Molecular Epidemiology and Genetic Characterization of Multi drug-Resistant *Acinetobacter baumannii*

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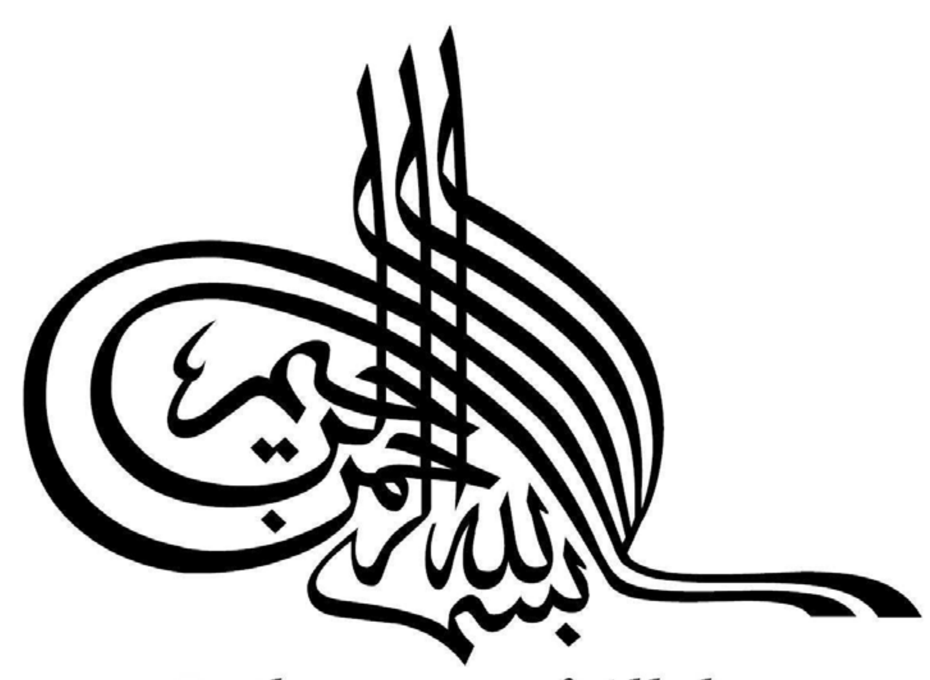

In the name of Allah,
the Most Beneficent,
the Most Merciful

Dedicated to my Beloved Parents and Husband

As an expression of gratitude for their unconditional love and support

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List of Abbreviations

A. baumannii	*Acinetobacter baumannii*
AMR	Antimicrobial resistance
API	Analytical profile Index
ATCC	American Type Culture Collection
CC	Clonal Complex
CHDL	Carbapenem hydrolyzing class D
CLSI	Clinical Laboratory Standards Institute
CSF	Cerebral spinal fluid
DNA	Deoxyribonucleic acid
ESBL	Extended spectrum beta lactamases
ERIC	Enterobacterial Repetitive Intergenic Consensus
EtBr	Ethidium bromide
ICU	Intensive care unit
KPC	Klebsiella pneumonia carbapenemase
LPS	Lipopolysaccharides
MBL	Metallo-beta-lactamases
MDR	Multi drug-resistance
MHB	Muller Hinton broth
MHA	Muller Hinton agar
MHT	Modified Hodge Test
MIC	Minimum Inhibitory concentration
MLST	Multi-locus sequence typing
NICU	Neonatal Intensive care unit
ORF	Open reading frame
PCR	Polymerase chain reaction
PDR	Pan drug-resistance
PFGE	Pulsed-field gel electrophoresis
PIMS	Pakistan Institute of Medical Science
PMQR	Plasmid mediated quinolones resistant
PNAG	poly-β-(1, 6)-N-acetylglucosamine
QS	Quorum sensing

REP PCR	Repetitive Element Palindromic Polymerase Chain Reaction
TBE	Tris boric acid EDTA buffer
TE	Tris EDTA buffer
tRNA	Transfer ribonucleic acid
TSA	Tryptic soy agar
UTI	Urinary tract infection
XDR	Extreme drug-resistance

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Abstract

A. baumannii infections are an emerging serious health issue, owing to the increasing development of antibiotic resistance across the globe including Pakistan. Particularly *A. baumannii* that produce extended spectrum beta-lactamases (ESBLs) and resist carbapenems are recognized threat to public health. These multi drug-resistant (MDR) *A. baumannii* result in serious life-threatening infections, increased treatment cost due to prolonged hospital stay, and increased mortality rate. However, a comprehensive national level data regarding *A. baumannii* is lacking. Keeping this in view, the present study encompass epidemiology, resistance traits, virulence markers and clonal diversity, to understand the current trend of antibiotic resistance and clonal relatedness which is necessary for planning therapeutic strategies.

In this study, a total of 375 isolates of *Acinetobacter* genus were obtained from the Microbiology laboratory of Pakistan Institute of Medical Sciences (PIMS), a tertiary care hospital in Islamabad, Pakistan. Several species of bacteria are included in this genus. Among them, *A. baumannii* is considered as the primary pathogen in clinical settings. We identified *A. baumannii* to be the most prevalent species (n=240, 64%) in our sample using various phenotypic and genotypic tests [colony morphology, Gram-staining, biochemical (API 20 NE kit) and genetic (*16S rRNA* and $bla_{OXA\text{-}51\text{-}like}$ genes) tests]. The basic demographic data of patients such as gender, age, specimen sites and hospital wards were also recorded. Antibiotic susceptibility assay was done for all isolates by disk diffusion method against a panel of antibiotics. Phenotypic assays for ESBLs, metallo beta-lactamases (MBLs), carbapenemases and AmpC were performed. PCR was carried out to detect ESBLs encoding genes, bla_{TEM}, $bla_{CTX\text{-}M}$ and bla_{SHV}, $bla_{M\text{-}PER}$, $bla_{M\text{-}GES}$, $bla_{M\text{-}VEB}$; MBLs carbapenemase encoding genes, bla_{VIM}, bla_{SIM}, bla_{NDM}, bla_{KPC}, bla_{AIM}, bla_{SPM}, bla_{BIC}, bla_{IMP}, bla_{DIM}, and bla_{GIM} ; ADC (Class C) β-lactamase; Oxacillinases included $bla_{OXA\text{-}51}$, $bla_{OXA\text{-}23}$, $bla_{OXA\text{-}24}$, $bla_{OXA\text{-}48}$ and $bla_{OXA\text{-}58}$. PCR was also done to detect resistance genes against aminoglycosides (*aadB, aphA6, aadA1,* and *aacC1)* and tetracycline (*tetA* and *tetB* genes) along with a set of virulence genes including *csgA*, *iutA*, *cnf1* and *cvaC*. PCR amplifications were performed for fluoroquinolone resistance genes (*qnrA, qnrB* and *qnrS*), insertion sequence *ISAba1* and outer membrane protein-encoding gene *CarO*. Phenotypic

efflux pump activity was analyzed by ethidium bromide cartwheel assay and PCR was done to detect efflux pump encoding genes. ERIC-PCR was performed for all *A. baumannii* isolates and dendrograms were made by bionumeric software (version 7.6) to investigate clonal relatedness among MDR isolates. Selected isolates were screened by PFGE analysis for clonal diversity. Multi-locus sequence typing (MLST) was carried out on subgroup of isolates. MLST sequences were uploaded to *A. baumannii* database (http://pubmlst.org/abaumannii/) for determination of the allele and subsequent sequence type (ST). Phylogenetic tree was generated by PHYLOVIZ software 2.0 to compare STs with clonal complexes. Demographic data revealed that most of the patients were adult males (59%) mostly admitted in intensive care unit (ICU) with high number of isolates extracted from tracheal secretions i.e. 48%. The phenotypic antibiotic susceptibility data exhibited that isolates were resistant to all classes (Penicllins, Cephalosporins, Fluoroquinolones, Aminoglycoside, Carbapenems and Tetracyclines) with tigecycline being the most effective with 50% of the isolates being susceptible. Among all isolates, 54% were multi drug-resistant (MDR), 33% were pan drug-resistant (PDR) and 13% were categorized as extreme drug-resistant (XDR). None of the isolates showed ESBL production using phenotypic DDST (Double disk synergy test). However, 59% of the isolates produced AmpC by AmpC disk test (n=141), 65% were carbapenemase producers using Modified hodge test (MHT) (n=157) and 42% were MBLs producers using MEM-EDTA test (n=101). Furthermore 31% of the isolates showed efflux pump activity (Ethidium bromide cartwheel method). The phenotypic assays were further confirmed with genotypic analysis, which showed varying results. The results regarding overall antibiotic resistance genes suggest that $bla_{OXA\text{-}51\text{-}like}$ was present in all of the isolates followed by AmpC-ADC (71%), *ISAba1* (31%), $bla_{\text{OXA-23-like}}$ (29%), bla_{IMP} (26%), *adeS* (21%), *aacC1* (20%), *qnrB* (17%), *tet B* (12%), bla_{NDM} (9%), $bla_{\text{OXA-48-like}}$ (8%), *qnrS* (7%) and bla_{VIM} (6%). Among the various virulence genes (*csgA*, *iutA*, *cnf1* and *cvaC*), only a single isolate carried *csgA*. Using ERIC-PCR, 240 isolates were grouped into eighteen clusters. A large number of isolates appeared as singleton and doubles suggesting high diversity among the *A. baumannii* isolates. In case of PFGE analysis, subset of isolates was divided into 10 pulsotypes belonging to MDR group. Among them, 3 major pulsotypes, B (n=23), C (n=16), and pulsotype H (n=15) were found in MDR *A. baumannii*. The MLST analysis showed the presence of four

different sequence types (STs) i.e. ST409, ST164, ST101 and ST241; all of which belong to Clonal Complex I (CC1), as per Pasteur scheme.

It is need of the hour to evaluate the presence of MDR *A. baumannii* in healthcare settings and understand the molecular epidemiology of these MDR *A. baumannii* to form strategies to decrease the overall burden of infections caused by these opportunistic bacteria. Hence, the present study provides comprehensive report on demography, resistotypes (phenotypic and genotypic) and clonal diversity of *A. baumannii* isolates present at the site of study. This information can be used for devising effective management strategies to counter the spread of *A. baumannii* infections.

Chapter 1

Introduction

1.1 *Acinetobacter* genus

Acinetobacter is the genus of Gram-negative bacteria. Its species are non-fermenting, catalase-positive and oxidase-negative Coccobacilli, usually present in the form of pairs and chains of different lengths (Juni, 2015). In 1911, Beijerinck discovered the *Acinetobacter* genus and named it as *Micrococcus calcoaceticus*. Later on, in the 1950s, it was given name as *Acinetobacter* (Almasaudi, 2018). The name originated from "akinetos"; a Greek word meaning immotile. In 1968, it was concluded that all formerly isolated species clustered as oxidase-negative fit exclusively to family "*Moraxella"* and were designated to genus *Acinetobacter* (Baumann, 1968). In 1971, the *Moraxella* and *Allied* bacterial taxonomy subcommittee recognized *Acinetobacter* genus officially based on Baumann's work (Gilardi, 1971).

1.2 Habitat

Members of the genus "*Acinetobacter"* are regarded as omnipresent organisms, which are recovered from nearly every sample taken from water surface or soil following enrichment culture (Doughari et al., 2009). They can reside on fingertips and on dry surfaces in contrast to other Gram-negative bacilli. It became apparent from replicated environmental settings that *A. baumannii* can live in dryness beyond 30 days (Houang et al., 1998). In physiological locations, these microbes inhabit digestive tract, skin, and oro-pharyngeal tract of patients (Munoz-Price and Weinstein, 2008). There are a range of foods where *A. baumannii* can survive and grow. It has been recovered from milk and milk-related products, fruits and uncooked vegetables (Almasaudi, 2018). Contrary to other species of the *Acinetobacter* genus, which are frequently isolated from the soil, water and animals, *A. baumannii* is more commonly found in in the hospital environment, particularly in intensive care units (ICUs) (Antunes et al., 2014). Furthermore, the seasonal differences have been displayed by *Acinetobacter* spp. in skin colonization in medical personnel. Higher infection prevalence was reported in summer in contrast to winter, showing the cyclic variation for *A. baumannii* (Chu et al., 1999).

1.3 *Acinetobacter baumanii*

Among the various strains of *Acinetobacter* species, *A. nosocomialis*, *A. baumannii* and *A. pettii* are prevalent in clinical settings (Al-Hasan, 2013). Among them, *A. baumannii* is considered as the main pathogenic specie in humans (Gonzalez-Villoria and Valverde-

Garduno, 2016). It is oxidase-negative, catalase-positive, immotile, non-fermenting and Gram-negative Coccobacilli (Peleg et al., 2008).

It is evident from studies that *A. baumannii* is associated with nosocomial infections resulting in increased mortality (Glew et al., 1977, Sobouti et al., 2020, Nasr, 2020). This pathogen causes a broad range of infections, particularly in terminally ill patients. It is most commonly linked with urinary tract infections (UTIs), bacteremia, ventilator-associated pneumonia (VAP), meningitis and wound infections (Peleg et al., 2008). Among these, pneumonia is a predominantly prevalent clinical condition, particularly within communities residing in tropical regions of Asia and Australia (Davis et al., 2014). Approximately half of infections caused by nosocomial pathogens are due to *A. baumannii* (Zilberberg et al., 2016). It generally infects or colonizes patients who are catheterized, and in outpatients it is associated with uncomplicated UTI (Falagas et al., 2015). It is listed as 10th most common microbial source of hospital-acquired bloodstream infections (Wisplinghoff et al., 2014). In injuries and traumatic patients with serious burns, *A. baumannii* often colonizes and produces infection (De Vos et al., 2016). Over the last few years, wound infections caused by *A. baumannii* have been associated with natural catastrophes (such as floods, earthquakes and war) because the hospitals have been overloaded without usual sanitation measures. *A. baumannii* got more attention during military activities in Iraq and Afghanistan when surgical and burn wound-related diseases and osteomyelitis were observed in troops returning to UK and USA (Sebeny et al., 2008). These isolates were mostly multi drug-resistant (MDR) and it was presumed that this organism could enter the injuries via previously colonized skin or by contamination with infected dust or soil. There is evidence, however, that wounded troops obtained the infection in field hospitals or subsequently in military clinics (Aleksic et al., 2014). Especially after neurosurgery, *A. baumannii* may trigger meningitis, which is a growing problem particularly if there are MDR strains. About 4% acute nosocomial meningitis cases are induced by *Acinetobacter*, and death in 70% of these patients has been observed (Almasaudi, 2018).

1.4 Risk factors associated with *A. baumannii*

Prolonged hospital stay, previous hospitalization, venous catheters, habitation in nursing homes, and compromised immune system are the risk factors linked with *A. baumannii* infection (Fishbain and Peleg, 2010). Unless causing infection in critically ill or

immunocompromised patients, this organism's virulence is considered low and it is most often linked with hospital-acquired infections (Jain and Danziger, 2004). However, the ability of *A. baumannii* to obtain and transfer antibiotic resistance and its existence in adverse conditions can lead to severe outcomes, particularly in people with immune suppression, surgery, burns and malignancy (Doughari et al., 2009). Through *A. baumannii* strains shedding into the adjacent environment, infected patients are regarded as major source of infection. In healthcare environment, it eventually contaminates the machinery, devices and can colonize the hospital staff as well. The colonization of sensitive patients and medical employees, ongoing persistence of antimicrobial and antiseptic resistance in the healthcare settings are the basic factors that cause recurrent outbreaks triggered by *Acinetobacter* species that are hard to contain (Alfandari et al., 2014). While cases of community-acquired *Acinetobacter* spp. diseases have been recorded, a key pathogenic function of these bacteria is certainly to induce hospital-acquired infections, primarily among patients in Intensive care unit (ICU) (Bergogne-Berezin and Towner, 1996). In addition, outbreaks in healthcare facilities and nursing homes has also been reported (Sengstock et al., 2010).

1.5 Clinical impacts of *A. baumannii* infections

Seriously ill patients with other co-morbidities in healthcare settings are frequently infected with *A. baumannii* and have a bad prognosis; thus, its real clinical effect is notorious and yet difficult to assess. Case-control surveys and matched mortality cohorts linked with *A. baumannii* infections showed mortality from 7.8% to 23% in healthcare environments, while 10% to 43% in ICUs (Falagas et al., 2006). One study suggests that mortality from *A. baumannii* increases with the duration of stay in hospitals (Falagas and Rafailidis, 2007). Mortality ranged from 3% to 67% in European epidemics induced by carbapenem-resistant strains (Kempf et al., 2012). The published trials revealed a rough mortality rate of 20-60% (Leão et al., 2016). *A. baumannii* are of great importance, but other species related to *A. baumannii* complex also have clinical importance. *Acinetobacter calcoaceticus-Acinetobacter baumannii* (ACB) complex members are generally MDR and responsible for significant epidemics in hospitals worldwide (Bianco et al., 2016). It is therefore proposed that ACB complex must be observed separately from other *Acinetobacter* species. Other species of *Acinetobacter* are not common source of human infections and hardly lead to epidemics. These are primarily isolated from patients

with serious underlying illnesses (Zander et al., 2014). Therefore, it is essential to distinguish among *A. baumannii* and other species, which is difficult in routine microbiological laboratories using standard bacteriological tests. In catastrophic mileu, *A. baumannii* has appeared as a key pathogen and is liable for multiple diseases. After an earthquake in Turkey in 1999, around 220 individuals were hospitalized and 18.6% acquired nosocomial diseases. The prevalence of *A. baumannii* among these patients was 31.2% with 2 pan drug-resistant (PDR) strains. On the contrary, the prevalence was 7.3% before the earthquake in the same hospital with no MDR strains (Öncül et al., 2002). Similarly, MDR *A. baumannii* were commonly reported during Iraq-Iran, Vietnam, and Gulf wars and were most prevalent Gram-negative bacteria from the first day of hospitalization. Armed forces health authorities recorded *A. baumannii* patients with positive blood cultures in camps providing medical facilities in Iraq, Afghanistan and Kuwait for wounded patients during 2002-2004 (Joly-Guillou, 2005). A study conducted in 2018 reported high prevalence of resistance to the common prescribed antibiotics in *A. baumannii* infections during 2006-2016 in both OECD (Organization for Economic Co-operation and Development) and non-OECD countries (Figure 1.1) (Xie et al., 2018)

Figure 1.1: Geographical distribution of the prevalence of antibiotics resistance in *A. baumannii* infections. n is the number of included studies per country (Xie et al., 2018).

1.6 Epidemiology and Prevalence of *A. baumannii* in Pakistan

A. baumannii is clinically important and remains in focus for studies in clinical samples because of the associated morbidity and mortality. In 2010, a study conducted in Karachi (Pakistan) reported 71% pan drug-resistance in *A. baumannii* isolated from neonates. They concluded that pan drug-resistant *Acinetobacter* infection is extremely fatal in neonates, particularly in premature and low-birth weight infants (Saleem et al., 2010). Another study reported 100% MDR *A. baumannii* with high prevalence of carbapenemases (Begum et al., 2013). A study reported that 65% carbapenem-resistant *A. baumannii* strains accounted for nosocomial infections in Pakistan (Hasan et al., 2014). Other studies have also reported remarkable phenotypic resistance to cephalosporins, fluoroquinolones, aminoglycosides, and piperacillin/tazobactam (Shamim et al., 2015, Sohail et al., 2016, Anwar et al., 2016). Hence, the aforementioned studies exhibit resistance patterns in *A. baumannii* in clinical settings. However, most of these studies were limited to phenotypic assays such as antimicrobial susceptibility testing and phenotypic tests for enzyme production e.g. MBLs and ESBLs. Although, the picture of antimicrobial resistance of *A. baumannii* is clear through multiple studies but there was a dire need for genetics elucidation of MDR *A. baumannii* to find out genetic basis of antibiotic resistance and its clonal diversity, which was underlined in the current study core objectives.

1.7 Virulence factors

The extent up to which a microorganism is pathogenic is called virulence. Studies based on genomics and proteomics have recognized several virulence factors that are responsible for *A. baumannii* pathogenesis (McConnell et al., 2013, Khazaal et al., 2020).

1.7.1 *Porins*

Adhesion is considered as the most critical determinant of virulence and is believed to involve a protein called as outer membrane protein (Omp). This protein makes channels for the transport of molecules through lipid bilayer called porins. OmpA is perhaps the most common porin in *A. baumannii* with a variety of biological characteristics and is a well-known virulence factor (Smith et al., 2007). OmpA is said to be associated with fibronectin to facilitate adherence and incursion within epithelial cells, while binding of

OmpA to factor H (human plasma) helps *A. baumannii* to avoid complementary lysis (Smani et al., 2014).

1.7.2 *Proteases & Phospholipases*

A. baumannii has the capacity to generate protease molecules, which adversely affect the patients and their metabolism during the course of infection (Antunes et al., 2011b). These proteases enhance the virulence of bacteria via degrading the host matrix (for penetration) and interfere with the host cell response to ensure the success of the pathogen in contact with the human host (Frees et al., 2013)

Phospholipases are virulence factors found in many pathogenic bacteria. Phospholipase A (PLA), phospholipase C (PLC) and phospholipase D (PLD) are three important types of phospholipases based on their cleavage site, however, only PLD and PLC are recognized in *A. baumannii* (Stahl et al., 2015).

1.7.3 *Hemolysis*

Hemolysis was earlier considered missing in *A. baumannii. A. haemolyticus* was the only *Acinetobacter* spp. that showed hemolysis (Peleg et al., 2008). Few *A. baumannii* isolates displayed little *in-vitro* hemolytic activity on Sheep Blood Agar (SBA) after the extended incubation period (Antunes et al., 2011a). Hemolytic activity and siderophores production together can cause quick iron reduction accessible to host.

1.7.4 *Surface Motility*

Another virulence factor thought to be missing earlier from *Acinetobacter* spp. was motility (Brisou and Prevot, 1954). Studies have revealed that genes encoding the secretion system for Type IV pili are present in *A. baumannii* (Peleg et al., 2008). Surface-associated movements are linked to these pili through their retraction and extension which is known as twitching motility (O'Toole and Kolter, 1998). Another study displayed that even though not pertinent to all, numerous strains of *A. baumannii* have ability to express this sort of motility phenotypically on agar plate surface (Antunes et al., 2011a). Pili not only help attachment and biofilms formation, but the combination of pili with receptors of the host cell can also cause inflammation by producing inflammatory intermediaries i.e. chemokines and cytokines (de Breij et al., 2009).

1.7.5 *Enzymes*

A. baumannii's virulence can also be ascribed to acid phosphatase, amino-peptidases, urease, and esterase enzymatic activities. Strong esterase activity, identified to hydrolyze short-chain fatty acid ester connections might have a role in its capacity to eradicate lipid tissue. *Acinetobacter*'s urease activity is changeable between distinct strains allowing the bacterial colonization of the stomach (Braun, 2008).

1.7.6 *Penicillin-binding proteins (PBPs)*

Penicillin-binding proteins (PBPs) are generally considered important for β-lactam resistance. However, PBP7/8 is also regarded as a virulence factor and is encoded by *pbpG* gene. The viability of *pbpG* mutant *A. baumannii* strain in human serum as well as rat infection model was reported to be significantly decreased (Russo et al., 2009). The PBP7/8 actually remodels the surface of bacterial cells in a manner, which protects it from defense components of the host (Russo et al., 2009).

1.7.7 *Quorum Sensing (QS)*

Another variable that contributes to virulence is quorum sensing (QS). This is a prevalent mechanism particularly among Gram-negative bacteria which involves acyl-homoserine lactone (AHL) like molecules which function like signal modulator for different bacterial physiological mechanisms (Miller and Bassler, 2001). There is a variation in quorum signals among *Acinetobacter* virulent and non-virulent strains that makes it difficult to distinguish them simply by quorum sensing. Mutation in *abaI* gene was noted with decreased biofilm formation in *Acinetobacter* strain, but their initial phenotype restored the capability of biofilm formation after the addition of exogenous AHL to *Acinetobacter* isolates. Therefore, QS signal molecules affect capacity to develop a biofilm that is an important virulence feature of sustainability and antibiotic resistance of *A. baumannii* (Bhargava et al., 2010). Studies have indicated different methods that interrupt QS or early gene expression related to QS for wider management of particular diseases related to bacteria. The goals for new therapies include quorum-sensing signals that can further reduce biofilm development and potentially other virulence factors (Sperandio, 2007). This interruption or hindrance of QS can be achieved through the use of synthetic compounds or plant extracts or enzymes that degrade AHL (Adonizio et al., 2008). These

antagonists disrupt the intracellular interaction of bacteria and thus hinder their ability of gene expression in a coordinated manner under the quorum sensing scheme (Rasko and Sperandio, 2010).

1.8 Resistance Mechanisms

Acinetobacter spp. have emerged as important MDR bacteria (Van Looveren et al., 2004). A number of resistance mechanisms are reported for *A. baumannii*, which are as follows:

1.8.1 *Genomic Resistance Island (GRI)*

Genetic analysis and comprehensive resistance annotation were undertaken on *A. baumannii* isolated from France where a resistance island of 86 kb was reported in an epidemic clone. From a total of 88 ORFs, 82 on this island were considered to have been acquired from Gram-negative bacteria such as *Pseudomonas* spp., *E. coli* and *Salmonella* spp. (Bonnin et al., 2011). Additionally, the G-C ratio was 52.8% in this region, compared to 38.8% of the remaining chromosome, suggesting a probable foreign source. A total of 86% of the recognized 52 resistance genes were located on *AbaR1* resistance island. These islands' genetic environments give more proof of genetic promiscuity *via* synchronization of broad-range host mobile genetic elements that include insertion sequences, transposons and Class 1 integrons (Fournier et al., 2006). Another GRI called *AbaR2* with size of 19.5 kb was identified in European clone II, which is thought to have been reduced from *AbaR1*, with *aacA4*; β-lactamase gene and aminoglycoside-modifying enzyme being observed as an integral component of class 1 (Iacono et al., 2008).

AbaR3 and *AbaR4* GRIs were identified in *A. baumannii* from America that have also been reported in clone I *A. baumannii* from Europe. The *AbaR3* is 64 kbp genomic island and contains 8 genes of resistance to β-lactam antibiotics, aminoglycosides and tetracyclines (Smith et al., 2007). *AbaR4* is 18 kbp resistance island carrying $bla_{\text{OXA-23}}$ carbapenemase gene flanked by *ISAba1* element at both ends (Kim et al., 2012). Australian isolates possessed *AbaR5* of 36 kbp in size (Post and Hall, 2009). The existence of these GRIs gives the international *A. baumannii* clones an MDR phenotype (Hamidian et al., 2013). Worldwide recognition of GRIs with similar nature and resistance characteristics in plasmids showed universal dissemination of these resistance

determinants, also termed as global clones. The existence of foreign genes denotes that *A. baumannii* has capability to acquire foreign DNA efficiently (Eijkelkamp et al., 2011).

1.8.2 *Resistance due to the presence of integrons, plasmids and transposons*

Initially, the only component necessary for the dissemination of resistance genes was believed to be the conjugative plasmids. Detection of transposons, incorporated into a chromosome or present on plasmids showed that plasmids alone were not the only component involved in the spread of resistance determinants (Devaud et al., 1982). Among genetic elements, integrin is one element associated with transposons. It is natural system of replication and expression that assimilates ORFs and transforms into functional genes (Rowe-Magnus and Mazel, 2001). Existence of these components in association with resistance markers in bacteria is suggestive of their role in the acquisition of antibiotic resistance. They have been found in environmental samples that had no antimicrobial contact, hence they are regarded as a gene capture system for not only providing antimicrobial resistance; but also for adaptation of the bacterial host and successive genome development (Nield et al., 2001). An integron essentially contains an integrase gene, *intI* and a proximal primary recombination site, *attI*. Gene cassette is either integrated or excised via integrase catalysed recombination between attI and/or attC sites. Integrase gene provides versatility in the integron genome by integration of casettes with different antibiotic resistance genes combination that allows bacteria to manage their multiple integron cassettes upon selective antibiotic pressure (Fluit and Schmitz, 1999). Class1 integron is the most prevalent of these 3 integral components in clinical antibiotic-resistant *A. baumannii* isolates (Turton et al., 2005). Antibiotic resistance in *A. baumannii* has also been attributed to non-integron components, either chromosomal or plasmid-encoded.

1.8.3 *Role of Insertion Sequences in Resistance*

Insertion sequences (IS) are part of mobile genetic elements, which function as the messenger of antibiotic resistance genes and enable their movement from chromosomes to plasmids. A powerful promoter is used by these sequences for resistance genes expression (Thapa, 2009). These IS components have been associated with many resistance genes and are accountable for two major activities. First, they are mobile and therefore transcribe transposase. Secondly, they have promoter regions that direct over-

expression of downstream resistance factors (Corvec et al., 2007). In short, these IS are considered important for acquisition and dissemination of antimicrobial resistance. More than thirty IS have been attributed to *Acinetobacter* spp., and underlie the evolution and resistance trait of this genus (Siguier et al., 2006). In case of *A. baumanni*, the most studied IS are *ISAba1, ISAba125 and ISAba3* (Mussi et al., 2005). Among them, the insertion sequence *ISAba1* predominates. Numerous genes such as $bla_{\text{OXA-51}}$/$bla_{\text{OXA-66}}$, $bla_{\text{OXA-23}}$ and $bla_{\text{OXA-58}}$ were reported to be inter-connected with this IS upstream. These carbapenemases associated with *ISAba1* increase the carbapenem resistance by many folds (Kuo et al., 2014). AmpC is chromosomally located intrinsic enzyme in *A. baumannii* and its expression is also associated with existence of *ISAba1* (Heritier et al., 2006). *ISAba1* elements, upstream of the *OXA* gene, have been significantly related to imipenem non-susceptible isolates (Alaei et al., 2016).

Among the IS, the IS*CR* (Insertion sequence common regions) are considered as important elements with the capability to capture and mobilize genes of antibiotic resistance. Additionally, they arrange clusters of antibiotic resistance related genes on plasmids and/or chromosomes (Toleman et al., 2006). They belong to the family of IS*91*-like elements and were initially discovered as 2.1 kb DNA elements in two complex class-1 integrons i.e. In6 and In7 (Stokes et al., 1993). A total of 21 members of this family have been reported (Schleinitz et al., 2010). Among them, *ISCR2* was predominantly present in *A. baumannii* isolates and has been associated with trimethoprim, tetracycline, chloramphenicol and sulphonamides resistance genes (Montana et al., 2017). Additionally, IS*CR11* was also reported in *A. baumannii* isolates from Germany harboring $bla_{\text{VIM-2}}$ gene (Toleman et al., 2004). These studies emphasize the capacity of genetic adaptability in *A. baumannii*. MDR strains appear to emerge from acquiring resistance factors from genus of other bacteria via a variety of processes that include transferring of plasmids, integrons and transposons. They lead to development of clusters of resistance genes called resistance islands as well as the aggregation of various mutations. Usually, *A. baumannii* appears as a bacterium with quite high genetic plasticity and is well-adjusted to current antimicrobials, hence an increased number of immunocompromised patients require use of broad-spectrum antibiotics for the treatment of infections with *A. baumannii*.

1.8.4 *Enzymatic Hydrolysis*

Hydrolysis of β-lactam antibiotics through several enzymes is the most widespread resistance mechanism in Gram-negative clinical isolates. The occurrence of such enzymes plays vital part in choice of suitable therapies like carbapenems, cephalosporins and penicillins (Bush et al., 1995). β-lactamases are the most common antimicrobial enzymes produced for the hydrolysis and breakdown of β-lactam antibiotics leading to resistance towards β-lactams. In *Acinetobacter*, β-lactam resistance is multifactorial. These factors include alteration in penicillin-binding proteins (PBPs) structure, enzymatic hydrolysis with β-lactamases, fluctuations in number and composition of porin and the efflux pump activity (Perez et al., 2007). The β-lactamases found in *A. baumannii* can be categorized using Ambler classification on the basis of their diverse functional groups i.e. serine and the metallo groups (Yanling et al., 2013).

1.8.4.1 *Ambler Class A β-lactamases:*

Class A-ESBLs have previously been reported in *A. baumannii*, while their exact prevalence is hampered by problems in laboratory identification, particularly due to the co-existence of *AmpC* gene (Pasterán et al., 2006). CARB-5, TEM-1 and TEM-2, narrow-spectrum β-lactamases have been detected in *A. baumannii*, however their clinical importance compared to other resistance markers was limited (Hujer et al., 2006). In France, the first recorded ESBL carbenicillinase called RTG-4 was recognized in *A. baumannii* that is an unusual ESBL because it effectively hydrolyzes cefepime, while on the other hand, it does not impact cefotaxime or ceftazidime (Potron et al., 2009). KPC β-lactamases also fall within the class A category. In Puerto Rico, *A. baumannii* isolates were reported to carry KPC in PCR-based assessment for β-lactam resistance (Robledo et al., 2010). Furthermore, *A. baumannii* has outlined a carbapenem-hydrolyzing GES enzyme that exhibits broad-spectrum characteristics, hydrolyzing oxyimino-cephalosporins. Dissimilarities in spectrum of substrates were noticed, however, some variants showed greater activity of carbapenemase as compared to others (Naas et al., 2008). Another GES-9 variant demonstrated an extended range of hydrolysis to aztreonam, which was found during carbapenemase screening in MDR *A. baumannii* isolated from Belgian hospitals (Bonnin et al., 2011). Most significant β-lactamase genes are variants of bla_{SHV}, bla_{TEM}, bla_{CTX-M}, bla_{VEB}, bla_{GES}, bla_{PER} and bla_{OXA} that confer

increased resistance against β-lactam antibiotics (Abrar et al., 2019, Naas et al., 2008, Perez-Llarena and Bou, 2009).

1.8.4.2 *Ambler Class B β Lactamases:*

Although this class is less frequently isolated from *A. baumannii* as compared to OXA enzymes, the hydrolytic impact of MBLs toward carbapenems is 100 to 1000 times more profound. They have the ability to degrade all β-lactams, including carbapenems, apart from monobactam and aztreonam that could be used for their identification in the laboratory (Poirel and Nordmann, 2006a). The following enzymes have been identified in *Acinetobacter* spp. from the MBL groups; SIM, VIM, IMP, GIM-1, NDM-1, and NDM-2 variants (Kaase et al., 2013). In contrast to Oxacillinases, MBLs were most frequently located on integrons. The majority of the MBL genes in *A. baumannii* were known to be associated with Class 1 integrons, frequently comprising a variety of resistance gene cassettes that encode enzymes with ability to modify aminoglycosides (Houang et al., 2003).

1.8.4.3 *Ambler Class C β-lactamases:*

Among numerous β-lactamases, the chromosomally encoded cephalosporinases (*AmpC*) are found in all *A. baumannii* strains. Uniform designation of Acinetobacter-derived cephalosporinases (ADC) for this family of cephalosporinases has been recommended (Heritier et al., 2006). There was a higher percentage of *AmpC* expression in isolates carrying both *ISAba1* and *AmpC*. The ability to regulate *AmpC* expression in combination with different IS components has turned out to be a crucial factor in *A. baumannii* cephalosporin resistance (Rezaee et al., 2013). *AmpC* Ser-90 phosphorylation has been found to negatively control β-lactamase activity in *A. baumannii* in addition to its ability to counteract imipenem's antimicrobial effect. These findings highlight the impact of antibiotic-resistant bacteria on future drug designing and current therapies (Lai et al., 2016).

1.8.4.4 *Ambler Class D β-lactamases:*

In Edinburgh (Scotland), the first carbapenemase OXA enzyme called ARI-1was observed in *A. baumannii* isolate. It was subsequently sequenced and termed as OXA-23. This enzyme plays an important part in global antimicrobial resistance of *A. baumannii*.

OXA-49 and OXA-27 are enzymes that are exclusively connected to cluster of bla_{OXA-23} gene (Bonomo and Szabo, 2006). OXA-58 and its variant OXA-97 are included in second category of Carbapenem hydrolyzing class D β-lactamases (CHDL). Bla_{OXA-58} gene was recognized in *Acinetobacter* strains globally by epidemiological studies. The bla_{OXA-58} genes are stated as linked with plasmid-encoded ISs (Poirel and Nordmann, 2006b). The 3rd CHDL group includes bla_{OXA-24} (encoding OXA-24, -25, -26, and -40) (Afzal-Shah et al., 2001). OXA-51, extra chromosomally encoded inherent β-lactamase is harbored by *Acinetobacter* having point mutation. Usually this enzyme has reduced carbapenemase activity levels but, effective promoters such as *ISAba1* or *ISAba9* may over-express and then impact the susceptibility of *Acinetobacter* to carbapenems (Poirel et al., 2010). In the last decade, new β-lactamases of OXA type have increased worldwide; and approximately 208 oxacillinases were illustrated in the year 2012. In 2013, 365 OXA enzymes were illustrated having 7 classes, 2 were narrow-spectrum oxacillinases while 5 were nominated as CHDLs (Périchon et al., 2014).

Among β–lactamases, four groups of CHDLs are known to exist in *A. baumannii* including three main acquired groups, bla_{OXA-23}, bla_{OXA-24} and bla_{OXA-58} while bla_{OXA-51} is a representative of the naturally occurring CHDLs (Al-Agamy et al., 2014).

1.8.5 *Non-enzymatic resistance mechanisms*

In *Acinetobacter,* β-lactam resistance is mainly triggered by the formation of β-lactamases. Other regimens include efflux pumps, deletion of porin channels and changes in the target site for antibiotic (Rahal, 2009).

1.8.5.1 *Efflux pumps:*

Efflux pumps expel single or multiple elements from inside to the outside of the cells (Paulsen et al., 1996). Transporters of antibiotics are grouped into distinct super families, which include ATP binding cassette (ABC), major facilitator (MF), Resistance-nodulation-cell division superfamily (RND), multidrug and toxic extrusion (MATE) and small multidrug resistance (SMR) family (Fournier et al., 2006). The genomic studies of *Acinetobacter* isolates have indicated that they encrypt a wide variety of multidrug efflux mechanisms. Two major types of efflux pumps are prevalent in clinical *A. baumannii* strains. First is AdeABC efflux system and belongs to the RND superfamily of

transporters. They are relatively non-specific and their expression is regulated by AdeRS two-component system (AdeS and AdeR TCS) and *ISAba1* insertion sequence (Lari et al., 2018, Marchand et al., 2004). The second efflux pump is encoded by *Tet* genes, which provide resistance towards tetracycline (Chopra and Roberts, 2001).

1.8.5.2 *Biofilm formation:*

A population of bacteria which aggregates by attaching with each other and surfaces along with embedding in the self-produced slimy extracellular matrix (known as extracellular polymeric substances, EPS), is called a biofilm. The biofilm is considered as a safe house where microorganisms can grow (Doi et al., 2015) and are protected against a variety of hazards such as macrophage attack and antimicrobial agents (Lebeaux et al., 2014). This protecting barrier generates a micro-environment in which bacterial cells can live safely in a broad array of conditions (Longo et al., 2014). Moreover, biofilms produce high-density microbes environment consequently facilitating parallel transmission of genetic material containing resistance or virulence genes (Aminov, 2011). This causes the spread and formation of new colonies. Additionally, the reservoirs formed in biofilms serve as a source of frequent hospital epidemics such as that of *A. baumannii*, which maintains its presence in hospitals for extended periods of time and is hard to eliminate (Loehfelm et al., 2008).

1.8.5.3 *Outer membrane proteins:*

They play an important part in resistance mechanisms. In *A. baumannii*, reduced expression of a number of porins is connected with antimicrobial resistance, which comprises of outer membrane proteins (OMPs), which display homology with OmpA porin of Enterobacteriaceae. CarO, a 43 kDa protein called OprD and Omp33/36 protein are three major OMPs linked with reduced susceptibility to carbapenems. OmpW, another OMP has displayed considerably reduced expression in *A. baumannii* isolates possessing resistance towards ceftriaxone (Vila et al., 2007). Disruption of the *OmpA* gene led to reductions in MICs of aztreonam, chloramphenicol and nalidixic acid, meaning that it was involved in antibiotics extrusion in combination with efflux units. It was also shown that OmpA increases survival and persistence by supporting the development of biofilms, surface motility and regulating the biogenesis of vesicles of the outer membrane in *A.*

baumannii (Clemmer et al., 2011). Six immunoreactive membrane proteins have been identified; OmpA, Omp34kDa, OprC, OprB-like, OXA-23, and ferric siderophore receptor protein. Notably, these proteins are highly abundant on the bacterial surface and involved in virulence, antibiotic resistance, and growth (Fajardo Bonin et al., 2014).

1.8.5.4 *Penicillin-binding proteins:*

Penicillin-binding proteins (PBPs) also typically give β-lactam resistance through changes in its target site for antibiotics (Gehrlein et al., 1991).

1.9 Antibiotics

After the discovery of penicillins, various classes of antibiotics have been developed which act on several pharmacological targets present within the microbes (Figure 1.2) (Bennett and Chung, 2001).

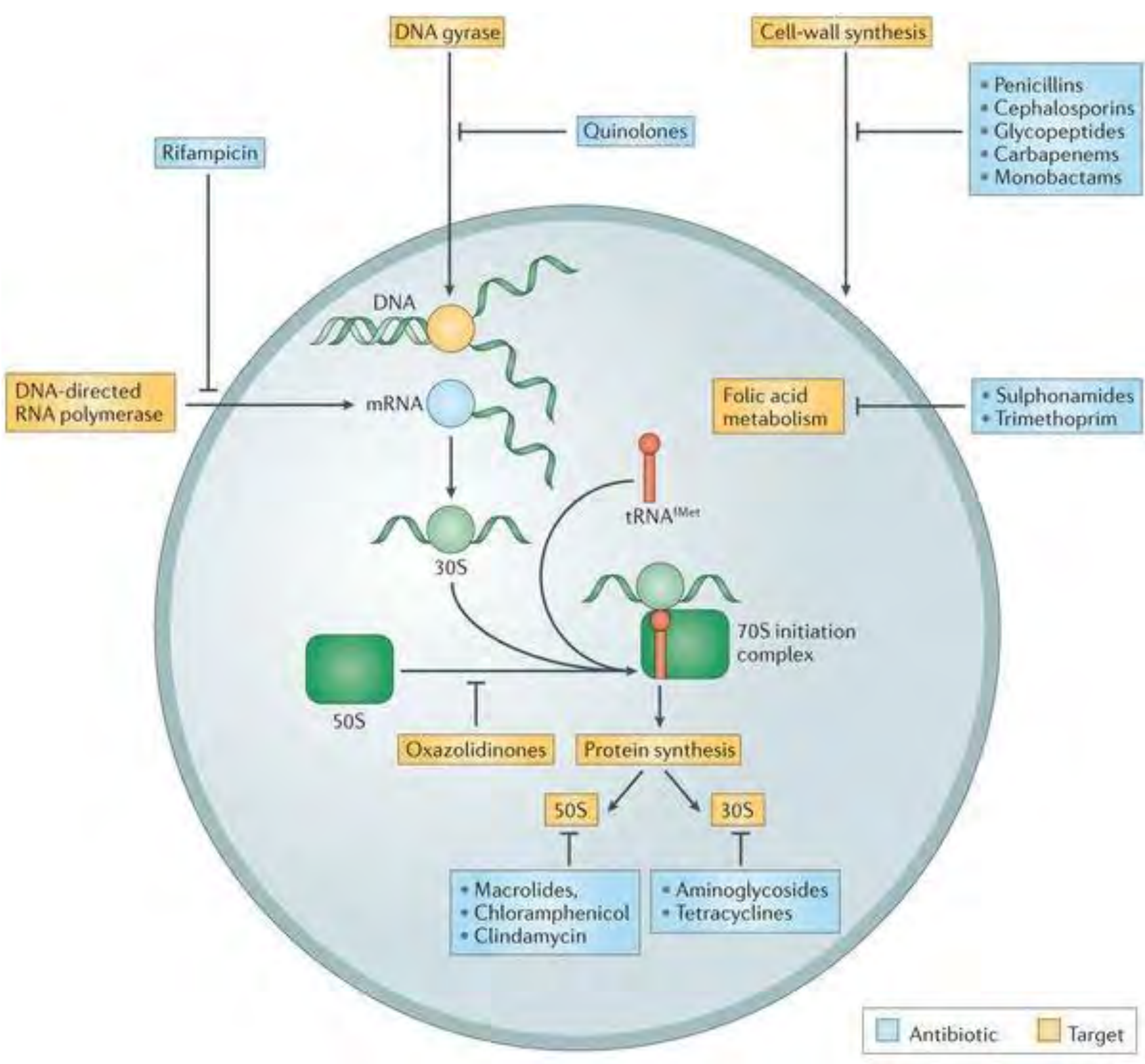


Figure 1.2. Antibiotic and their various pharmacological targets (Coates et al., 2002)

1.10 Resistance towards Various Antibiotics

A. baumannii is resistant towards several classes of antibiotics, the mechanistic details of which are as follows:

1.10.1 *Aminoglycosides:*

Aminoglycoside resistance occurs due to the enzymatic modification of hydroxyl and amino groups by Aminoglycoside Modifying Enzymes (AMEs). This results in weak binding between aminoglycosides and ribosomes that permits the survival of bacterial isolates (Vakulenko and Mobashery, 2003). AMEs present in *A. baumannii* include Adenyltransferases (ANT), Phosphoryltransferases (APH) and the Aminoglycoside Acetyltransferases (AAC) (Zavascki et al., 2010). The geographical variations in the

frequency of specific genes of AMEs in clinical isolates of *Acinetobacter* have been observed (Seward et al., 1998). AAC (60) I-ad was isolated from *Acinetobacter* spp., which amongst the aminoglycoside acetyltransferases, contributes a significant part in amikacin resistance. In addition, plasmid-encoded RNA methylases like Arma were identified in *Acinetobacter* isolates, which by 16S rRNA alteration arbitrate resistance for most aminoglycosides. The *Arma* gene was recognized upstream to the complex integration of Class 1 (Galimand et al., 2005). The AdeABC efflux pump is less efficient in transporting kanamycin and amikacin owing to its hydrophilic nature while kanamycin and gentamicin have also been recorded as substrates of AbeM pump belonging to MATE family (Su et al., 2005).

1.10.2 *Quinolones and Fluoroquinolones:*

There has been a rise in the proportion of *Acinetobacter* isolates with coupled resistance to all quinolones, β-lactams and aminoglycosides (Gupta et al., 2002). Mutations were also recorded widely in *A. baumannii* at quinolones and fluoroquinolones target sites. Specifically, mutations in *GyrA* and *ParC* genes in clinical isolates enhance MICs for ciprofloxacin (Vila et al., 2007). Additional methods for resistance of quinolone in *A. baumannii* comprise of active MATE AbeM, the SMR and efflux by the RND efflux pumps. All of these mechanisms related to ciprofloxacin resistance are chromosomally encoded (Coyne et al., 2011). Fluoroquinolones and quinolones work by binding with bacterial topoisomerase IV and gyrase. In Enterobacteriaceae, specific mutations in the Quinolone Resistance Determining Region (QRDR) of DNA gyrase and topoisomerase IV have been identified to affect quinolones and fluoroquinolones susceptibility (Nordmann and Poirel, 2005).

1.10.3 *Tetracyclines:*

Tetracyclines have bacteriostatic activity and act by binding reversibly to the 30S ribosomal subunit and hindering the translation of proteins. Tetracycline resistance can be regulated by ribosomal protection or efflux pumps. Determinants of *tetA* and *tetB* are particular efflux proteins that expel tetracyclines from the bacterial cell. Protection of ribosome is facilitated by widespread *TetM* determinant, both of which are well associated with *A. baumannii* isolates (Perez et al., 2007). Glycylcyclines, a derivative of tetracycline with some structural modifications, have substantial antimicrobial activity

against MDR *A. baumannii* (Kulah et al., 2009). The AdeABC efflux pump plays a part in the resistance to tigecycline. The increased expression of *adeABC* locus is correlated to 3 fold rise in MIC of tigecycline (Bonnin et al., 2013).

1.10.4 *Polymyxins:*

The peptide antibiotics colistin and polymyxin B have been used commonly as a last resort therapy of MDR *Acinetobacter* infections. Unfortunately, the colistin resistance in *Acinetobacter* isolates is alarming for public health (Goverman et al., 2007, Qureshi et al., 2015a, Nurtop et al., 2019). The primary mechanisms of resistance of these antibiotics comprise of decreasing net negative charge of outer-membrane protein by modifying lipid A, removing peptides through a wide range of efflux-pumps and proteolytic cleavage of antibiotic. Mutations in *pmrAB* two-component system has been attributed to resistance towards colistin in *A. baumannii* (Adams et al., 2009). A transcriptomic study revealed a total of six genes in colistin resistance in *A. baumannii*. These genes encode PmrAB two-component regulatory enzymes, PmrC (a lipid A phosphoethanolamine transferase), a glycosyltransferase, a poly-β-1,6-*N*-acetylglucosamine deacetylase, and a putative membrane protein. It is of note that the colistin-resistant strains had modified lipid A structure by the addition of phosphoethanolamine. Moreover, the resistance genes were found to be associated with either lipopolysaccharide biosynthesis or electrostatic changes in the bacterial cell membrane. Hence, it was observed that lipopolysaccharide modification was the principal mode of acquisition of colistin resistance in *A. baumannii* strains (Park et al., 2015).

1.10.5 *Other Antibiotics, Disinfectants and Heavy Metals:*

Hetero-resistant, genetically identical sub-clones or sub-populations of bacteria, which are more resistant than the original parent clone, are a threat to the use of several classes of antibiotics, disinfectants and heavy metals. It will be necessary to analyze and monitor the effect of hetero-resistance (Pournaras et al., 2005).

Macrolide and chloramphenicol are antibiotics which act through inhibition of protein synthesis. The resistance to the former is considered inherent to *Acinetobacter*. However, Tn1548-associated plasmid-mediated macrolide resistance was also noted. *Acinetobacter* also harbors *mel* and *mph* genes that encode efflux pump and macrolide-modification

enzyme, which render the drug ineffective (Galimand et al., 2005). Moreover, chloramphenicol resistance has been attributed to genes located on integrons such as *cmlA* and *cat*, the former encode efflux pump while later produce an enzyme that modifies chloramphenicol (Peleg et al., 2008).

Acinetobacter species have also developed resistance towards anti-metabolite class of antibiotics such as sulfonamide and trimethoprim. Resistance to sulfonamides in *Acinetobacter* species is common due to the presence of *sul1* gene, which encodes efflux-based sulfonamide resistance mechanism, and is constantly associated to Class 1 integrons, that are common in *Acinetobacter* species (Bonomo and Szabo, 2006). Trimethoprim acts on folate formation, is sometimes regulated by *dfr* genes which are broadly disseminated in most Gram-negative bacteria. In *Acinetobacter*, trimethoprim resistance might be due to the higher expression of inherent efflux pumps (Coyne et al., 2011).

Heavy metal, dye and disinfectants resistance is primarily regulated by inherent efflux pumps. Genome analysis of *Acinetobacter* found the presence of numerous operons with efflux encoding genes that give resistance to various disinfectants and heavy metals (Fournier et al., 2006). Some mechanism of resistance by antibiotic class in *Acinetobacter baumannii* are tabularized below in Table 1.1.

Table 1.1: Mechanism of resistance by antibiotic class in *Acinetobacter baumannii* (Esterly et al., 2011).

Antibiotic Class	Major Mechanisms of Resistance	Protein or Enzyme
β-lactams	β-lactamases	
	cephalosporinase	AmpC
	ESBLs	CTX-M SHV TEM
	carbapenemase	OXA IMP VIM SIM KPC
	OMPs	CarO OprD
	Efflux pumps RND	AdeABC
	Target binding site alteration PBPs	PBP
Tetracyclines and Glycyclines	Efflux pumps	TetA TetB
	RND	AdeABC AdeIJK
	Target binding site alteration	TetM
Fluoroquinolones	Target binding site alteration DNA gyrase Topoisomerase IV	GyrA ParC
	Efflux pumps RND	AdeABC AbeM
Aminoglycosides	AME acetyltransferases nucleotidyltransferases phosphotransferases	Gene cassettes within integron
	Target binding site alteration 16S rRNA methylase	ArmA RmtA RmtB RmtC RmtD
	Efflux pumps RND	AdeABC

1.11 Newer Antibiotics for *A. baumannii*

The emergence of resistance against existing antibiotics and the development of newer antibiotics runs like hand-in-glove. In case of MDR *A. baumannii*, the choice of antibiotic has been reduced to a single drug i.e. colistin. Unfortunately, there have been reports of resistance against this one too (Qureshi et al., 2015b, Li et al., 2006). This is the reason WHO positioned *A. baumannii* in the top priority pathogens for which further research is required for the identification of newer antibiotics.

In *A. baumannii*, the Two-Component Systems (TCSs) facilitate survival in unfavorable environments *via* sensing and adapting to the changing environmental milieu. Therefore,

this TCS system is believed to play an important role in its resistance and virulence. Keeping in view its importance, this TCS presents itself as a potential novel drug target for eradication of *A. baumannii* (De Silva and Kumar, 2019). Some of the inhibitors of TCS are tabularized below in Table 1.2.

Table 1.2: Small molecule inhibitors of TCS (De Silva and Kumar, 2019)

TCS	Organism(s)	Inhibitor	Inhibitory action
AlgR1/AlgR2	*Pseudomonas aeruginosa*	Thiazole derivatives	Inhibition of AlgR1 phosphorylation and AlgR2 kinase activity
WalKR	*Staphylococcus aureus* *Bacillus subtilis*	Walkmycin B Waldiomycin	Inhibition of autophosphorylation of WalK
		Walrycin A Walrycin B	Inhibition of phosphotransfer from WalR
QseCB	Enterohemorrhagic *E. coli* (EHEC)	LED209	Inhibition of autophosphorylation of QseC
PhoPQ	*Salmonella sp.*	Radicicol	Activity against PhoQ
VanSR	*Enterococcus faecium*	Thiazole derivatives	Inhibition of phosphotransfer to VanR

Various newer drugs alone or in combination are being reported for the management of MDR Gram-negative pathogens (Karaiskos et al., 2019). They are tabularized as follows in Table 1.3.

Table 1.3: Newer antibiotics and their combination for the management of MDR Gram-negative bacteria (Karaiskos et al., 2019)

Name	Class	Structure
Ceftolozane-Tazobactam	β-lactam / β-lactamase inhibitor	

Ceftazidime-Avibactam	β-lactam / β-lactamase inhibitor	
Meropenem-vaborbactam	Non-β-lactam / β-lactamase inhibitor	

Plazomicin	Aminoglycoside	
Eravacycline	Tetracycline	

1.12 Isolation, Recognition and Molecular Typing

The clinically significant species of *Acinetobacter* grow well at 37°C on routinely used microbial growth media with characteristic cream or white colour colony on blood agar. Bluish to bluish-grey colonies are generated by species of *Acinetobacter* on Eosin methylene blue agar (Doughari et al., 2012). On MacConkey agar, *Acinetobacter* colonies appear as colourless, violet or light lavender because they are incapable of lactose fermentation. Enrichment of cultures with an oxygenated broth medium with low pH (such as acetate and nitrate as carbon and nitrogen source) were suitable for the detection of *Acinetobacter* species from different environmental and clinical samples. A selective and differential medium such as Leeds *Acinetobacter* medium was suggested to facilitate isolation of species of clinical significance from a variety of bacterial populations in hospital settings (Jawad et al., 1994). For identification to species-level, commercial microbiology systems are routinely used in clinical laboratories. These include PhoenixTM, Vitek ®, MicroScan WalkAway® and API 20NE systems (Lee et al., 2015). The inventory of such commercial products is restricted and substrates utilized in these bacterial recognition systems were not specifically intended for *Acinetobacter* (Dijkshoorn et al., 1993).

Various molecular techniques have been developed and evaluated for the identification to species level. These are ribotyping, *rpoB* gene sequencing, 16S-23S rRNA, Restriction fragment length polymorphism (RFLP), Amplified fragment length polymorphism (AFLP), Amplified ribosomal DNA restriction analysis (ARDRA) and tRNA spacer

fingerprinting (Dolzani et al., 1995). AFLP and ARDRA are currently regarded as the most reliable techniques for identifying *Acinetobacter* species, with the availability of a profile library for clinical as well as reference strains. tRNA fingerprints too are regarded suitable for general species identification but do not distinguish *A. baumannii* species at the genomic level. The 16S-23S ribosomal RNA gene sequence typing along with ribotyping is regarded suitable to distinguish strains related to the ACB complex, but not for species other than ACB complex. Over last few years, these molecular techniques have considerably enhanced knowledge of clinical and epidemiological significance of *Acinetobacter* strains. They are restricted primarily to reference laboratories as they are too laborious for normal diagnostic laboratories (Peleg et al., 2008).

Typing methods to distinguish bacterial isolates belonging to different species are important epidemiological tools used in infection control and prevention. *A. baumannii* is associated with healthcare infections and is responsible for high mortality and morbidity. Because of its clinical importance, it is important to know about the molecular typing methods used for the confirmation of outbreaks caused by *A. baumanni* as well as the identification of sequence types of the isolates belonging to different geographical areas (Johnson et al., 2016). Old typing methods based on phenotypes, such as biotype, serotype and antibiogram have been used in the past. But now the molecular methods are being used for enhanced surveillance and detection of outbreaks (Sabat et al., 2013). Currently, advanced methods that are being used for molecular typing are pulsed-field gel electrophoresis (PFGE), Multi-locus variable-number tandem repeat analysis (MLVA), Multi-locus sequence typing (MLST) and Enterobacterial Repetitive Intergenic Consensus Polymerase chain reactions (ERIC-PCR). Genetic relatedness of the isolates can be checked by the MLST and PFGE. PFGE and MLVA are used to discriminate between isolates (Johnson et al., 2016).

Aim of the Study

The aim of the current study was to assess the molecular basis of antibiotic resistance and clonal diversity in clinical isolates of *Acinetobacter baumannii*.

Objectives

- Isolation, characterization and antibiotic susceptibility profiling of clinical isolates of *A. baumannii*.
- To analyze the β-lactam resistance through phenotypic and PCR based methods.
- To detect resistance genes of non-β lactam antibiotics (fluoroquinolones, aminoglycosides and tetracyclines), efflux pumps and virulence encoding genes in study isolates.
- To elucidate the clonal relatedness by ERIC PCR and genetic diversity of subset of isolates through Pulsed-field gel electrophoresis and Multi-locus sequence typing.

Chapter 2

Materials & Methods

2.1 Sample collection

A total of 375 *Acinetobacter* isolates [based on sample size calculation formula; $n=(z)^2 p (1 - p) / d^2)=306$] were obtained during January 2014 - June 2015 from the Microbiology laboratory of Pakistan Institute of Medical Sciences (PIMS), a tertiary care hospital in Islamabad, Pakistan. These isolates were obtained from outdoor and indoor patients admitted in various wards including ICUs, medical and surgical wards. Ethical approval to undertake the study was obtained from Bio-Ethical Committee (BEC) of Quaid-i-Azam University Islamabad, Pakistan.

2.2 Demography

Demographic information such as age, gender and the clinical source was collected from the medical record of the patients.

2.3 Identification of *A. baumannii* Isolates

Standard microbiological techniques were used for the species identification as follows:

2.3.1 *Colony Morphology*

Initially, isolates were grown on MacConkey agar and blood agar medium. MacConkey agar differentiates lactose fermenters from non-fermenters. Blood agar was used for the growth of non-pigmented and non-hemolytic colonies. Incubation was carried out for 24-48 hours at 37˚C.

2.3.2 *Gram staining*

A thin smear was formed by moving the bacterial culture suspension on a slide and was fixed by passing the slide through a flame. Firstly, a reagent crystal-violet was applied and removed with distilled water after 1 minute, and Gram's iodine solution was applied. After 1 minute, the iodine solution was dispensed and washed with water. Depending on discoloration, a few drops of alcohol were added and washed with water after 5-30s exposure. Smear was counterstained for 40-60s with a Safranin solution (few drops). Lastly, the solution was washed with water and slide was air-dried after removing surplus water with blotting paper and observed under microscope.

2.3.3 *Biochemical & Molecular Identification*

The kit based biochemical testing was performed for the identification of the bacteria as follows:

API 20NE kit:

API 20 NE kit (Biomeriuex,France) is a standardized scheme for the identification of Gram-negative non-Enterobacteriaceae members (O'Hara, 2005). Kit contains 20 biochemical tests as shown in Table 2.1. There are 20 microtubes with dried substrates in one strip. Suspension of bacteria (0.5 McFarland equivalent) was formed by placing 3-5 isolated colonies in 5ml of normal saline. To obtain a homogeneous bacterial suspension, it was thoroughly emulsified. The bacterial suspension was inoculated in wells. The colour changed due to metabolic activity of isolates during incubation either spontaneously or after addition of reagents. Depending on the test requirement, tubes and cupules were filled. By overlaying mineral oil, some tests were incubated anaerobically. After incubation at 37°C for 24 hours, and addition of reagents to respective wells, results were observed. To calculate numerical profile, tests were divided into groups of three and a value equal to 1, 2 or 4 was given to each positive reaction according to the place in its group, first, second or third, respectively. The resultant digit from 0 and 7 was obtained from the sum of these 3 values (0 for negative reaction). A numerical profile of seven digits was documented and compared with the Analytical Profile Index given in the API booklet. The seven-digit numerical profile for *A. baumannii* is 0041073.

Table 2.1: Biochemical tests in API 20NE Kit

Test	Reaction	Positive	Negative
$NO_3 \rightarrow NO$ $NO_2 \rightarrow N_2$	Reduction of Potassium nitrate	Colourless Red/Pink	Red (NITI+NIT2) Colourless (Zn)
TRP	Production of Indole from tryptophan	Yellow	Pink
GLU	Glucose Fermentation	Blue/Green	Yellow
ADH	Arginine hydrolysis	Yellow	Orange/Pink/Red
URE	Urea hydrolysis	Yellow	Orange/Pink/Red
ESC	Aesculin hydrolysis	Yellow	Grey/Brown/Black
GEL	Gelatin hydrolysis	No pigment diffusion	Diffusion of black pigment
PNPG	p-nitrophenyl-ßD galactopyranoside hydrolysis	Colourless	Yellow
GLU	Glucose Assimilation	Transparent	Opaque
ARA	Arabinose assimilation	Transparent	Opaque
MNE	Mannose assimilation	Transparent	Opaque
MAN	Mannitol assimilation	Transparent	Opaque
NAG	N-acetyl-glucosamine assimilation	Transparent	Opaque
MAL	Maltose assimilation	Transparent	Opaque
GNT	Gluconate assimilation	Transparent	Opaque
CAP	Caprate assimilation	Transparent	Opaque
ADI	Adipate assimilation	Transparent	Opaque
MLT	Malate assimilation	Transparent	Opaque
CIT	Citrate assimilation	Transparent	Opaque
PAC	Phenyl-acetate assimilation	Transparent	Opaque
Oxidase	Cytochrome oxidase	Colourless	Purple

2.3.4 *Molecular Identification via Detection of 16S rRNA and bla$_{OXA-51-like}$ genes*

For ribotyping, the universal primers from the previously reported study were used to amplify the 16S rRNA gene, a conserved marker of bacteria as shown in Table 2.2 (Fredriksson et al., 2013). The *bla*$_{OXA-51-like}$ is an intrinsic gene, whose presence has been used for specie level identification of *A. baumannii* (Chen et al., 2010, Hu et al., 2007).

Boil colony method was used for the isolation of bacterial DNA as described previously (Vaneechoutte et al., 1995). Pure colony was suspended in 50 µl sterile water and heated for 15 min at 100°C. Centrifugation was done for 4 min at 7,000rpm and supernatant was stored at -20°C. All PCR products (details are given below) were electrophoresed using 2% agarose gel at 100V for 60 minutes. Gels were analyzed under UV illuminator (Syngene, USA).

For PCR, the reaction mixture was prepared in 25 µl reaction volumes with 12.5 pmol of each Primer (Table 2.2), 3µl of extracted DNA, 1X PCR buffer comprising 1.5 U of Taq polymerase, 200 µM of each deoxynucleoside triphosphate, 1.5 mM $MgCl_2$. Program for multiplex PCR was as follows: initial denaturation for 3 min at 94°C, and then 35 cycles of denaturation for 45s at 94°C, annealing for 45s at 60°C, and initial extension at 72°C for 1 min, with a final extension at 72°C for 5 min (Turton et al., 2006).

Table 2.2: Primers used for molecular identification of *A. baumannii*

Gene	Primer Sequence (5' – 3')	Product size (bp)
16S rRNA	F-AGAGTTTGATCMTGGCTCAG R-TACGGYTACCTTGTTACGACTT	150
bla$_{OXA-51-like}$	F-TAATGCTTTGATCGGCCTTG R-TGGATTGCACTTCATCTTGG	351

2.4 Phenotypic Assays

Various phenotypic assays were performed to explore the resistance pattern of *A. baumannii* isolates

2.4.1 *Disk diffusion method*

Antibiotic susceptibility assay was done using the Kirby-Bauer disk diffusion method as described previously (Bauer et al., 1966). To achieve density equal to 0.5 Mcfarland standard, suspension of colonies from overnight culture was formed in normal saline. The bacterial lawn was prepared on the plate of Mueller Hinton Agar (MHA Oxoid, UK), utilizing a sterile swab by rotating the plate at 60° three times. Using sterile forceps, various antibiotic disks (Table 2.3) were separated from the dispenser and carefully put on MHA plate. Overnight incubation was done at 37°C. Inhibition zones were calculated around the disks after 16-24 hrs of incubation. *E. coli* ATCC 25922 was used as a reference strain.

Table 2.3: List of antimicrobial agents used for susceptibility testing

Antimicrobial Category	Antimicrobial agent	Code	Potency
Penicillins + ß-lactamase inhibitors	Piperacillin/tazobactum	TZP	110 µg
	Amoxicillin/clavulanic acid	AMC	30 µg
Extended-spectrum Cephalosporins	Cefotaxime	CTX	30 µg
	Ceftriaxone	CRO	30 µg
	Ceftazidime	CAZ	30 µg
Carbapenems	Imipenem	IPM	10 µg
Aminoglycosides	Amikacin	AK	30 µg
	Tobramycin	TOB	10 µg
Fluroquinolones	Ciprofloxacin	CIP	5 µg
Folate pathway inhibitors	Trimethoprim Sulfamethoxazole	SXT	25 µg
Polymyxins	Colistin	CT	10 µg
	Polymyxin B	PB	10 µg
Tetracyclines	Tetracycline	TE	30 µg
	Minocycline	MN	30 µg
Glycylcyclines	Tigecycline	TGC	15 µg

2.4.2 *Broth Microdilution Method*

All isolates were processed for MICs by the broth microdilution method (Jorgensen and Turnidge, 2015). MICs for ceftriaxone, levofloxacin, ampicillin, chloramphenicol, amikacin, rifampicin, colistin and imipenem were determined. Colonies suspension from an overnight culture in normal saline was made with a density comparable to 0.5 Mcfarland. Broth microdilution MICs testing was performed in polystyrene 96 well round-bottomed micro-titre plates. Isolates suspensions with OD625 = 0.08–0.1 (0.5 McFarland) was used. Mueller Hinton broth (MHB) was used to prepare dilution of the above-mentioned antibiotics till concentration of 512mg/L. Tested concentrations included the antibiotic breakpoints, 256mg/L, 128mg/L, 64mg/L, 32mg/L, 16mg/L,

8mg/L, 4mg/L, 2mg/L, 1mg/L and 0.5mg/L. In 11th well, 50 µl of inoculum was added. Un-inoculated MHB was added as a standard to the 12th well. The 50 µl of antibiotic solution was added to first well and then 50µl was transferred from this well to the next well, mixed and added 50µl from each well to the next well, until 11th well. Placed the lid carefully and incubated at 37°C 18-24 hours. The results were measured using micro-titre plate reader.

2.4.3 ***Assays for the β-lactamases***

The phenotypic assays for the detection of various types of β-lactamases were performed as follows:

2.4.3.1 *Double disk synergy test (DDST) for ESBLs*

DDST is used for ESBLs detection (Drieux et al., 2008). Ceftazidime (30 µg), ceftriaxone (30 µg) and amoxicillin/clavulanic acid (20/10 µg) antibiotic discs were used as previously described (Miles and Amyes, 1996). In the center, amoxicillin/clavulanic acid disc was placed, while ceftazidime (30 µg) and ceftriaxone (30 µg) at a distance of 15mm were placed on either side. In addition, cefoxitin (30µg) disc was put on the same plate to evaluate cephamycin susceptibility. The ESBL test was termed positive if there was a visible expansion of the border of the cephalosporin inhibition zone towards the inhibitor or inhibition of bacterial growth was noted where the two antibiotics diffused together.

2.4.3.2 *IMP-EDTA Test for MBLs*

The test isolates were grown on the MHA plates as suggested in the literature (Lee et al., 2001). Two imipenem discs (10 µg) were positioned on the bacterial lawn with a 20 mm distance. In one of the imipenem discs, 10 µl of 0.5 M EDTA (Appendix I) was added and incubated overnight. Isolates displaying an increase of ≥7 mm in the imipenem-EDTA disk inhibition zone as compared to imipenem disk alone was regarded as MBL positive result.

2.4.3.3 *AmpC disk test for AmpC-β- lactamases*

Cefoxitin-resistant strains were subjected to AmpC disc test for AmpC β-lactamase production following the previous protocol (Black et al., 2005). Bacterial lawn of *E. coli* ATCC25922 was made on MHA plate. On the agar surface, a cefoxitin disk (30µg) was

placed and a blank disk soaked with bacterial suspension was positioned in such a manner that it almost touched it. This was followed by incubation of plate overnight at temperature 37 °C. Flattening or indentation of inhibition zone around cefoxitin disc near the disc with the test strain was labeled as positive for AmpC β-lactamase production. An unaltered zone was considered as negative.

2.4.3.4 *Modified Hodge Test (MHT) for Carbapenemases*

The inoculum (control *E. coli* 25922 strain) was uniformly distributed on the MHA plate. After air drying, 10 µg carbapenem disc was applied. The carbapenem-resistant test organisms were streaked from the disc's bottom to the plate's bottom. On a single plate, 3 organisms were tested. The plates were then placed in an inverted position and incubated for 24 hours at temperature 37°C. The indentation of *E. coli* 25922, which grows along the test organism within the diffusion zone was considered as a positive result showing the production of carbapenemase (Lee et al., 2001).

2.4.4 *Efflux Pump Activity*

Phenotypic assay for efflux pump activity was done using the Ethidium bromide Cartwheel method as described earlier (Martins et al., 2013). The EtBr-agar cartwheel (EtBrCW) technique is a practical methodology for assessing the existence of enhanced efflux activity in large number of clinical bacterial isolates. Based on their ability to extrude EtBr, this technique enables comparison of distinct isolates. Cultures of the bacterial isolates to be tested were prepared overnight in nutrient broth and their concentration adjusted to 0.5 McFarland standard the following day. Two Trypticase Soy Agar (TSA) plate sets comprising EtBr concentrations from a range of 0.0 to 2.5 mg/L were used. The plates were divided by radial lines into 12 sectors that form a cartwheel pattern. EtBr-TSA plates were swabbed starting from the center of the plate to the margin for adjusted bacterial cultures. Incubation of TSA plates was carried out at 37ºC for 16 hours followed by its observation under a gel-imaging system (or a U.V. transilluminator). The minimum EtBr concentration producing the fluorescence was recorded for bacterial mass and the photograph of TSA plates was taken. Cartwheel ethidium bromide assay showing loss of florescence of bacterial culture was positive result for efflux activity while culture with florescence was considered negative result for this assay.

2.5 Genetic Characterization for Antibiotic Resistance Genes

The genetic characterization of the isolates for the carriage of various resistance related genes was performed as follows:

2.5.1 *Detection of β-lactamase genes*

Detection of classes of β-lactamases was done by Polymerase chain reactions (PCR) (Appendix VI).

2.5.1.1 *Extended-spectrum β-lactamases (ESBLs) encoding genes*

Multiplex PCR was intended for bla_{TEM} and bla_{SHV} genes as previously stated (Lal et al., 2007). Detection of $bla_{CTX\text{-}M}$ groups including $bla_{CTX\text{-}M\text{-}group\text{-}1}$, $bla_{CTX\text{-}M\text{-}group\text{-}9}$, $bla_{CTX\text{-}M\text{-}group\text{-}8/25}$ was performed with annealing at 60°C, using primers as previously described (Dallenne et al., 2010). A set of multiplex PCR was performed to amplify the $bla_{M\text{-}PER}$, $bla_{M\text{-}GES}$, $bla_{M\text{-}VEB}$ β-lactamase genes with annealing at 57°C as mentioned previously (Dallenne et al., 2010) (Appendix VI). The primer sequences are tabularized in Table 2.4.

2.5.1.2 *Metallo β-lactamase encoding genes:*

Multiplex PCR was performed for the screening of 10 MBL genes; bla_{IMP}, bla_{VIM}, bla_{SPM}, bla_{GIM}, bla_{KPC}, bla_{BIC}, bla_{AIM}, bla_{GIM}, bla_{SIM}, and bla_{DIM} as described earlier (Poirel et al., 2011).

2.5.1.3 *AmpC–ADC β-lactamase encoding gene:*

A set of primers was used for ADC (Class C) β-lactamase gene amplification by following the previous protocol (Ruiz et al., 2007).

2.5.1.4 *Carbapenemase encoding genes (Oxacillinases):*

The amplification of oxacillinases i.e. $bla_{OXA\text{-}51}$, $bla_{OXA\text{-}23}$, $bla_{OXA\text{-}24}$, $bla_{OXA\text{-}48}$ and $bla_{OXA\text{-}58}$ was done as described previously (Woodford et al., 2006).

2.5.2 *Fluoroquinolone resistance genes:*

For the detection of fluoroquinolone resistance genes (*qnrA, qnrB* and *qnrS*), the primer sequences were obtained from a previous study (Sedláková et al., 2014).

2.5.3 *Tetracycline resistance genes:*

PCR method was used to study the presence of *tetA* and *tetB* genes as described earlier (Maleki et al., 2014).

2.5.4 *Aminoglycosides resistance genes:*

For aminoglycoside-modifying enzymes (AMEs), amplification of *aadB, aphA6, aadA1,* and *aacC1* was performed as described earlier (Aliakbarzade et al., 2014).

2.5.5 *Efflux Pump:*

The presence of genes encoding efflux pump (*adeR and adeS genes*) was evaluated using PCR according to the previous study (Beheshti et al., 2014).

2.5.6 *Insertion sequence ISAba 1:*

Amplification of insertion sequence *ISAba1* was done as described earlier (Heritier et al., 2006).

2.5.7 *Detection of Outer membrane protein*

We also investigated a possible porin-mediated mechanism relating to the carbapenem resistance-associated outer membrane protein, CarO. Amplification of outer membrane protein-encoding gene *CarO* was performed using primers and protocol as described previously (Yang et al., 2015).

Table 2.4: Primer sequences and product size of resistance genes

Category	Genes	Primer Sequence (5'– 3')	Product Size (bp)
ESBLs	*bla*$_{SHV}$	F- TCAGCGAAAAACACCTTG R- TCCCGCAGATAAATCACC	471
	bla$_{TEM}$	F- CTTCCTGTTTTTGCTCACCCA R- TACGATACGGGAGGGCTTAC	717
	bla$_{CTX\text{-}M\text{-}group\text{-}1}$	F-TTAGGAARTGTGCCGCTGYAb R-CGATATCGTTGGTGGTRCCATb	688
	bla$_{CTX\text{-}M\text{-}group\text{-}9}$	F-TCAAGCCTGCCGATCTGGT R-TGATTCTCGCCGCTGAAG	561
	bla$_{CTX\ M\text{-}825}$	F-AACRCRCAGACGCTCTACb R-TCGAGCCGGAASGTGTYATb	326
	bla$_{M\text{-}GES}$	F-AGTCGGCTAGACCGGAAAG R-TTTGTCCGTGCTCAGGAT	399
	bla$_{M\text{-}PER}$	F-GCTCCGATAATGAAAGCGT R-TTCGGCTTGACTCGGCTGA	520
	bla$_{M\text{-}VEB}$	F-CATTTCCCGATGCAAAGCGT R-CGAAGTTTCTTTGGACTCTG	648
MBLs	*bla*$_{IMP}$	F-GGAATAGAGTGGCTTAAYTCTC R-GGTTTAAYAAAACAACCACC	232
	bla$_{VIM}$	F-GATGGTGTTTGGTCGCATA R-CGAATGCGCAGCACCAG	390
	bla$_{SPM}$	F-AAAATCTGGGTACGCAAACG R-ACATTATCCGCTGGAACAGG	271
	bla$_{NDM}$	F-GGTTTGGCGATCTGGTTTTC R-CGGAATGGCTCATCACGATC	621
	bla$_{KPC}$	F-CGTCTAGTTCTGCTGTCTTG R-CTTGTCATCCTTGTTAGGCG	798
	bla$_{BIC}$	F-TATGCAGCTCCTTTAAGGGC R-TCATTGGCGGTGCCGTACAC	537
	bla$_{AIM}$	F-CTGAAGGTGTACGGAAACAC R-GTTCGGCCACCTCGAATTG	322
	bla$_{GIM}$	F-TCGACACACCTTGGTCTGAA R-AACTTCCAACTTTGCCATGC	477
	bla$_{SIM}$	F-TACAAGGGATTCGGCATCG R-TAATGGCCTGTTCCCATGTG	570
	bla$_{DIM}$	F-GCTTGTCTTCGCTTGCTAACG R-CGTTCGGCTGGATTGATTTG	699

AmpC-ADC β-lactamase	*bla*$_{AMPC-ADC}$	F-CCGCGACAGCAGGTGGATA R-TCGGCTGATTTTCTTGGTT	395
Carbapenemase	*bla*$_{OXA-51-like}$	F-TAATGCTTTGATCGGCCTTG R-TGGATTGCACTTCATCTTGG	353
	bla$_{OXA-23-like}$	F-GATCGGATTGGAGAACCAGA R-ATTTCTGACCGCATTTCCAT	501
	bla$_{OXA-24-like}$	F-GGTTAGTTGGCCCCCTTAAA R-AGTTGAGCGAAAAGGGGATT	246
	bla$_{OXA-58-like}$	F-AAGTATTGGGGVTTGTGCTG R-CCCCTCTGCGCTCTACATAC	599
Fluoroquinolones	*qnrA*	F-AGAGGATTTCTCACGCCAGG R-TGCCAGGCACAGATCTTGAC	580
	qnrB	F-GGMATHGAAATTCGCCACTG R-TTTGCYGYYCGCCAGTCGAA	264
	qnrS	F-GCAAGTTCATTGAACAGGGT R-TCTAAACCGTCGAGTTCGGCG	428
Tetracyclines	*tetA*	F-GCGCGATCTGGTTCACTCG R-AGTCGACAGGCGCCGGC	164
	tetB	F-CGTGAATTTATTGCTTCGG R-ATACAGCATCCAAAGCGCAC	206
Aminoglycosides	*aphA6*	F-ATGGAATTGCCCAATATTATTC R-TCAATTCAATTCATCAAGTTTTA	797
	aadA1	F-ATGAGGGAAGCGGTGATCG R-TTATTTGCCGACTACCTTGGTG	792
	aadB	F-ATGGACACAACGCAGGTCGC R-TTAGGCCGCATATCGCGACC	534
	aacC1	F-ATGGGCATCATTCGCACATGTAG R-TTAGGTGGCGGTACTTGGGTC	456
Efflux pump	*adeS*	F-ACTGTTATCTTCTGTGGCTGTA R-GTGGACGTTAGGTCAAGTTCTG	477
	adeR	F-AAACGGTTGGGAAGTATTA R-ATGGCTATCTACGGTTCG	544
Insertion sequence	*ISAba1*	F-ATGCAGCGCTTCTTTGCAGG R-AATGATTGGTGACAATGAAG	390
Outer membrane protein	*CarO*	F-ATGAAAGTATTACGTGTTTTAGTG R-TTACCAGTAGAATTACACCAACT	729

2.6 Genetic Characterization of Virulence Genes

The genetic characterization for virulence-related genes including *csgA* , *iutA*, *cnf1* and *cvaC* was performed by using PCR method as described previously (Darvishi, 2016). DreamTaq PCR Master Mix (Thermo Fisher Scientific., USA) was used that comprises of dNTPs, Taq polymerase, $MgCl_2$ and suitable buffer. A total of 25 µl reaction mixture was available in each PCR tube that comprised of master mix (12.5 µl), forward and reverse primer solution (1 µl each in a final concentration of 200 nM), DNA (1 µl of ~ 200 ng/µl concentration) and nuclease free water. Following were the conditions for PCR: initial denaturation for 5 min at 94°C, followed by 30 cycles of 1 min denaturation at 94°C, 1 min annealing at 54°C, 1 min extension at 72°C, followed by a final extension at 72 °C for 10 min. The amplified products were separated by electrophoresis as described in section 2.7.12. Primers sequence details are shown in Table 2.5.

Table 2.5: Primer sequences and Product size for virulence genes

Genes	**Primer Sequence (5'-3')**	**Product Size (bp)**
cnf1	F-AAGATGGAGTTTCCTATGCAGGAG R-ATTCAGAGTCCTGCCCTCATTATT	498
csgA	F-ACTCTGACTTGACTATTACC R-AGATGCAGTCTGGTCAAC	200
cvaC	F-CACACACAAACGGGAGCTGTT R-CTTCCCGCAGCATAGTTCCAT	680
iutA	F-GGCTGGACATCATGGGAACTGG R-CGTCGGGAACGGGTAGAATCG	300

2.7 Clonal Analysis and Molecular Typing

The typing was performed as follows:

2.7.1 *Entero-bacterial Repetitive Intergenic Consensus-Polymerase Chain Reactions (ERIC-PCR)*

For *A. baumannii* isolates clonal relatedness, REP-PCR specific primers ERIC-1R (5′-ATGTAAGCTCCTGGGGATTCAC-3′) and ERIC-2 (5′-AAGTAAGTGACTGGGGTGAGCG-3′) were used as described earlier (Hasan et al., 2014).

2.7.1.1 *DNA extractions*

DNA extraction was done by boil colony method as described earlier in section 2.3.4.

2.7.1.2 *ERIC-PCR*

Amplification was carried out in sterile polypropylene tubes (0.2 ml). The reaction mix (50 µl) comprised of DNA template (2 µl), each primer (2 µl of 10 µM concentration) and 25 µl of 2X PCR Master Mix (Thermo Scientific). The conditions for amplification were: 94°C for 10 min.; 30 cycles of 94°C for 1min followed by annealing for 1 min at 45°C and extension for 2 min at 72°C followed by the final extension for 16 min at 72°C.

2.7.1.3 *Gel electrophoresis*

Analysis of amplicons was done using agarose gel (1.5 %) and 1X TAE buffer at 110 Volts for 80 minutes. Amplicon size was measured using the 100bp DNA Marker (Bio-Rad Inc., USA).

2.7.1.4 *DNA fingerprinting and Dendrogram using bionumeric software*

All gels were visualized and DNA fingerprints were evaluated by bionumeric software version 7.6 (Applied Maths, Belgium). Relatedness between the patterns of DNA was determined by Dice similarity coefficients calculated by using bionumeric software (Applied Maths, Belgium) that was based on band positions. The patterns of ERIC-PCR with 80 percent dice similarity coefficients were taken as genetically relevant and labeled as clonal type. A dendrogram was developed to represent similarities in the matrix using

the unweighted pair group technique with arithmetic averages (UPGMA). The results interpretation was based on criteria described in previous study (Tenover et al., 1995).

2.7.2 *Pulsed Field Gel Electrophoresis (PFGE)*

PFGE is a somewhat laborious and expensive technique, a subset of 110 MDR strains comprising of diverse resistotypes was selected. These were digested by XbaI and subjected to PFGE following previously defined protocol (Seifert et al., 2005).

2.7.2.1 *Plug Preparation*

Labeling of 2 ml sterilized microfuge tubes with corresponding strain numbers was done and cell lysis-PK solution (1.5 ml) was dispended in each tube. The plugs were injected in the microfuge tube after solidification, placed in floating stand and incubated at 54°C in shaking water bath (Julabo SW 23, Allentown, PA, USA) with speed setting ~10% for 2 hours

2.7.2.2 *Plug Washing*

The tubes were taken from the water bath and the plug was moved on clean parafilm. A 2 mm slice from the plug was cut by using clean single-edged razor and dropped into microfuge tubes that were pre-labeled. These slices were washed using sterile water (Type 1, 750 µl) for 15 minutes. Water was removed from microfuge tube by vacuum pump and sliced and washed again followed by washing thrice with 750µl TE (Appendix III). After removal of TE, restriction enzyme (REact 2, 750µl) (Invitrogen, Mississauga Ontario, Canada) was added and permitted to stand for 15 minutes.

2.7.2.3 *Digestion of Plugs*

After removing REact 2, in each slice XbaI master-mix (200 µl) was added. Slices were immersed in the solution and incubated for 2 hours at 55°C.

2.7.2.4 *Gel Preparation and Gel Run*

One gram of ultrapure agarose (Invitrogen) was weighed and dissolved in 100 ml of 0.5X TBE (Appendix II) buffer. A microwave oven was used to melt agarose and kept at 55°C. Slices were removed from the incubator after incubation. The *XbaI* master mix was removed and 500 µl 0.5X TBE was added in each microfuge tube and incubated at 25°C

for 5 minutes. Slices were removed from 0.5X TBE after incubation and were aligned on comb along with *S. braenderup* (H9812) as standard. The sequence of samples aligned on the comb was recorded on PFGE worksheet. Slices were dried for 5 minutes. The casting stand was installed and 20 well combs were inserted into the casting stand. 1% molten agarose was softly poured into the casting stand and solidified for 30 minutes. The chamber of electrophoresis was filled with newly prepared 2000 ml of 0.5X TBE solution and CHEF-DR III (Bio-Rad Laboratories, Inc. Hercules, CA, USA) was adjusted to the following setting and was turned on:

- Block 1
- 2 seconds of Initial switch time
- 40 seconds of Final switch time
- 24 hours Run time
- Volts/cm 6.0 V/cm
- 120° of Included angle

CHEF-DR III water pump was turned on. After the buffer flows over the rubber tubing, the chiller attached was switched on and the temperature was adjusted to $14^{o}C$. In the electrophoresis chamber, as temperature of buffer reached to $14^{o}C$, comb was removed from the gel softly. The gel along with its platform was separated from the casting stand and placed in casting platform of the electrophoresis chamber. The chamber top was locked and power supply was turned on.

2.7.2.5 *DNA fingerprinting and Gels analysis*

Bionumeric 6.0 software (Applied-Maths, Ghent, Belgium) was used to evaluate the DNA patterns acquired with the PFGE methods after conversion and normalization. DNA patterns resemblance on the basis of band positions were assessed from Dice similarity coefficients calculated by the software. The PFGE patterns having Dice similarity coefficients more than 80% were labeled genetically correlated and PFGE type was assigned. A dendrogram was made by means of the unweighted pair group method with arithmetic averages (UPGMA) to reveal relationships in the matrix. The results were interpreted as described earlier (Tenover et al., 1995).

2.7.3 *Multi-locus Sequence Typing (MLST)*

The MLST was carried out in accordance with the Pasteur scheme by amplifying seven *A. baumannii* housekeeping genes. The primers for MLST profiling were accessed from the *A. baumannii* MLST scheme website (http:// pubmlst.org/abaumannii/info/primers_pasture.shtml).

2.7.3.1 *DNA extraction using QIAGEN kit*

The genomic DNA extraction was performed by the QIAGEN kit (Appendix IV) (QIAamp DNA Mini Kit, QIAGen, Valencia, CA, USA) following manufacturer's instructions.

2.7.3.2 *PCR amplification (Pasture scheme)*

The PCR was carried out in a 50 µl reaction mixture comprising 2 µl of sample DNA, 1 µl of each of the primers (10 µM) and 25 µl of 2X PCR Master Mix (Thermo Scientific). The following PCR conditions were applied: denaturation for 2 minutes at 94°C, followed by 35 cycles of 94°C for 30 seconds, 50°C for 30 seconds and 72°C for 30 seconds and a 5 minutes final extension at 72°C in TAKARA™ PCR Thermal Cycler (TaKaRa Bio Inc., USA). The analysis of amplicons was done by agarose gel electrophoresis using 1% agarose gel containing 1X TAE buffer at 110 Volts for 30 minutes. After electrophoresis, the gel was placed in a container having a 3X GelGreen™ staining buffer and placed on a shaker for 30-45 minutes. The gel was removed carefully and retained in a container for de-staining for 5 minutes with water. 100bp DNA Marker (Thermo scientific) was used to measure amplicon size. The products amplified were sent for Sanger Sequencing. The pasture scheme was used for primers sequence, PCR amplification, product purifications and sequencing. Details are shown in Table 2.6.

Table 2.6: Primers sequences and Product size for housekeeping genes for pasture scheme

Gene	Primer Sequence (5'-3')	Product Size (bp)
cpn60	F-ACTGTACTTGCTCAAGC R-TTCAGCGATGATAAGAAGTGG	405
fusA	F-ATCGGTATTTCFGCKCATYGAT R-CCAACATACKYTGWACACCTTTGTT	633
gltA	F-AATTTACAGTGGCACATTAGGTCCC R-GCAGAGATACCAGCAGAGATACACG	485
pyrG	F-GGTGTTGTTTCATCACTAGGWAAAGG R-ATAAATGGTAAAGAYTCGATRTCACCMA	297
recA	F-CCTGAATCTTCYGGTAAAAC R-GTTTCTGGGCTGCCAAACATTAC	372
rplB	F-GTAGAGCGTATTGAATACGATCCTAACC R-CACCACCACCRTGYGGGTGATA	330
rpoB	F-GGCGAAATGGCAGTGAAGAACCA R-GAAGTCCTTCGAAGTTGTAACC	456

2.7.3.3 *Gel electrophoresis*

The amplified products were analyzed by gel electrophoresis on 1 % agarose followed by ethidium bromide staining and analyzed in UV illumination using the EZ Gel Doc system (Bio-Rad, USA).

2.7.3.4 *PCR purification and Sequencing*

ABI 3130 Genetic Analyzer (Life Technologies, USA) was used to purify and sequence PCR amplicons' sense and anti-sense strands by using ABI BigDye terminator cycle sequencing ready reaction kit according to recommendations by the suppliers (Appendix V).

2.7.3.5 *Sequence query, Database by the combination of loci*

After sequencing, the sequences were uploaded to *A. baumannii* sequence database (http://pubmlst.org/abaumannii/) for the determination of allele and sequence type (ST). A phylogenetic tree was generated by PHYLOVIZ software 2.0 to compare STs with clonal complexes.

Overall this study can be summarized into following sections as described in Figure 2.1.

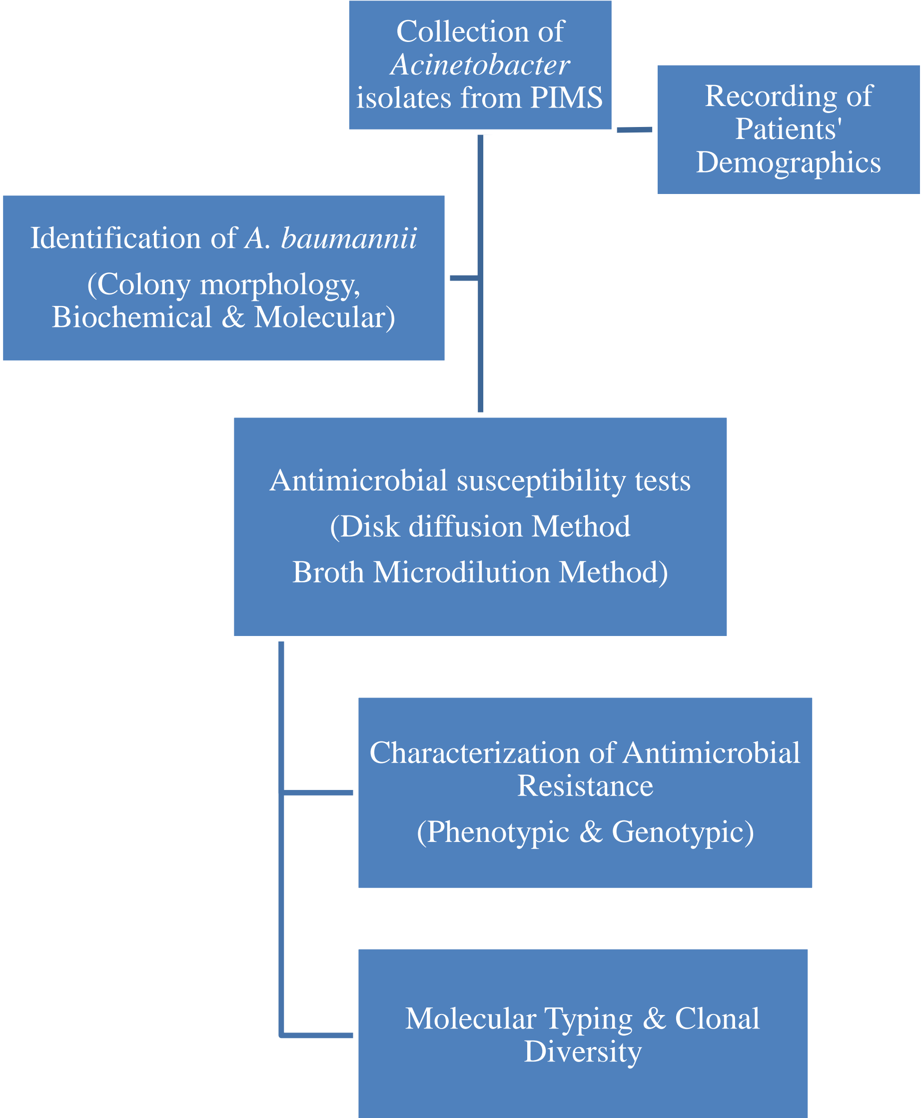


Figure 2.1 Strobe Diagram of Methodology representing collection, identification, susceptibility testing, molecular characterization and clonal typing of study isolates.

Chapter 3

Identification, Demography & Antibiotic susceptibility of MDR *A. baumannii*

3.1 INTRODUCTION

Acinetobacter baumannii, an opportunistic pathogen, has developed a reputation for its involvement in nosocomial infections, mainly in immuno-compromised patients in Intensive care units (ICUs). Its ability to persist for longer periods in hospital environment makes its dissemination imminent. It can cause serious infections including pneumonia, bacteremia, meningitis, urinary tract infections, and has been linked to high morbidity and mortality, with mortality rates as high as 60% in vulnerable patients (Blanchard et al., 2014). *A. baumannii* is regarded as omnipresent organism, and in healthcare settings it can colonize the gastrointestinal tract, skin, and oro-pharyngeal tracts of patients (Munoz-Price and Weinstein, 2008) while in case of equipment they have been isolated from arterial blood pressure devices, ventilator, respirometers, humidifiers, urinals and sinks. The hospital staff's skin, as well as patient beddings can also be a source of dissemination of *A. baumannii* in healthcare facilities (Shamsizadeh et al., 2017).

Although opportunistic, the infections caused by *A. baumannii* pose a challenge due to their capability to develop resistance against all antibiotics. A number of studies from Pakistan have described significant resistance in *A. baumannii* against cephalosporins, fluoroquinolones, aminoglycosides, and piperacillin/tazobactam (Shamim et al., 2015, Sohail et al., 2016, Anwar et al., 2016). The ever increasing antibiotic resistance has led to the emergence of MDR *A. baumannii*. One of the major underlying reasons for the emergence of MDR bacteria is the misuse and overuse of antibiotics (Ahmed et al., 2019). Besides that, the poor infection prevention and control also indirectly contributes to the increase in antibiotic resistance (Wall, 2019). Carbapenems are recommended for treating MDR isolates, but there is a significant rise in carbapenem-resistant *A. baumannii* (CRAB) which has resulted in it being declared as one of the top priority pathogen by the World Health Organization (WHO) (Tacconelli et al., 2018). Polymixins are the last-resort for treating carbapenem-resistant infections, while resistance against this class of antibiotics is also on the rise leaving no therapeutic options for the treatment of serious nosocomial infections. In the current study, we have evaluated the antibiotic susceptibility patterns of clinical *A. baumannii* isolates along with the patient demographic data and have identified the MDR isolates.

3.2 RESULTS

3.2.1 Identification of *A. baumannii* Isolates

The results of various techniques used for the identification of *A. baumannii* isolates are as follows:

3.2.1.1 *Colony Morphology & Gram Staining*

The morphological identification revealed pale yellow colonies of *A. baumannii* on MacConkey agar, which suggested the non-lactose fermenting bacteria (Figure 3.1a). Mucoid smooth colonies with non-hemolytic activity appeared on blood agar (Figure 3.1b). In Gram-staining, the bacteria appeared as pink short rods known as Coccobacilli, displaying a Gram-negative reaction (Figure 3.1c).

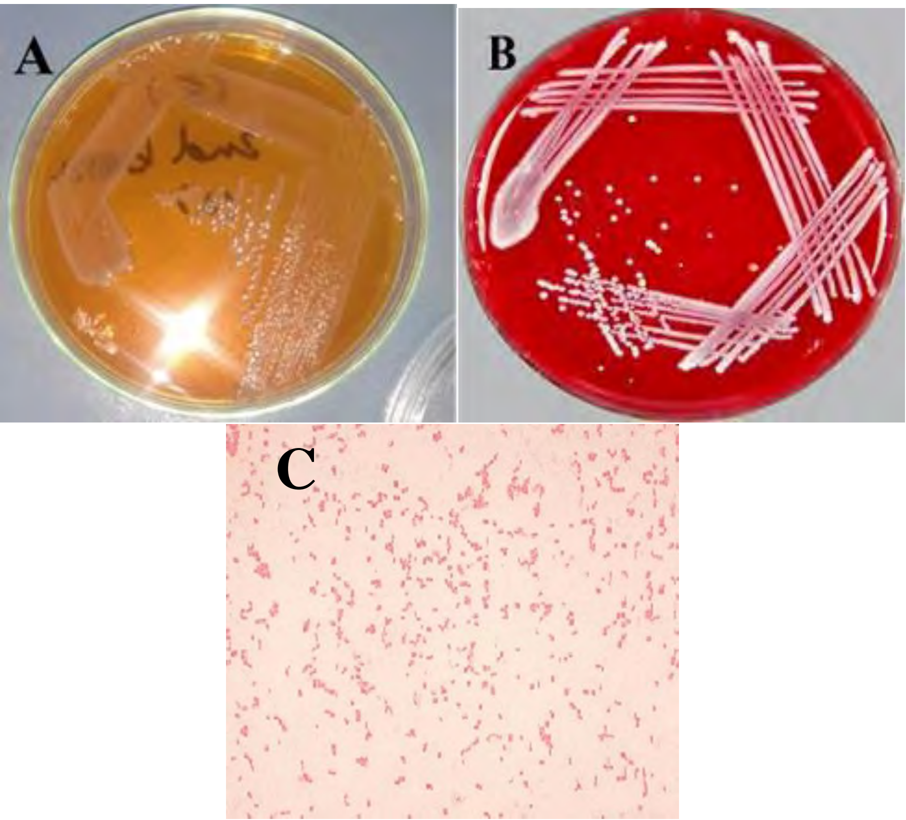


Figure 3.1 Identification of isolates through cell morphology and Gram staining
The figure depicts: A) Pale yellow colonies of *A. baumannii* on MacConkey, B) Mucoid smooth surface colonies on blood agar and C) Pink short rods in Gram's staining.

3.2.1.2 *Biochemical Identification*

API 20 NE kit was used for the biochemical identification of *A. baumannii*. Isolates showed positive activity for Arabinose, Adipate, Caprate, Malate, Citrate and Phenyl-acetate biochemical tests as shown in Figure 3.2. Tests are separated into groups of 3 and a number 1, 2 or 4 is indicated for each. By adding the values corresponding to positive reactions within each group, a 7-digit number is obtained. The seven-digit numerical profile for *A. baumannii* is 0041073.

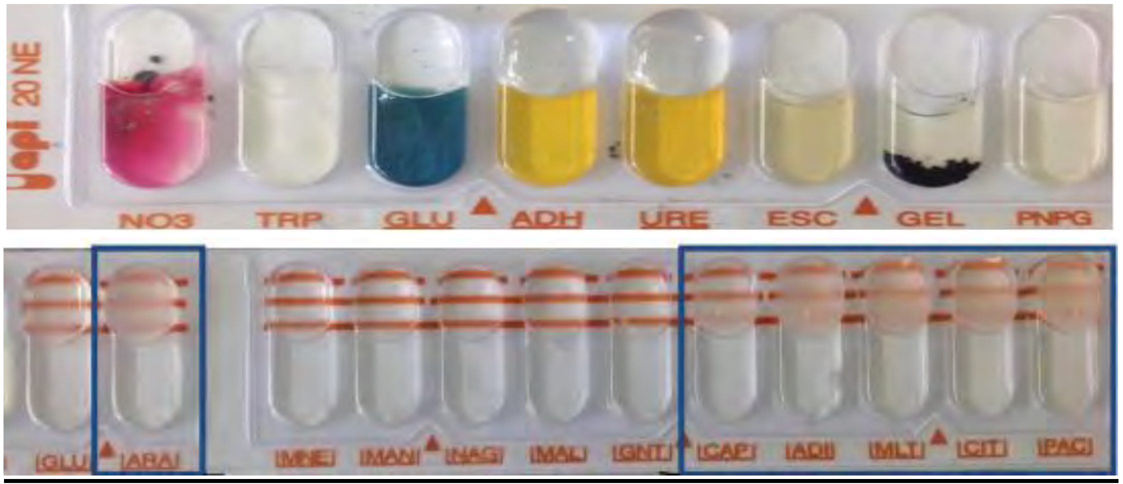


Figure 3.2 Biochemical identification of isolates using API 20 NE kit. Arabinose, Caprate, Adipate, Malate, Citrate and Phenyl-acetate biochemical tests (in blue box) were positive for *A. baumannii*.

3.2.1.3 *Molecular Identification via detection of 16S rRNA and bla$_{OXA-51-like}$ genes*

A. baumannii isolates were further confirmed by 16S rRNA and *bla*$_{OXA-51-like}$ genes. All (100%) isolates were positive for 16S rRNA and *bla*$_{OXA-51-like}$ genes (Figure 3.3).

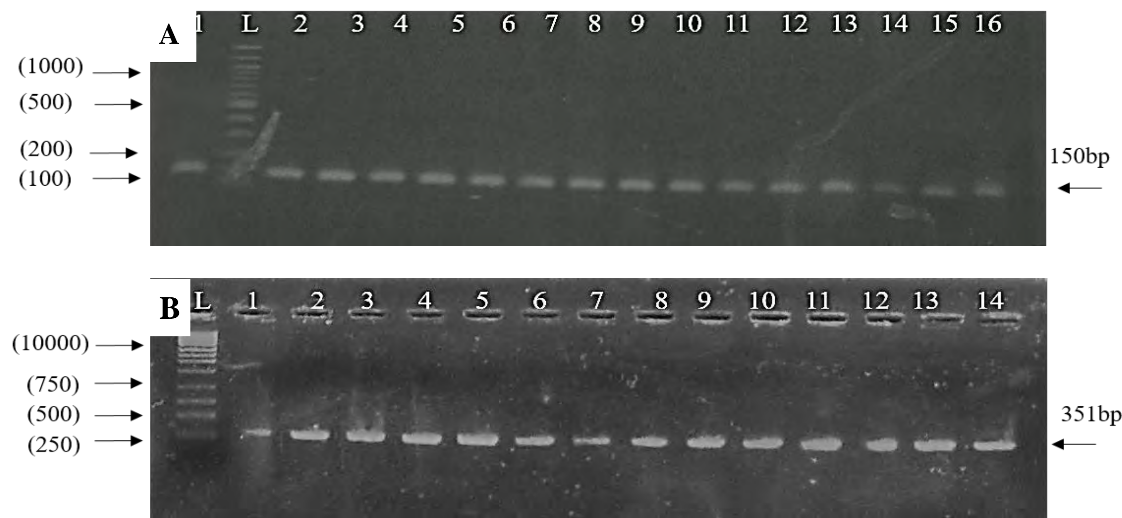


Figure 3.3 Representative gel images of PCR products of 16S rRNA and *bla*$_{OXA-51-like}$ genes for *A. baumannii* isolates.

A) Lane L shows 100 bp DNA ladder, Lanes 1-16 represent PCR products of 150bp for 16S rRNA gene; B) Lane L shows 1Kb DNA ladder, Lanes 1-14 showing PCR products for *bla*$_{OXA-51-like}$ gene of 351bp.

Out of 375 isolates, 240 were confirmed as *A. baumannii* based on their morphological, biochemical and molecular characteristics.

3.2.2 Demography

On the basis of gender distribution, 59% isolates were from male patients while 41% were from female patients (Figure 3.4).

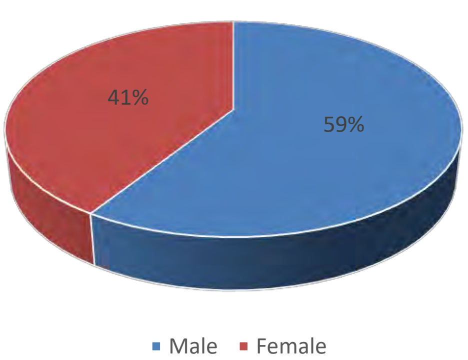


Figure 3.4 Gender-wise distribution of *A. baumannii* isolates

The figure shows gender-wise distribution of *A. baumannii* infection. The higher incidence was noted in males i.e. 59%, while 41% of total were isolated from female patients.

Patients were classified into four groups on the basis of their age. Patients with age <18 years were in group I, 18 to 35 years were assigned group II, 36 to 55 years were listed in group III, while >55 years as group IV. The decreasing order of prevalence was found to be 44%, 30%, 15% and 11% for groups II, III, IV and I respectively as shown in Figure 3.5. The difference between the groups was not significant (p-value 0.447) .

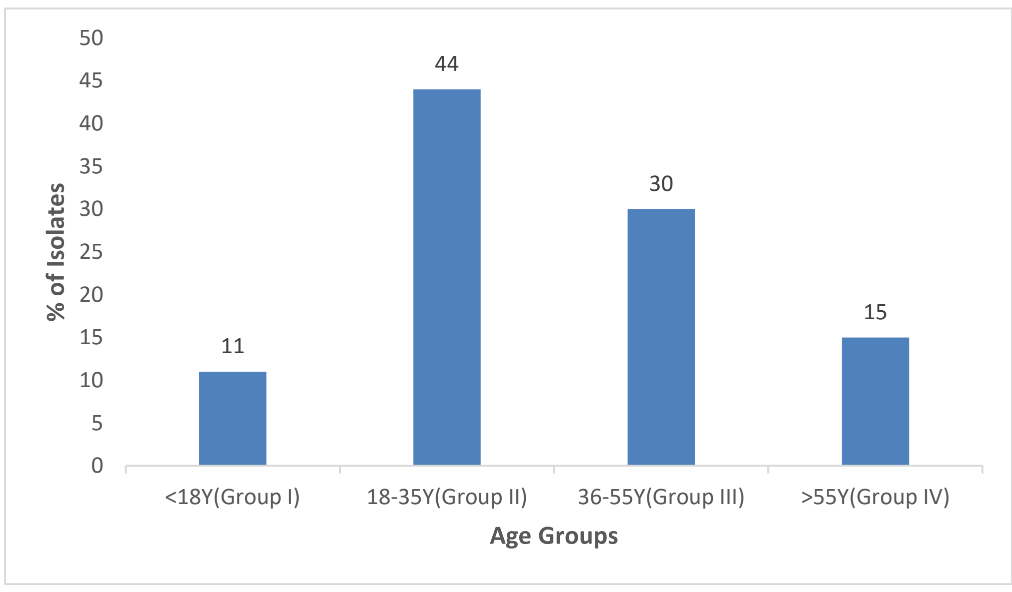


Figure 3.5 Age-wise division of *A. baumannii* isolates.

The figure depicts the age-wise distribution of isolates. The prevalence of isolates was 44%, 30%, 15% and 11% in group II, III, IV and I, respectively.

Among *A. baumannii* isolates, the highest number of isolates were from patients in medical ICU i.e. 42% followed by 26% from emergency. The overall prevalence in various wards is shown in Figure 3.6.

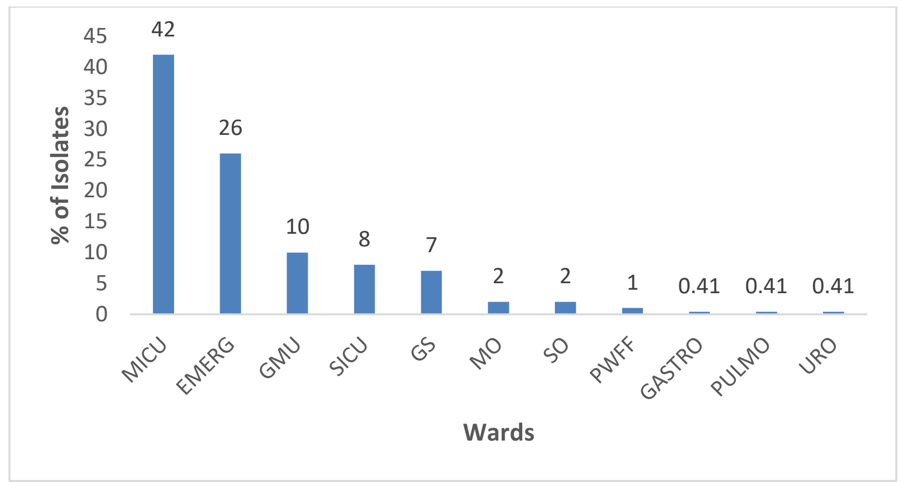


Figure 3.6: Distribution of *A. baumannii* isolates in different wards of the hospital

The figure depicts the distribution of *A. baumannii* in various wards of hospital. The highest prevalence of isolates was from MICU: Medical ICU followed by EMERG: emergency and GMW: General medicine ward, MO: Medical outpatient department, GS: General surgery, MICU: Medical intensive care unit, GASTRO: Gastrology, PULMO: Pulmonology, PWFF: Private ward first floor, SICU: Surgical intensive care unit, URO: Urology.

Among the variety of specimen, highest number of *A. baumannii* were isolated from tracheal secretions and pus samples with prevalence of 48% and 25% respectively. The distribution of *A. baumannii* among various specimen is shown in Figure 3.7.

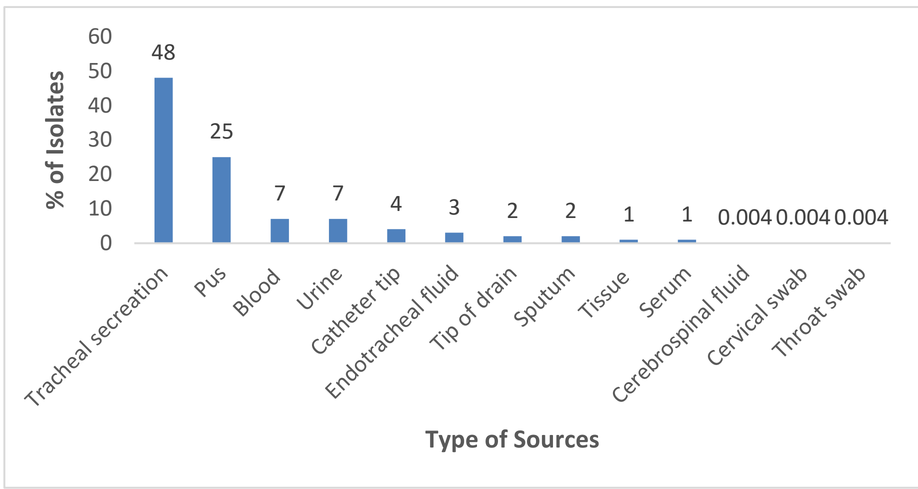


Figure 3.7 Distribution of *A. baumannii* isolates in various specimen taken from patients.

The figure shows the distribution of *A. baumannii* isolates in various clinical specimen. The most prevalent source was tracheal secretions followed by pus, blood, urine, catheter tip, endotracheal tube, tip of drain, sputum, serum, tissue and cerebrospinal fluid.

3.2.3 Antibiotic Resistance Profiles

Antibiotics susceptibility of clinical isolates of *A. baumannii* was checked by the Kirby-Bauer's disc diffusion method. A representative image of the assay is shown in Figure 3.8.

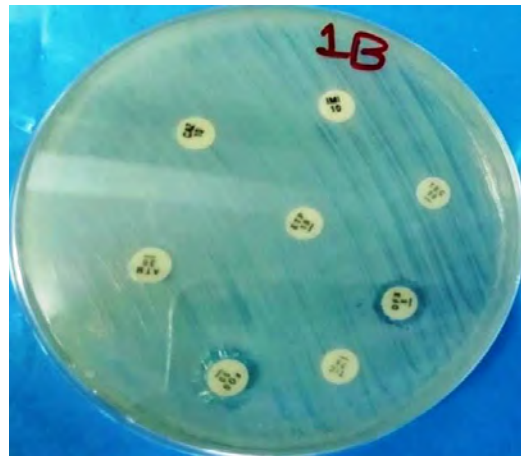


Figure 3.8 Representative diagram of antibiotic susceptibility pattern of *A. baumannii* isolate.

The figure depicts the representative antibiotic susceptibility pattern of *A. baumannii* isolates. The majority of isolates have shown high resistance towards all of the tested groups of antibiotics.

Isolates in this study displayed the highest prevalence of resistance against cephalosporins including ceftazidime, ceftriaxone, and cefotaxime with 96%, 94% and 68% resistance respectively (Figure 3.9). Among carbapenem, highest resistance was seen against imipenem with 96% resistant isolates. High resistance was also seen against amoxicillin+clavulanic acid with 95% of isolates being resistant. In case of aminoglycosides, highest resistance was observed against amikacin (93%) as compared to tobramycin (74%). Among fluoroquinolones more resistance was noticed against levofloxacin (90%) as compared to ofloxacin (67%). This was followed by sulfamethatrioxole with 87% resistant isolates followed by tazobactam displaying 78% resistance. High resistance was also observed against aztreonam (77%) followed by chloramphenicol (61%). In case of tetracyclines, 40% isolates were found to be resistant to minocycline, while 50% of the isolates showed resistance to tigecycline.

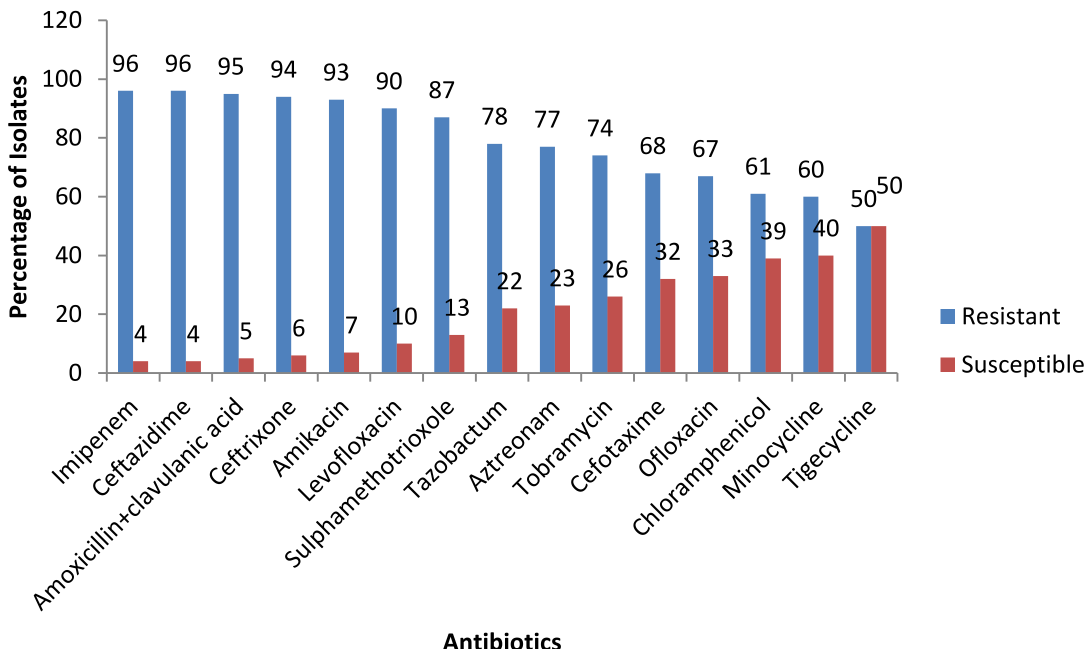


Figure 3.9 Prevalence of resistance in *A. baumannii* against various antibiotics

The figure depicts the susceptibility pattern of *A. baumannii* isolates against various classes of antibiotics.

Based on their resistance profiles, the isolates were categorized as multi drug-resistant (MDR) (lack of susceptibility to at least 1 agent in 3 or more antimicrobial classes), extremely drug-resistant (XDR) (lack of susceptibility to at least 1 agent in all but 2 or fewer antimicrobial classes (i.e. susceptibility to only 1 or 2 categories)) and pan drug-resistant (PDR) (lack of susceptibility to all agents in all antimicrobial classes) according to already published criteria (Magiorakos et al., 2012). Among all isolates, 54% isolates were MDR, 13% were categorized as XDR, while 33% were PDR as shown in Figure 3.10.

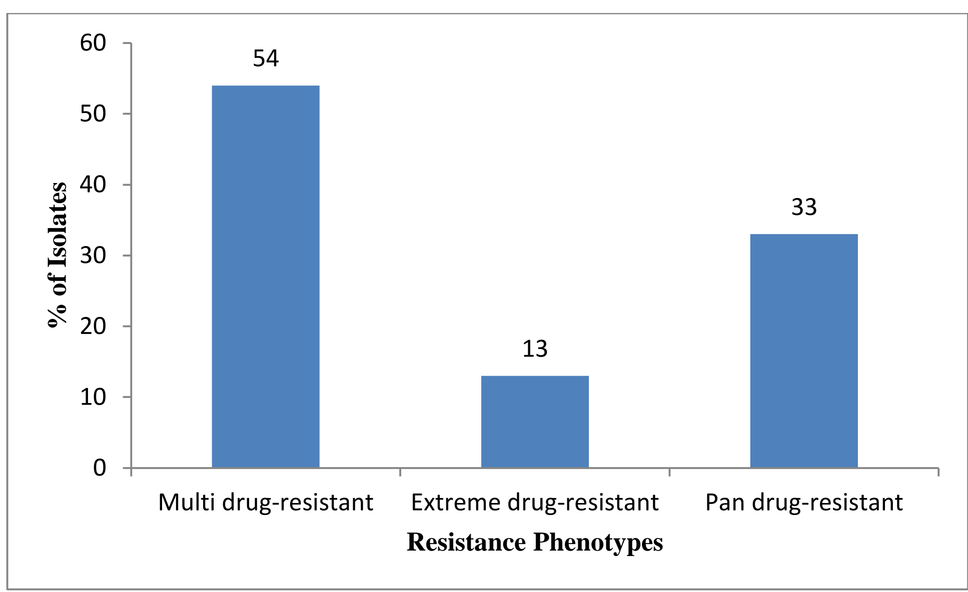


Figure 3.10 Prevalence of MDR, XDR and PDR in *A.baumannii*.

The figure depicts the classification of resistance of *A. baumannii* isolates based on phenotypic antibiotic susceptibility tests. The highest percentage (54%) of isolates were MDR. XDR and PDR were 13% and 33%, respectively.

3.2.3.1 *Minimum inhibitory concentrations*

Broth microdilution method was performed to measure the MICs of levofloxacin, ceftriaxone, tetracycline, ampicillin, chloramphenicol, imipenem and colistin against study isolates.

All MDR isolates showed high MICs against ceftrioxone, 30% isolates showed MICs of 512 mg/L, while 30% isolates had MICs of 256 mg/L, 36% had 128 mg/L, 2% had 64 mg/L and 2% had 32mg/L. MICs for levofloxacin, ampicillin and chloramphenicol were recorded as 512 mg/L, 256 mg/L,128 mg/L, 64 mg/L, 32mg/L, 16mg/L and 8mg/L in 2%, 10%, 33%, 22%, 23%, 8% and 2% isolates respectively. For colistin 12% isolates showed MIC value of 128 mg/L, while 22% isolates had 64 mg/L, 30% had 32mg/L, 20% had 16mg/L, 12% had 8mg/L and 4% had 4mg/L. In case of tetracycline 5% isolates showed MIC value of 256 mg/L, while 19% isolates had 128 mg/L, 21% had 64mg/L, 27% had 32mg/L, 18% had 16mg/L, 8% had 8mg/L and 2% had 4mg/L. MIC against imipenem in all MDR isolates was >512 mg/L. The overall susceptibility data is summarized below in Table 3.1.

Table 3.1: MIC of *A. baumannii* isolates for different antimicrobials

Antimicrobials	**Breakpoints values in mg/L with the percentage of isolates against each breakpoint**									**MIC (mg/L)**
	>512	512	256	128	64	32	16	8	4	
Levofloxacin		2	10	33	22	23	8	2		64
Ampicillin		2	10	33	22	23	8	2		64
Chloramphenicol		2	10	33	22	23	8	2		64
Ceftriaxone		30	30	36	2	2				256
Tetracycline		5	19	21	27	18	8	2		64
Imipenem	100	0	0	0						0
Colistin				12	22	30	20	12	4	32

3.3 DISCUSSION

In clinical settings, *A. baumannii* strains have emerged as resistant to all available therapeutic options creating problems for clinicians (Gonzalez-Villoria and Valverde-Garduno, 2016). In current study, 240 isolates (64%) were confirmed as *A. baumannii* using various tests. This high representation supports the WHO priority pathogen list, which advocates that local healthcare authorities should also join the common global goal to control and eradicate *A. baumannii* (Tacconelli et al., 2018, Lim et al., 2019, Pormohammad et al., 2020, Ayobami et al., 2019). On the other hand, the remaining 36% of isolates is also a significant number and should be further investigated, which is beyond the focus of current study. They may belong to species like *A. nosocomialis* and *A. petti*, which are also reported to be prevalent in clinical settings (Al-Hasan, 2013).

Demographic information provides valuable insight into various contributing factors involved in the occurrence and spread of any disease. Our data revealed that most of the isolates were obtained from male (59%) patients, which is similar to an earlier report exhibiting 61% from male patients (Khurshid, 2018). Although literature reveals higher prevalence values for female patients as well (Rebic et al., 2018) but predominant reports are suggestive of vulnerability for males (Arjuna et al., 2017, Liu et al., 2016). It is of note that males are more susceptible to development of infection, especially after trauma, due to depressed cell-mediated immunity (Offner et al., 1999, Angele and Faist, 2002, Angele et al., 2014). Males in this part of the world are more prone to trauma and its associated hospitalization because they pre-dominantly work outside their homes (Munir et al., 2019). This could possibly underlie their higher potential for *A. baumannii* infection. Age is another critical determinant for development of disease. Our data revealed that the subjects (44%) aged between 18-35 years were more prone to *A. baumannii* infection. Some previous reports have suggested highest frequency of isolates in neonates (ul Ain et al., 2019, Begum et al., 2013) and higher age (mean = 52 years) subjects (Khurshid et al., 2017). It can be understood by relating the fact that *Acinetobacter* is well known as a nosocomial pathogen (Valencia et al., 2009). Hence both of the aforementioned age groups are more prone to development of infection due to increased hospitalization because of several factors like birth (de Crespigny and Savulescu, 2014), immunity (Chandra, 1989) and age-associated morbidities (Nowossadeck, 2012). Taken together, it appears that trauma (for adults) and

hospitalization (for neonates and aged) make them vulnerable towards *A. baumannii* infection (Bochicchio et al., 2001). Hence, comparison of reason for hospitalization could appear to be the critical determinant of concluding variable demographic outcomes reported among various studies. Although, this reason was not observed in present study, highest number of isolates (42%) from Intensive care unit (ICU) shed light on the importance of adult age–trauma–ICU nexus in the occurrence of *A. baumannii* infection in present study. This nexus is further strengthened by second highest isolates (26%) from the emergency ward in present study. A search of literature revealed several other studies, which also report maximum isolation (40%) from the ICU (Khan et al., 2012, Begum et al., 2013). Moreover, our specimen data revealed that pre-dominant isolates were obtained from the tracheal secretion samples i.e. 48%. This is in line with various national (ul Ain et al., 2019, Khurshid, 2018, Begum et al., 2013) and international reports (Shanthi and Sekar, 2009, Khan et al., 2012). The highest ratio of *A. baumannii* isolates in respiratory samples has been shown previously (Uwingabiye et al., 2016). It can be explained in a manner that *Acinetobacter* species target high fluid content of body; the highest of which exposed to outside is the respiratory tract thus making it an ideal site for *A. baumannii* to inhabit (Obeidat et al., 2014). Additionally, this respiratory route also favors the transmission of bacteria among the subjects; the reason probably underlying its rapid spread in the community.

The clinically important *A. baumannii* has developed resistance to almost all available therapeutic options, with high prevalence of MDR strains worldwide (Xie et al., 2018). Antibiotic resistance phenotypes are categorized into MDR, XDR and PDR, which make it difficult to manage infection control and thus increase treatment challenges for clinicians (Swe-Han et al., 2017). Our data revealed that all isolates were MDR. In similar lines, other studies have reported high percentages of MDR clinical isolates i.e. 97% (Hasan et al., 2014) and 100% (Begum et al., 2013). It is interesting to note that in current study we found a lower percentage of XDR i.e. 13% as compared to previously reported 29% XDR while a higher percentage of PDR i.e. 33% as compared to previously reported 21% PDR (Marturano and Lowery, 2019). This may be due to emerging resistance against newer classes of antibiotics which may have led to phenotypic change in isolates from XDR to PDR over time.

Our antibiotic susceptibility data revealed that a high number of isolates were resistant to cephalosporins (68-97%) which is comparable with previous reports from Pakistan describing 98-100% resistance (Irfan et al., 2011, Begum et al., 2013, Asif et al., 2018, Shamim et al., 2015, Hasan et al., 2014). In current study, 67-90% resistance was observed against fluoroquinolones which is higher than the previously reported level of 74% (Hasan et al., 2014). In case of aminoglycosides, (65-93%) resistance was observed which is much higher than previously reported 71-83% (Anwar et al., 2016). The highest susceptibility of 50% was noted in case of tigecycline. In agreement with our results, some earlier studies also reported higher susceptibility (lower resistance) for tetracyclines (Hasan et al., 2014), especially tigecycline (Hassan et al., 2010). Taken together, these reports are indicative of an increasing trend of resistance against various classes of antibiotics in *A. baumannii* and higher therapeutic potential of tetracyclines against it. This is also suggestive of lack of resistance development against tigecycline over the years by this pathogen, the underlying cause of which is worth investigation. It was noted that the resistance level in clinical Gram-negative isolates was particularly elevated for fluoroquinolones, aminoglycosides, cephalosporins, and carbapenems probably due to excessive use of these drugs (Breijyeh et al., 2020, Sedláková et al., 2014). Presumably, the low resistance levels against tetracycline can be attributed to its uncommon use in clinical practice nowadays. The development of antibiotic resistance has been described to follow the need base evolutionary concept or antimicrobial selection pressure (MacLean and San Millan, 2019). Bacteria develop new resistance mechanisms depending upon the mechanism of action of antibiotics in use; and loses already developed resistance mechanism upon discontinuation of use in clinical practice (Heilmann et al., 2005). Hence, our data support that the old prototype antibiotics, which are no more utilized in clinics nowadays, may be a therapeutic option for difficult to manage *A. baumannii*. It is worth mentioning that clinical resistance has started to emerge for tigecycline, especially in *Acinetobacter baumannii* and Enterobacteriaceae, mainly in MDR strains involving the efflux pump mechanism (Sun et al., 2013). Hence, in order to prolong the usefulness of this effective medicine, it should now be used cautiously in clinics involving avoidance of long term monotherapy and continuous monitoring of efflux pump mediated resistance. Additionally, the drug is known for its toxicity (Vandecasteele et al., 2018, Yang et al., 2020), therefore it should be used with caution in patients.

Apart from the bacterial susceptibility test, the MIC values (using broth microdilution method) were also obtained against several routinely used antibiotics to check the phenotypic resistance patterns in the isolates. In similarity with the disc susceptibility test, the penicillins, cephalosporin, carbapenems, fluoroquinolone and aminoglycoside representative drugs displayed higher MIC values, which is in accordance with the local study (ul Ain et al., 2019). As expected, the tetracycline displayed better MIC range among various isolates. Apart from these antibiotics, colistin was also assessed in this assay, which belongs to the class of polymixin B and is considered as last resort for the treatment of MDR Gram-negative infections particlualry CRAB but its use is limited due to toxicity (Pogue et al., 2017). Although, it exhibited highest susceptibility but resistant isolates also showed comparatively higher MIC values. In conformity, colistin resistance in *Acinetobacter* strains has also been reported (Bahador et al., 2018). This is an alarming situation and demands strict adherence to antibiotic treatment guidelines in order to avoid the spread of this resistance.

Chapter 4

Phenotypic and Genotypic Characterization of *A. baumannii* for Resistance and Virulence Markers

4.1 INTRODUCTION

The phenomenon of antimicrobial resistance was identified soon after the discovery of first antibiotic decades ago (D'Costa et al., 2011). There are various antimicrobials resistance mechanisms that contribute to resistance against various classes of antibiotics in bacteria (Coyne et al., 2011). Of various mechanisms by which bacteria can escape the effects of an antibiotic, the enzymatic inactivation is the conventional mechanism of antibiotic resistance. MDR *A. baumannii* have been associated with the production of various β-lactamases such as, CTX-M, TEM, SHV, IMP, VIM, KPC, OXA and NDM enzymes. *AmpC* gene, first cloned in 2000, also known as bla_{ADC} (*Acinetobacter*-Derived Cephalosporinase), is another enzyme responsible for the resistance in *A. baumannii* (Karah et al., 2017).

Another mechanism by which *A. baumannii* can illustrate antimicrobial resistance is efflux pumps. There are two major types of efflux pumps prevalent in clinical *A. baumannii* strains. One is the AdeABC efflux system that belongs to the resistance-nodulation-cell division (RND) superfamily of transporters. They are relatively non-specific and their expression is regulated by AdeRS two-component system (i.e. AdeS and AdeR TCS) and *ISAba 1* insertion sequence (Lari et al., 2018, Marchand et al., 2004). The other efflux pump is encoded by *Tet* genes, which provides specific resistance towards tetracycline (Chopra and Roberts, 2001).

Virulence factors in bacteria are responsible for its ability and the extent of disease it may cause in host. The ability of *A. baumannii* as a successful nosocomial pathogen is due to expression of numerous virulence factors. Virulence traits of *Acinetobacter* spp. such as polysaccharide, capsule and the adhesive capability of epithelial cells through fimbriae are same as of other Gram-negative bacteria (Braun and Vidotto, 2004). In the current study the phenotypic tests and molecular detection of various antibiotic resistance genes including markers for ESBL, carbapenemases, AmpC, PMQR and virulence markers was performed in order to understand the contribution of these factors to antibiotic resistance and to determine their prevalence in MDR *A. baumannii* isolates.

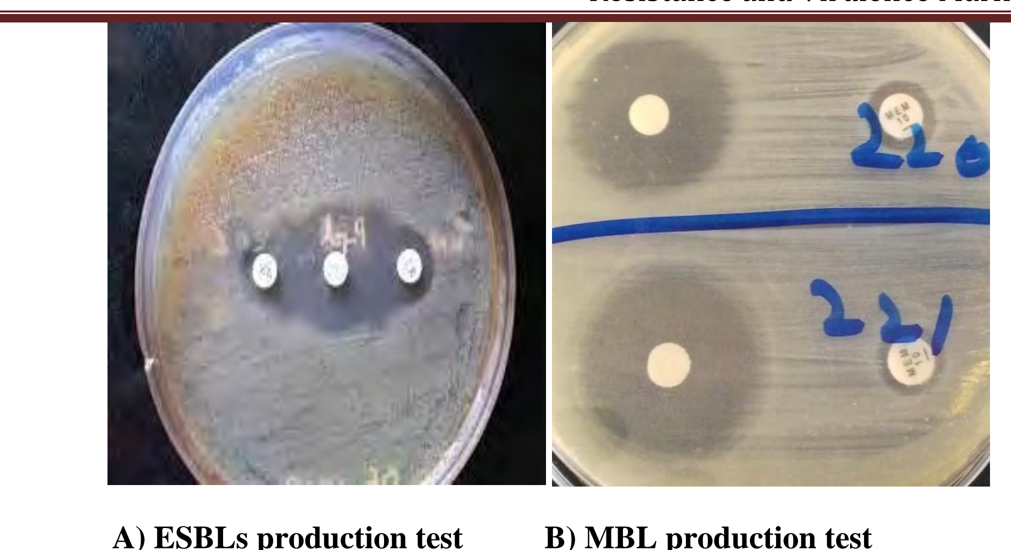
4.2 RESULTS

4.2.1 Phenotypic Assays for the Detection of Resistance

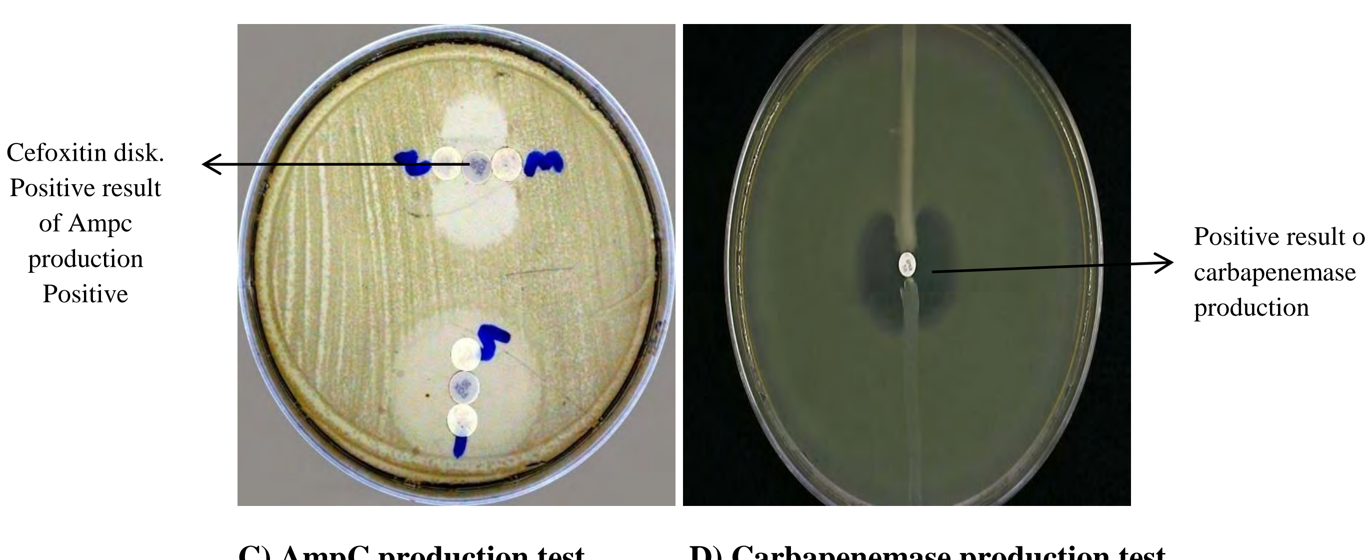
The results of various phenotypic assays are as follows:

4.2.1.1 *Assays for the β-lactamases*

Phenotypic assays were carried out to check enzyme production. Double disk synergy test was used for Extended Spectrum β-Lactamases (ESBLs) detection. An enlargement of zone of inhibition around cephalosporin disc towards clavulanic acid confirmed ESBL production as shown in Figure 4.1(A). Meropenem-Ethylenediamine tetra acetic acid (MEM-EDTA) test was used for the detection of MBLs production. An increase of >7mm in the zone diameter of MEM-EDTA combined disk as compared to meropenem disk confirmed MBLs production (Figure 4.1(B)). AmpC enzyme production was tested by AmpC disk test and a reduction in the zone of cefoxitin disk indicated AmpC production (Figure 4.1(C)). Modified hodge test was carried out to detect the presence of carbapenemase enzymes where a clover leaf-like indentation confirmed carbapenemase production (Figure 4.1(D)).



A) ESBLs production test **B) MBL production test**



C) AmpC production test **D) Carbapenemase production test**

Figure 4.1 Diagrammatic representation of phenotypic test for the production of various β-lactamase enzymes.

The figure depicts A) Double disk synergy test for ESBLs production. B) MEM-EDTA combined disk test for MBLs production C) AmpC disk test for AmpC enzyme production D) Modified-hodge test for carbapenemase detection.

The results of phenotypic assays for the detection of various types of β-lactamases are as follows: None of the isolates was found to be an ESBL producer using the double disk synergy test. Results of MEM-EDTA combined disk test showed that 42% (n=101) isolates were positive, while 58% (n=139) were negative. AmpC disk test used for AmpC

enzyme detection showed that 59% (n=141) isolates were positive, while 41% (n=99) were negative. Of all the isolates, 65% (n=157) were positive and 35% (n=83) were negative for carbapenemase production using Modified-hodge test (Figure 4.2).

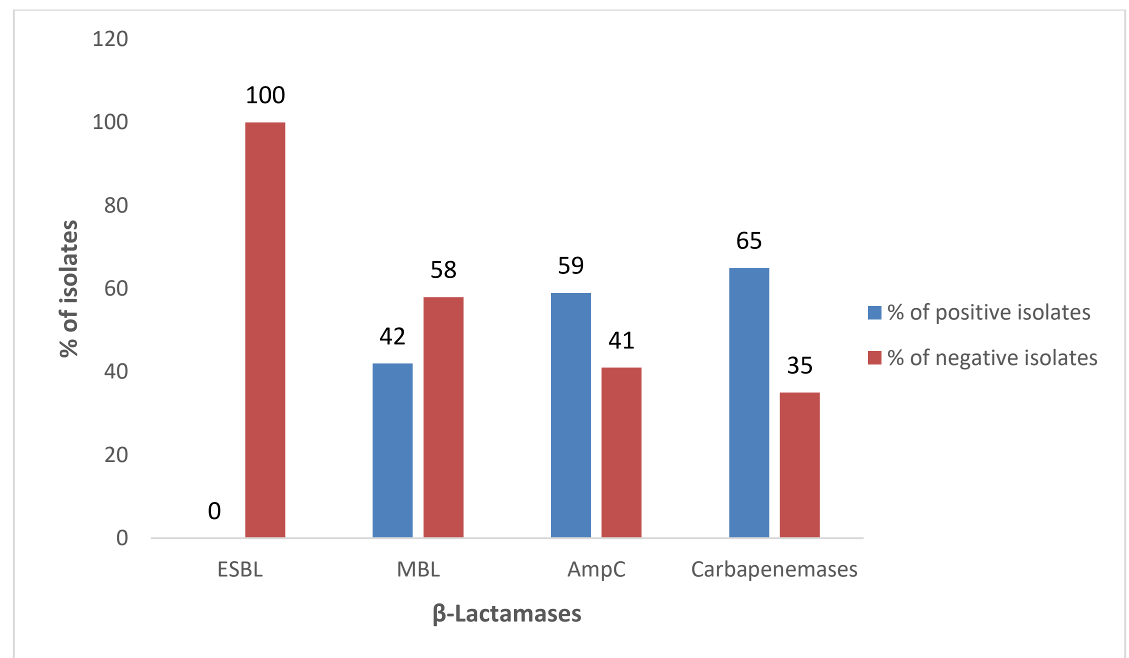
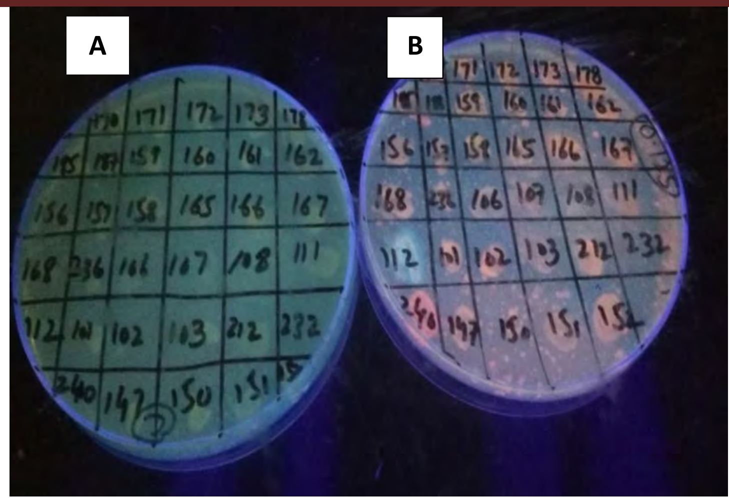


Figure 4.2 Phenotypic presence of ESBLs, AmpC, MBLs and Carbapenemases in isolates.

The figure shows the prevalence of positive and negative isolates for the production of ESBLs, MBLs, AmpC and Carbapenemases. Highest prevalence was of Carbapenemase enzymes followed by AmpC and MBLs, while none of the isolates produced ESBLs.

4.2.1.2 *Efflux Pump activity*

The EtBr-agar cartwheel (EtBrCW) technique was used to evaluate the efflux activity. The results showed that 31% (n=74) isolates were positive for this test while 69% (n=166) displayed negative result as shown in Figure 4.3.



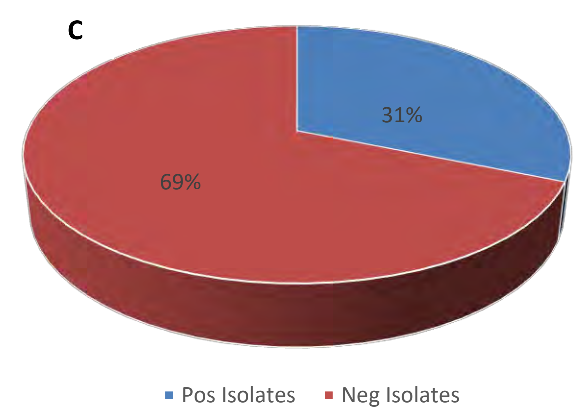


Figure 4.3 Efflux Pump assay for *A. baumannii* isolates.
A) Cartwheel ethidium bromide assay without ethidium bromide and test plate B) Cartwheel ethidium bromide assay showing loss of florescence of bacterial culture positive result for efflux activity while culture with florescence showing negative result for this assay and C) Prevalence of isolates for the presence and absence of efflux pump. Majority of isolates (69%) were found to be negative.

4.2.2 Genetic Characterization for Antibiotic Resistance Genes

The presence or absence of genes responsible for antibiotic resistance was observed as follows:

4.2.2.1 *Detection of β-lactamases genes*

Results for the detection of classes of β-lactamases, using Polymerase chain reactions (PCR) are as follows:

4.2.2.1.1 *ESBLs encoding genes:*

The PCR results demonstrated that three isolates carried *bla*$_{\text{SHV}}$ gene and two isolates carried *bla*$_{\text{TEM}}$ genes, while six isolates harbored *bla*$_{\text{M-PER}}$ gene. Only one isolate was positive for *bla*$_{\text{M-GES}}$ and none of the isolates was positive for *bla*$_{\text{M-VEB.}}$ Five isolates carried *bla*$_{\text{CTX-M-Group-9}}$ genes and four isolates harbored *bla*$_{\text{CTX-M-Group-1}}$ genes. Control strain DA48896 for *bla*$_{\text{CTX-M-Group-1}}$, *bla*$_{\text{SHV}}$, and *bla*$_{\text{TEM}}$ (Nahid et al., 2017) and DA48911 was used for *bla*$_{\text{CTX-M-Group-9}}$. The representative gel diagram is shown in Figure 4.4.

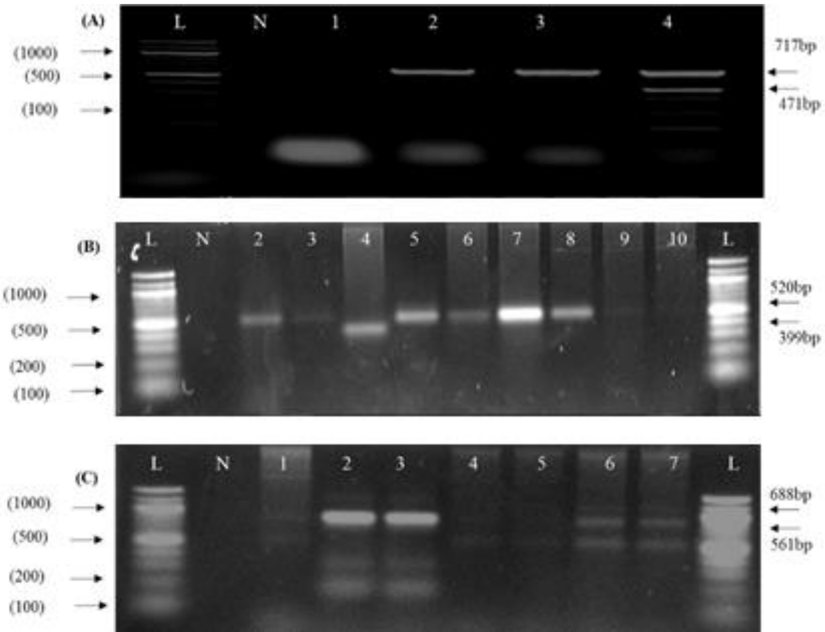


Figure 4.4 Representative gel images for amplification products of ESBLs encoding genes

The figure shows: **(A)** Lane L;100bp ladder, Lane N; Negative control, Lanes 2; Positive control for *bla*$_{\text{TEM}}$, Lanes 3-4, PCR product for *bla*$_{\text{TEM}}$ of size 717bp and in lane 4, PCR product of 471bp for *bla*$_{\text{SHV}}$; **(B)** Lane 2, 3, 5, 6, 7, 8, 9, PCR product of size 520bp for *bla*$_{\text{M-PER}}$; Lane 4, PCR product of size 399bp for *bla*$_{\text{GES}}$; **(C)** Lane 2; Positive control and lane 3, PCR product of 688bp for *bla*$_{\text{CTX-M-group-1}}$and Lane 6,7, PCR product of size 561bp for *bla*$_{\text{CTX-M-group-9}}$.

4.2.2.1.2 *MBLs encoding genes:*

Multiplex PCR sets were carried out for 10 carbapenemase encoding genes *bla*$_{\text{DIM}}$, *bla*$_{\text{GIM}}$, *bla*$_{\text{IMP}}$, *bla*$_{\text{VIM}}$, *bla*$_{\text{NDM}}$, *bla*$_{\text{SPM}}$, *bla*$_{\text{AIM}}$, *bla*$_{\text{SIM}}$, *bla*$_{\text{KPC}}$ and *bla*$_{\text{BIC}}$. PCR products of sizes 188bp, 383bp were observed for *bla*$_{\text{IMP}}$, and *bla*$_{\text{VIM}}$ respectively. Fifteen isolates out of 240 were positive for *bla*$_{\text{VIM}}$ and sixty-three were positive for *bla*$_{\text{IMP}}$. Second multiplex

PCR was done for the detection of genes encoding bla_{KPC}, bla_{BIC} and bla_{NDM}. PCR products of sizes 798bp, 621bp and 537bp were observed for bla_{KPC}, bla_{NDM} and bla_{BIC} respectively. Twenty-one isolates out of 240 were positive for bla_{NDM}, five for bla_{KPC} and six isolates were positive for bla_{BIC}. Another multiplex PCR was done for the detection of genes encoding bla_{AIM}, bla_{GIM}, bla_{SIM}, and bla_{DIM}. PCR products of sizes 471bp for bla_{SIM} and 321bp for bla_{AIM} were amplified. Four isolates were positive for bla_{AIM} gene, only one isolate harbored bla_{SIM}, while none of isolates were positive for bla_{SPM}, and bla_{DIM} genes. Control strains used were, DA48819 for bla_{NDM}, DA49027 for bla_{VIM}, *K. pneumoniae* (K-10) for bla_{KPC}, ATCC4852272 for bla_{IMP}. Controls were not available for bla_{AIM} and bla_{SIM} and their identity needs to be confirmed with sequencing. The representative gel diagram is shown in figure 4.5.

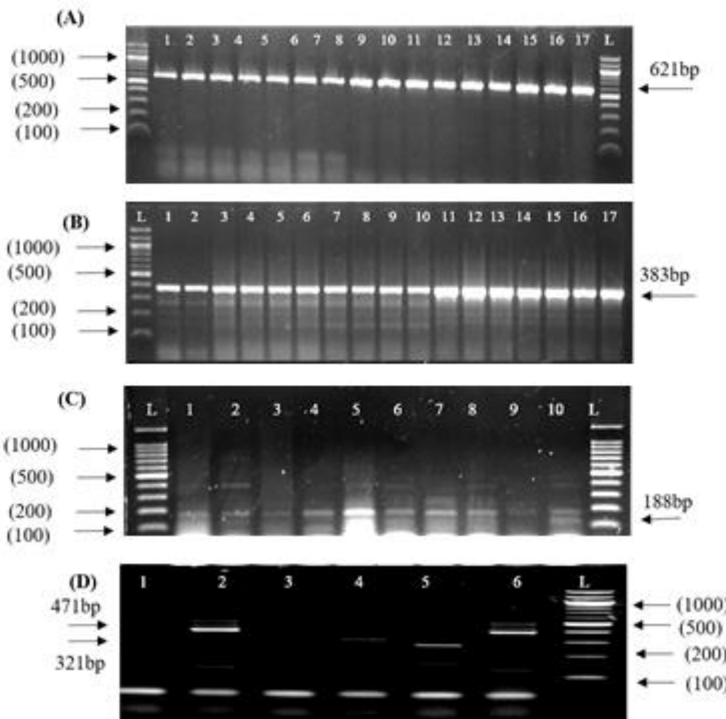


Figure 4.5 Representative gel images for amplification products of MBLs encoding genes The figure shows: **(A)** Lane 1; Positive control, and Lanes 2-17 show PCR product of size 621bp for bla_{NDM}; **(B)** Lane1; Positive control and Lanes 2-17 show PCR product of size 383bp for bla_{VIM}; **(C)** Lane 1; Positive control and Lanes 2-10 show PCR product of size 188bp for bla_{IMP};**(D)** Lane 2 and 6 show PCR products for bla_{AIM} gene of size 321bp and lane 5 shows PCR product for bla_{SIM} of size 471bp while L shows DNA ladder 100bp.

4.2.2.1.3 *AmpC–ADC β lactamase encoding gene:*

PCR was carried out for the detection of *AmpC–ADC* β-lactamases encoding gene. PCR products of sizes 395bp for *AmpC–ADC* gene were observed (Figure 4.6). Of all isolates, 71% (n=170) isolates were positive for *AmpC-ADC* gene.

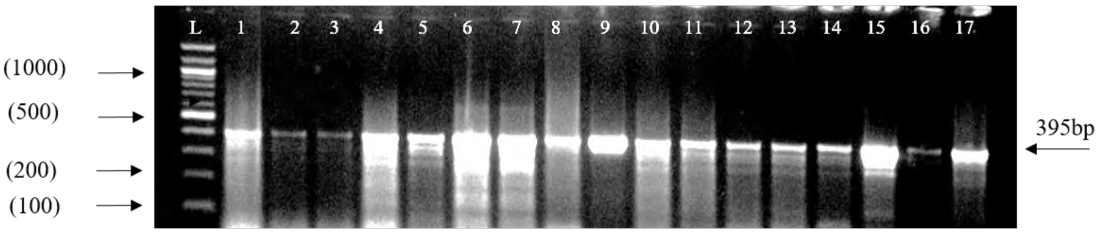


Figure 4.6 Representative gel image for amplification products of *AmpC–ADC* encoding genes.

The figure shows Lanes 1-17 showing PCR product for *AmpC–ADC* of size 395bp. L has DNA ladder of 100bp.

4.2.2.1.4 *Carbapenemase (Oxacillinases) encoding gene:*

PCR was carried out for the detection of carbapenemase encoding genes. *bla*$_{\text{OXA-51-like}}$ gene was present in all study isolates. This gene is regarded as inherent marker for *A. baumannii* strains identification. Seventy isolates carried *bla*$_{\text{OXA-23-like}}$ gene with *bla*$_{\text{OXA-51-like}}$ genes and 19 carried *bla*$_{\text{OXA-48-}\textit{like}}$ with *bla*$_{\text{OXA-51-like}}$ genes, while all isolates were negative for *bla*$_{\text{OXA-24-like}}$ and *bla*$_{\text{OXA-58-like}}$ genes (Figure 4.7).

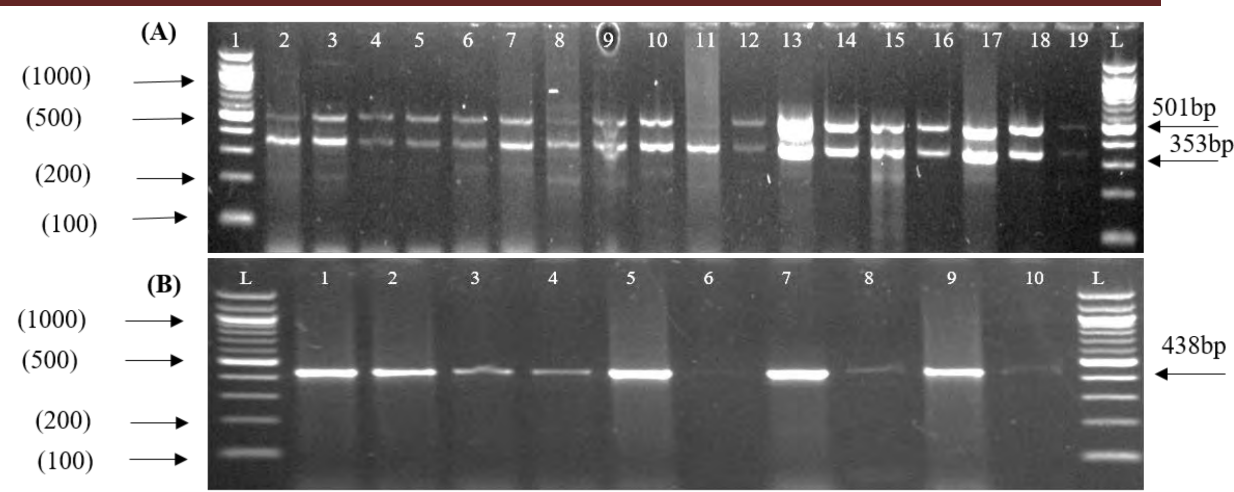


Figure 4.7 Representative gel images for amplification products of Carbapenemases (Oxacillinases) encoding gene encoding genes. (A) Lane 1-19 shows PCR products of sizes 353bp and 501bp for $bla_{\text{OXA-51-like}}$ and $bla_{\text{OXA-23-like}}$ respectively; **(B)** Lanes 1-5 and 7-10 represent PCR products of $bla_{\text{OXA-48}}$ of 438bp.

4.2.2.2 *Insertion sequence ISAba1 encoding gene*

PCR was conducted for the detection of insertion sequence *ISAba1*. A total of 73 (31%) out of 240 isolates carried *ISAba1*-$bla_{\text{OXA-51}}$ combination (Figure 4.8). Out of 73 *ISAba1* positive isolates, 67 were resistant to carbapenems.

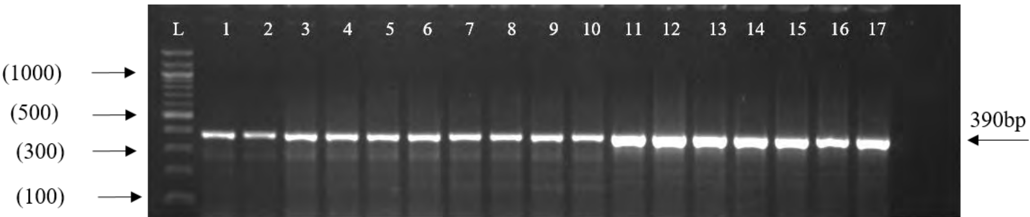


Figure 4.8 Representative gel images for amplification products of *ISAba1* encoding gene.

The figure shows PCR product for insertion sequence *ISAba1* in lanes 1-17, of size 390bp. L shows DNA ladder of 100bp.

4.2.2.3 *Fluoroquinolones resistance genes*

Multiplex PCR was performed for fluoroquinolones resistance genes i.e. *qnr*A, *qnr*B and *qnr*S in all *A. baumannii* isolates (Figure 4.9). PCR products of sizes 580bp, 264bp and 599bp were observed for *qnrA*, *qnrB* and *qnrS* respectively as shown in Figure 4.9. Out of 240, 42 (18%) were positive for *qnrB,* 16 (7%) were positive for *qnrS* and 10 (4.2%)

isolates were positive for *qnrA*. DA48819 was used as a control strain for *qnrS* and *qnrB*.

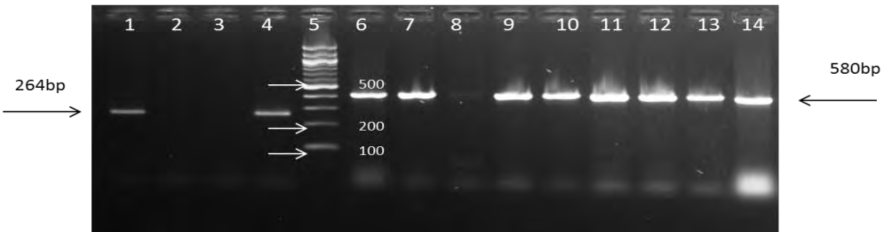


Figure 4.9 Representative gel images for amplification products of of *qnrA and qnrB* genes

The figure shows: Lane 1,4 shows PCR products for *qnrB* of size 264bp while lane 6-14 shows *qnrA* gene of size 580bp.

4.2.2.4 *Tetracycline resistance genes*

Multiplex PCR was done to detect tetracycline resistance genes in all *A. baumannii* isolates. PCR products of sizes 164bp and 206bp were observed for *tetA* and *tetB*, respectively (Figure 4.10). Out of 240, 28 isolates were positive for *tetB* and 2 for *tetA* gene.

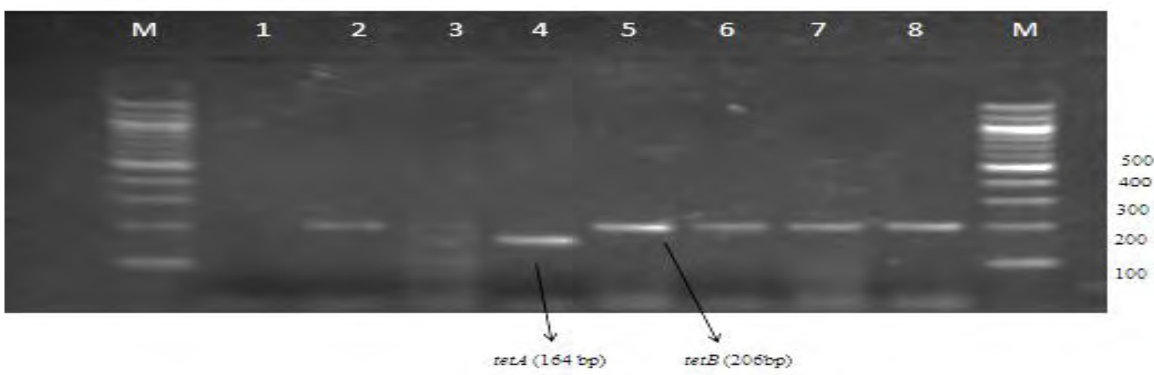


Figure 4.10 Representative gel images for amplification products for *tetA* and *tetB* genes.

The figure shows PCR products for *tetA* of size 164bp in Lane 2, 4and *tetB* of size 206bp in lanes 2, 5, 6, 7 and 8. Lane M contains DNA marker of 100bp.

4.2.2.5 *Aminoglycosides resistance genes*

The multiplex PCR was performed to detect aminoglycosides resistance genes i.e. *aphA6* and *aadB*, *aadA1*, *aacC1* in *A. baumannii* isolates (Figure 4.11). Out of 240 isolates, 20% were positive for *aacC1*, 19% for *aadA1*, 1% for *aadB* and 0.42% for *aphA6*.

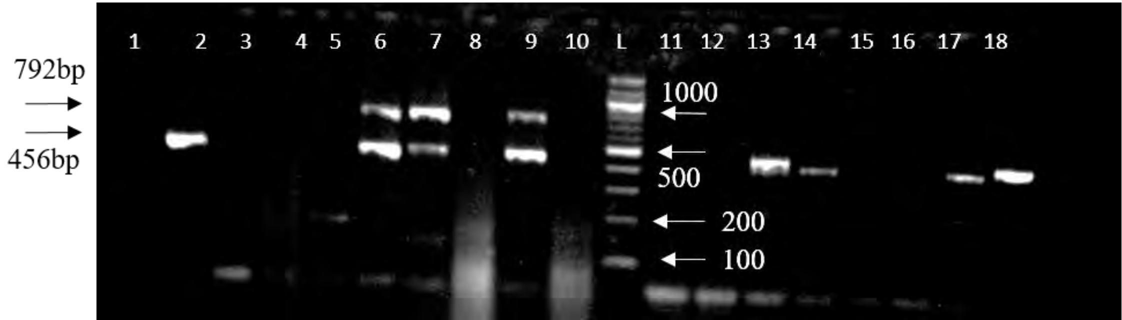


Figure 4.11 Representative gel images for amplification products for *aadA1* and *aacC1* genes.

The figure shows PCR products for *aadA1* gene of size 792bp in lanes 6 and 7, while lanes 2, 6, 7, 9, 13, 14, 17 and 18 represent PCR product for *aaaC1* gene of size 456bp.

4.2.2.6 *Efflux pump Related* (*adeR* and *adeS*) genes

Multiplex PCR was performed to detect *adeR,* and *adeS* in *A. baumannii* isolates (Figure 4.12). Out of 240 isolates, *AdeR* was found in 34 (14%) while *adeS* was found in 50 (21%) isolates.

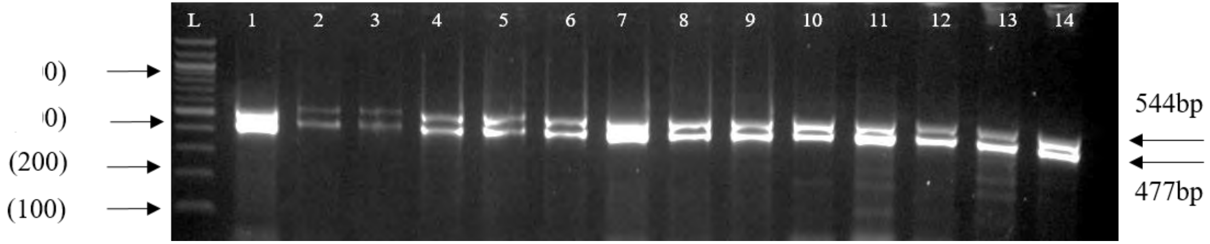


Figure 4.12 Representative gel images for amplification products for *adeR and adeS* genes.

The figure shows PCR products in lanes 1-14 represent PCR products for genes *adeR* and *adeS* with sizes of 544bp and 477bp respectively.

4.2.2.7 *Detection of outer membrane encoding CarO gene*

Amplification of outer membrane encoding *CarO* gene was done by PCR. None of the isolates were positive for *CarO* gene.

4.2.2.8 *Overall Antibiotic Resistance Genes (ARGs) Prevalence*

In the study isolates, the highest prevalence was of *bla*$_{\text{OXA51-like}}$ i.e. 100% followed by *AmpC*-ADC (71%), *ISAba1* (31%), *bla*$_{\text{OXA-23-like}}$ (29%), *bla*$_{\text{IMP}}$ (26%), *adeS* (21%), *aac1* gene (20%), *anr B* (17%), *tet B* (12%), *bla*$_{\text{NDM}}$ (9%) *bla*$_{\text{OXA-48-like}}$ (8%), *qnrS* (7%) and *bla*$_{\text{VIM}}$ (6%). The overall prevalence of all ARGs is shown in figure 4.13.

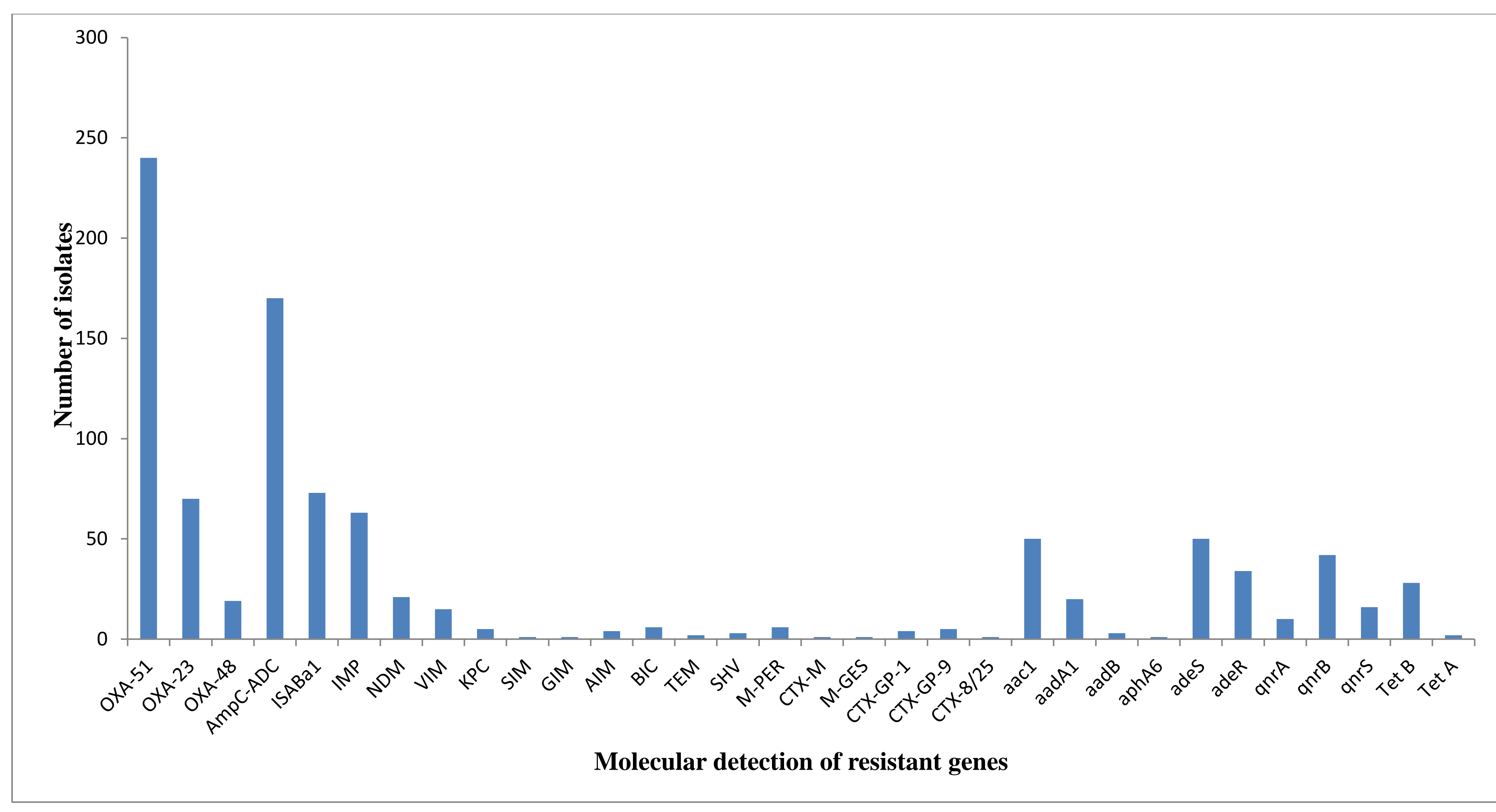


Figure 4.13: Molecular detection of antibiotic resistance genes in *A. baumannii* isolates.

4.2.3 Genetic Characterization for Virulence Genes

PCR was performed to detect *csgA*, *iutA*, *cnf1* and *cvaC* genes in *A. baumannii* isolates. Only one isolate harbored PCR product of size 200bp for *csgA* gene (Figure 4.14). Other three genes were absent in all *A. baumannii* isolates.

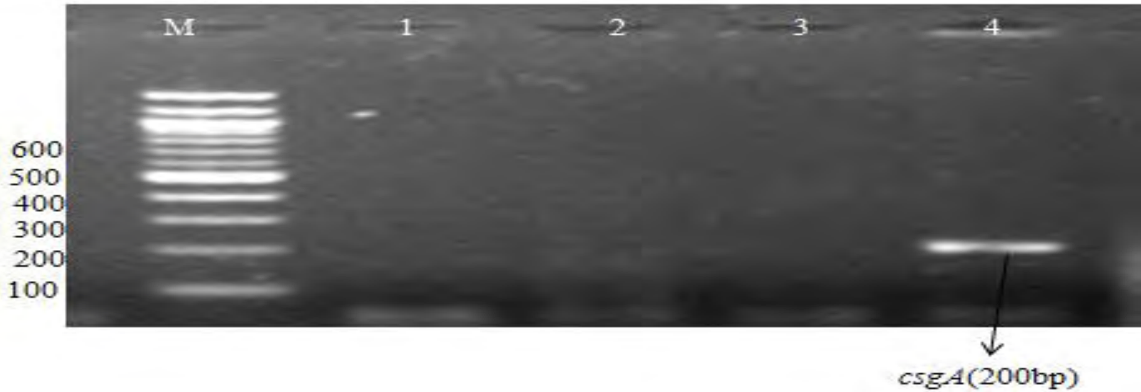


Figure 4.14 Representative gel images for amplification products for Virulence Genes.

The figure shows PCR product for *csgA* gene of size 200bp in lane 4.

4.3 DISCUSSION

In MDR *A. baumannii* clinical isolates, β-lactamase production constitutes an important mechanism of resistance. Ambler class A, class B, class C, and class D β -lactamases present diverse enzymes including ESBLs, MBLs, AmpC β-lactamases and oxacillinases, respectively. Among all, ESBL producers play a vital role in inducing resistance against recommended β-lactam antibiotics in clinical settings (Abrar et al., 2019). On the contrary, none of the isolates in the present study was found to be ESBL producer using double disc synergy test. Similar findings were reported earlier from Islamabad, Pakistan (Begum et al., 2013). DDST is not the only method for ESBLs detection in Gram-negative clinical isolates (Datta et al., 2004). There are various other methods, which include inhibitor potentiated disc diffusion, MIC reduction test and phenotypic confirmatory disc diffusion test (Chaudhary and Aggarwal, 2004). None of these methods has 100% accuracy for confirmation of ESBLs production therefore it is necessary to rely on molecular methods (Nasim et al., 2004). In partial support of our phenotypic assay, the molecular screening for ESBLs gene showed extremely low prevalence of ESBLs encoding genes i.e. $bla_{\text{M-PER}}$ (n=6), bla_{SHV} (n=3), bla_{TEM} (n=2), and $bla_{\text{M-GES}}$ (n=1). Along similar lines, the CTX-M which is pre-dominant ESBL type reported in clinical isolates from Pakistan (Habeeb et al., 2013) was found to be completely absent in a local study conducted on *A. baumannii* clinical isolates (Hasan et al., 2014). Furthermore, the comparison of ESBL gene variants with the data obtained from other nearby countries like Iran (Vahidi Emami et al., 2018, Abdar et al., 2019, Safari et al., 2015, Sharif et al., 2014) and Iraq (Ghaima, 2018) revealed higher carriage rates by the isolates. Variation in ESBLs genes reported in different studies is most likely due to different regions of the studies, the difference in prevalence rate of strains, sample size and study designs.

Our phenotypic data revealed that around 60% of isolates were positive for the production of AmpC enzyme, which can be attributed to its intrinsic status. A local study has also previously reported its high prevalence (Asif et al., 2018). Our genotypic data also exhibited that 71% of the isolates harbored this gene. These high figures related to encoding and production of AmpC β lactamase are in line with existing literature (Liu and Liu, 2015, Corvec et al., 2003). Hence, in case of AmpC β lactamase, no significant discrepancy was noted in phenotypic – genotypic as well as regional comparisons. Moreover, literature revealed that co-production of AmpC β lactamases with ESBLs can hamper the phenotypic detection of ESBLs in clinical isolates (Rajni

et al., 2008). In this regard, the partial discrepancy noted in phenotype and genotype of ESBL could be due to higher AmpC production. However, further work is needed to confirm this notion.

Our phenotypic data regarding MBLs class of β-lactamases revealed 42% positive isolates while a much higher prevalence of 83% was reported in another local study (Anwar et al., 2016) whereas another study exhibited wider range (15% to 28%) of MBL producers (John and Balagurunathan, 2011). These variations could be due to studies being carried out in different clinical environments and different patient numbers. Our genetic characterization revealed that among various variants of MBL genes, bla_{IMP} gene was highly prevalent (26%) followed by bla_{NDM} (9%) and bla_{VIM} (6%). Among other MBLs in our study, low prevalence of bla_{AIM} gene, bla_{SIM}, and bla_{GIM} genes was observed. Comparative analysis with national studies revealed variable reports exhibiting similar (Hasan et al., 2014) and different (Akhtar et al., 2018, Khurshid, 2018) outcomes. Similar analysis was obtained upon comparing with international reports including alike (Alkasaby and El Sayed Zaki, 2017, Erfani et al., 2017) and different (Ramadan et al., 2018, Amin et al., 2019) results. As mentioned earlier, such differences could probably be due to regional differences, demographic features, sample size and study designs. This also creates an alarming situation due to two reasons; one is that MBLs not only confer resistance to carbapenems but also to all β lactam as well as fluoroquinolones and aminoglycosides. Secondly, horizontal gene transfer of these β-lactamase genes has been previously observed in nosocomial infection by such pathogens (Kabbaj et al., 2012). Hence, specific attention is needed for this particular resistance trait.

OXA-type-β-lactamases are most common carbapenemase found in species of Gram-negative bacteria like *A. baumannii* (Zarrilli et al., 2009). Our phenotypic MIC data revealed that carbapenem resistance was highly prevalent in our study isolates and carbapenemases production was observed in majority of isolates. This can be attributed to intrinsic $bla_{oxa\text{-}51\text{-}like}$ gene found in all isolates (100%), which has been used as a standard for the identification of *A. baumannii*. The other variants of this gene were found to be 31% for $bla_{OXA\text{-}23\text{-}like}$ while none of isolates carried $bla_{OXA\text{-}24\text{-}like}$ and $bla_{OXA\text{-}58\text{-}like}$ genes. The comparative analysis with previously reported data revealed similar (Gokmen et al., 2016) and partially divergent (Handal et al., 2017, Rezaei et al., 2018, Wang et al., 2013) outcomes. The $bla_{OXA\text{-}51\text{-}like}$ encoding genes possess strong association

with *ISAba1* insertion sequence (Chaulagain et al., 2012) which acts as upstream promoter for enhancing the expression of oxacillinases (Turton et al., 2006).

The emergence of MDR *Acinetobacter* has been a global concern, which has also been attributed to the presence of efflux pump with a ability to expel out a variety of antibiotics such as quinolones, aminoglycosides, fluoroquinolones, tetracyclines, macrolides, chloramphenicol, erythromycin and tigecycline (Esterly et al., 2011). Therefore, the phenotypic EtBr-agar cartwheel (EtBrCW) technique was used to check efflux activity (Martins et al., 2013). Our data revealed that 31% of isolates possesses this resistance system. This data on phenotypic efflux activity in *A. baumannii* from Pakistan has not been reported previously, and this highlights the aspect of efflux pump mediated resistance as an important contributor to resistance in clinical *A. baumannii* strains. Genetic characterization of isolates was done to know more about the prevalent efflux systems in the study isolates. Our data revealed that *adeR*, *adeS* and *ISAba 1* genes were found in 7%, 16% and 31% of isolates, respectively. This is suggestive of predominant role of *ISAba1* insertion sequence in efflux pump mediated resistance, which also co-relates with the phenotypic assay. In conformity, the important role of this insertion sequence in AdeABC system was also proposed earlier in *A. baumannii* isolates (Ardebili et al., 2014). On the contrary, TCS was also reported to be the major regulator of AdeABC multidrug efflux pump (Marchand et al., 2004) in *A. baumannii*. This is suggestive of intra-specie genetic diversity and highlights the importance of genetic characterization for determining the resistance traits. Additionally, it is worth mentioning that search of literature did not reveal any report on the comparison of efflux pump related phenotypic and genotypic characterization in same *A. baumannii* isolates.

Tetracycline is a bacteriostatic antibiotic. Its resistance mechanism has been primarily attributed to *tet*-genes encoded efflux pump (Møller et al., 2016). Among the various *tet*-genes, *tetA* and *tetB* have been reported to be crucial for tetracycline resistance (Maleki et al., 2014). The genotypic characterization revealed that only a small number of isolates possessed genes for *tetA* (2) and *tetB* (28), while none of the isolates had co-carriage of these genes. This is suggestive of presence of different *tet*-genes in our local strains and warrants further investigation.

In the current study, *A. baumannii* resistance to fluoroquinolone was found to be 90% in case of levofloxacin and 67% for ciprofloxacin. These *qnr* genes protect DNA gyrase and topoisomerase

IV from inhibiting action of quinolones thereby decrease its susceptibility (Khayat et al., 2017, Esterly et al., 2011). In our study, we have focused on *qnr* genes because of the potential of horizontal gene transfer, which involves the transfer of genetic material to organisms other than its progeny. Hence, the process leads to rapid endowment of resistance mechanism among microbes in a community thereby rendering their eradication more difficult. Our genetic characterization data revealed that out of 240, 10 (4.2%) isolates were positive for *qnr*A, 42 (18%) positive for *qnrB* and 16 (7%) were positive for *qnrS*. These percentages are very low as compared to the antibiotic susceptibility results. This needs further investigation regarding the role and contribution of chromosomal genes, *gyrA, gyrB*, and *parC* mutation of target proteins i.e. DNA gyrase and topoisomerase IV). In similar lines, altered target site has been reported to be the predominant mechanism of quinolone resistance in clinical *A. baumannii* isolates (Geisinger et al., 2019).

Aminoglycosides is another important class of antibiotics used for the treatment of *Acinetobacter* spp. infections. Our susceptibility data exhibited that highest resistance was observed against amikacin (93%) as compared to tobramycin (74%). The resistance to aminoglycosides have been reported to occur because of several mechanisms (Doi et al., 2016), Of these, the enzymatic modification is considered as the most crucial and was focused in the present study. Our data revealed the prevalence of *aadA1* (8%) and *aacC*1 (20%) genes. In similar studies conducted in various cities of Iran, higher prevalence were noted for *aphA6* (60-98%) and *aacC1* (63-100%) genes (Moniri et al., 2010, Aliakbarzade et al., 2014, Shafigh et al., 2018). Despite the fact that both countries have similar phenotypic resistance data as mentioned above, *A. baumannii* clinical isolates varied in the genetic resistance traits. This is suggestive of need to perform genetic characterization of prevalent microbial strains for the confirmation of resistance genes in any geographical region. The phenotypic data is not the reliable measure of carriage of the resistance genes.

Virulence factors play a vital role in pathogenesis (transmission, binding and invasion) of disease caused by *A. baumannii* (Doi et al., 2015). Some of the significant virulence factors of *A. baumannii* involved in human clinical infection are curli fibers (*csg*), cytotoxic necrotizing factor (*cnf*), colicin V production (*cvaC*), and aerobactin (*iutA*) (Eijkelkamp et al., 2014, Eraç et al., 2014) but their genetic distribution in clinical isolates has been reported in limited studies (Liu et

al., 2018). Recognition of these underlying virulence genes has great epidemiological significance and may help the physicians to control spreading of infectious diseases (Darvishi, 2016). Keeping this in view, the presence of these genes (*csgA, iutA, cnf1 and cvaC*) was evaluated in *A. baumannii* isolates. It is interesting to note that only single isolate carried *csgA* gene. This is suggestive of low virulence of strains included in the study. However, search of literature neither revealed any local report on virulence gene nor fate (morbidity or mortality) of *A. baumannii* infections in order to confirm our results. Furthermore, there may be PCR limitations, lack of controls, human errors and risk of cross contamination contributing to the low detection of virulence genes in study strains. On the contrary, studies from nearby country (Iran) exhibit the presence of all four genes with variable percentages (Daryanavard and Safaei, 2015, Momtaz et al., 2015). Both countries i.e. Pakistan and Iran, share border and there is regular movement of their countrymen across this border. Despite of this observation, both countries have variable outcomes in virulence genes as well as previously described resistance genes. Hence, it is reiterated that pathogen control strategy should be handled in case-to-case fashion. Global data is useful in developing general understanding of the subject matter but may not reflect the ground reality of disease at grass root level. Moreover, antibiotic resistance and virulence are reported to be closely related and are affected by various factors such as bacterial types, ecological niche, host and correlation of virulence and resistance mechanisms. The negligible representation of virulence genes in present study could be due to their suppression by high antibiotic resistance genes load, an interaction worth further investigation.

Chapter 5

Clonal Analysis and Molecular Typing of *A. baumannii*

5.1 INTRODUCTION

Various typing methods including ribotyping, plasmid typing, Pulsed-field gel electrophoresis (PFGE), Enterobacterial Repetitive Intergenic Consensus Polymerase Chain Reaction (ERIC-PCR), Multi-locus sequence typing (MLST) are important epidemiological tools used for analyzing the genetic relatedness among isolates of the same species. Typing methods based on genetics are more accurate than conventional methods because of their higher discriminatory power (Pérez-Losada et al., 2013).

ERIC-PCR fingerprinting is a molecular method that defines relatedness based upon 127-bp imperfect palindromes that occur in multiple copies in the genomes of enteric bacteria and Vibrios (Wilson and Sharp, 2006). It has a high degree of discrimination compared to traditional methods such as biotyping, serotyping and antibiogram (Waturangi et al., 2012) and its discriminative power is comparable to that of PFGE. It is a convenient and fast method, therefore, is more suitable than ribotyping, and is more cost-effective than other DNA fingerprint methods (Aljindan et al., 2018). PFGE is considered as gold standard for large epidemiological investigations, and is highly discriminative based on the migration of DNA fragments, used in epidemiological studies (Kaufmann and Pitt, 2018), although it is technically complex, time and cost demanding technique (Rafei et al., 2014).

Multi-locus sequence typing (MLST) is based on variation in seven loci nucleotides and is, therefore, a reliable technique for analyzing the genetic relationship between bacterial isolates (Enright and Spratt, 1999). The data generated by MLST is explicit and portable and can, therefore, be implemented in various laboratories without affecting the results (Jolley et al., 2004). It could be used theoretically for both global epidemiological studies and micro-scale epidemic assessment (Tomaschek et al., 2016). In the current study, we have explored the clonal relationship among MDR *A. baumannii* isolates using above mentioned techniques.

5.2 RESULTS

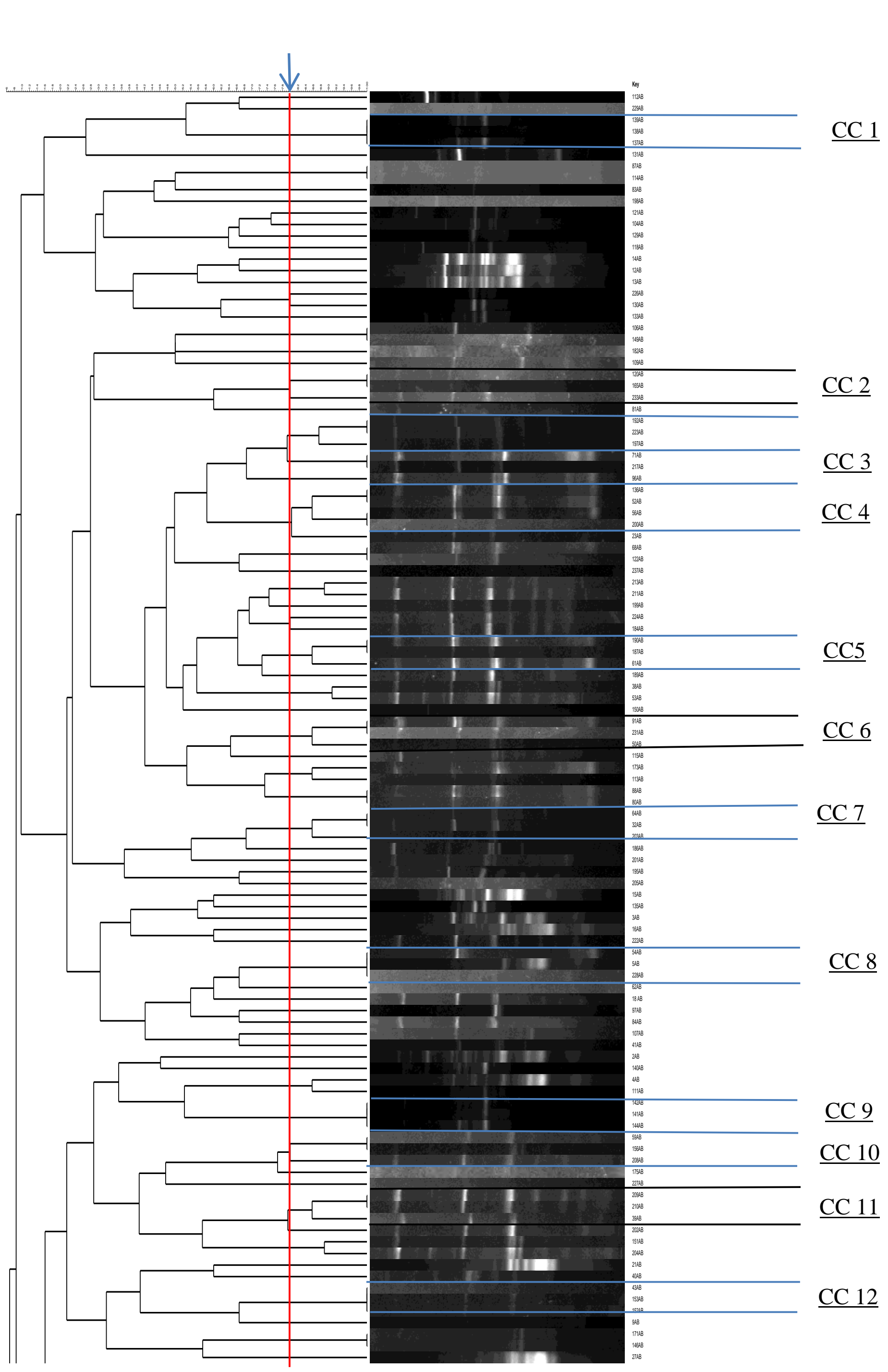
The results obtained for the clonal analysis and molecular typing *A. baumannii* isolates are as follows:

5.2.1 *ERIC-PCR*

The genetic profiles of 240 *A. baumannii* isolates were evaluated by ERIC-PCR which revealed a number of different clonal types. Cluster analysis of $bla_{\text{OXA-51-like}}$ positive isolates showed that they could be grouped into eighteen clusters. The matrix of DICE similarity coefficient generated a dendrogram derived from the unweighted pair group method with arithmetic mean (UPGMA) algorithm. The dendrogram showed heterogeneous profiles with similarity varying from 80% to ~100%. *A. baumannii* isolates were grouped into clusters on the basis of their similar band patterns with cutoff point to be at least 80% similarity as shown in Figure 5.1.

ERIC-PCR showed a high degree of genetic variability among isolates, with heterogeneous patterns, out of which twenty-seven patterns (consisting of 2-3 isolates) showed ~100% similarity. A large number of isolates appeared as singleton and doubles suggesting high diversity among the *A. baumannii* isolates.

CC 13 was the largest clonal cluster comprising of 5 isolates with similarity ranging from 80% to 100% followed by CC4 that contained 4 isolates. While CC1, CC8, CC9, CC12, CC16 and CC17 all comprised of 3 isolates each with ~100% similarity. Clusters CC2, CC3, CC5, CC6, CC7, CC10, CC11, CC14, CC15 and CC18, all comprised of 3 isolates each with similarity between 80% to ~100%. All other isolates showed variable ERIC-PCR patterns with >15% difference in UPGMA geometric dice coefficient. Twelve isolates were non-typable which could be due to lack of amplification. All the isolates were MDR with highly divergent clonal profiles, isolated from different hospital wards and clinical sources reflecting the dissemination of different clones in the same sites of a hospital throughout the study time.


CC 1
CC 2
CC 3
CC 4
CC5
CC 6
CC 7
CC 8
CC 9
CC 10
CC 11
CC 12

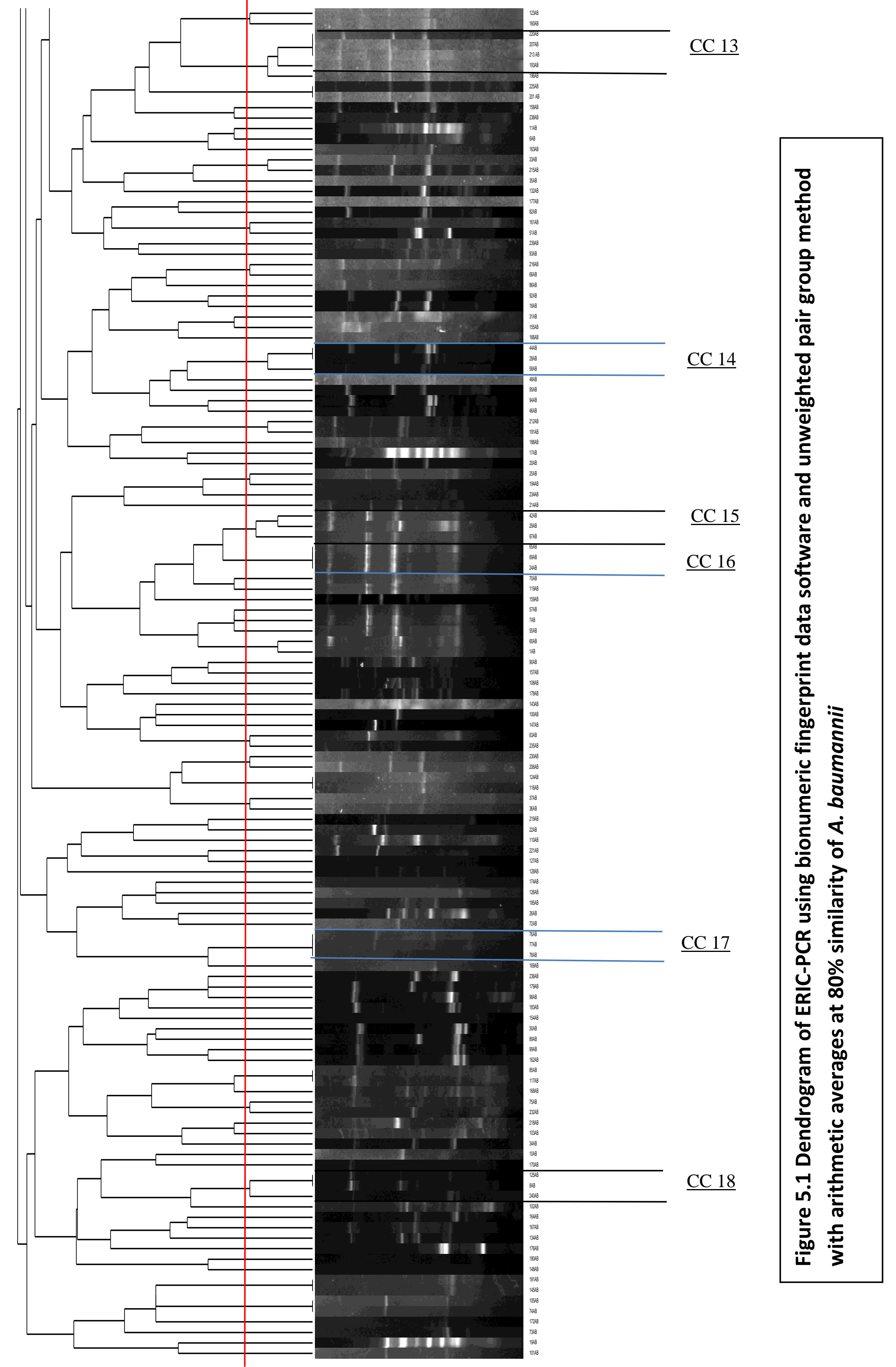


Figure 5.1 Dendrogram of ERIC-PCR using bionumeric fingerprint data software and unweighted pair group method with arithmetic averages at 80% similarity of *A. baumannii*

5.2.2 *PFGE*

Clonal relation among subset of isolates was also analyzed by PFGE. Ten pulsotypes were generated as shown in Figure 5.2. Pulsotype B was the largest group having 23 isolates with band pattern similarity ranging from 80% to ~100%. Pulsotype B included carbapenemase producers, where 8 isolates carried *bla*$_{IMP}$ gene while *bla*$_{VIM}$ gene co-prevalence with *bla*$_{IMP}$ genes was in 4 isolates. Co-prevalence of *bla*$_{VIM}$, *bla*$_{SHV,}$ and *bla*$_{IMP}$ genes was shown in one isolate. Members of this group had varying resistance profile. Majority of members of this group were isolated from patients in medical ICU, and most of these were from pus source.

The second-largest group was pulsotype C containing 16 isolates with similarity between 80% to ~100%. Among this group, high prevalence of *bla*$_{IMP}$ gene was observed, along with 2 isolates also carrying *bla*$_{OXA-48}$ gene.

Pulsotype H contained 15 isolates with band pattern similarity between 80% to ~100%. This group showed high prevalence of insertion sequence *ISAba 1*, most isolates were obtained from female patients and clinical source was pus.

Pulsotype G contained 14 isolates with similarity ranging between 90% to ~100%. This group contained *bla*$_{IMP}$ producers isolated from urine and blood. Seven members of this group were isolated from patients in medical ICU. Pulsotype F contained 10 isolates with similarity ranging between 80% to ~100%. Majority if isolates were from females admitted in medical ICU.

Pulsotypes A and E comprised 9 and 3 isolates respectively, each with band pattern similarity ranging between 90% to ~100%. All isolates had varying range of resistance profiles. Majority of isolates of these groups were isolated from patients in medical ICU.

Pulsotype I and D contained 8 and 7 isolates respectively with similarity ranging between 80% to ~100%. High percentages of isolates were from medical ICU for both groups. Among β-lactamase producers *bla*$_{IMP}$*,* *bla*$_{VIM}$, *bla*$_{TEM,}$ and *bla*$_{OXA-48}$ genes were more prominent from pulsotype D, while pulsotype H represented the highest number of isolates with *ISAba1* carriage. Pulsotype J included 4 isolates which were from male patients with carriage of *bla*$_{IMP}$ gene.

Seven isolates were assigned as unique isolates by PFGE analysis. PFGE pulsotypes along with isolates demographics and resistotypes are shown in Table 5.1.

PFGE

PFGE

65 70 75 80 85 90 95 100

A

B

C

D

E

Key	Source	Warrd	Gender	Age	Resistance Genes	Antibiotic Resistance
106	T.sec	Med ICU	F	42 Y	VIM,SHV	CRO,CAZ,AMC.ATM,SCF,IMP,AK,TOB,LEV,CHL,MH
108	T.sec	Med ICU	M	24 Y	VIM	CAZ,AMC,CTX,TZP,PB,AK,MH,OFX,LEV
58	T.sec	Med ICU	M	25Y	ISAb a 1	CRO,TOB,CAZ,AMC,IMP,AK,LEV,AK
125	ETT	SICU	F	26Y	IMP.NDM	CRO,CAZ,TOB,PB,IMP,AMC,SCF,MH,LEV
240	T.sec	MW6	M	27Y	VIM	CRO,CAZ,TZP,ATM,LEV,OFX,AK,IMP,PB,MH
71	Pus	Med ICU	F	80Y	AdeS,ISA ba 1	CRO,TZP,CAZ,SCF,AK,LEV,IMP,CTX,ATM PB
124	T.sec	Med ICU	F	25Y	NDM,IMP	CRO,CAZ,AMC,ATM,PB,LEV,SCF,IMP,TOB
104	Urine	Med ICU	F	40Y	NDM	CRO,CAZ,AMC,ATM,IMP,SCF,TZP,CHL
110	T.sec	SICU	M	46Y	NDM,VIM	IMP,CAZ,CRO,TZP,SCF,TOB,AK,OFX,CHL,CTX,
217	Pus	SICU	M	17 Y	VIM,IMP	CRO,CAZ,TZP,IMP,TOB,AK,LEV,ATM,AMC
233	Pus	SICU	M	58Y	VIM,IMP	CRO,CAZ,TZP,AMC,IMP,LEV,AK,ATM,PB
225	Pus	Med ICU	M	12 Y	VIM,IMP	CRO,TZP,IMP,PB,CAZ,AMC,LEV,AK,ATM,MH,
117	Pus	Med ICU	F	15 Y	IMP	CTX,AMC,CAZ,IMP,AK,TOB,MH,SCF,OFX
122	T.sec	SICU	M	62 Y	IMP	CRO,CAZ,TZP,SCF,TOB,AK,PB,AMC,IMP,LEV
226	T.sec	Med ICU	M	58 Y	IMP	CRO,CAZ,SCF,AMC,IMP,ATM,AK,PB,LEV
89	Pus	Emergency	M	27Y	ISAb a1	CRO,CAZ,TZP,AK,PB,SCF,OFX,AMC,IMP
107	Blood	Mw3	M	59Y	VIM	AMC,CTX,CAZ,TOB,IMP,MH,TGC,TOB,AK
62	Pus	Med ICU	M	40Y	IMP	CAZ,CRO,AMC,IMP,TZP,SCF,PB,LEV,AK
120	Pus	Emergency	F	40Y	NDM,OXA48	SCF,AMC,IMP,CRO,CAZ,TZP,AK,OFX
202	Pus	Emergency	F	43Y	IMP	CRO,CAZ,SCF,TZP,OFX,IMP,AMC,TOB
105	T.sec	Med ICU	M	34Y	VIM,IMP,SHV	AMC,CRO,CAZ,TZP,LEV,IMP,AK,SCF,TOB
81	T.sec	Med ICU	M	65 Y	IMP	CAZ,CRO,OFX,TZP,IMP,TOB,AK,SCF,AMC,ATM
82	T.sec	MW5	M	28Y	ISA ba 1	CAZ,CRO,AMC,IMP,TZP,PB,AK,SCF,ATM,LEV
83	Pus	Emergency	M	24Y	ISA ba 1	CAZ,CRO,TZP,TOB,OFX,IMP,AK
239	Blood	MED ICU	F	8 Y	IMP	TOB,CAZ,CTX,AMCM,AK,PB,IMP,LEV
131	Pus	Med ICU	F	28Y	NDM,IMP	CRO,AMC,IMP,SCF,TOB,PB,TZP,LEV,CHL
53	T.sec	Med ICU	M	30Y	AdeR	CAZ,CRO,TZP,SCF,IMP,PB,LEV,AK,
61	T.sec	Med ICU	F	16Y	ISAb a 1	CRO,CAZ,AMC,TOB,AK,IMP,SCF,LEV,
51	Blood	MW6	M	25Y	AdeS	CAZ,AMC,TOB,IMP,MH,SCF,CTX,AK,LEV
127	T.sec	Emergency	M	55Y	NDM,IMP	CRO,CAZ,IMP,TOB,AK,SCF,AK,PB,LEV
172	C.tip	Med ICU	F	25Y	NDM	IMP,CRO,CAZ,SCF,AMC,TOB,AK,LEV,PB
227	Blood	Emergency	M	40Y	IMP,VIM	CRO,TZP,CAZ,CTX,AMC,IMP,LEV,AK,
126	T.sec	Med ICU	F	30Y	IMP	CRO,CAZ,TOB,SCF,IMP,AMC,ATM,LEV,AK,
129	T.sec	G.Medicine	F	6 Y	IMP,OXA48	CRO,TZP,SCF,AMC,IMP,CAZ,ATM,TGC,LEV
130	T.sec	G.surgery	M	10 Y	IMP	CAZ,IMP,CTX,AMC,ATM,TOB,AK,SCF,LEV,PB
133	T.sec	Med ICU	F	30Y	IMP,SIM	IMP,TOB,AMC,CAZ,TZP,CTX,SCF,AK,LEV
158	Pus	SICU	M	40Y	IMP	IMP.CAZ,CRO,NDM,OFX,TOB,AK,SCF,LEV
136	T.sec	Emergency	M	29Y	IMP,OXA 48	CRO,CAZ,AMC,TZP0,OFX,CHL,IMP,PB,AK,
72	T.sec	Surgery	M	65Y	ISA ba 1	CAZ,CTX,CRO,AMC,IMP,SCF,TZP,TOB,AK,LEV
74	T.sec	Med ICU	F	55Y	AdeS,ISAb a 1	CRO,CAZ,TOB,SCF,IMP,LEV,AMC,ATM,AK
77	T.sec	Med ICU	F	24 Y	AdeS,ISA ba1	CAZ,CRO,TZP,TOB,AK,IMP,AMC,ATM,LEV
79	T.sec	SICU	M	25Y	IMP	CRO,CAZ,SCF,TOB,AK,AMC,ATM,SCF,LEV
80	T.sec	SICU	M	25Y	ISA ba 1	CAZ,CTX,AMC,CRO,IMP,LEV,SCF,PB,AK
75	Pus	Emergency	M	30Y	ISA ba 1	CAZ,CRO,TZP,SCF,AK,TOB,LEV,AMC,ATM,MH
78	Pus	Med ICU	M	20Y	IMP	CAZ,CRO,AMCMAK,IMP,ATM,LEV,SCF,PB,LEV
84	Pus	Emergency	M	54Y	ISAba 1	CAZ,CTX,TOB,AMC,IMP,TOB,PB,LEV,AK,
65	T.sec	Med ICU	M	25Y	IMP	CRO,CAZ,AMC,TZP,AK,TOB,LEV,IMP,PB
73	T.sec	Surgery	M	33Y	ISA ba 1	CRO,CTX,CAZ,SCF,IMP,TOB,AMC,ATM,LEV
11	T.sec	Med ICU	F	40Y	ISAba 1	TZP,AMC,CRO,CAZ,SCF,PB,TGC,MH,CHL,LEV
116	T.sec	Med ICU	M	40Y	IMP	CTX,CAZ,AMC,PB,TGC,OFX,IMP,SCF,TZP,AK
188	Blood	MedICU	M	40Y	IMP	CRO,CAZ,AMC,TZP,IMP,AK,TOB,LEV,MH
48	Pus	Med ICU	F	43Y	AdeS,ISAb a 1	CRO,CAZ,AMC,TZP,SCF,TOB,AK,IMP,LEV
12	C.tip	Med ICU	M	6Y	ISAba1	CAZ,TZP,IMP,AMC,SCF,TOB,AK,CTX,LEV,PB
229	Tip of d.	Med ICU	M	30Y	VIM,IMP	CRO,CAZ,AMC,SCF,ATM,LEV,PB,IMP,MH
102	T.sec	Med ICU	M	40Y	TEM,OXA 48	CRO,CAZ,AMC.ATM,OFX,AK,IMP,SCF,TOB,CHL,TZP
209	Pus	SICU	F	53 Y	IMP	CRO,CAZ,IMP,TZP,LEV,AK,AMC,ATM,PB
230	ETT	Surgery	F	22Y	IMP	IMP,ATM,AMC,CAZ,CTX,LEV,TOB,AK,MH,
119	T.sec	Med ICU	F	16Y	NDM,IMP	CAZ,CTX,AMC,OFX,SCF,IMP,TGC,TOB,AK

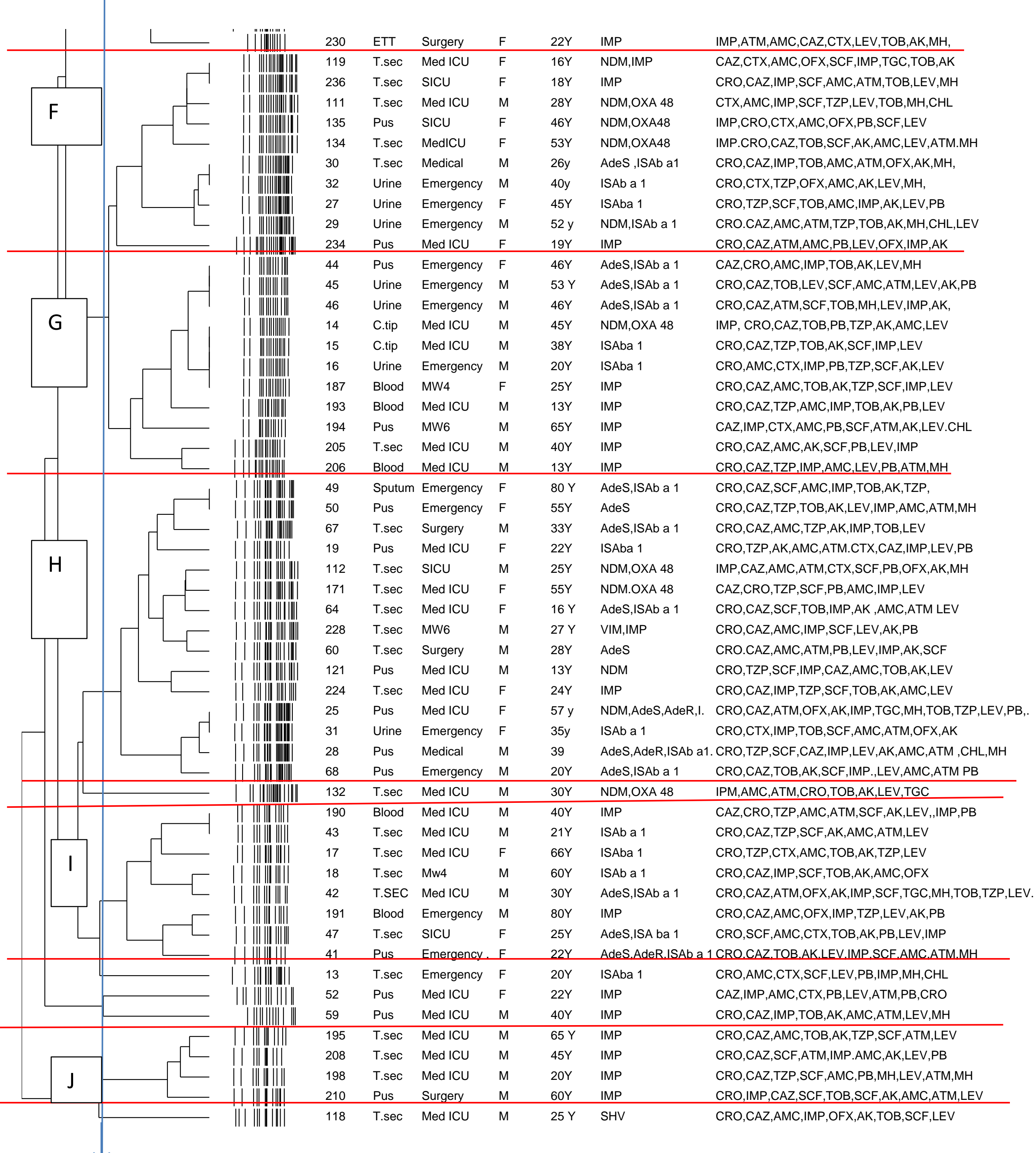


Figure 5.2 Dendrogram of *A. baumannii* isolates along with demographic features, pulsotype, clonal complex, resistance genes, and antibiotic resistance profiles.

Table 5.1: PFGE pulsotypes along with isolates demographics and resistotypes.

Pulsotype	No of Isolates	Source	Ward	Gender	Resistotypes	Genotypic Resistant Markers
A	9	Tracheal secretion (6) Pus (1) Urine (1) Endotracheal tube (1)	Medical ICU (6) SICU (2) MW6 (1)	M (4) F (5)	MDR	bla_{VIM}, bla_{SHV} (1) bla_{VIM} (2) bla_{IMP}, bla_{NDM} (1) *ISABa1* (1) bla_{IMP}, bla_{NDM}(2) *adeS*, *ISAba1* (1) bla_{NDM}, bla_{VIM} (1)
B	23	Pus (10) Tracheal secretion (8) Blood (4) Catheter tip (1)	Medical ICU (12), SICU (2), Emergency (6) Medical wards (3)	M (16) F (7)	MDR	bla_{VIM}, bla_{IMP} (3) bla_{IMP} (6) *ISAba1* (4) bla_{VIM} (1) bla_{NDM}, bla_{OXA48}(1) bla_{VIM}, bla_{IMP}, bla_{SHV} (1) bla_{NDM}, bla_{IMP} (2) *adeR* (1) bla_{NDM} (1) bla_{IMP}, bla_{VIM} (1)
C	16	Tracheal secretion (12) Pus (4)	Medical ICU (6) G.Medical (1) G.Surgery (3) SICU (3) Emergency (3)	M (11) F (5)	MDR	bla_{IMP} (6) bla_{IMP}, bla_{OXA-48} (2) bla_{IMP}, bla_{SIM} (1) *adeS*,*ISABa1* (2)
D	7	Blood (1) Pus (2) Respiratory sites (3) Tip of drain (1)	Medical ICU (5) Surgery (1) SICU (1)	M (4) F (3)	MDR	bla_{IMP} (3) *adeS*,*ISABa1* (1) *ISABa1* (2) bla_{VIM} $bla_{,IMP}$ (1) bla_{TEM}, bla_{OXA-48} (1)
E	3	Tracheal secretion (1), Pus (1), Endotracheal tracheal tube (1)	Medical ICU (1) Surgery (1) SICU (1)	M (1) F (2)	MDR	bla_{IMP} (2) $bla_{OXA\ 48}$ (1) bla_{TEM} (1)
F	10	Tracheal secretion (5) Urine (3) Pus (2)	Medical ICU (4), SICU (2) Medical ward (1) Emergency (3)	M (4) F (6)	MDR	$bla_{NDM}$$bla_{,IMP}$ (1) bla_{IMP} (2) bla_{NDM}, bla_{OXA-48} (3) *adeS*,*ISBa1*(1) *ISABa1* (2) bla_{NDM},*ISABa1*(1)
G	14	Urine (3) Pus (3) Catheter tip (2) Blood (4) Tracheal secretion (2)	Emergency (4) Medical ICU (7) Medical wards (3)	M (12) F (2)	MDR	*adeS*,*ISABa1* (3) bla_{NDM} , bla_{OXA-48} (1), *ISABa1* (2) bla_{IMP} (8)
H	15	Tracheal secretion (7) Pus (6) Sputum (1) Urine (1)	Emergency (4) Medical ICU (6) Surgery (2) SICU (1) Medical wards (2)	M (7) F (8)	MDR	*adeS*,*ISABa1* (6) *adeS* (2) *ISABa1* (2) bla_{NDM}, bla_{OXA-48} (2) bla_{VIM}, bla_{IMP} (1) bla_{NDM} (1)

						bla$_{IMP}$ (1) *bla*$_{NDM}$,a *deS,a deR* (1) a*deS,a deR,ISABa1* (1)
I	8	Tracheal secretion (5) Blood (2) Pus (1)	Medical ICU (4) Emergency (2) SICU (1) Medical ward (1)	M (5) F (3)	MDR	*bla*$_{IMP}$ (2), *ISABa1* (3), *adeS, ISABa1* (3),
J	4	Tracheal secretion (3) Pus (1)	Medical ICU (3) Surgery(1)	M (4)	MDR	*bla*$_{IMP}$ (4)

5.2.3 *MLST*

Representative six isolates from most prevalent hospital sites and sample sources were selected based upon their DNA band patterns by ERIC-PCR and PFGE analysis. Amplification of seven housekeeping genes (*cnp 60, fus A, glt A, pyr G, rec A, rpi B*, and *rpo B*) by using pasture scheme was done. Amplified products were purified and then sequenced. STs were assigned by combining allelic profiles following the MLST database (http://pubmlst.org/ abaumannii/). Demographic data, resistance phenotypes and genotypic resistance markers for these six isolates are shown in Table 5.2. Of these 6 isolates, two belonged to ST241, two belonged to ST164, one isolate was ST101 and one belonged to ST409. Allelic profiles of these isolates with their sequence types (STs) are given in Table 5.3.

Table 5.2: Demographic details, phenotypic, genotypic resistance, allelic profile.

Isolate ID	Source	Ward	Gender	Age	Resistance Phenotypes	Genotypic Resistant markers	Allelic profile	ST
227	Blood	Emergency	M	40Y	CAZ-TZP-CAZ-CTX-AMC-IMP-LEV-AK	bla_{IMP} bla_{VIM}	40-3-15-2-40-4-4	241
74	Tracheal secretions	Med ICU	F	55Y	CRO-CAZ-TOB-SCF-IMP-LEV-AMC-ATM-AK	*adeS* *ISABa 1*	40-3-15-2-40-4-4	241
188	Blood	Med ICU	M	40Y	CRO-CAZ-AMC-TZP-IMP-AK-TOB-LEV-MH	bla_{IMP}	40-3-7-2-40-4-4	164
206	Blood	Med ICU	M	13Y	CRO-CAZ-TZP-IMP-AMC-LEV-PB-ATM-MH	bla_{IMP}	40-3-7-2-40-4-4	164
25	Pus	Med ICU	F	57Y	CRO-CAZ-ATM-OFX-AK-IMP-TGC-MH-TOB-TZP-PB-LEV	bla_{NDM} *adeS* *adeR*	1-1-1-1-2-1-1	409
118	Tracheal secretions	Med ICU	M	25Y	CRO-CAZ-AMC-IMP-OFX-AK-TOB-SCF-LEV	bla_{SHV}	1-1-1-2-1-1-1	101

Table 5.3: Sequence Types by combination of seven loci

Isolate ID	*Cnp60*	*fusA*	*gltA*	*pyrG*	*recA*	*rpiB*	*rpoB*	ST
227	40	3	15	2	40	4	4	241
74	40	3	15	2	40	4	4	241
188	40	3	7	2	40	4	4	164
206	40	3	7	2	40	4	4	164
25	1	1	1	1	2	1	1	409
118	1	1	1	2	1	1	1	101

Same sequence types that belonged to different clusters of PFGE and ERIC-PCR were observed. Then further clonal relation among STs found in current study with global clonal groups and their single locus variant analysis was done by using PHYLOVIZ 2.0 software. The data analyzed using PHYLOVIZ 2.0 provided a closer look at the STs found in CC1 and SLVs. ST409 is sub-founder of clonal complex CC1. ST164, ST101, and ST241 represented the further sub-founders of this clonal complex as shown in Figure 5.3.

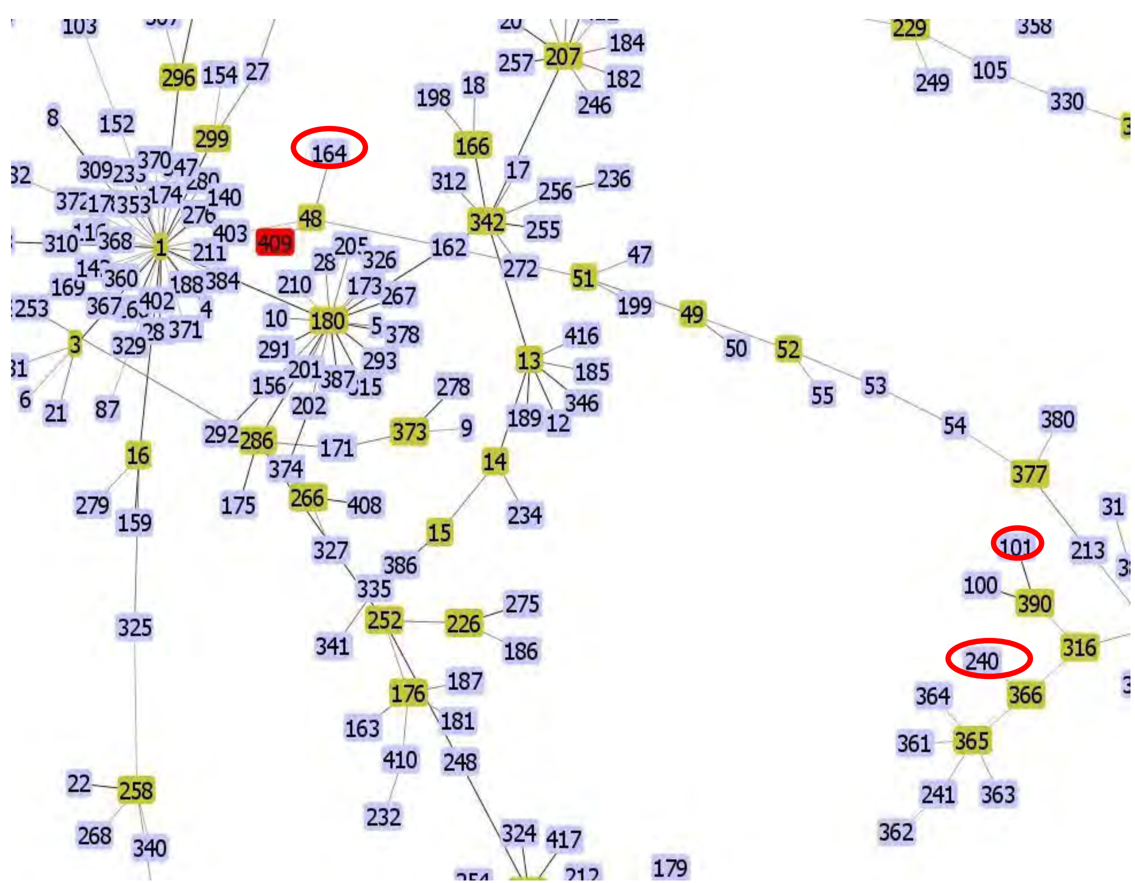


Figure 5.3 The images generated using PHYLOVIZ 2.0 gives a closer look at the STs found in CC1: ST409 (red circle) sub-founder of clonal complex CC1. ST164, ST101 and ST241 in red circles represent the further sub-founders of this clonal complex.

5.3 DISCUSSION

In clinical settings, the investigation of clonal diversity among *A. baumannii* provides useful data to understand local epidemiology and help in the implementation of effective control programs (Wang et al., 2013). ERIC-PCR results of this work showed that study isolates were heterogeneous. Most of the isolates were scattered throughout the dendrogram and a few were grouped as clusters. A total of 240 isolates were clustered in eighteen types. The most prevalent type was CC13 (5 isolates) followed by CC4 (4 isolates). Search of literature revealed a local study, reporting 9 different epidemiological clones (Hasan et al., 2014), which is again indicative of clonal diversity within country. In similarity with our scenario, few studies from Iran also revealed inconsistent findings: 7 cluster types (Heidari et al., 2018), 10 clusters types (Maleki et al., 2014) and 14 distinct ERIC-types (Falah et al., 2019). A study from Saudi Arabia grouped *A. baumannii* into 7 cluster types (Aljindan et al., 2018). A Turkish report grouped 33 strains into 2 clusters (Ece et al., 2015) while a Brazilian study reported wide clonal diversity of 38 clones of *A. baumannii* isolates (Viana et al., 2011). Another study showed different findings where 29 strains were grouped into 10 ERIC-PCR clonal complexes while 11 strains had no bands (Maleki et al., 2017). Most of our studied strains were isolated from ICU which may indicate clonal spread with tracheal secretions as a source of sample indicating high prevalence in immune-compromised patients and with indulged devices like tracheal tubes. The other isolates clustered into distinct clades that could not be formally grouped indicating that the spread/persistence of *A. baumannii* at PIMS is multi clonal.

In case of PFGE analysis, sub-set of isolates was divided into 10 pulsotypes belonging to MDR group. Among them, 3 major pulsotypes, pulsotype B (n=23), pulsotype C (n=16), pulsotype H (n=15) made up almost half of all MDR *A. baumannii*. These clones appeared to be isolated primarily from tracheal secretion and pus of patients in medical ICU and harbored bla_{IMP} gene. These findings are in accordance with the previous studies with major clones from medical and surgical ICU with high prevalence of carbapenemase genes (Zarrilli et al., 2004, Raka et al., 2009). Furthermore, the dendrogram showed that despite the commonality of medical ICU ward, the resistotypes of isolates varied within clonal clusters. In other words, the isolates belonging to the same groups had varying resistance profiles in the same sites of a hospital. This is probably

because majority of the isolates harbored resistance markers that can be transferred via mobile genetic elements including transposons, integrons and plasmids and result in wide dissemination.

In our study, all clonal groups in ERIC-PCR were not consistent with isolates distribution through PFGE analysis. Furthermore, isolates belonging to the same groups through ERIC-PCR and PFGE analysis had varying resistance profiles.

Acinetobacter baumannii has surfaced as the primary pathogen causing hospital-acquired infection. This success as a pathogen has been attributed to its ability to remodel its genome. In clinical settings, a variety of epidemic clonal complexes have been reported across the world, which are further divided into diversified strains of *A. baumannii* (Wen et al., 2014). Numerous epidemic clonal complexes of *A. baumannii* have been reported globally, each of which contains a subset of diversified strains. These epidemic strains generally belong to three international clones (IC) i.e. I, II and III (Rafei et al., 2014). According to the Pasteur scheme, these genotypes are named as clonal complex (CC) 1, 2 and 3, respectively (Gaiarsa et al., 2019). With the introduction of newer antibiotics, distinct lineages or sub-lineages within each clonal complex arose with significant heterogeneity. These differences help differentiate close relatives causing outbreaks or dissemination at the local, national or global level. Reports suggest CC2 to be the dominant clonal type in Asian countries (Kim et al., 2013, Qu et al., 2016, Tada et al., 2015). In present study, six isolates were selected for MLST analysis based on their clonal complex patterns determined by ERIC-PCR and PFGE analysis. The analysis revealed four different Sequence types (STs) i.e. ST 409, ST164, ST101 and ST241. In contradiction to aforementioned notion, all of these STs belonged to CC1, according to Pasteur scheme. The ST409 is sub-founder of clonal complex CC1, while ST164, ST101 and ST241 were further sub-founders of this clonal complex. As for previous studies from Pakistan, most of the studies have investigated resistance in *A. baumannii* by phenotypic methods. A study from Faisalabad reported the epidemiological typing of clinical *A. baumannii* isolates with different results by ERIC-PCR and MLST. Different findings from our study with 8 diverse clonal groups by ERIC-PCR and STs (ST642, ST589, ST2, ST600, ST338, ST103, and ST615) in clinical *A. baumannii* isolates were reported (Khurshid, 2018). More recent data revealed entirely different sequences types (including newer ones) belonging to both CC1 and CC2 (Khurshid, 2018, Khurshid et al., 2020), which is suggestive of existence of two major clonal types i.e. CC1 and CC2 in the country. The

difference could be due to different healthcare settings from different cities, which may have different circulating clones and sequence types. However, to the best of our knowledge, the sequence types (ST241, ST164, ST409, and ST101) reported in this study have not been reported earlier from Pakistan. These STs belong to a CC1, clonal complex with successful global dissemination, and identification of these STs is suggestive of presence of CC1 at the site of study. Furthermore, the difference could be due to limited number of isolates used for sequence type determination. In order to compensate this limitation, the isolates belonging to most prevalent wards, and different clonal clusters were selected for clonal typing. However, further work is required to confirm our deduction.

Chapter 6

General Discussion

Acinetobacter baumannii is one of the ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter species*) pathogens, a major concern due to their ability to acquire antibiotic resistance hence leading to increased morbidity and mortality. World Health Organization (WHO) has recognized *A. baumannii* as a serious threat to public health and has included it in the list of "Priority pathogens" with the need for the development of new antimicrobial agents (Marturano and Lowery, 2019). Wide ranges of intrinsic and acquired mechanisms attributing to the higher resistance rate in *Acinetobacter* spp. have been reported by researchers. It is directly and indirectly linked with enhanced rate of mortality and morbidity (Poirel et al., 2017). Different proportions of multi drug-resistant (MDR) strains of *Acinetobacter* spp. have been isolated and reported from diverse areas of the world. Its ability to develop and acquire resistance genes is responsible for high prevalence of MDR *A. baumannii*. There is need to understand molecular epidemiology of *A. baumannii* to devise strategies for prevention and control of MDR infections. Very limited information is available regarding molecular epidemiology and genetic characterization of *A. baumannii* in Pakistan. The present study gives an insight into its current epidemiology, resistance traits (both phenotypic and genotypic), virulence and clonal diversity, which may help in devising appropriate preventive and therapeutic strategies against this pathogen.

We found that *Acinetobacter baumannii* showed resistance to almost all classes of antibiotics and all the isolates were found to be MDR which is an alarming situation as these MDR isolates can result in unmanageable infections. One third of these MDR strains were found to be PDR *A. baumannii*, further complicating the situation as these may even lead to treatment failures resulting in increased mortality. Presence of these serious antibiotic-resistant infections in developing countries is particularly worrisome as they can easily disseminate and help in emergence of true pathogens (Paterson and Bonomo, 2005). In developing countries like Pakistan, with no set protocols to handle MDR isolates and substandard hygienic conditions these infections are prone to become an epidemic. It is appropriate time to highlight MDR *A. baumannii* presence in healthcare settings and introduce protocols for handling such infections. It is need of the hour to deeply analyze this emerging issue to re-evaluate the primary antimicrobial therapy, as first-line therapies have become ineffective in the case of MDR isolates.

Misuse/abuse of these antibiotics is resulting in not just treatment failures but also contributing in evolution of isolates under antibiotic pressure. In Pakistan, most of the healthcare facilities do not have access to susceptibility testing and rely upon the preset protocols for treatment. Up to date information on susceptibility in these situation can help identify the best treatment options and reduce misuse/overuse of antibiotics. Among tested antibiotics, tigecycline appeared to be an effective option in vitro, with treatment potential against *A. baumannii* isolates. Therefore, in order to prolong the usefulness of this effective medicine, it is recommended to use this drug cautiously in clinics involving avoidance of long-term monotherapy and continuous monitoring of efflux pump mediated resistance. Additionally, the respiratory route appears to be the primary physiological habitat of *A. baumannii*, making it highly contagious. Hence, it is recommended to take protective measures especially involving vulnerable inhalation route, particularly in the ICU, the apparently prevalent hospital site for *A. baumannii*.

We found variation in results of phenotypic–genotypic assays for *A. baumannii* isolates. Search of literature revealed enormous debate on the efficiency of these assays. In this regard the genotypic-based methods although costly and require more labor, are considered more accurate for the detection of antimicrobial resistance. However, phenotypic assays are considered more useful in determining antibiotic susceptibility. Our phenotypic–genotypic comparison data suggest that the use of molecular PCR-based methods for reliable detection of resistance traits is much more efficient as compared to the phenotypic methods and should be included in diagnostics wherever possible.

A. baumannii was considered only a final recipient of carbapenemase genes from Enterobacteriaceae and *P. aeruginosa*, but the discovery of bla_{NDM} changed this perception. It not only acquired bla_{NDM}, but also resulted in the genetic manipulation that led to widespread dissemination of this carbapenemase (Dortet et al., 2014). *A. baumannii* as a collector of antimicrobial resistance genes is not only troublesome as it results in MDR phenotype but also because this reservoir can play a role in successful dissemination of these genes. A wide range of antibiotic resistance markers were identified in our isolates, which confirm its reservoir status and need further investigations of the genetics structure of these genes. The deficiency of regular monitoring programs at national level, insufficient infection control programs, lack of facilities and unsuitable approaches for diagnostics add to the development of resistance to antibiotics in

bacteria. Hence, the dissemination of highly resistant isolates in hospitals calls for establishment of systemic surveillance network for monitoring genetic basis of antibiotics resistance mechanisms and spread of these resistant strains (Ahmad, 2018, Abrar et al., 2019).

Clonal complexes recognized via ERIC-PCR were heterogeneous, most of the isolates were scattered throughout the dendrogram and a few were grouped as clusters. This shows dissemination of multiple clones inside the hospital. PFGE analysis revealed some major clones and CC1 was found to be major clonal complex. CC1 is a clade with global dissemination showing its potential for spread (Higgins et al., 2010), hence measures should be taken to control its further spread inside the hospital. We need to investigate all these various clones on genetic level to understand their origin and setup one single handling protocol which is suitable for all these variants.

The primary limitation of the study was that it was performed at a single tertiary care hospital, which is insufficient to provide bigger picture of *A. baumannii* and its associated infections in the country. Furthermore, clonal typing along with sequence types was performed in limited number of samples, which can be further explored to screen the clonal diversity.

The findings from current studies indicate dissemination of MDR *A. baumannii* in healthcare setting, especially in ICU indicating a complete lack of in-place handling protocol or inefficient following of protocol by the hospital staff. Training of personnel is of utmost importance in ICU where a substantial number of MDR *A. baumannii* were recovered. We also found an increasing trend of PDR phenotype, suggesting misuse/abuse of antibiotics creating a pressure for strains to adopt and mutate. A plethora of antibiotic genes make these isolates a serious threat and renders current therapies ineffective. Research and development of new antimicrobials is of utmost importance to manage these infections but at the same time it is essential to introduce strategies for accurate identification, timely reporting and effective infection control strategies of these various MDR strains, as this is the only way of dealing with this issue at hand.

Chapter 7

Concluding Remarks

7.1 Summary of the work presented in this thesis

In the current study, *A. baumannii* were isolated from a variety of samples taken from both male and female patients of diverse age groups. High frequency of antibiotic resistant phenotypes were observed; 54% MDR, 13% XDR and 33% PDR *A. baumannii.* The phenotypic antibiotic susceptibility data exhibited that isolates showed resistance to all classes (Penicillins, Cephalosporins, Fluoroquinolones, Aminoglycoside, Carbapenems and Tetracyclines) with tigecycline being the most effective with 50% of the isolates being susceptible. Among MDR isolates, 65% were carbapenemase producers, 59% AmpC and 42% MBL producers. $Bla_{\text{oxa-51-like}}$ was found to be intrinsic in all isolates. The results showed that 31% isolates were phenotypically positive for efflux pump activity while 21% isolates carried *adeS* and 14% *adeR* genes. The most prevalent ESBL gene and carbapenemase gene was $bla_{\text{M-PER}}$ and bla_{IMP}, respectively. Other ESBL genes identified were bla_{SHV}, bla_{TEM}, bla_{GES} and variants of $bla_{\text{CTX-M}}$ and MBLs included bla_{VIM}, bla_{KPC}, bla_{NDM}, bla_{BIC}, bla_{SIM} and bla_{AIM}. A high prevalence of *AmpC–ADC* gene i.e. 71% was observed. Seventy isolates carried $bla_{\text{OXA-23-like}}$ with $bla_{\text{OXA-51-like}}$ and 19 carried $bla_{\text{OXA-48-}like}$ with $bla_{\text{OXA-51-like}}$, while all isolates were negative for $bla_{\text{OXA-24-like}}$ and $bla_{\text{OXA-58-like}}$ genes. Among fluoroquinolone resistant genes, *qnrB* (18%) was found to be more prevalent than *qnrS* (7%) and *qnrA* (4%). For tetracycline resistant genes, 12% isolates carried *tetB* while only two isolates were *tetA* positive. In case of Aminoglycosides, 20% isolates were positive for *aacC1* followed by *aadA1* (19%). None of the isolates was positive for virulence genes, *iutA*, *cnf1* and *cvaC* and only a single isolate carried *csgA*.

Using ERIC-PCR, 240 isolates were grouped into eighteen clusters. A large number of isolates appeared as singleton and doubles showing high diversity among the isolates. The clonal analysis by PFGE showed that there were three major pulsotypes, pulsotype B (n=23), pulsotype C (n=16), pulsotype H (n=15). MLST analysis of six isolates revealed four different sequence types ST409, ST164, ST101 and ST241 belonging to CC1, according to Pasteur scheme. Two isolates of sequence type ST164 carried bla_{IMP} while of two ST241 isolates one harbored bla_{IMP} with bla_{VIM}, and one carried *adeS* and *ISAba1* genes.

In the current study, we have reported a high MDR prevalence with an increase in the prevalence of PDR isolates representing emergence of resistance against classes of antiobitics. The study highlights the differences in the efficiency of phenotypic-genotypic methods in the detection of

antibiotic resistance mechanisms. The analysis of clonal relatedness showed there is presence of multiple clones in the hospital with STs confirming this observation. We found isolates belonging to STs including ST409, ST164, ST101 and ST241 which belong to CC1, the presence of these STs have not been previously reported from Pakistan.

7.2 Future Directions

Future studies are required for queries generated during the study and remain un-answered. In this regard, the aforementioned adult age–trauma–ICU nexus in the occurrence of *A. baumannii* infection needs to be confirmed. Hence, instead of including various wards of hospital, the more focused and in-depth study from ICU isolates shall be conducted to know more about contributing factors of *A. baumannii* infection. Keeping in view the low prevalence of virulence markers, the fate of *A. baumannii* infections shall be monitored in order to rationalize the study for identification of new virulence markers. Further investigations are required to explore all non-enzymatic resistance mechanisms of bacteria including contribution of porins, efflux pumps and other outer membrane proteins to the overall antibiotic resistance in *A. baumannii.* There is a need to study the variants of genetic markers and variations in the promoter regions of resistance genes, efflux pumps and porins to see their expression especially in PDR strains. Whole genome sequence of these isolates may give information regarding phylogenetic linkages. We explored the clonal relatedness and sequence types at a tertiary care hospital but plasmid-mediated resistance islands need to be studied as well, which might be involved in enhancing the antibiotic resistance in strains prevalent at site of present study. Furthermore, the more extensive study on clonal typing is also needed to confirm the diversity of *A. baumannii* at the site of study.

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Prevalence of aminoglycoside and tetracycline resistance genes and their association with virulence factors in clinical isolates of *Acinetobacter baumannii*

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Abstract

High prevalence of antibiotic resistance in ***Acinetobacter baumannii***(***A. baumannii***) is a serious threat to modern health care system due to its inherent and acquired resistance mechanism.It is difficult to treat and becoming resistant to various classes of antibiotics. The aim of the study was to investigate aminoglycoside and tetracyclineantibiotic resistancegenes in ***A. baumannii*** and their correlation with virulence factors. A total of 240 clinical ***A. baumannii*** isolates collected from Pakistan Institute of Medical Sciences (PIMS) were preceded for the present study. Initially isolates were identified by using standard microbiologicaltechniques. Antibiotic susceptibility assay was performed using Kirby Bauer disk diffusion method. Polymerase chain reaction (PCR) was done for screening of antibiotic resistance genesand virulence factors. PCR results showed that among aminoglycoside resistance genes20%, 8%, 1% and 0.42% of isolates were found positive for ***aaC1***, ***aadA1***, ***aadB*** and ***aphA6***genes respectively. In case of tetracycline resistance genes, 13% isolates were positive for ***tetB*** and 1% for ***tetA***. Among virulence factors, only one isolate harbored***csgA*** gene and none of the isolates were positive for***iutA***, ***cnf1*** and ***cvaC.*** It was concluded that due to residing on same genetic elements antibiotic resistance genes suppress the effect of virulence factors.

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Introduction

Acinetobacter baumannii (*A. baumannii*) is a Gram-negative, catalase-positive, oxidase negative, non-motile, and aerobic in nature. It is worldwide reported as hospital-associated bacteria (Lin ***et al***., 2014). ***A. baumannii*** commonly causes bacteremia, pneumonia, meningitis and urinary tract infections (Al Anazi ***et al.,*** 2014). In 2009-2010 in United State, National Healthcare Safety Network (NHSN) discovered 1.8% infections caused by ***A. baumannii*** (Sievert ***et al***., 2013). ***A. baumannii*** is recognized as one of the important bacteria in ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumonia*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter species*) pathogens by Infectious Diseases Society of America (IDSA) (Bush ***et al***., 2010). Due to multidrug resistance and long persistence in hospitals, ***A. baumannii*** became a serious threat for hospitalized patients. Nowadays in many hospitals carbapenem resistance (CR) is accounting as hallmark for extreme drug resistance (XDR) ***A. baumannii***strains. Except some aminoglycosides, tigecycline and polymyxins these CR strains show resistance to all routinely used antibiotics. Colistin is used as a drug of choice to treat complicated ***A. baumannii*** infection but their efficacy is yet to be determined (Chang ***et al***., 2015).

Virulence factors play vital role in transmission, binding and invasion of the ***A. baumannii*** and cellular damages (Doi ***et al***., 2015). Pathogenesis of diseases caused by ***A. baumannii***is derived from the presence of latent virulence genes. Some of the significant virulence factors of ***A. baumannii*** involved in human clinical infection are curli fibers (csg), cytotoxic necrotizing factor (cnf), colicin V production (cvaC), and aerobactin (iutA) (Eraç ***et al***., 2014;Eijkelkamp ***et al***., 2014). Screening of virulence factors in ***A. baumannii*** clinical isolates provides some great epidemiological outcomes to help medical practitioners and clinicians to control dissemination of infectious diseases caused by this bacterium. So it is mandatory to develop some control measures to overcome infections caused by this aggressive bacterium (Darvishi ***et al***., 2016).

According to the best of our knowledge, no previously published datais available about the virulence factors and antibiotic resistance genes (aminoglycoside, tetracycline) in clinical ***A. baumannii***isolates from Pakistan. In Pakistan, limited studies reported phenotypic antibiotics resistance in***A. Baumannii*** (Hassan ***et al***., 2014; Saba ***et al***., 2015).Therefore the present study focuses on the distribution of virulence factors and aminoglycosides, tetracycline resistance genes pattern in clinical***. Baumannii*** isolates collected from tertiary care hospital PIMS Islamabad.

Materials and methods

Samples collection and processing

A total of 375isolates were collected Microbiology Laboratory at Pakistan Institute of Medical Sciences (PIMS), Islamabad Pakistan during January 2014-June 2015. The isolates were characterized by colony morphology on MacConkey and blood agar. Isolates were Gram-stained and later confirmed by biochemical tests using API 20NE Kit (biomeriuex, USA). Ethical approval was taken from bioethics committee Quaid-i-Azam University (QAU) Islamabad Pakistan.

Antibiotic susceptibility profiling

Antibiotic susceptibility assay was done on Muller Hinton agar by usingKirby Bauer disk diffusion method (Bauer ***et al***., 1966). Antibiotics susceptibility was checked for two groups of antibiotics including tetracycline (tigecycline (15µg), minocycline (30µg)) and aminoglycoside (amikacin (30µg), tobramycin (10µg) (Oxoid, UK). Result interpretation was done according to CLSI guidelines 2015. ***E.coli*** ATTCC 25922 was used as a quality control strain.

DNA extraction

DNA extraction was carried out using the boiling method according to the previously published protocol (Vaneechoutte ***et al***., 1995).

Polymerase Chain Reaction (PCR)

Primer sequences for antibiotic resistance genes and virulence genes used in this study were chosen from previously published data (Maleki ***et al***., 2013;

Aliakbarzade ***et al***., 2014; Darvishi ***et al***., 2017). Primers details are shown in Tables 1 and 2. PCR was carried out to detect antibiotic resistance genes, tetracycline resistance genes, ***tet*B** and ***tet*A**, aminoglycoside resistance genes such as, ***aacC*1**, ***aadA*1**, ***aadB*** and ***aphA*6** and virulence genes such as ***iutA***, ***cvaC*** and ***cnf*1**. The PCR parameter was set as; initial denaturation was carried out for 5 minutes at 95°C, 33 cycle each consisting of 30 seconds at 94°C second denaturation, annealing temperature for duplex PCRs for***tet*B**, ***tet*A** (56°C), ***aacC*1**, ***aadA*1**(52°C), ***aadB***, ***aphA*6**(55°C) and multiplex PCR for***iutA***, ***cvaC, csgA,*** and ***cnf*1** was(58°C) respectively, initial extension was at 72°C for 1 minute and final extension at 72°C for 10 minutes. PCR product was run on 1% agarose gel and photographed using gel documentation system (Syngene, Germany).

Statistical analysis

Statistical analysis for determining the ***p***-value was carried out to find a significant association between antibiotic resistance genes and their association with virulence factors.

This was done through chi-square analysis utilizing the scientific software GraphPad Prism version 7.03 to estimate statistical significance. ***p***-value less than 0.05 was considered statistically significant.

Results

Isolates identification

Non pigmented colonies of ***A. baumannii*** appeared on MacConkey agar after incubation at **37°C for 24** hours, as shown in Figure 1.

Table 1. Primers used for the detection of tetracycline and aminoglycoside resistance genes.

Primer	Primer sequence	Product size (bp)	Reference
tetA	F: GCG CGATCTGGTTCACTCG R: AGTCGACAGYRGCG CCGGC	164	MH Maliki ***et al***., 2013
tetB	F: CGTGAATTTATIGCTTCGG R: ATACAGCATCCAAAGCGCAC	206	
aphA6	F: ATGGAATTGCCCAATATTATTC R: TCAATTCAATTCATCAAGTTTTA	797	Aliakbarzade ***et al***., 2013
aadA1	F: ATGAGGGAAGCGGTGATCG R: TTATTTGCCGACTACCTTGGTG	792	
aadB	F: ATGGACACAACGCAGGTCGC R:TTAGGCCGCATATCGCGACC	534	
aacC1	F: ATGGGCATCATTCGCACATGTAGG R: TTAGGTGGCGGTACTTGGGTC	456	

Biochemical tests results

Out of 375 a total of 240 isolates were confirmed as ***A. baumannii*** after colony morphology and through biochemical identification tests.

Antibiotic susceptibility profile of study isolates

All study isolates were evaluated for antibiotic susceptibility profiles, among which high resistance was examined against amikacin (93%), followed by tobramycin (65%), minocycline (59%) and tigecycline (50%). Isolates were highly susceptible to tigecycline (50%) followed by minocycline (41%) and tobramycin (17%) as shown in Figure 2. The data was highly significant with ***p***-value <0.0001. Molecular detection of antibiotic resistance genes (tetracycline, aminoglycoside), and virulence genes. All the study isolates were screened for the presence of antibiotic resistance genes and virulence genes through PCR as shown in Figure 3.

Prevalence of tetracycline and aminoglycoside resistance genes

Among all study isolates, the prevalence of tetracycline resistance genes was 13% for ***tet*B**, 1% for

*tet*A, while in case of aminoglycoside resistance genes, 20% for ***aacC***1, 8% for ***aadA***1, and 0.42% isolates were found positive for ***aphA***6 gene, as shown in Figure 4.

Prevalence of virulence factors

Among all the isolates none was positive for ***iutA***, ***cvaC*** and ***cnf1***. Only single isolate carried ***csg***A virulence factor. Correlation between antibiotic resistance genes and virulence genes was highly significant (*p*-value <0.0001).

Table 2. Primers used for virulence factors (***cnf1***, ***csga***,***cvaC***and***iutA***).

Primer	Primer sequence	Product size (bp)	Reference
Cnf1	F: AAGATGGAGTTTCCTATGCAGGAG R: CATTCAGAGTCCTGCCCTCATTATT	498	Darvishi ***et al.,*** 2017
csgA	F: ACTCTGACTTGACTATTACC R: AGATGCAGTCTGGTCAAC	200	
cvaC	F: CACACACAAACGGGAGCTGTT R: CTTCCCGCAGCATAGTTCCAT	680	
iutA	F: GGCTGGACATCATGGGAACTGG R: CGTCGGGAACGGGTAGAATCG	300	

Discussion

The current study reports high resistance against tetracycline and aminoglycoside in ***A.baumannii*** isolates and is the first report of tetracycline and aminoglycoside resistance genes associated with virulence factors in ***A. baumannii***from Pakistan. In Pakistan, most of the studieshave been limited to phenotypic antibiotic resistance and no comprehensive study is available on the genetic basis of tetracycline and aminoglycoside resistance and its association with virulence factors.

A.baumanniihas the ability to attain new function due to open pan-genome. Due to the excessive use of antibiotics ***A. baumannii***infections remain persistent for long period of time in clinical settings. Moreover, this organism has evolved as a drug-resistant pathogen and some of its virulence factors facilitate its survival in hospital environment (Imperi ***et al***., 2011).In present study, among tetracycline, minocycline was highly resistant (59%) followed by tigecycline(50%) showing different resistance patterns from previously reported studies from Pakistan (Hassan ***et al.,***2014; Begum ***et al***., 2013).In case of aminoglycoside, highest resistance was observed in 93% of isolates for amikacin followed by tobramycin 65% depicting close resemblance with other reports from Pakistan (Fakhuruddin***et al***., 2017).

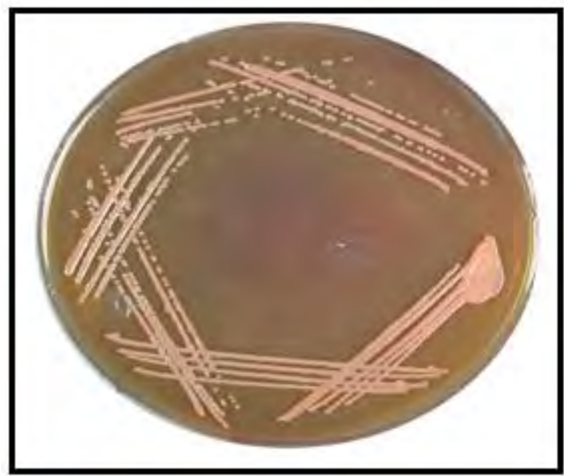

Fig. 1. Colony morphology of ***A. baumannii*** on MacConkey agar.

The current study showed that among tetracycline resistance genes ***tetB*** (13%) had high prevalence rate than ***tetA*** which was 1% while from Pakistan no data is still available on genetic basis of these antibiotic resistance genes. In present study, a low prevalence of ***tetA*** and ***tetB*** genes were reported as compared to another study (Maleki ***et al***., 2014).Similar patterns of differences were observed in two other studies with

tetA in 95% and ***tetB*** in 62% isolates while another study reported 100% presence of***tetB*** while none of the isolates carried ***tetA*** gene (Asadollahi ***et al***., 2012; Farsiani ***et al***., 2015). These discrepancies could be due to geography of the studied areas and variations between clinical samples. Low prevalence of resistance genes may be due to presence of non-enzymatic mechanisms (porins, efflux pumps) which has not been evaluated in current study.

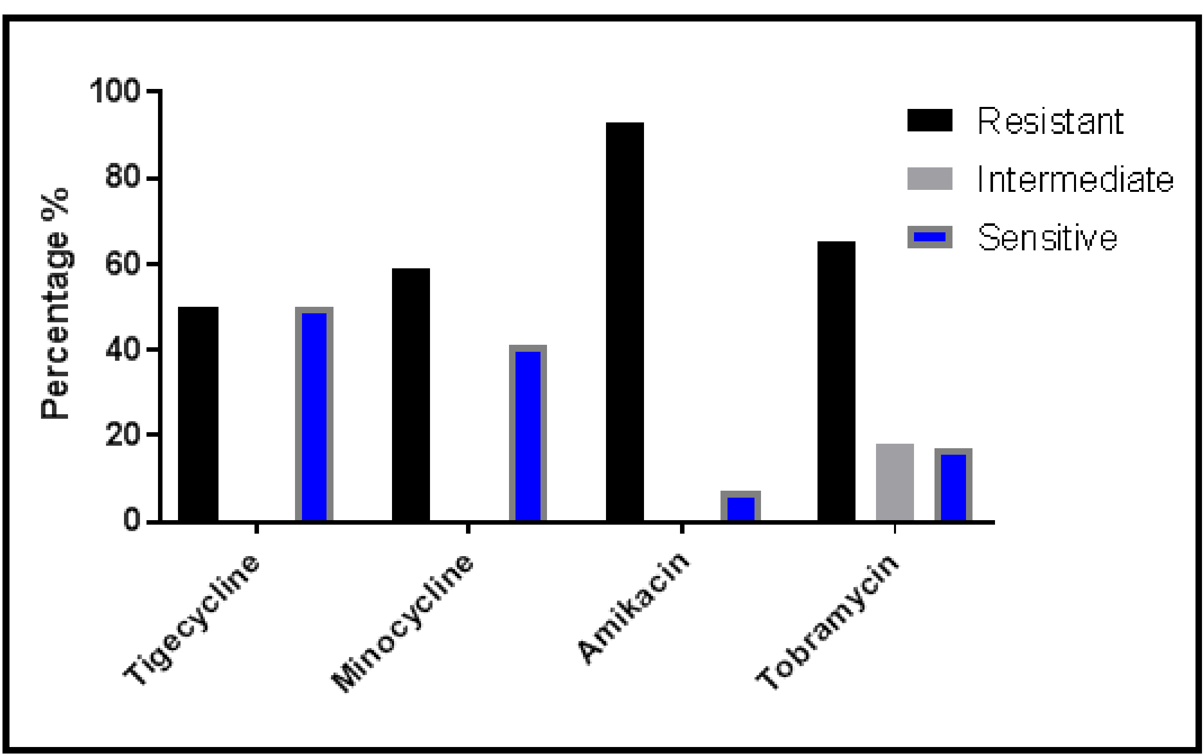

Fig. 2. Antibiotic susceptibility profile of study isolates.

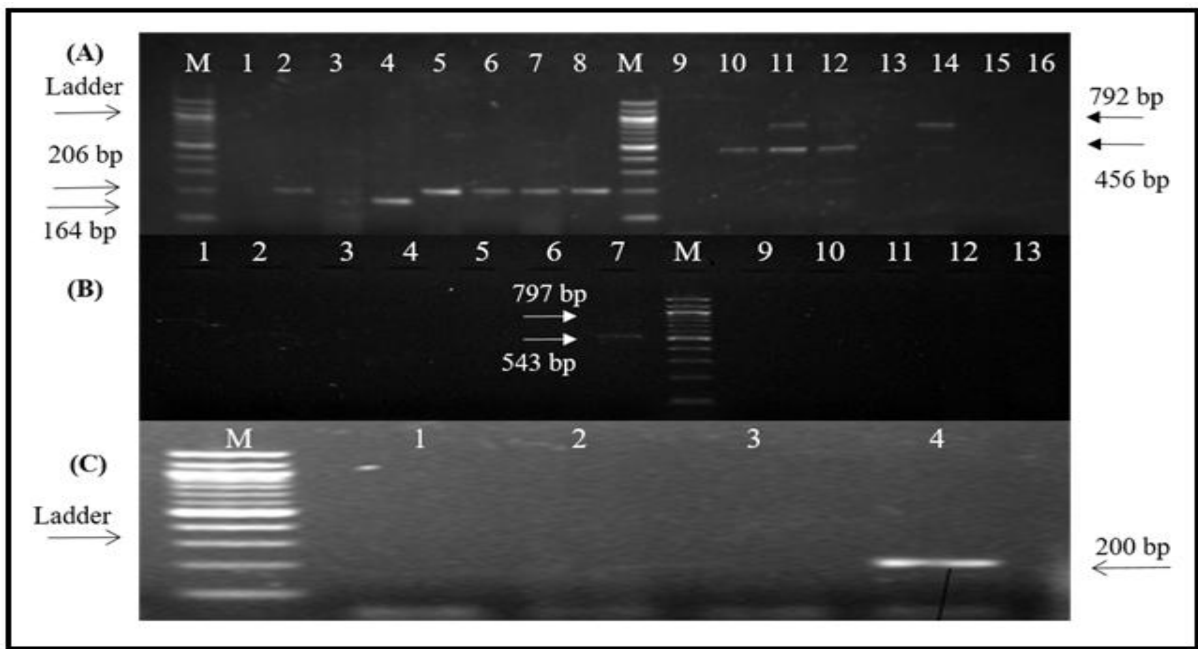

Fig. 3. Representative gel images showing PCR products for detection of Antibiotic resistance genes (A) ***tet***B gene (206bp), ***tet***A (164bp gene), aadA1 gene (792bp), aacC1(456bp) (B)***aph***A6 gene (797bp), ***aad***B gene (543bp) (C)***csg***A gene (200bp).

A. ***baumannii*** develops resistance against aminoglycosides by producing aminoglycoside modifying enzymes (AME). Present study reported aminoglycoside resistance genes ***aacC1*** in 20%, ***aadA1*** in 8%, ***aadB*** in 1% and ***aphA6*** in 0.42% isolates of ***A. baumannii***. Prevalence rate detected in our study were not in agreement with the previous study conducted byHujar ***et al*** (Hujer ***et al.,*** 2006).Another study reported aminoglycoside resistance genes in clinical isolates of ***A. baumanniiaph6, aacC1, aadA1*** and ***aadB***with65%, 63.3%, 41.7% and 3.3% prevalence rate (Moniri ***et al***., 2010). A study conducted by Asadollahi***et al*** in Iran reported***aphA6***, ***aadA1*** and ***aadB*** in 17.3%, 17.3% and 4.3% isolates respectively (Asadollahi ***et al***., 2012). Farsiani ***et al*** in 2015 also reported aminoglycoside resistance genes with different prevalence rate as compared to the present study (Farsiani ***et al.,*** 2015***)***.The contradictions found between present study and all the previous studies may be due to variations in clinical samples, handling, and geographical conditions. Differences may be caused due to the loss of resistance genes residing on plasmids.

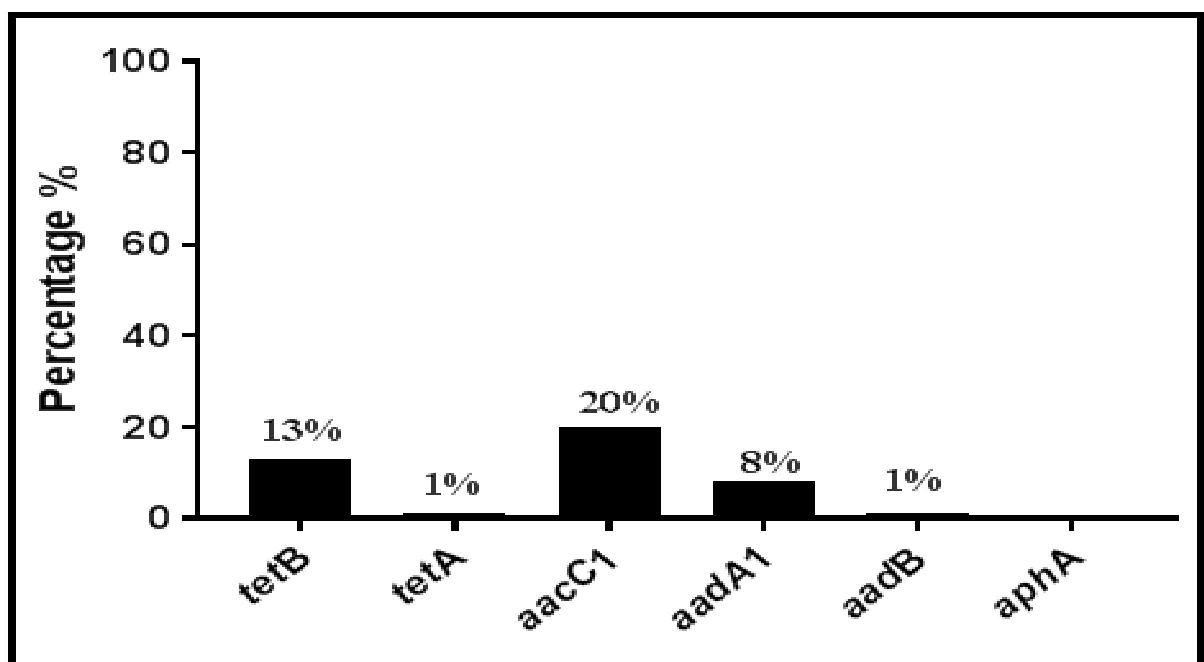

Fig. 4. Prevalence of Antibiotic resistance genes.

Cnf1, ***csgA***, ***iutA*** and ***cvaC***are significant virulence factors that arefound in ***A. baumannii***.

The present study reported that only single isolate carried ***csgA*** gene, while all of the isolates were negative for the remaining genes.In 2015 studies conducted by Daryanavard ***et al*** and Momtaz ***et al*** reported virulence factors with high prevalence rate in clinical strains of ***A. baumannii***. Daryanavard ***et al*** detected ***csgA***, ***iutA***, ***cnf1*** and ***cvaC*** with a prevalence rate of 55%, 30%, 40% and 10% respectively (Daryanavard ***et al***., 2015). Likewise Momtaz ***et al*** also found all these genes with high prevalence rate as compared to our finding. They reported ***cnf1*** in 35%, ***iutA*** in 19%, ***cvaC***in 21.48% and ***csgA*** in 12.39% clinical isolates of ***A. baumannii*** (Momtaz ***et al***., 2015)**.** Variations in prevalence of antibiotics resistance genes with virulence factors may involve the suppression of virulence genes due to high antibiotic resistance.

Conclusion

It was concluded that antibiotic resistance genes and virulence factors are in a complex relationship with each other. Antibiotic resistance genes can suppress the expression of virulence factors if both reside on the same genetic element.

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