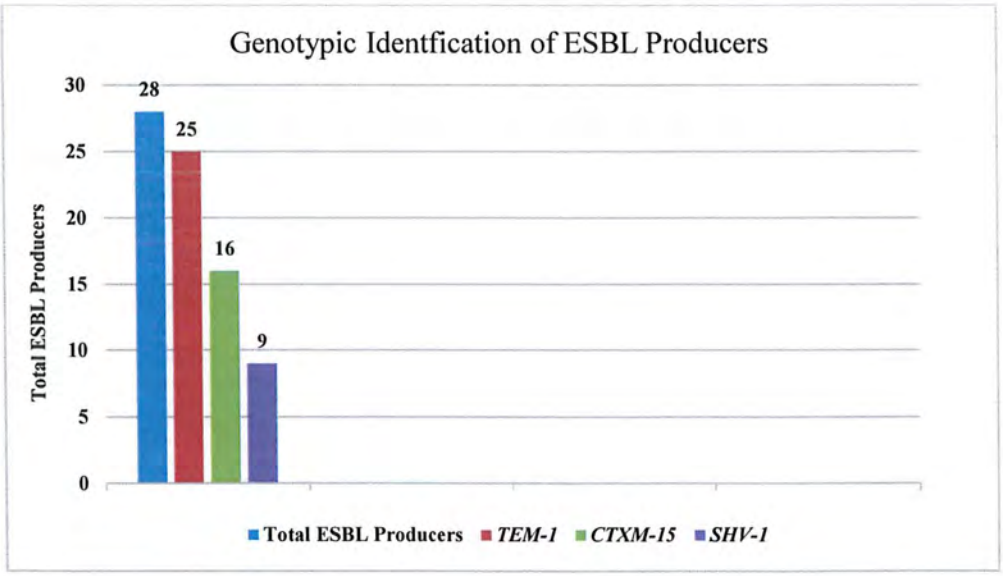

Figure 4.15: Bar graph shows that out of 28 ESBL producers, 25 isolates have *TEM-1*, 16 isolates have *CTXM-15* and 9 isolated have *SHV-1* gene.

4.6 Biofilm Formation in *A. baumannii*

Biofilm of 100 isolates of *A. baumannii* was formed on flat bottomed 96-well microtiter plate and optical density was measured at 630nm and 492nm with the help of ELISA plate reader. Out of 100 strains 35 strains were strong biofilm formers, 60 strains were moderate biofilm formers and 5 strains were weak biofilm formers.

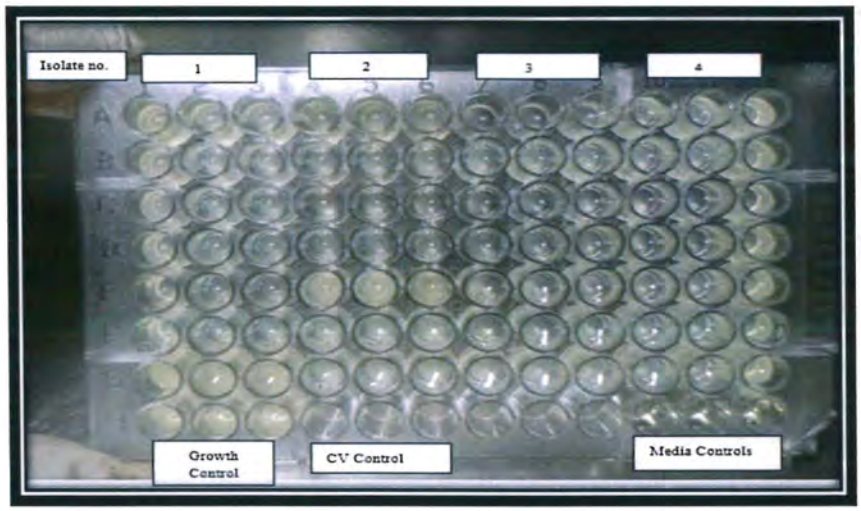

Figure 4.16: Shows biofilm formation before staining with crystal violet.

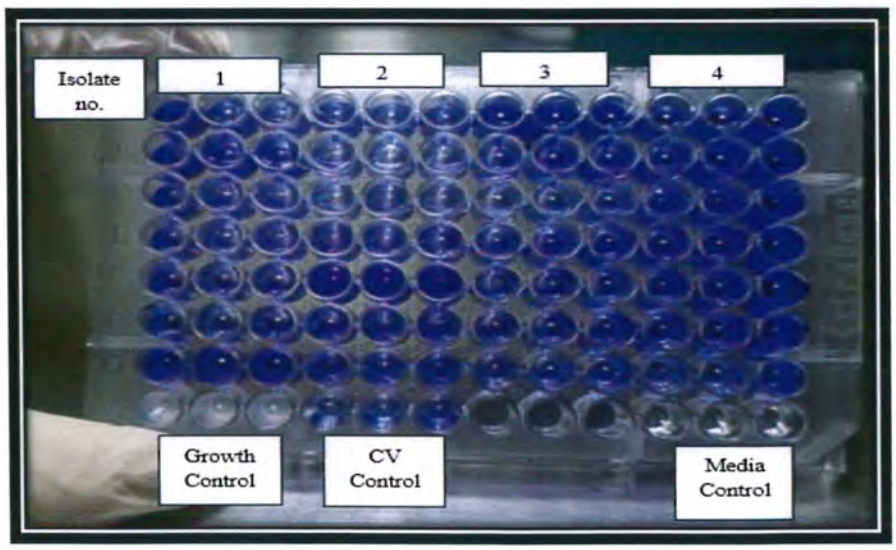

Figure 4.17: Shows biofilm formation after staining with crystal violet.

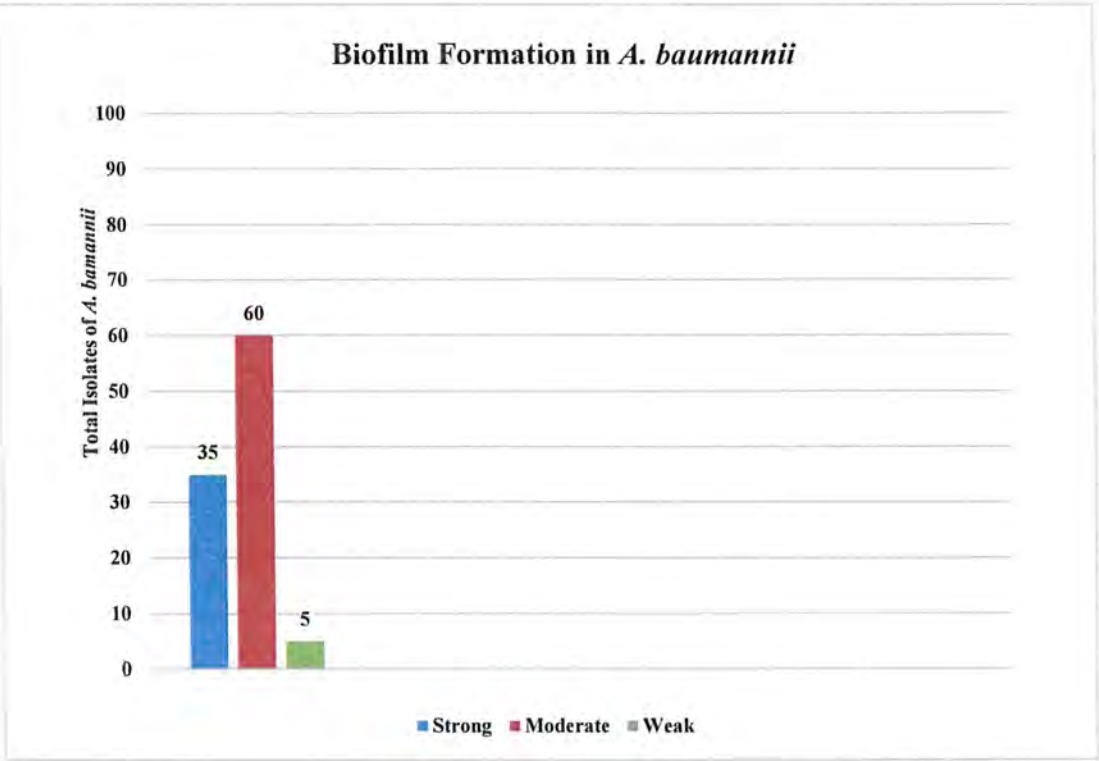

Figure 4.18: Bar graph shows biofilm formation in *A. baumannii*. Out of 100 isolates 35 isolates were strong biofilm formers, 60 isolates were moderate biofilm formers, and 5 isolates were weak biofilm formers.

4.7 Minimum Inhibitory Concentration (MIC-P)

After incubation of 24 hours test tubes were checked for turbidity. The last test tube with no visible growth was considered as minimum inhibitory concentration. The MICs of 71, 72, 58 and 92 isolates were 2, 2, 4 and 2µg/ml respectively.

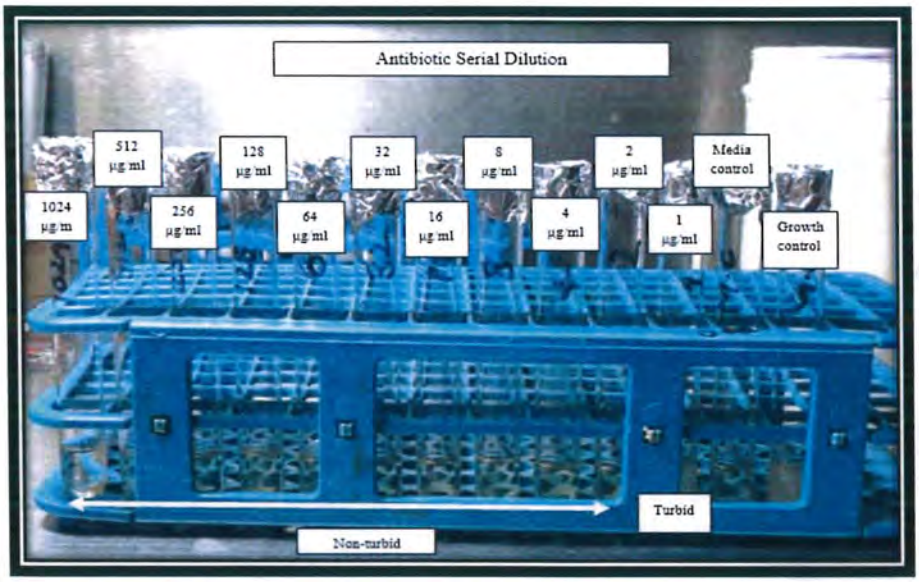

Figure 4.19: Shows minimum inhibitory concentration (MIC-p) of colistin of selected sensitive isolate. MIC-p value of colistin is 2µg/ml.

4.8 Minimum Inhibitory Concentration of Biofilm (MIC-b)

After 24 hours of incubation turbidity was checked. The last well with no visible growth was considered as minimum inhibitory concentration of biofilm (MIC-b). The MIC-b values of 71, 72, 58 and 92 were 4, 2, 4 and 4µg/ml respectively.

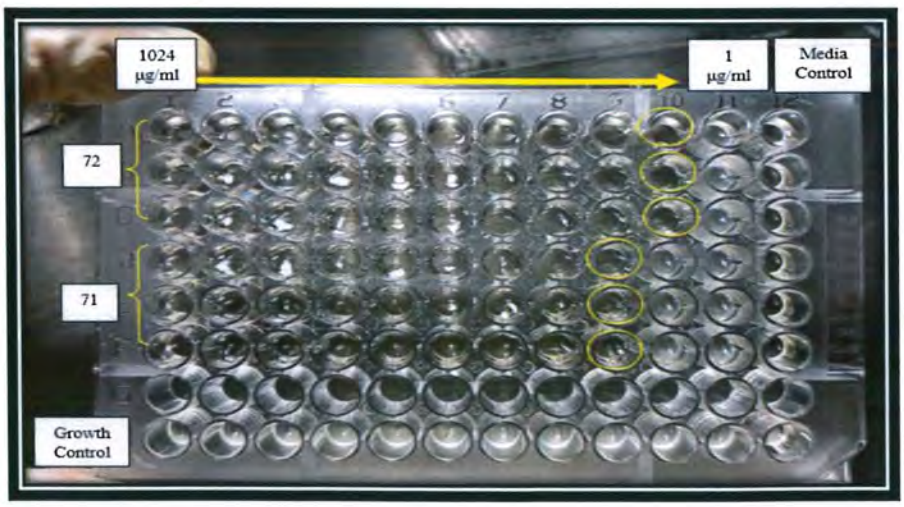

Figure 4.20: Indicates MIC-b value of colistin for isolate no. 72 and 71. Encircled wells show MIC-b value. Both media and growth controls are also shown.

4.9 Minimum Regrowth Concentration of Biofilm (MRC/MBIC)

After 24 hours of incubation turbidity was checked and wells with no visible growth were considered for MRC. MRC values for 71, 72, 58 and 92 were 8, 16, 16 and 16µg/ml respectively.

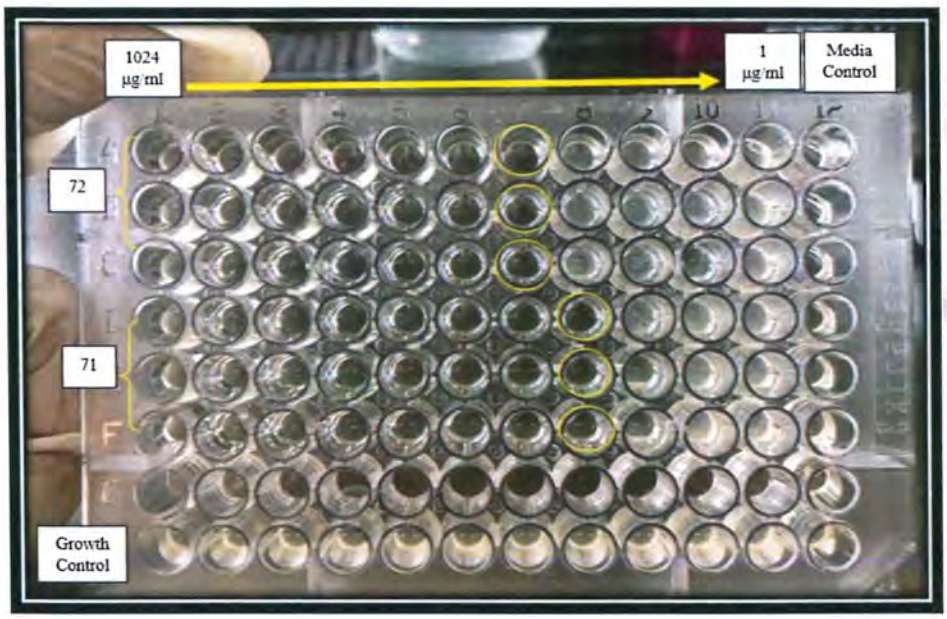

Figure 4.21: Indicates MRC value of colistin for isolate no. 72 and 71. Encircled wells show MRC value. Both media and growth controls are also shown.

4.10 Minimum Biofilm Eradication Concentration (MBEC)

After 24 hours of antibiotic addition on preformed biofilm wells were scraped and streaked on TSA. Next day petri plates with no visible growth were considered for MBEC. The MBEC values for 71, 72, 58 and 92 were 128, 64, 128 and 256µg/ml respectively.

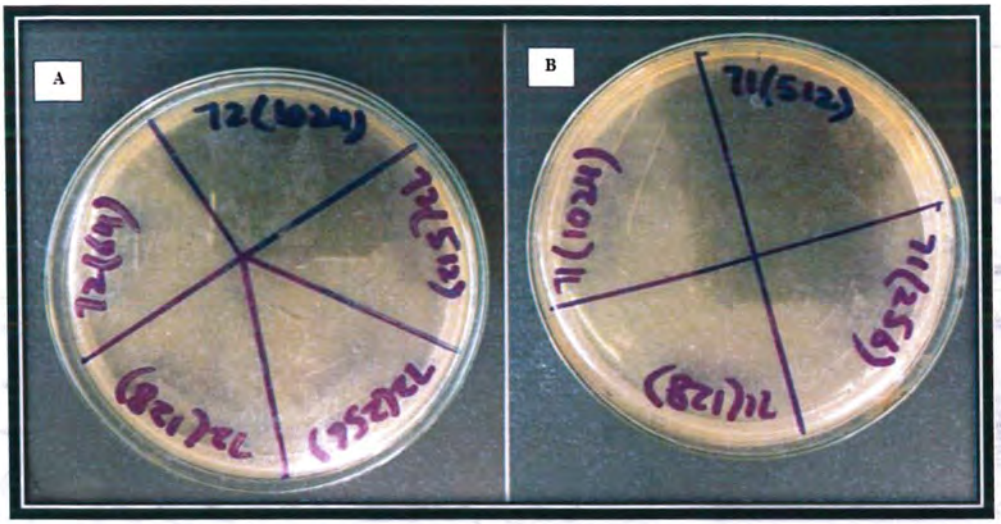

Figure 4.22: (A) indicates no visible growth of isolate no. 72 from 1024µg/ml to 64µg/ml and (B) indicates no visible growth of isolate no. 71 from 1024µg/ml to 128µg/ml.

Table 4.2: Shows MIC-p, MIC-b, MRC/MBIC and MBEC values of colistin against selected isolates of *A. baumannii*.

Sr no.	Isolate no.	Antibiotic	Susceptibility	MIC-p (µg/ml)	MIC-b (µg/ml)	MRC/MBIC (µg/ml)	MBEC (µg/ml)
1	71	Colistin	Sensitive	2	4	8	128
2	72	Colistin	Sensitive	2	2	16	64
3	58	Colistin	Sensitive	4	4	16	128
4	92	Colistin	Sensitive	2	4	16	256

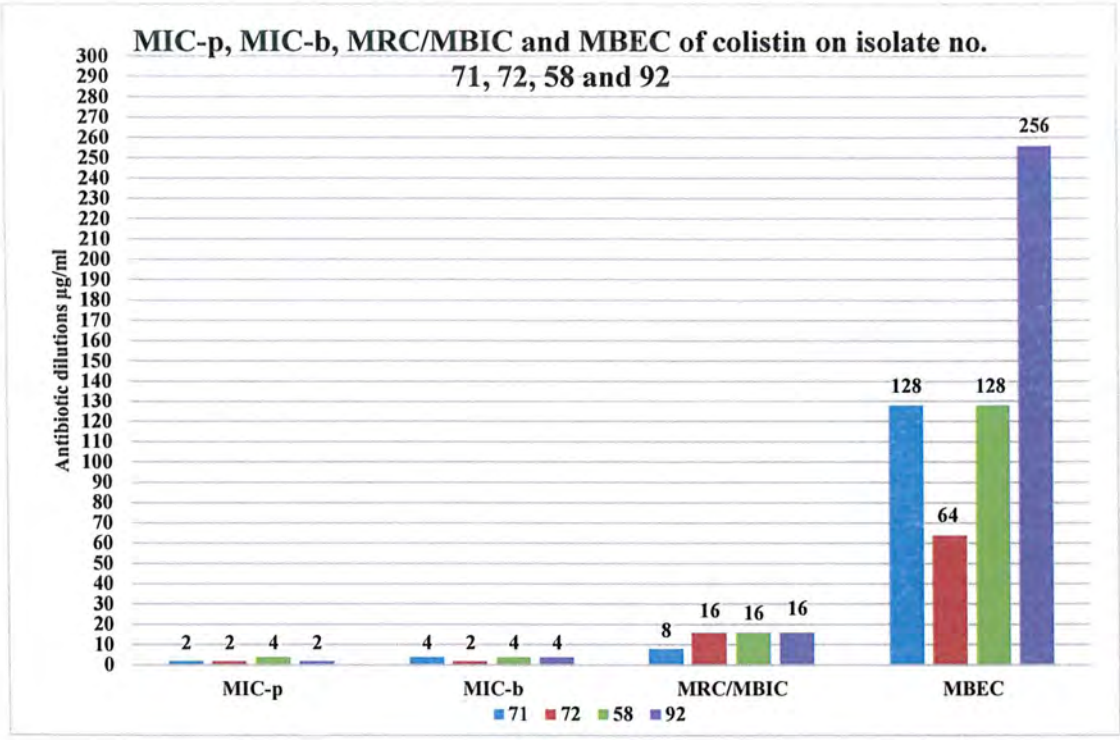

Figure 4.23: Bar graph shows MIC-p, MIC-b, MRC/MBIC and MBEC values of Colistin for isolate no. 71,72, 58 and 92

DISCUSSION

Acinetobacter baumannii is an important troublesome pathogen that is commonly linked with nosocomial infections. The recent rise in incidence, which has been associated with infected soldiers of armed forces coming back from battle zones, as well as a substantial rise in the occurrence of MDR strains, has increased the significance of this new opportunistic pathogen. The colonization and biofilm formation of *A. baumannii* on biotic and abiotic substrates leads to chronic infections, antimicrobial resistance and transmission of pathogen. The EPS layer of biofilm that encapsulates cells allows bacteria to tolerate harsh conditions and escape from antibiotic therapy. Therefore, the current therapeutic approaches are not successful against infections caused by *A. baumannii*.

For the evaluation of biofilm formation and screening of beta lactamase genes 100 strains of *A. baumannii* were collected from Pakistan Institute of Medical Sciences (PIMS) Islamabad. Clinical strains of *A. baumannii* were collected from multiple sources like sputum, blood, wounds, urine, endotracheal aspirates, and nasal swabs. All 100 strains of *A. baumannii* were sensitive to colistin. Out of 100 strains 5% strains were sensitive to tobramycin and amikacin, 43% strains showed sensitivity to tetracyclins, 3% strains were sensitive to imipenem and meropenem and resistance towards ceftazidime, cefixime and ceftriaxone were 100%. 22% strains were phenotypically positive for AmpC test (Irum *et al.*, 2022).

In this research I checked the extended spectrum beta lactamase (ESBL) production in 100 isolates of *A. baumannii*. Out of 100 strains only 28% isolates were ESBL producers and remaining 72% were non-ESBL producers. Sinha *et al* also conducted the similar study in India on 150 isolates of *A. baumannii* for ESBL detection and the percentage of ESBL production was 28% (Sinha *et al.*, 2007). Similar research was also conducted in Adesh Institute of Medical Sciences and Research on 116 clinical strains of *A. baumannii* and ESBL production was seen in 27% isolates (Kaur and Singh 2018). Ghaima and Kais conducted research on *A. baumannii* isolates from burn wounds and reported that 61%

isolates were ESBL producers. Mina *et al.*, reported that out of 203 clinical isolates of *A. baumannii* 62% were ESBL producers (Zarabadi *et al.*, 2021).

TEM-1, *SHV-1* and *CTXM-15* are the most common genes found in *A. baumannii*. Extended spectrum beta lactamases (ESBLs) which are derivatives of the *TEM-1*, *CTXM-15* and *SHV-1* enzymes, have emerged as a result of the extensive usage of various novel beta-lactam antibiotics in past few decades. They can hydrolyze a broad range of beta-lactam antibiotics except for carbapenem drugs. All these three enzymes belong to Ambler Class A β-lactamases. They are also known as serine-dependent enzymes, and they can be inactivated by Clavulanate or tazobactam. One of the objectives of this research was to screen *TEM-1*, *CTXM-15* and *SHV-1* genes in 28 isolates of *A. baumannii* that also showed ESBL production phenotypically. Out of 28 strains 25 strains were positive for *TEM-1* gene, 16 strains were positive for *CTXM-15* gene and 9 strains were positive for *SHV-1* gene. So, the percentages of *TEM-1*, *SHV-1* and *CTXM-15* were 89%, 32% and 57% respectively.

Our results were consistent with another study conducted in Iran where they found occurrence of *TEM-1* and *CTXM-15* was 52% and 43% respectively (Ranjbar *et al.*, 2018). Another study was conducted in Tehran and they found 42% prevalence of *TEM-1* gene in *A. baumannii* (Abdar *et al.*, 2019). Thahab *et al* conducted research on *A. baumannii* for detection of extended spectrum beta lactamases and he detected 25% prevalence of *SHV-1* gene and 8.3% prevalence of *TEM-1* gene (Thahab *et al.*, 2013). Al-Agamy *et al.*, conducted research on 40 strains of *A. baumannii* of which 87% strains were positive for *TEM-1* gene (Al-Agamy *et al.*, 2014). Another study was also conducted for detection of ESBL genes on 100 clinical isolates of *A. baumannii* and they detected 58% *SHV-1* gene and 20% *TEM-1* gene (Safari *et al.*, 2015). There may be multiple reasons for prevalence difference in different parts of the world, but the most common reasons include use of any specific antibiotic at specific geographical location, different types of sample sources, diagnostic facilities and co-resistance to other antimicrobial classes.

Recent research suggests that the ability of *A. baumannii* isolates to form biofilms is linked to key virulence factors, increasing bacterial survival in a way that is different

from multi drug resistant phenotypes. The ability of *A. baumannii* to produce biofilms and its long-term survival on both living and non-living substrates are linked with its high level of antibiotic resistance. Biofilm formation of *A. bumannii* on different medical devices such as ventilators and catheters cause many chronic infections.

Evaluation of biofilm formation of *A. baumannii* was also another objective of this study. Biofilm of *A. baumannii* was formed on 96-well microtiter plate as described by Christensen *et al.*, 1985. After biofilm formation microtiter plate was washed and fixed with methanol. After that biofilm was stained by 2% crystal violet as described in Christensen's biofilm formation assay. Optical density was recorded at 492nm and 630 nm. Out of 100 isolates 35% were strong biofilm formers, 60% were moderate biofilm formers and only 5% were weak biofilm formers.

Same study was also conducted in Tehran where they formed biofilm of 75 clinical isolates of *A. baumannii* on 96-well microtiter plate same as described by Christensen. Out of 75 only 18% isolates were strong biofilm formers, 10% were moderate ,47% were weak and 25% were non-biofilm formers (Ali *et al.*, 2014). Chen *et al* also conducted the similar research in Central South University of China where they checked biofilm formation in clinical strains of *A. baumannii collected* from different kind of specimens. Out of 92 strains only 54.3% strains were strong biofilm formers (Chen *et al.*, 2020).

Ebrahim and his colleagues investigated biofilm formation of *A. baumannii* strains collected from three different hospitals of Tehran. Out of 156 isolates 3% were non-biofilm formers, 13% were strong biofilm formers while 91% isolates were moderate biofilm formers and 49% isolates formed weak biofilm on 96-well microtiter plate (Ebrahim *et al* 2016). Dheepa *et al* checked biofilm formation and their results showed that out of 50 isolates 60% were biofilm formers and remaining 40% were non-biofilm formers (Dheepa *et al.*, 2011).

Biofilm can also be formed on test tubes but reading of results and classification of isolates into strong, moderate and weak biofilm formers can be complicated. Biofilm formation on 96-well microtiter plate is the most reliable, precise, sensitive and reasonable method. Results of all above mentioned studies showed that most of the

strains of *A. baumannii* have ability to form biofilm and it acts as a major virulence factor for promoting antibiotic resistance in *A. baumannii*.

Minimum inhibitory concentration is the minimum amount of drug required to inhibit the visible growth of bacteria. Colistin resistance in many strains of *A. baumannii* have been reported worldwide. But still colistin and polymyxin B are effective up to some extent against *A. baumannii*. Colistin acts on Lipid A of lipopolysaccharide layer and disrupt plasma membrane of Gram-negative bacteria. Polymyxins are considered as last-resort antibiotics against *A. baumannii*. Irum *et al* performed Kirby-Bauer disc diffusion test for colistin and reported that all 100 strains of *A. baumannii* were sensitive to colistin. Another objective of this study was to check minimum inhibitory concentration of Colistin against *A. baumannii*. MIC-p of isolate no. 71, 72, 58 and 92 were performed by test tube method as described in CLSI guidelines 2019. After 24 hours of incubation test tubes with no visible growth were considered as minimum inhibitory concentration of colistin. The MIC-p values of isolate no. 71, 72, 58 and 92 were 2, 2, 4, 2µg/ml. According to CLSI guidelines 2019 the MIC-p value of colistin is >4µg/ml against *A. baumannii*. So, our results showed that all these four strains were sensitive to colistin.

Similar study was also performed in Hamadan University of Medical Sciences and their MIC-p values of colistin were ranging between 0.5-1µg/ml (Bardbari *et al.*, 2018). Teikyo University School of Medicine conducted similar research on MDR *A. baumannii* and their MIC value was 2µg/ml (Sato *et al.*, 2021). Another study was also conducted at Ahram Canadian University to determine minimum inhibitory concentration of colistin against MDR *A. baumannii* and they reported that MIC values of 55% isolates were ranging between 4-1024µg/ml (Sherif *et al.*, 2021).

In this research minimum inhibitory concentration of biofilm was also determined. Biofilm of isolate no. 71, 72, 58 and 92 was formed on 96-well microtiter plate and after 24 hours of incubation different dilutions of colistin was added into the microtiter plate. After overnight incubation the last well with no visible growth was considered as MIC-b of colistin. The MIC-b values of isolate no. 71, 72, 58 and 92 were 4, 2, 4 and 4 respectively. The MIC-p and MIC-b values of isolate no. 72 and 58 were same. Sato *et al* also determined the MIC-b of colistin against MDR *A. baumanni* and their MIC-b values

were 4, 16 and 32µg/ml (Sato *et al.*, 2021). Their MIC-b values are 8 and 16 folds more than MIC-p and in our study the MIC-b values of 2 isolates are 2 folds more than MIC-p.

Another objective of this study was to check minimum regrowth concentration (MRC). After performing MIC-b recovery media was added in same microtiter plate and again incubated it overnight. Next day wells with no visible growth were considered as MRC. MRC is basically the minimum amount of drug required to inhibit regrowth of bacterial cells. MRC/MBIC values of isolate no 71, 72, 58 and 92 were 8, 16, 16 and 16 µg/ml. MRC values of colistin were more than MIC-p and MIC-b. Wang *et al* also performed similar type of research to check the effect of azithromycin and fluoroquinolones on *Stenotrophomonas maltophilia* in china. MRC/MBIC values were ranging between 1024µg/ml and 2048µg/ml even MRC/MBIC values of some isolates were more than 2048 µg/ml. Quaid I Azam University also conducted similar type of research to check the effect of ciprofloxacin at sub-MIC level on uropathogenic *Escherichia coli* and their MRC/MBIC values >2048 µg/ml (Rafaque *et al.*, 2020). The huge difference in MRC values in our results and other studies may be due to difference in antibiotic resistance profile, difference in antibiotic permeability in biofilm form and each bacteria behave differently towards different antibiotics.

Last objective of this study was to determine minimum biofilm eradication concentration (MBEC) of colistin against *A. baumannii.* MBEC is minimum concentration of an antibiotic required to eradicate biofilm completely. Different dilutions of colistin were added on preformed biofilm and next day clear wells were streaked on TSA. The MBEC values of isolate no 71, 72, 58 and 92 were 128, 64, 128 and 256µg/ml respectively. MBEC values of all isolates were greater than MRC/MBIC, MIC-b and MIC-p. So high concentration of colistin is required to completely eradicate the biofilm of *A. baumannii* present on inner mucosal linings and medical devices.

CONCLUSIONS

According to World Health Organization *Acinetobacter baumannii* is a troublesome nosocomial pathogen and comes first on the critical priority list for novel antimicrobial drugs. It is resistant to almost all currently available antimicrobial agents making it a superbug in clinical settings particularly in intensive care units. In our study a number of antibiotics from different classes have been tested but only colistin was 100% effective against MDR *Acinetobacter baumannii*. Biofilm formation of *Acinetobacter baumannii* on both living and non-living surfaces facilitates its attachment and long-term persistence. It also promotes antimicrobial resistance and makes therapeutic approaches more complicated. This study also highlighted that colistin can inhibit biofilm formation and even complete biofilm eradication was also observed when high concentrations of colistin were used. Due to adverse side effects of colistin on kidneys alternative therapeutic strategies are urgently needed. So, researchers should conduct further studies to explore the effect of combination therapies, organic compounds, bacteriophage therapies, nanoparticles, and their combinations with other antimicrobial agents on biofilm of *A. baumannii*.

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APPENDICES

A.1 Nutrient Agar Composition	
Ingredients	**Gram/Liter**
Yeast Extract	**2g**
Lab Lemco Powder	**1g**
Agar	**15g**
Sodium Chloride	**5g**
Peptone	**5g**

A.2 Luria Bertani Agar Composition	
Ingredients	**Gram/Liter**
Trypton	**10g**
Yeast Extract	**5g**
Sodium Chloride	**5g**
Agar	**15g**

A.3 Tryptic Soy Agar Composition	
Ingredients	**Gram/Liter**
Peptic Digest of Soybean Meal	**5g**
Sodium Chloride	**5g**
Pancreatic Digest of Casein	**15g**

Agar	**15g**

A.4 MacConkey Agar Composition	
Ingredients	**Gram/Liter**
Peptone	**20g**
Lactose	**10g**
Bile Salt	**5g**
Sodium Chloride	**5g**
Brom Cresol Purple	**0.01g**

A.5 Muller Hinton Agar Composition	
Ingredients	**Gram/Liter**
Starch	**1.5g**
Beef dehydrated infusion	**2.0g**
Casein hydrolysate	**17.5g**

A.6 Catalase Test Reagent Composition	
Ingredients	**Amount**
Hydrogen Peroxide	**3% (10 Volume Solution)**

A.7 Oxidase Test Reagent Composition	
Ingredients	**Amount**

Distilled Water	**10ml**
Tetramethyl-p-phenylenediamine	**0.1g**

A.8 Indole Test Reagent Composition	
Ingredients	**Gram/Liter**
Tryptone	16g
Yeast Extract	10
Sodium Chloride	5g

A.9 Simmons Citrate Agar Composition	
Ingredients	**Gram/Liter**
Ammonium dihydrogen phosphate	**1.0g**
Sodium chloride	**5.0g**
Magnesium sulphate (heptahydrate)	**0.2g**
Sodium citrate	**2.0g**
Dipotassium phosphate	**1.0g**
Bromothymol blue	**0.08g**
Agar	**15g**

A.10 Urea Agar Base Composition	
Ingredients	**Gram/Liter**

peptone	1.0g
Glucose	1.0g
Sodium Chloride	5.0g
Monopotassium Phosphate	2.0g
Phenol Red	0.012g
Agar	15g

A.11 Phosphate Buffer Saline Composition	
Ingredients	
Na2HPO4	0.7g
KH2PO4	0.12g
KCl	0.1g
NaCl	4g
Distilled Water	500ml

A.12 Triple Sugar Iron Test Composition	
Ingredients	Gram/Liter
Lab-Lemco' Powder	3g
Yeast Extract	3g
Peptone	20g
Sodium Chloride	5g
Lactose	10g

Sucrose	**10g**
Glucose	**1g**
Ferric Citrate	**0.3g**
Sodium Thiosulphate	**0.3g**
Phenol Red	**0.024g**
Agar	**12g**

A.13 70% Methanol Composition	
Ingredients	**Amount**
Absolute Methanol	**70ml**
Distilled Water	**30ml**

A.14 2% Crystal Violet Solution Composition	
Ingredients	**Amount**
Crystal Violet Powder	**2g**
95% Ethyl Alcohol	**20ml**
Distilled Water	**80ml**

A.15 95% Ethanol Composition	
Ingredients	**Amount**
Absolute Ethanol	**95ml**
Distilled Water	**5ml**

A.16 Normal Saline Composition	
Ingredients	**Amount**
NaCl	**0.9g**
Distilled Water	**100ml**

A.17 10X TBE Buffer Composition	
Ingredients	**Gram/500ml**
EDTA	**4.65g**
Boric Acid	**29g**
Tris Base	**54g**

10X TE Buffer Composition	
Ingredients	**Gram/Liter**
EDTA	**2.92g**
Tris-HCL	**15mM**

Loading Dye Composition	
Ingredients	**Amount**
Bromophenol Blue	**0.25g**
Distilled Water	**7ml**

Glycerol	**3ml**

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