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Phenotypic and Genotypic Typing of Carbapenem-Resistant

Acinetobacter baumannii Isolates from Lebanon.

By

Siwar Sleiman Haidar

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THESIS APPROVAL FORM

Student Name: Siwar Haidar I.D. #: 201300755

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School: Arts and Sciences

The undersigned certify that they have examined the final electronic copy of this thesis and approved it in Partial Fulfillment of the requirements for the degree of:

MS in the major of Biological Sciences

Thesis Advisor's Name: Sima Tokajian

Signature: [redacted] Date: 28 / 5 / 2021
Day Month Year

Committee Member's Name: Brigitte Wex

Signature: [redacted] Date: 28 / 5 / 2021
Day Month Year

Committee Member's Name: Roy Khalaf

Signature: [redacted] Date: 28 / 5 / 2021
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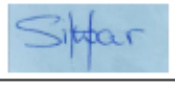
Signature: 

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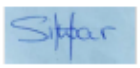
Signature: 

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Phenotypic and Genotypic Typing of Carbapenem-Resistant *Acinetobacter baumannii* Isolates from Lebanon

Siwar Sleiman Haidar

ABSTRACT

Infections caused by Carbapenem-Resistant *Acinetobacter baumannii* (CRAB) have become an essential healthcare-associated problem. We used whole-genome sequencing (WGS) to characterize *A. baumannii* collected from a hospital in Lebanon. The genotypic relatedness was assessed by pulsed-field gel electrophoresis (PFGE), multi-locus sequence typing (MLST), and whole-genome SNP-based phylogenetic analysis (wg-SNP). *A. baumannii* PCR-based replicon typing (AB-PBRT) was also used to analyze the plasmid content. PFGE demonstrated that most isolates that belonged to a unique clonal type were assigned to ST2 of the international clone II. Phenotypic antimicrobial susceptibility testing revealed that the isolates were extensively drug-resistant (XDR). Intrinsic β-lactam resistance genes, including bla_{ADC25} (*Acinetobacter*-Derived Cephalosporinase) and the bla_{OXA66} (a variant of bla_{OXA51}), were among the common resistance determinants. AB-PBRT, which categorizes *A. baumannii* plasmids into homology groups (GRs) based on the nucleotide homology of their respective replicase genes, showed that the isolates were mainly positive for GR2 and GR6. *A. baumannii* is a nosocomial pathogen that is difficult to control. WGS analysis showed the relatedness of the studied CRAB isolates and mobile genetic elements and revealed the enhanced transmission prospects of *A. baumannii* between and among patients. WGS and interventional trials are needed to define and prevent the spread of XDR microbial pathogens.

Keywords: *Acinetobacter baumannii*, CRAB, WGS, PFGE, Outbreak.

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LIST OF ABBREVIATIONS

A. baumannii: *Acinetobacter baumannii*
MDR: Multidrug-resistant
IC: International clone
XDR: Extensively drug resistant
PDR: Pan-drug resistant
ESBLs: Extended Spectrum β-Lactamases
AmpC: Ampicillin class C
OXA: Oxacillinases
MIC: Minimum inhibitory concentrations
CRAB: Carbapenem resistant *A. baumannii*
ADC: *Acinetobacter*-derived cephalosporinase
HGT: Horizontal gene transfer
Rep: Replication
Mob: transfer
ABPBRT: *Acinetobacter baumannii* PCR-based replicon typing
GR: Homology groups
OriV: Origin of replication
TA: Toxin-antitoxin system
RND: Resistance-nodulation-division
MATE: Multidrug and toxic compound extrusion
MFS: Major facilitator superfamily
SMF: Small multidrug resistance family
OMP: Outer membrane protein
LPS: Lipopolysaccharide
T5SS: Type 5 secretion system
T2SS: Type 2 secretion system
Sec: general secretion pathway
T6SS: Type 6 secretion system
OMV: Outer membrane vesicles
DAP: 1,3-diaminopropane
LOS: Lipooligosaccharide
AHL: Acyl homoserine lactoses
BAPs: Biofilm associated proteins
TC: Two component
Csu: Chaperone/usher pilus system
EPS: Exopolysaccharide
SNPs: Single nucleotide polymorphisms
DTA: Deep tracheal aspirate
CLSI: Clinical and laboratory standards institute
TSA: Trypticase soy agar
PCR: Polymerase chain reaction
PFGE: Pulse field gel electrophoresis

PT: Pulsotypes
TSB: Tryptone soy broth
OD: Optical density
SD: Standard deviation
LB: Luria broth
WGS: Whole genome sequencing
RAST: Rapid annotation using subsystem technology
MLST: Multi-locus sequence typing
VFDB: Virulence factor database
ST: Sequence type
AAC: Aminoglycoside acyltransferases
APH: Aminoglycoside phosphotransferases
LPS: Lipopolysaccharide
LOS: Lipooligosaccharide

Chapter One

Introduction

1.1 Overview

The Gram-negative non-fermenter *Acinetobacter baumannii* is a coccobacillus with most nosocomial and community-acquired infections being instigated by this pathogen (Asif et al., 2018). It is linked to a variety of clinical manifestations and infections involving the soft tissue and skin, urinary tract, blood, and respiratory system (Morris et al., 2019). It has simple nutritional requirements, can grow under different temperatures and pH values, and is highly resistant to decontaminating and sterilizing agents. It can form biofilms on both animate and inanimate objects (including hospital mechanical equipment) and can endure on abiotic surfaces for approximately five months (Nowak & Paluchowska, 2016). It is a multidrug-resistant (MDR) organism (McConnell et al., 2013), and has eight main clones disseminating across the globe, known as international clones (IC). IC1 and IC2 are related to MDR hospital outbreaks (Salloum et al., 2018; Hua et al., 2017; Antunes et al., 2014).

1.2 Antimicrobial Resistance

MDR isolates of *A. baumannii* were classified as being resistant to three classes of antimicrobial agents namely: fluoroquinolones, cephalosporins, and aminoglycosides. Extensively drug-resistant (XDR) isolates, however, are resistant to the former classes and

to carbapenems, whereas the pan drug-resistant (PDR) isolates were additionally resistant to polymyxins (Manchanda et al., 2010).

1.2.1 Resistance Genes

1.2.1.1 Extended Spectrum β-Lactamases (ESBLs)

A. baumannii produces enzymes that inactivate β-lactam antibiotics such as carbapenemases, β-lactamases and ampicillin class C (AmpC). β-lactamases are classified into Ambler A to D (Lowings et al., 2015). Development of ESBL producers was linked to the increased use of many β-lactam antibiotics. These enzymes hydrolyze a broad spectrum of β-lactam antibiotics and were detected in many Gram-negative bacteria (Abdar, et al., 2019).

1.2.1.1.1 OXA. Class D β-lactamases are penicillinases that can disintegrate and induce resistance to penicillin and oxacillin and are referred to as oxacillinases (OXA) (Evans & Amyes, 2014). These enzymes have 95 variants that can either be chromosomal or plasmid-encoded, differ in their affinity to imipenem and meropenem, have low hydrolytic activity against carbapenems, and *in vivo* expression is linked to the increase in the minimal inhibitory concentrations (MIC) to carbapenems (Evans & Amyes, 2014).

Different OXA carbapenemases were detected in *A. baumannii* including the OXA-23, OXA-(24)-40 and OXA-58 (Livermore & Woodford, 2006). OXA-23 carbapenemase was first detected in *A. baumannii* recovered from a clinical setting in the UK followed by worldwide dissemination and rise of carbapenem resistant *Acinetobacter baumannii* (CRAB) (Perez et al.,

2007; Mugnier et al., 2010; Al-Agamy et al., 2016; Chen et al., 2018). OXA-23 enzymes hydrolyze oxacillin, piperacillin, aminopenicillins, aztreonam, oxyimino cephalosporins and carbapenems (Evans & Amyes, 2014).

1.2.1.1.2 TEM. The original TEM-type ESBLs known to confer resistance to penicillins, were designated as TEM-1 and TEM-2 (Paterson & Bonomo, 2005). In *A. baumannii*, the presence of TEM-1 β-lactamase was shown to be responsible for resistance to sulbactam (Krizova et al., 2013). TEM-2 β-lactamase is less common but exhibits similar biochemical properties (Paterson & Bonomo, 2005). To date, there are over 100 described variants of TEM-type ESBLs (Paterson & Bonomo, 2005). The majority of TEM-type resistance genes are ESBLs, however, several TEM derivatives showed a decreased affinity to β-lactamase inhibitors, with little hydrolytic activity towards the extended spectrum cephalosporins, and are thus not classified under the ESBL TEM-type derivatives (Paterson & Bonomo, 2005).

Cephalosporins are antibiotics that target penicillin-binding proteins. First, second, and third generation cephalosporins, and penicillin are hydrolyzed by TEM enzymes, but are less effective with ceftazidime and cefotaxime (oxyimino-cephalosporins). Variants of TEM showing improved hydrolytic activities towards the newer cephalosporins (fourth and fifth generation) have also emerged (Palzkill, 2018).

1.2.1.1.3 *Acinetobacter*-derived cephalosporinase (ADC) Genes. ADC genes are cephalosporinase-encoding genes active against cephalosporins including ceftazidime and cefotaxime but excluding carbapenems and cefepime (Jeon, et al., 2015; Rao, et al., 2020). AmpC β-lactamase cephalosporinases are usually active against carbapenems. However, few broad-spectrum enzymes, including a derivative of the ADC gene (ADC-68), hydrolyze carbapenems more readily than other derivatives (Oinuma, et al., 2019). *bla*$_{ADC25}$, another ADC derivative, was recently shown to induce resistance towards second and third generation cephalosporins (Freitas, et al., 2019). Resistance to penicillin, cefazolin, cefoxitin, cephalothin, and β-lactamase inhibitor-β-lactam combinations is mediated by chromosomal AmpC β-lactamase cephalosporinases detected in *Enterobacteriaceae*, as well as other organisms such as *A. baumannii* (Jacoby, 2009).

1.2.2 Plasmids

Many resistance phenotypes developed by *A. baumannii* occur through horizontal gene transfer (HGT) (Carattoli, 2013). To date, plasmid-typing techniques are categorized based on their conserved backbones that are associated with replication (Rep) or transfer (Mob) backbones (Bertini et al., 2010; Lean and Yeo, 2017).

Classifying *A. baumannii* plasmids based on replication was developed by Bertini et al. (2010), and the approach was designated as *Acinetobacter baumannii* PCR-based replicon typing (ABPBRT). Using ABPBRT revealed the presence of 19 homology groups (GR1-GR19) (Bertini et al., 2010). Phylogenetic analysis and alignment of a large group of *A. baumannii* rep proteins showed the presence of four other homology groups

designated and were designated as GR20-GR23 (Cameranesi et al., 2017; Lean and Yeo, 2017; Matos et al., 2019).

Four modules are part of *A. baumannii*'s plasmid architecture namely: replication, stability, mobilization/transfer, and accessory modules (Brovedan et al., 2020). In the replication module, an origin of replication (*oriV*) and the rep proteins that bind to the *ori* site including Rep-3 superfamily encoding genes are present (Garcillán-Barcia et al., 2011; Lean and Yeo, 2017). Stable maintenance of the *A. baumannii* plasmids are mediated primarily through a toxin-antitoxin (TA) system, and which is in the stability module (Salto et al., 2018). On the other hand, plasmid mobilization is thought to be one of the most important mechanisms mediating HGT (Furuya and Lowy, 2006). An *oriT*, in the transfer module, is enough for a plasmid to be classified as one that is mobilizable, since self-transmissible plasmids have support through their own helper functions mediating spread (Salto et al., 2018). Plasmids that are transmissible may also have their own relaxase as a means of transfer (Brovedan et al., 2020). Finally, the accessory modules in *A. baumannii* are well-characterized as they carry the resistance determinants (Carattoli, 2013).

An alternative classification approach was also recently developed by Brovedan et al. (2020), based on plasmid size distribution, and placing the plasmids in two groups: small (<20kb) or large plasmids (>20kb). Majority of sequenced *A. baumannii* genomes have plasmids less than 20kb in size (Hamidian et al., 2012), such as the Rep-3 superfamily and pRAY-type and its derivatives (Lean and Yeo, 2017). The Rep-3 superfamily plasmids were grouped under GR20 based on replicon typing and have *mob* genes (Lean and Yeo, 2017). The pRAY plasmid was first recovered from an *A baumannii* isolated from South

Africa, designated as SUN, and had an undefined clonal origin. SUN was isolated through carrying aminoglycoside resistance determinant (gentamycin, tobramycin, and kanamycin) and that was linked to the *aadB* gene (Segal and Elisha, 1999). pRAY plasmids do not contain a recognizable *rep* gene (Brovedan et al., 2020).

Most of these large plasmids, were found to belong to the GR6 homology group according to the rep-based classification (Bertini et al., 2010). These large plasmids have their own mobilization machinery and carry several resistance genes and were linked to the dissemination of resistance determinants against carbapenems and aminoglycosides (Hamidian et al., 2014).

1.2.3 Efflux Pumps

Efflux pumps in *A. baumannii* were classified under four groups: resistance-nodulation-division superfamily (RND), multidrug and toxic compound extrusion family (MATE), major facilitator superfamily (MFS), and small multidrug resistance family transporters (SMF) (Lee et al., 2017; and Lin and Lan, 2014).

1.2.3.1 RND Superfamily. Three-component pumps that have a wide range of substrates, and all have in common the periplasmic tripartite and the inner and outer membrane components (Marchand et al., 2004). AdeABC efflux system is chromosomally encoded (Dijkshoorn et al., 2007), and confers resistance to aminoglycosides (Magnet et al., 2001), tigecycline (Ruzin et al., 2007), and to non-fluoroquinolone antibiotics (Higgins et al., 2004). AdeABC efflux system expression is regulated by a two-component regulatory system, AdeRS (Marchand et al., 2004). Several mutations in *adeS* (sensor protein integrated

in the membrane) were linked to chloramphenicol, erythromycin, β-lactams, aminoglycosides, and tetracycline through inducing AdeABC overexpression (Sun et al., 2016).

AdeFGH (regulated by AdeL) and AdeIJK (regulated by AdeN) are also RND pumps that are associated with resistance to tigecycline (Damier-Piolle et al., 2008). AdeL and AdeN overproduction was linked to RND pumps mediated multidrug resistance (Coyne et al., 2010; Rosenfeld et al., 2012).

1.2.3.2 MATE Family. The chromosomally encoded *abeM* efflux pump (Dijkshoorn et al., 2007) (H^+-coupled multidrug efflux pump) induces resistance to fluoroquinolones and imipenem. Overexpression of *abeM* was associated with a decreased susceptibility to gentamicin, erythromycin, quinolones, kanamycin, trimethoprim, and chloramphenicol (Su et al., 2005), and imipenem (Lee et al., 2017).

1.2.3.3 MFS Superfamily. Efflux pumps such as CmlA, CraA, and ABAYE_0913 were each found associated with chloramphenicol resistance and TetA with tetracycline resistance (Fournier et al., 2006; Li L. et al. (2016); Roca et al., 2009; Ribera et al., 2003). On the other hand, AmvA, another efflux pump system, conferred resistance to several classes of antimicrobial agents including benzalkonium chloride, erythromycin, methyl viologen, and acriflavine (Rajamohan et al., 2010), while AbaF was linked to fosfomycin resistance (Sharma et al., 2016). A1S_1535, however, was linked to chloroxylenol, kanamycin, oxytetracycline, gentamycin, 1,10-phenanthroline

and chloramphenicol resistance, and A1S_2795, was the first MFS associated with sulfathiazole resistance.

1.2.3.4 SMF Family. AbeS, a chromosomally encoded efflux pump, was associated with resistance to many antimicrobial agents such as macrolides, quinolones, and chloramphenicol. The correlation between antimicrobial resistance and AbeS was further studied through deletion of the *abeS* and complementation analyses, with observed decrease in resistance to the three above mentioned antimicrobials (Srinivasan et al., 2009).

1.3 Virulence Factors

Many virulence determinants are associated with *A. baumannii*. These include outer membrane proteins, cell envelope factors, phospholipases, secretion systems, outer membrane vesicles, metal acquisition systems, motility, quorum sensing and formation of pellicles and biofilms.

1.3.1 Outer Membrane Proteins. OmpA is an outer membrane protein in *A. baumannii* linked to decrease in membrane permeability (Sugawara and Nikaido, 2012). OmpA, located on outer membrane vesicles or the cell surface of *A. baumannii*, was found to confer cytotoxicity when interacting with eukaryotic cells by adhering and binding to surface death receptors (Ahmad et al., 2016). Following internalization, the OmpA localized in the nucleus or the mitochondria (Choi et al., 2005, 2008; Rumbo et al., 2014), and induced proapoptotic signals, by activating proteins from the Bcl-2 family and releasing factors that induced apoptosis as well as cytochrome C (Choi et al., 2005). In the nucleus however, it induced host DNA degradation in a manner

similar to that of DNase I (Choi et al., 2007; Choi et al., 2008; Rumbo et al., 2014). OmpA additionally decreased the efficiency of complement-mediated killing via factor H binding by attaching to extracellular matrix proteins such as fibronectin (colonization of lung epithelia) (Kim et al., 2009; Smani et al., 2012).

1.3.2 Cell Envelope Factors. The lipopolysaccharide (LPS) found in the outer membrane of Gram-negative bacteria is another virulence factor. The LPS in *A. baumannii* induced decreased susceptibility to human serum, increasing the survival potential in the host. In animal models, the LPS was linked to proinflammatory responses (Knapp et al., 2006), in addition to its role in attachment and colonization (Haseley et al., 1997).

The capsule, another important virulence determinant in *A. baumannii,* is made of tightly grouped repetitive sugars protecting the organism against harsh conditions such as disinfection and dryness, phagocytosis, and antimicrobial agents (Singh et al., 2019; Geisinger and Isberg, 2015). There are more than 100 capsular types in *A. baumannii* (Kenyon and Hall, 2013).

1.3.3 Phospholipases. Enzymes involved in the hydrolytic activities against human cell membrane phospholipids. The two main phospholipases that were studied in *A. baumannii* are Phospholipase C and D. Phospholipase D helps the pathogen to survive in the serum while phospholipase C induces toxicity to epithelial cells (Camarena et al., 2010).

1.3.4 Autotransporters and Secretion Systems. *Acinetobacter* trimeric autotransporter adhesin (Ata), a trimeric autotransporter, was identified as being a member of the type V secretion system (T5SS). The T5SS facilitates adherence to collagen by AtaA and biofilm formation (Weber et al., 2017). The type II secretion system (T2SS) on the other hand, which is the main terminal branch of the general secretion pathway (sec), is used to deliver many effector proteins; sec equips the proteins that need to be secreted with an N-terminal secretion signal through the inner membrane of the pathogen. The sec general secretory pathway facilitates translocation or integration of proteins in the cytoplasmic membrane (Tsirigotaki et al., 2017). The signal peptide is then removed as the mature proteins are folded and released into the periplasmic space (Sandkvist, 2001).

The Type VI secretion system (T6SS) helps bacteria in creating an ideal niche by eradicating other bacteria. Its main function is to inject toxins into the opposing bacteria, inhibiting main cell functions (Hachani et al., 2016). T6SS was also detected in *A. baumannii* and mediates the release of the effector proteins including nucleic acids, peptidoglycans, and phospholipases (Shneider et al., 2013) through the action of hemolysin co-regulated protein (Hcp) and valine glycine repeat protein (VgrG) which combine to create the tail tube and the capping spike, respectively, (Leiman et al., 2009). These effector proteins released by *A. baumannii* T6SS usually provide self-protection against peptidoglycan hydrolases and nucleases secreted to target other organisms (Elhosseiny and Attia, 2018).

1.3.5 Outer Membrane Vesicles (OMV). Originally**,** *A. baumannii* OMVs were thought to be the secretion platform for outer membrane protein A (OmpA). There are two types of OMVs based on the size: small vesicles (less than 100nm) and large (200nm-500nm). Both types were found to bud from the surface, with the large ones only budding through the septa of dividing bacteria (Weber et al., 2017). The small OMVs however, are formed from membrane protrusions after losing the OM lipoprotein-peptidoglycan interaction (Roier et al., 2016). OMVs have a protective role from the host's innate immune system (Beceiro et al., 2013). In murine pneumonia models, OMV was found to mediate the translocation of OmpA to the cell's cytoplasm (Weber et al., 2017; Jin et al., 2011). *In vitro* studies of *A. baumannii* OMVs exhibited several enzymatic activities such as production of phospholipase C to induce toxicity to epithelial cells, hemolytic activity, and leukotoxicity (the capability of changing the shape of a granulocyte by lysing the cell and introducing toxins into the cell membrane) (Jha et al., 2017). Once inside the host, a proinflammatory response and host cell cytotoxicity are elicited (Li et al., 2018).

1.3.6 Micronutrient Acquisition Systems. Five different types of siderophores (iron-chelating molecules) are used by the pathogen for iron acquisition in the host (Morris, et al., 2019). Acinetobactin is the most common and well-characterized siderophore of *A. baumannii*. Acinetobactin increases persistence within the host epithelial cells leading to cell damage and was

abundant in clinical MDR isolates of *A. baumannii* as opposed to isolates that were avirulent (Ali et al., 2017).

Calprotectin chelates divalent metal ions such as manganese, zinc, and others, inducing bacterial death through sequestering the metals, which is also known as nutritional immunity. Survival through calprotectin and nutritional immunity is through a number of factors such as ZigA and ZnuABC (Nairn et al., 2016). ZigA, a metallochaperone with GTPase activity required for growth under zinc-limiting conditions, and ZnuABC (zinc transporter) are part of a highly efficient system for scavenging zinc (Moore et al., 2014; Nairn et al., 2016). In a murine pneumonia model, calprotectin growth inhibition was associated with ZnuABC and ZigA upregulation (Hood et al., 2012). Loss of ZigA was also linked to the decrease in the dissemination from the lungs following infection in a murine pneumonia model (Nairn et al., 2016).

1.3.7 Motility. *A. baumannii* is a non-motile but can move through a process known as twitching motility using the type IV pili (Wilharm et al., 2013). Surface-associated motility was also detected in *A. baumannii*. This form of motility resembles the flagellum-dependent swarming motility of *Pseudomonas aeruginosa* and involves the formation of 1,3-diaminopropane (DAP), production of lipooligosaccharide (LOS), and quorum sensing (Kearns, 2010). The DAP may function as a signaling molecule regulating surface motility through quorum sensing (Harding et al., 2018).

1.3.8 Quorum Sensing. Quorum sensing mediates cell to cell communication. It is a signaling mechanism that allows coordination between the microorganisms using autoinducers (signaling molecules) (Whiteley et al., 2017). Antibiotic production, plasmid transfer, motility, and biofilm formation are all enhanced when cells are in close proximity (Bhargava et al, 2010; Whiteley et al., 2017).

Acyl homoserine lactoses (AHLs) are autoinducers and are hormone-like compounds that act to induce biofilm formation and motility (Saipriya et al., 2020). AbaI-AbaR is a two-component system and is part of quorum sensing (Tang et al., 2020). AbaI is an autoinducer synthase that generates AHL signal molecules. AHL binds to AbaR (receptor protein), which will bind to a lux-box promoter and further regulate expression of other genes such as the ones linked to the *csu* pili and surface associated motility (Monem et al., 2020). AbaR binding to the AHL, activates AbaI and which in turn mediates more AHL production (Subhadra et al., 2016). The AbaI-AbaR complex as a result, by activating AHL production, controls biofilm formation (Saipriya et al., 2020) and multidrug resistance, by upregulating *AbaI* and *AbaR* genes, as well as efflux pump genes and biofilm formation genes in *A. baumannii* clinical strains (Subhadra et al., 2016; Tang et al., 2020).

1.3.9 Biofilm Formation. Biofilms are groups of one or more types of organisms growing together on different surfaces. Biofilm formation could facilitate resistance against immune system clearance, antibiotics, and desiccation (Doi et al., 2015). In *A. baumannii* host-pathogen interactions could be enabled

through biofilm formation (Greene et al., 2016). Biofilm associated proteins (BAPs) are found on the cell surface and allow the formation of the biofilm matrix by attaching to abiotic surfaces and mediating intercellular adhesion in response to harsh environmental conditions (Loehfelm et al., 2008; Fattahian et al., 2011).

Moreover, BfmRS is a two-component (TC) regulatory system (*bfmR* – response regulator, and *bfmS* – sensor kinase) that controls the expression of the chaperone/usher pilus system (Csu). Csu pili mediate attachment to abiotic surfaces and are protein filaments produced through the CsuAB-A-B-C-D-E chaperone-usher secretion system (Tomaras et al. 2008; Gaddy and Actis, 2009). The BfmRS TC system mediates exopolysaccharide (EPS) formation to protect *A. baumannii* from antibiotics by regulating the K locus gene expression (Greene et al., 2016). *bfmS* deletion led to an increase in resistance to ciprofloxacin and a decrease to imipenem, while *bfmR* deletion led to an increase in resistance to colistin and meropenem (Russo et al., 2016).

1.3.10 Pellicle Formation. The pellicle is bacterial aggregation or clustering that occurs on liquid suspensions at the air-liquid interface. It floats on the surface and requires an increased organization as there is no solid surface for adherence and motility. The air-liquid interface is an advantageous niche providing nutrients from the liquid and oxygen from the air (Chabane et al., 2014), and allows for bacterial transmission (Krasauskas et al., 2019).

Krasauskas et al. (2019) also showed that BfmR promotes pellicle formation (Krasauskas et al., 2019).

1.4 Aims

In Lebanon around 77-95% of *A. baumannii* isolates recovered from clinical settings were carbapenem resistant, with $bla_{\text{OXA-23-like}}$, $bla_{\text{OXA-24}}$, and $bla_{\text{OXA-58}}$ being among the most commonly found resistance genes (Hammoudi et al., 2015; Al Atrouni et al., 2016; Dagher et al., 2019; Moghnieh et al., 2019). More recently however, Makke et al. (2020) revealed 95% resistance to carbapenems, along with resistance to other antimicrobial agents including quinolones, aminoglycosides, and cephalosporins, while Kanj et al. (2018) showed an increase in $bla_{\text{OXA-23-like}}$ and $bla_{\text{OXA-51-like}}$ resistance determinants within two separate outbreaks.

Outbreaks linked to international clonal lineage ST2 were commonly reported in Lebanon with high resistance profiles due to the increase in carbapenem treatment (Ballouz et al., 2017), with 60-90% of *A. baumannii* isolates collected in Lebanon over recent years belonging to the ST2 clone (Jamal et al., 2018; Osman et al., 2020). On the other hand, Rafei et al. (2015), studied *A. baumannii*'s prevalence in animals and environmental sources. International clones 1 and 10 were detected in water and animals revealing that animals could be a potential reservoir for *A. baumannii* and contribute to the spread of new and emerging carbapenemase genes.

The aims of this study are to:

- Investigate and predict the phenotypic antibiotic resistance profiles of collected isolates.

- Determine the biofilm and pellicle formation capabilities of the collected isolates.
- Perform plasmid typing to characterize the plasmids present in the isolates.
- Detect the presence of different resistance encoding genes through *in silico* analysis.
- Examine the virulence factors present in the *A. baumannii* isolates collected.
- Perform comparative genome analysis amongst several strains of *A. baumannii*.
- Use genome sequence data to construct a genome-based phylogeny to identify different clusters.
- Examine the phylogenetic relatedness of the isolates using PFGE, pan-genome analysis, and recombination hotspot analysis, demonstrating a probable outbreak.

Chapter Two

Materials and Methods

2.1 Specimen collection

Between January and November 2020, a total of 46 *A. baumannii* isolates were collected from clinical settings in Lebanon. The isolates were designated as ABS 1– 48. ABS 4 and 8 were excluded from the study. The age range of the patients was 15-93 years old, distributed equally between males and females (Table 1).

Table 1. Demographic information. DTA, deep tracheal aspirate; M, Male; F, Female. A dash is designated where the information was unavailable.

Label	Site of Infection	Date	Sample ID	Age	Sex	Accession Number
ABS1	Wound	6 January	1	93	M	PRJNA700083
ABS2	Urine	15 January	2	34	M	PRJNA700120
ABS3	Pus	17 January	3	58	M	-
ABS5	Wound	18 January	4	61	M	-
ABS6	-	10 January	5	-	F	PRJNA700122
ABS7	-	10 January	6	-	M	PRJNA700123
ABS9	-	10 January	7	-	M	PRJNA700127
ABS10	*DTA	16 January	8	58	F	PRJNA719634
ABS11	-	-	9	-	-	-
ABS12	Blood, DTA	15 February	10	75	F	PRJNA719637

ABS13	Nasal	4 June	11	18	F	PRJNA719641
ABS14	DTA	7 June	12	87	F	PRJNA719657
ABS15	Stool	7 June	11	18	F	PRJNA719658
ABS16	Blood	7 June	13	80	F	PRJNA719659
ABS17	DTA	7 June	14	59	F	PRJNA719660
ABS18	DTA	22 June	15	80	F	PRJNA719663
ABS19	Wound	15 June	16	73	M	PRJNA719664
ABS20	Stool	13 June	17	70	F	PRJNA719665
ABS21	Wound	15 June	16	73	M	PRJNA719666
ABS22	Stool	12 June	18	90	F	-
ABS23	Wound	12 June	19	80	F	PRJNA719667
ABS24	Wound	26 June	16	73	M	PRJNA719643
ABS25	Stool	8 July	20	15	M	-
ABS26	Blood	18 July	21	71	M	PRJNA719644
ABS27	Blood	3 August	22	74	F	-
ABS28	Wound	25 July	23	87	M	PRJNA719646
ABS29	Wound	24 July	24	84	F	-
ABS30	Wound	24 July	25	88	M	PRJNA719648
ABS31	DTA	8 August	26	67	M	-
ABS32	DTA	19 August	27	69	M	-
ABS33	DTA	19 August	28	90	F	-
ABS34	DTA	19 August	29	83	F	-
ABS35	DTA	14 August	30	87	M	-

ABS36	DTA	29 August	31	82	F	-
ABS37	Urine	5 September	32	89	M	-
ABS38	Stool	4 September	33	27	F	-
ABS39	Sputum	8 September	34	27	F	-
ABS40	Blood	29 September	35	70	F	-
ABS41	DTA	30 September	36	59	M	-
ABS42	DTA	22 September	37	80	F	-
ABS43	DTA	14 September	38	31	M	PRJNA719650
ABS44	DTA	11 October	39	80	M	-
ABS45	Wound	5 October	40	73	F	-
ABS46	Stool	26 October	41	80	M	-
ABS47	DTA	19 October	42	68	M	-
ABS48	DTA	23 October	43	72	M	-

*DTA, deep tracheal aspirate; M, Male; F, Female. (-): missing information.

2.2 Antimicrobial susceptibility testing

Disk diffusion assay, following the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2019), on Mueller-Hinton Agar was used to study resistance patterns. Twelve antibiotics covering 8 main classes were tested and included: aminoglycosides (gentamicin, tobramycin and amikacin), quinolones (ciprofloxacin), glycylcyclines (tigecycline), polymyxins (colistin), cephalosporins (ceftazidime and cefepime), carbapenems (imipenem and meropenem), penicillin with β-lactamase inhibitor (piperacillin/tazobactam), and sulfonamides (Sulfamethoxazole+Trimethoprim).

2.3 Bacterial DNA Extraction

Isolates were cultivated on Trypticase Soy Agar (TSA) and single colonies were used for DNA extraction using the Nucleospin® Tissue kit (Macherey-Nagel, Germany) following the manufacturer's instructions.

2.4 Plasmid Typing

Replicon typing was done following the procedure developed by Bertini et al. (2010) in which 19 homology groups could be determined from the 27 known *A. baumannii* replicase genes (*rep*) and based on nucleotide sequence similarities. Six multiplex PCR assays were needed to deduce *rep* types and using the primers in Table 2.

Table 2. Multiplex PCR assays, primer sequences, and expected amplicon sizes.

Multiplex	Group	Primer	Sequence (5'-3')	Amplicon Size (bp)
1	GR1	GR1FW	CATAGAAATACAGCCTATAAAG	330
		GR1RV	TTCTTCTAGCTCTACCAAAAT	
	GR2	GR2FW	AGTAGAACAACGTTTAATTTTATTGGC	851
		GR2RV	CCACTTTTTTTAGGTATGGGTATAG	
	GR3	GR3FW	TAATTAATGCCAGTTATAACCTTG	505
		GR3RV	GTATCGAGTACACCTATTTTTTGT	
2	GR5	GR5FW	AGAATGGGGAACTTTAAAGA	220
		GR5RV	GACGCTGGGCATCTGTTAAC	
	GR18	GR18FW	TCGGGTTATCACAATAACAA	676
		GR18RV	TAGAACATTGGCAATCCATA	
	GR7	GR7FW	GAACAGTTTAGTTGTGAAAG	885

		GR7RV	TCTCTAAATTTTTCAGGCTC	
3	GR9	GR9FW	GCAAGTTATACATTAAGCCT	191
		GR9RV	AAAAATAAACGCTCTGATGC	
	GR4	GR4FW	GTCCATGCTGAGAGCTATGT	508
		GR4RV	TACGTCCCTTTTTATGTTGC	
	GR11	GR11FW	GGCTATTCAAAACAAAGTTAC	852
		GR11RV	GTTTCCTCTCTTACACTTTT	
4	GR12	GR12FW	TCATTGGTATTCGTTTTTCAAAACC	165
		GR12RV	ATTTCACGCTTACCTATTTGTC	
	GR10	GR10FW	TTTCACTAGCTACCAACTAA	371
		GR10RV	ACACGTTGGTTTGGAGTC	
	GR13	GR13FW	CAAGATCGTGAAATTACAGA	780
		GR13RV	CTGTTTATAATTTGGGTCGT	
5	GR8	GR8FW	AATTAATCGTAAAGGATAATGC	233
		GR8RV	GACATAGCGATCAAATAAGC	
	GR14	GR14FW	TTAAATGGGTGCGGTAATTT	622
		GR14RV	GCTTACCTTTCAAAACTTTG	
	GR15	GR15FW	GGAAATAAAAATGATGAGTCC	876
		GR15RV	ATAAGTTGTTTTTGTTGTATTCG	
6	GR16	GR16FW	CTCGAGTTCAGGCTATTTTT	233
		GR16RV	GCCATTTCGAAGATCTAAAC	
	GR17	GR17FW	AATAACACTTATAATCCTTGTA	380
		GR17RV	GCAAATGTGACCTCTAATATA	

	GR6	GR6FW	AGCAAGTACGTGGGACTAAT	662
		GR6RV	AAGCAATGAAACAGGCTAAT	
	GR19	GR19FW	ACGAGATACAAACATGCTCA	815
		GR19RV	AGCTAGACATTTCAGGCATT	

2.5 Pulse Field Gel Electrophoresis (PFGE)

A. baumannii DNA plugs were prepared according to Seifert et al. (2005). Briefly, the restriction enzyme ApaI (Thermo Fisher Scientific, MA, USA) was used to digest bacterial DNA for 1.5h at 37°C. On 1% Seakem Gold gel, the DNA fragments were separated for 14h using Bio-Rad Laboratories CHEF DR-III system (Bio-Rad Laboratories, Inc, CA, USA) with initial switch time of 7s and final switch time of 20s. *Salmonella enterica* subsp. *enterica serovar Braenderup* (ATCC® BAA664TM) was used a s reference using XbaI restriction enzyme (Thermo Fisher Scientific, MA, USA). Gels were stained using ethidium bromide and were visualized under UV light. BioNumerics software version 7.6.1 (Applied Maths, St-Martens-Latem, Belgium) was used to analyze fingerprints and generate dendograms. The pulsotypes (PT) were clustered according to the band-based coefficient in BioNumerics with an optimization and tolerance of 1.5%. A difference in three or more bands was used as a cutoff to cluster isolates into PTs (Tenover et al., 1995).

2.6 Crystal-violet biofilm formation assay

Crystal violet biofilm formation assay was performed on all the isolates as previously described by Singh et al., (2020), with some modifications. Briefly, bacterial cultures grown overnight in Tryptone Soy Broth (TSB) were equilibrated to an optical density

(OD) of 0.02 at 600nm, and 100 µL from each inoculum was transferred to each well of a non-treated U-bottom PVC 96-well plate (Corning Inc., NY, USA). The plates were sealed with paraffin and incubated in a humid environment at 37°C for 24h. To remove planktonic cells the wells were washed with 150µL of autoclaved water followed by the addition of 15µL of 0.1% w/v crystal-violet stain. The stained wells were left for 30min, followed by removal of the stain, and washing with 150µL of autoclaved water. 200µL of 96% ethanol was added to each well for de-staining, and this was left for 30 min. a fresh 96-well flat bottom plate (Corning Inc., NY, USA) was obtained and 125µL from each well was transferred to new wells. Absorbance was measured at 590nm. The experiment was done with three replicates for each isolate including a negative control. The biofilm strength was categorized as non-adherent ($OD \leq ODc$); weakly adherent ($ODc < OD \leq 2$ x ODc); moderately adherent ($2x\ ODc < OD \leq 4 \times ODc$); or strongly adherent ($4 \times ODc < OD$). The optical density cut-off value (ODc) = average OD of negative control + 3x standard deviation (SD) of the negative control (Bardbari et al., 2017).

2.7 Pellicle formation assay

Pellicle formation assay was performed as described by Chabane et al. (2014) with some modifications. Briefly, bacterial cultures grown overnight in Tryptone Soy Broth (TSB) were equilibrated to an OD of 0.02 at 600nm. Next, 1mL of the new inoculum was transferred to a glass tube (18mm x 150mm) containing 5mL of Luria broth (LB) supplemented with 1% glucose, followed by incubation at 37°C in the dark for 72h. The formation of a pellicle was seen as an opaque layer at the air-liquid surface and classified as thick (++), moderate (+) or absent (0) (Chabane et al., 2014).

2.8 Whole-genome sequencing and assembly

A total of 22 isolates were chosen for WGS. These isolates were chosen based on highest purity and concentration of DNA after DNA extraction. Genomic libraries were constructed using the Nextera XT DNA library preparation kit with dual indexing (Illumina). The libraries were sequenced on an Illumina MiSeq with 500 bp x 2 read length. *de novo* genome assembly was performed using Spades Genome Assembler Version 3.9.0 (Bankevich et al. 2012), reads were trimmed using Trimmomatic (Bolger et al., 2014), and the quality was assessed using Samtools (Li et al., 2009), BedTools (Quinlan and Hall, 2010) and BWA-MEM (Li, 2013) for 10 of the isolates. Reference-based assembly was performed on 12 isolates using BioNumerics software version 7.6.1 (Applied Maths, St-Martens-Latem, Belgium) with the default settings.

2.9 Genome annotation

RAST server was used to annotate the assembled genomes (http://rast.nmpdr.org) (Aziz et al., 2012). Multi Locus Sequence Typing (MLST) was performed using the MLST 2.0 server on the CGE database (CGE- www.genomicepidemiology.org) (Larsen et al., 2012). Antibiotic resistance genes were identified using ResFinder (CGE- www.genomicepidemiology.org) at a 90% identity (ID) threshold (Zankari et al., 2012). The virulence factors database was obtained using *AB* viresDB (https://acba.shinyapps.io/ABviresDB/) (Xie et al., 2020), and determined in the sequenced genomes using MyDBFinder 2.0 (CGE- www.genomicepidemiology.org) and Virulence Factor Database (VFDB) (http://www.mgc.ac.cn/VFs/) (Liu et al., 2019). Capsule and lipopolysaccharide serotype predictions were performed using Bautype web-

based server for *A. baumannii* locus typing (http://bautype.net/Acinetobacter_baumannii/home/) (Hua et al., 2020).

2.10 wg-SNP-based phylogenetic analysis

SNP-based analysis was performed on CSIPhylogeny v1.4 (CGE-www.genomicepidemiology.org) using default settings (Kaas et al., 2014). The platform calls and filters SNPs performs a site validation, and deduces a phylogeny based on the concatenated alignment of the SNPs. The genomes (both *de novo*-assembled and reference-based assembled) were aligned using CSIPhylogeny with default parameters and *A. baumannii* ACICU as a reference genome (Accession # NZ_CP031380). The phylogenetic tree was created using iTOL (https://itol.embl.de/) (Letunic and Bork, 2019). The tree being unrooted and branch lengths hidden.

2.11 Pan-genome analysis

Genomes were annotated using Prokka version 1.13 with a similarity cutoff e-value of 10^{-6} and minimum contig size of 200 bp (Seemann, 2014). Annotated GFF3 files were piped into Roary version 3.12.0 choosing a minimum blastp identity of 95 and a core gene prevalence of >99% in all the isolates (Page et al., 2015). A maximum-likelihood phylogenetic tree based on the core genome alignment was constructed using RaxML (Seemann). The resulting phylogenetic tree, along with the recombination hotspots, were visualized on Phandango V1.1.0 (Hadfield et al., 2018).

2.12 Recombination analysis

For the 10 *de novo* assembled genomes, the core genome alignment file from Snippy was used as input into Gubbins version 2.2.1 to identify recombination events in the core genes (Stamatakis, 2006), and a maximum-likelihood tree was generated using RAxML

(Seemann). It was then used to visualize a clean core genome SNP-based tree and identify recombination hotspots with high SNP densities. The resulting phylogenetic tree, along with the recombination hotspots were visualized on Phandango V1.1.0 (Hadfield et al., 2018).

Chapter Three

Results

3.1 Susceptibility profiling

Antibiotic susceptibility testing was performed on all 46 *A. baumannii* isolates. Resistance to the aminoglycosides, gentamicin, amikacin, and tobramycin, was observed in 95.7% (n=44), 86.9% (n=40), and 95.7% (n=44), of the isolates, respectively. Similar resistance profiles were seen with the β-lactam/β-lactamase inhibitor piperacillin/tazobactam (91.3%, n=42) as well as with the tested cephalosporins (ceftazidime, cefepime; 93.5%, n=41), and carbapenems (imipenem 93.5%, n=43, meropenem 84.8%, n=39). ABS3, ABS9, ABS42 and ABS46 showed intermediate resistance to piperacillin/tazobactam, while ABS3 to ceftazidime and cefepime. Quinolone and fluoroquinolone resistance was observed in 93.5% (n=43) and 89.1% (n=41) of the isolates, respectively. All isolates were susceptible to colistin and the lowest detected resistance was against tigecycline detected (17.4%; n=8). Based on the above and according to Magiorakos et al. (2012), 93.5% (n=43) of the isolates were extensively drug resistant (XDR) being resistant to at least one antibiotic in all except two or less antimicrobial categories. All isolates can be considered carbapenem resistant (Figure 1).

3.2 Molecular typing

In silico multi-locus sequence typing (MLST) was performed on all the sequenced isolates through determining the allelic profiles of seven house-keeping genes (*cpn60*, *fusA*, *gltA*, *pyrG*, *recA*, *rplB*, and *rpoB*). Among the 22 isolates, 95.45% (n=21) had the

sequence type ST2 with an allelic profile of 2-2-2-2-2-2-2. The remaining (4.54%; n=1) were typed as ST78; allelic profile: 25-3-6-2-28-1-29 (Figure 2).

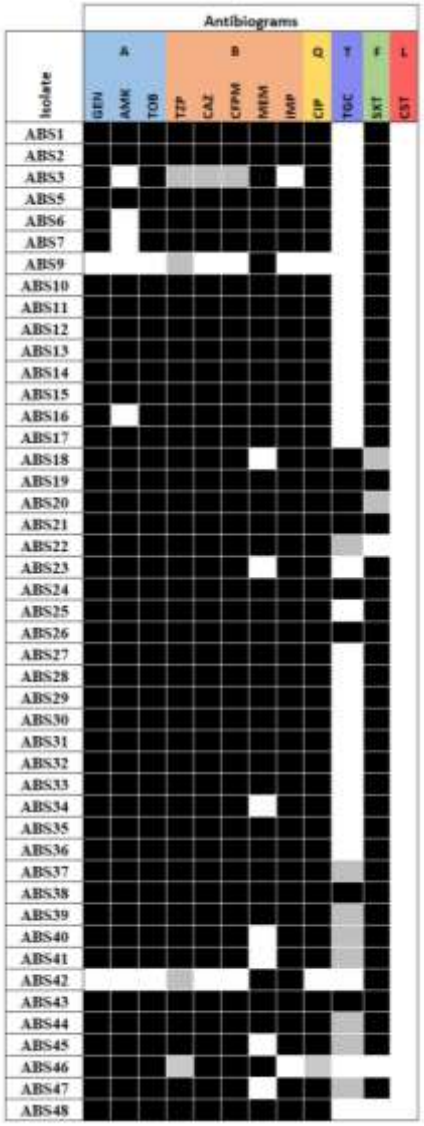


Figure 1. Phenotypic determination of antimicrobial susceptibility using the disc-diffusion assay. A: aminoglycosides, B: β-lactams, Q: fluoroquinolones, T: tetracyclines, F: folate pathway inhibitors, L: lipopeptides. Antibiotics: GEN: gentamicin, AMK: amikacin, TOB: tobramycin, TZP: piperacillin/tazobactam, CAZ: ceftazidime, CFPM: cefepime, IMP: imipenem, MEM: meropenem, CIP: ciprofloxacin, TGC: tigecycline, SXT: sulfamethoxazol-trimethoprim, CST: colistin. Black: resistant; Grey: intermediate; White: sensitive.

3.3 Resistance genes

In silico detection of resistance determinants was done using ResFinder. Four different aminoglycoside inactivating enzymes and their variants were detected. Aminoglycoside acetyltransferases (AAC): *aac(6')-Ib-cr* and *aac(6')-Ian* were found in 18% (n=4) and 4.5% (n=1) of the isolates, respectively. Aminoglycoside phosphotransferases (APH) however, and its variants were also detected with (*aph(3'')-Ib* and *aph(6)-Id* being the most prevalent (n=9; 40.9% (n=9) for each).

The intrinsic β-lactam resistance genes including bla_{ADC25} (*Acinetobacter*-Derived Cephalosporinase) and the bla_{OXA66} (a variant of bla_{OXA51}) were also among the common resistance determinants being detected in 90.9% (n=20) and 95.5% (n=21) of the isolates, respectively. Several additional carbapenem-hydrolyzing Class D β-lactamases were detected such as bla_{OXA260} and bla_{OXA480} ,bla_{TEM1D}, a class A β-lactamases (Figure 2).

3.4 Homology group typing

A. baumannii PCR-based replicon typing approach (AB-PBRT) was used to determine the homology group. Majority of the sequenced isolates were typed as GR2 (86.3%; n=19) and (50%; n=11) GR6 (Figure 2).

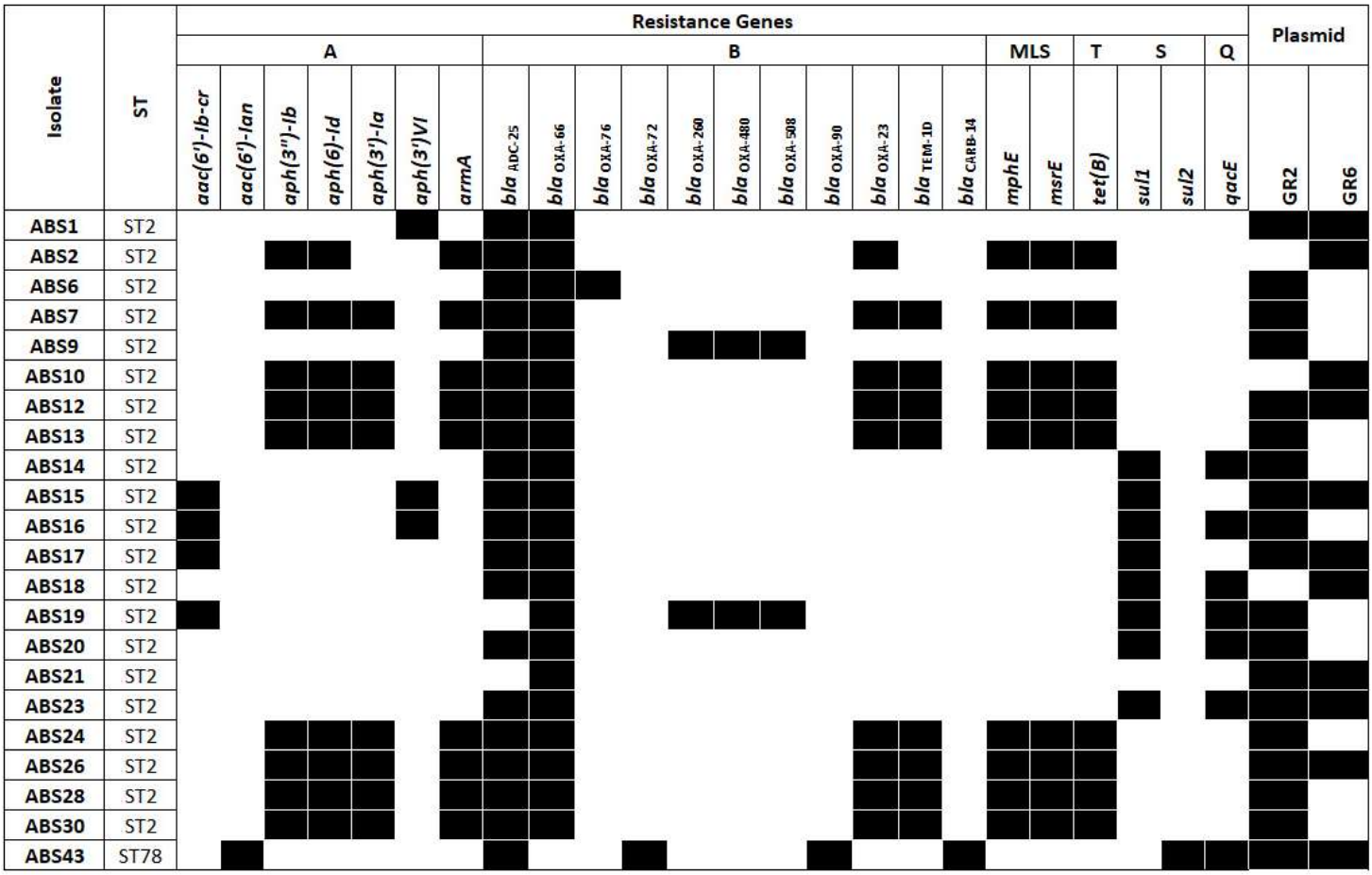


Figure 2. Resistance determinants and homology groups detected by *A. baumannii* PCR-based replicon typing approach (AB-PBRT). Antibiotic resistance: A: aminoglycoside resistance genes, B: β-lactam resistance genes, S: sulfonamide resistance genes, MLS: macrolide-lincosamide-streptogramin resistance genes, T: tetracycline resistance genes, Q: quaternary ammonium compounds. Black: present; White: absent.

3.5 Virulence factors

Virulence and pathogenesis in *A. baumannii* can be attributed to several different factors and mechanisms including: adherence, biofilm formation, enzymes, iron uptake mechanisms, regulatory mechanisms, and immune evasion. Biofilm formation could be linked to several different factors and which were detected in the isolates undertaken in this study and included: *adeF* (membrane fusion protein), *adeG* (inner membrane transporter), and *adeH* (outer membrane channel protein) belonging to the RND-type adeFGH efflux system (CARD, https://card.mcmaster.ca/), *bap* (biofilm-associated protein) cell surface protein, β-1,6-linked *N*-acetylglucosamine (PNAG) surface polysaccharide genes (*pgaA, pgaB, pgaC*, and *pgaD*), and regulatory factors (*bfmR* – response regulator, and *bfmS* – sensor kinase) (Gaddy and Actis, 2009). Outer membrane protein A (OmpA), the enzymes phospholipase C (*plcC*) and D (*plcD*), and quorum sensing genes *abaI* (acyl-homoserine-lactone (AHL) synthase) and *abaR* (interacts with the AHL produced by *abaI* and controls its gene expression) were also among the detected virulence determinants (Figure 3).

3.6 Capsule and lipopolysaccharide typing

Major virulence determinants found in *A. baumannii* are the capsule and the lipopolysaccharides (Singh et al., 2019). Capsular typing revealed 22 types and KL39 (36.4; n=8) and KL9 (36.4%; n=8) were the two most common. Moreover, OCL1, represented by two variants OCL1c and OCL1d, lipopolysaccharide was detected in all except ABS9 and ABS19 which instead had OCL11 (Figure 3).

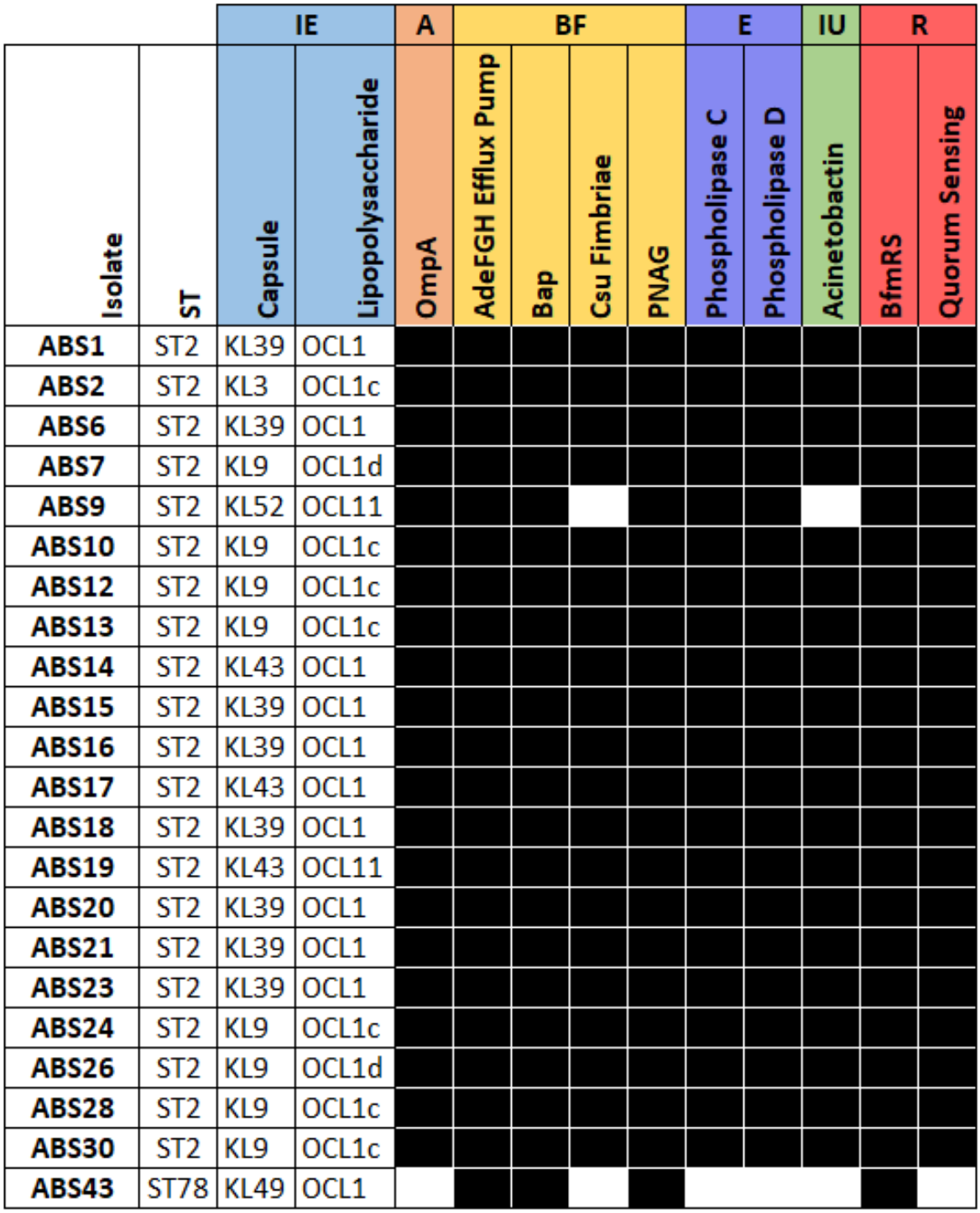


Figure 3. Virulence determinants in *A. baumannii*. ST: sequence type; IE: immune evasion as linked to the type of capsule and the lipopolysaccharide; A: adherence; OmpA: outer membrane protein A; BF: biofilm formation including AdeFGH Efflux pump, Bap: biofilm associated proteins, Csu fimbriae: chaperone-usher pili, and PNAG: β-1,6-linked *N*-acetylglucosamine; E: Phospholipase C and D; IU: iron uptake Acinetobactin; R: regulation including two-component regulatory system BfmRS and quorum sensing. Black: present; White: absent.

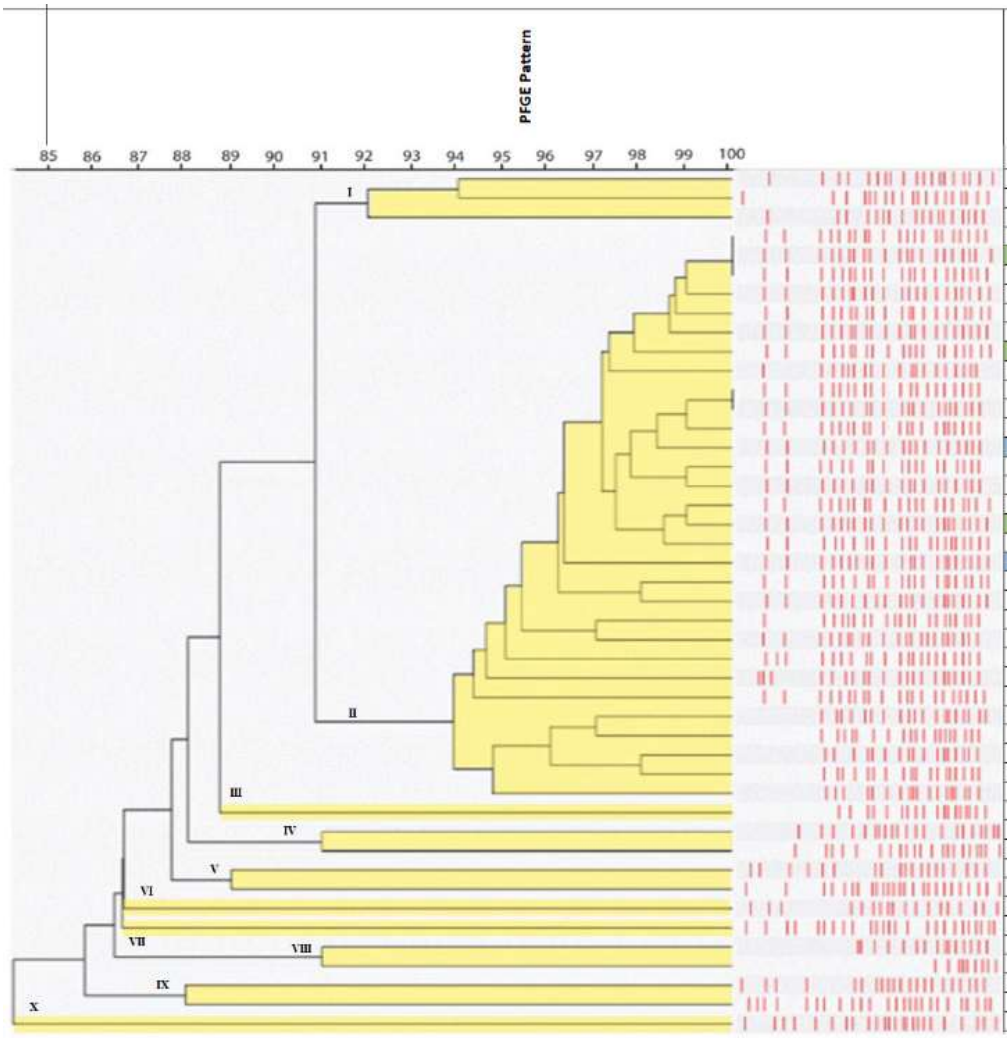
3.7 PFGE

Twenty-six different pulsotypes (PTs) were identified using the difference in three or more bands as the cutoff for each cluster (Tenover et al., 1995). A percentage similarity of greater than 83% was seen in all the isolates. Grouping revealed ten clusters, which were designated as cluster I–X. Cluster II included all the isolates with sequence type ST2, except for ABS9. Isolates recovered from the same patient (ABS19, 21, and 24; ABS13 and 15) exhibited similar PFGE patterns. We could not detect any pattern or correlation between the PT, sequence type, or time of isolation (Figure 4).

3.8 Biofilm and pellicle formation assays

The biofilm, and based on strength, could be categorized as being: non-adherent ($OD \leq ODc$), weakly adherent ($ODc < OD \leq 2 \times ODc$), moderately adherent ($2x\ ODc < OD \leq 4 \times ODc$), or strongly adherent ($4 \times ODc < OD$). The optical density cut-off value (ODc) = average OD of the negative control + 3x the standard deviation (SD) of the negative control (Bardbari et al., 2017). Among the 46 tested isolates, all except five (ABS44 – ABS48) were biofilm formers, with the majority categorized under moderate adherence., with the average detected OD (590nm) being 1.326 ± 0.467.

On the other hand, pellicle formation, an opaque layer at the air-liquid surface, was also classified as thick (++), moderate (+) or absent (0) (Chabane et al., 2014). Seven isolates were observed to have thick pellicles, 25 were moderate, and 14 were negative (Figure 4).



Isolate	PT	ST	Biofilm	Pellicle	Patient Number	Sex	Age	Specimen Type	Date of Isolation
ABS41	1	-	++	+	35	M	59	DTA	30/9/2020
ABS44	2	-	-	+	38	M	80	DTA	11/10/2020
ABS40	3	-	+	+	34	F	70	Blood	29/9/2020
ABS20	4	2	++	-	16	F	70	Stool	13/6/2020
ABS21	5	2	++	+	15	M	73	Wound	15/3/2020
ABS23	5	2	++	-	18	F	80	Wound	12/6/2020
ABS17	5	2	++	-	14	F	59	DTA	7/6/2020
ABS26	5	2	++	-	20	M	71	Blood	18/7/2020
ABS25	5	-	++	+	19	M	15	Stool	8/7/2020
ABS24	5	2	+	-	15	M	73	Wound	26/6/2020
ABS2	6	2	++	+	2	M	34	Urine	15/1/2020
ABS1	6	2	++	+	1	M	93	Wound	6/1/2020
ABS12	6	2	++	+	10	F	75	DTA	15/2/2020
ABS10	6	2	++	+	8	F	58	DTA	16/2/2020
ABS13	6	2	++	+	11	F	18	Nasal	4/6/2020
ABS11	6	-	++	+	9	-	-	-	-
ABS14	6	2	++	+	12	F	87	DTA	7/6/2020
ABS16	7	2	+	-	13	F	80	Blood	7/6/2020
ABS19	7	2	++	+	15	M	73	Wound	15/6/2020
ABS39	8	-	++	+	33	F	27	Sputum	8/9/2020
ABS15	9	2	++	++	11	F	18	Stool	7/6/2020
ABS29	9	-	++	-	23	F	84	Wound	24/7/2020
ABS30	9	2	++	+	24	M	88	Wound	24/7/2020
ABS5	10	-	++	++	4	M	61	Wound	18/1/2020
ABS7	11	2	++	++	6	M	-	-	10/1/2020
ABS42	12	-	++	+	36	F	80	DTA	22/9/2020
ABS6	12	2	++	+	5	F	-	-	10/1/2020
ABS22	12	-	++	-	17	F	90	Stool	12/6/2020
ABS28	13	2	++	-	22	M	87	Wound	25/7/2020
ABS38	14	-	++	+	32	F	27	Stool	4/9/2020
ABS34	15	-	++	+	28	F	83	DTA	19/8/2020
ABS37	15	-	++	++	31	M	89	Urine	5/9/2020
ABS35	15	-	++	++	29	M	87	DTA	14/8/2020
ABS33	16	-	++	+	27	F	90	DTA	19/8/2020
ABS3	17	-	++	-	3	M	58	Pus	17/1/2020
ABS47	18	-	-	-	41	M	68	DTA	19/10/2020
ABS43	19	78	++	+	37	M	31	DTA	14/9/2020
ABS48	20	-	-	+	42	M	72	DTA	23/10/2020
ABS36	21	-	++	+	30	F	82	DTA	29/8/2020
ABS46	22	-	-	-	40	M	80	Stool	26/10/2020
ABS27	23	-	++	-	21	F	74	Blood	3/8/2020
ABS32	23	-	++	++	26	M	69	DTA	19/8/2020
ABS9	24	2	++	+	7	M	-	-	10/1/2020
ABS31	25	-	+	-	25	M	67	DTA	8/8/2020
ABS45	26	-	-	++	39	F	73	Wound	5/10/2020

Figure 4. PFGE pattern of the isolates undertaken in this study using ApaI restriction enzyme. PT: pulsotype, sequence type (ST), sex (M – male, F – female), age, specimen type (DTA: deep tracheal aspiration). Dashed line (-): missing data. Under biofilm: "-"nonadherent, "+"weakly adherent, and "++" moderately adherent. Pellicle, "-" not detected, "+"moderate thickness, " ++" thick pellicle. BioNumerics software v7.6.1 was used to construct the dendogram.

3.9 wg-SNP-based phylogenetic analysis

Whole-genome SNP-based phylogenetic analysis, using *A. baumannii* ACICU as a reference (Accession # NZ_CP031380), placed the isolates within 10 main groups (Figure 5).

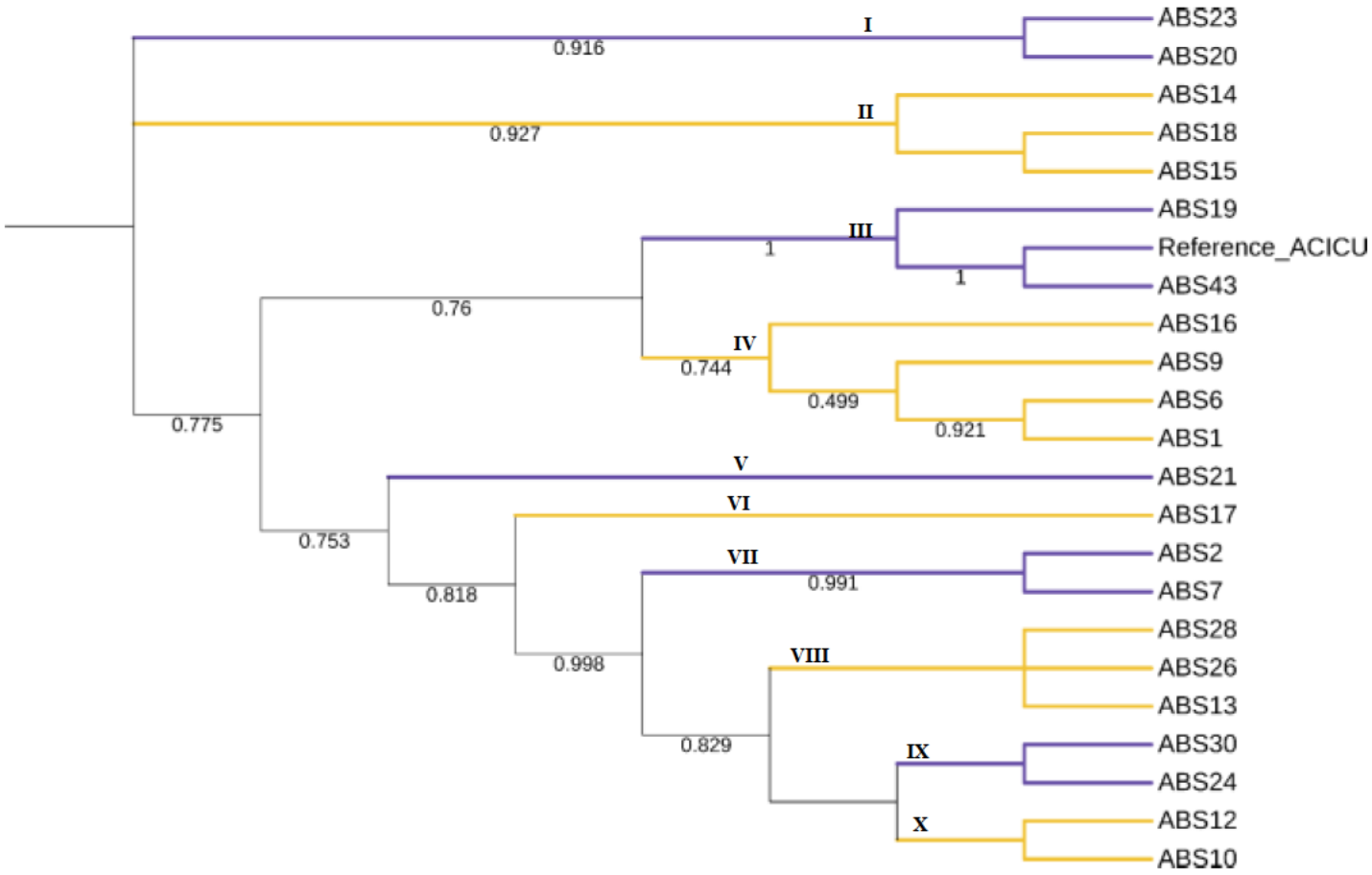


Figure 5. Phylogenetic analysis of *A. baumannii*. wgSNP-based phylogenetic tree of the 22 sequenced isolates was generated using CSIPhylogeny. The maximum likelihood phylogeny of all the isolates with their bootstrap values were observed using iTOL. Each alternating color indicates a cluster. Clusters are labeled I – X.

3.10 Pan-genome analysis

A total of 7,624 coding genes were detected following pan-genome analysis and were classified as: 1,306 core genes (common between 99% – 100% of the isolates), 693 soft-core (common between 95% – 99%), and accessory genes. The accessory genes included 1,856 shell genes that were common between 15% – 95% of the isolates and 3,769 cloud genes common between 0% – 15% of the isolates (Figure 6).

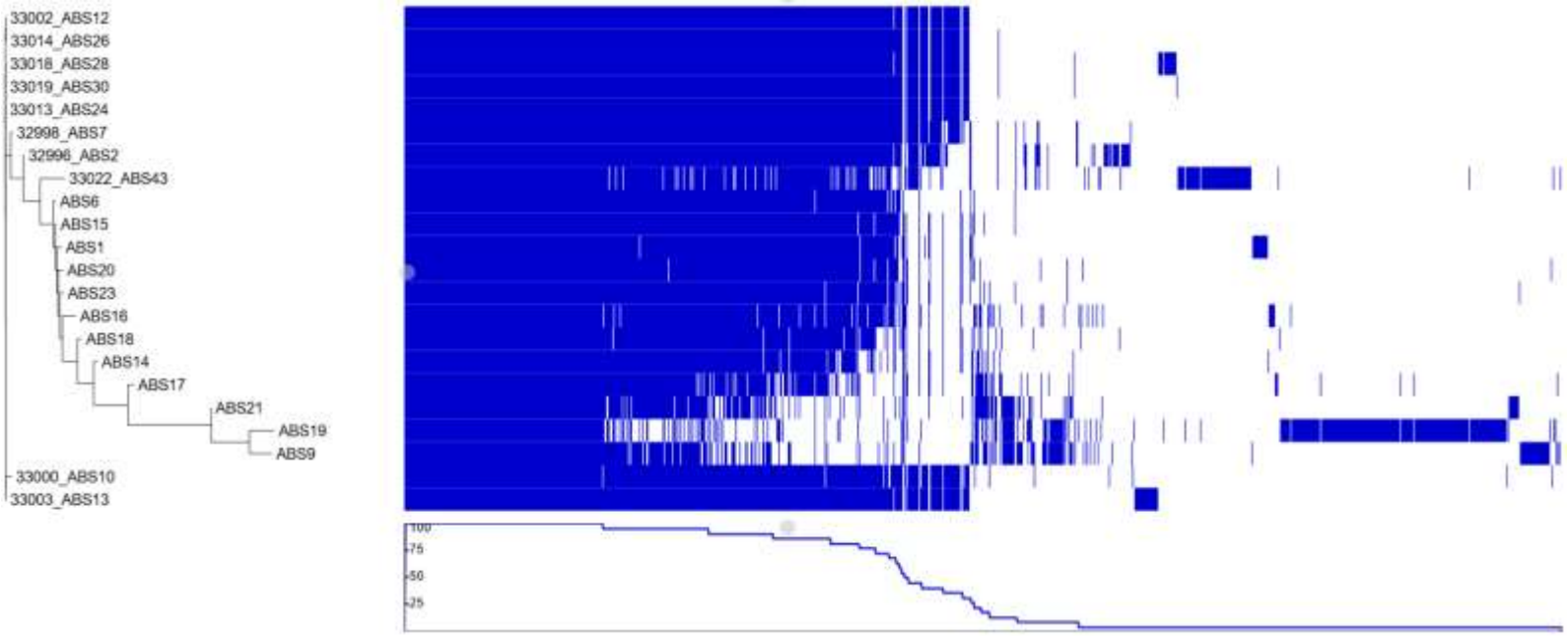


Figure 6. Pan-genome similarity matrix. The core and accessory genes were used to create a pan-genome similarity matrix using Roary. The phylogenetic relatedness is observed in blue as presence and white fragments as absence.

3.11 Recombination analysis

Snippy, a tool for rapid bacterial SNP calling and core genome alignments, was used with the 10 *de novo* assembled genome (ABS2, ABS7, ABS10, ABS12, ABS13, ABS24, ABS26, ABS28, ABS30, and ABS43). High recombination frequency regions found in the core genome were studied using Gubbins (Croucher et al., 2015). Gubbins identifies loci containing elevated densities of base substitutions while in parallel constructing a phylogeny based on the putative point mutations outside of these regions and which can be used to reconstruct models of short-term bacterial evolution. Seven recombination hotspots of significance were detected and designated as A-G (Figure 7). A peak was identified as a recombination hotspot when the density of the SNPs was greater than one. A, C, and F had densities greater than two and were further analyzed.

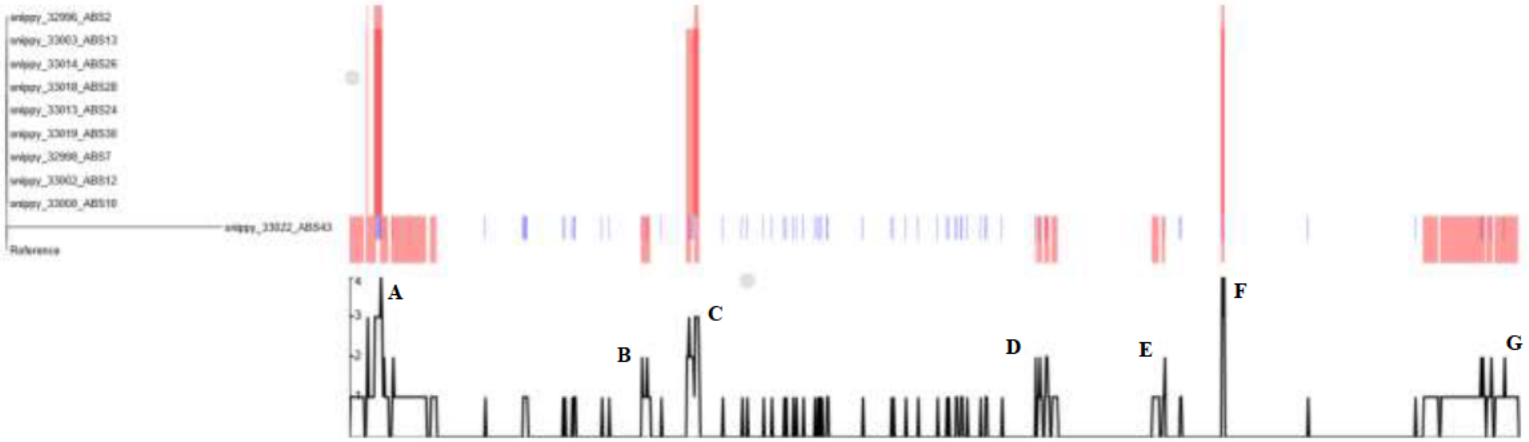


Figure 7. Recombination densities found in the core genomes using Gubbins. A maximum likelihood phylogenetic tree was constructed using Snippy and based on the SNPs found in the core genome. The recombination densities found between the isolates are shown in red (predicted recombinations that are happening on internal branches indicating shared recombinations between several isolates of common ancestry) and blue (recombination on terminal branches indicating recombinations unique to individual isolates). The graph at the bottom shows the density of the SNPs in relation to their genome position. Regions A, C, and F were further analyzed.

Chapter Four

Discussion

A. baumannii has acquired through horizontal gene transfer a large number of virulence and resistance determinants. Over the past ten years it was continuously being linked to nosocomial outbreaks and epidemics, disseminating globally and within cities and healthcare settings (Agodi et al., 2014; Handal et al., 2017). We aimed in this study to phenotypically and genotypically characterize 46 MDR *A. baumannii* isolates collected between January and November 2020 from 43 patients admitted to a single hospital in Lebanon. In Lebanon, 76.5% of *A. baumannii* isolates were found to be carbapenem resistant (Al Atrouni et al., 2016; Moghnieh et al., 2019), while it was shown to reach 95% in a more recent study by Makke et al. (2020). Resistance detected in Lebanon was higher than that reported in Italy (45.7%; Principe et al., 2014), and Saudi Arabia (69%; Al Sultan et al., 2015). We also detected a number of carbapenemase encoding genes such as bla_{OXA23} and bla_{OXA66}, and which was confirmed through the disc diffusion assay with 97.7% showing resistance to imipenem.

WGS, PFGE, and MLST were performed to determine the clonality and genotypic relatedness between the isolates undertaken in this study. Our results revealed the prevalence of the ST2 clone. ST2 was recently linked in Lebanon to an outbreak in a tertiary care hospital, representing 92.9% of the *A. baumannii* nonduplicate CRAB isolates (Osman et al., 2020). It is noteworthy that between 60-90% of *A. baumannii* isolates recovered from Lebanon over recent years were identified to be of ST2 sequence type, and which was in harmony with our results and confirming the prevalence of this clone

(Jamal et al., 2018). ST2, linked to the international clonal lineage IC II (Fitzpatrick et al., 2016), was associated with outbreaks in health settings in Greece (Pournaras et al., 2017), Italy (Agodi et al., 2014), Thailand (Molter et al., 2016), and in Lebanon (Al Atrouni et al., 2016; Osman et al, 2020). Out of 194 CRAB isolates recovered in Greece, 77.8% were ST2 and IC II lineage (Pournaras et al., 2017), while it was 76.5% in Lebanon (Al Atrouni et al., 2016).

Many emerging resistance patterns in *A. baumannii* could be traced to horizontal gene transfer (HGT) (Carattoli, 2013). Sequence analysis of *A. baumannii* plasmids showed differences in size and genetic content when compared to other bacteria, and accordingly a different plasmid classifying scheme was developed (Bertini et al., 2010; Towner et al., 2011). Plasmids from *A. baumannii* were categorized based on their replicase proteins (Rep), and which was the basis of the current replicon typing scheme. Using this approach we detected 19 homology groups and we designated them as GR1-GR19, with GR2 and GR6 being the most prevalent. Most of the sequenced isolates were positive for GR2 (86.3%; n=19) and 50% (n=11) for GR6. In a study by Towner et al. (2011), GR2 (72.9%) and GR6 (96.8%) were also among the most ubiquitously found homology groups among *A. baumannii*, with GR6 (51%) being commonly recovered from hospitals in China (Chen et al., 2018). We had eight isolates harboring GR2 and GR6 homology groups (36.4%), three had only GR6 and 11 only GR2. Interestingly, GR2 plasmids do not carry mobilization determinants but could be mobilized along with GR6 (Blackwell and Hall, 2019).

Using PFGE, we detected 26 different PTs, with the difference defining a PT being three bands or more (Tenover et al., 1995). All the ST2 isolates, except for ABS9, were

clustered together. ABS9 exhibited a different resistance pattern being susceptible to most of the tested antimicrobial agents. However, *A. baumannii* may exhibit different PFGE banding patterns although being linked clonally (Kanamori et al., 2016; Salipante et al., 2015). ABS19, ABS21, and ABS24 were collected one week apart, while ABS13 and ABS15 were recovered from the same patient three days apart, and yet all showed banding patterns.

In *A. baumannii* host-pathogen interactions could be enhanced through biofilm formation (Greene et al., 2016). Baps are found on the cell surface and allow the formation of the biofilm matrix by attaching to abiotic surfaces and mediating intercellular adhesion in response to harsh environmental conditions (Loehfelm et al., 2008; Fattahian et al., 2011). Biofilm formation could facilitate resistance against immune system clearance, antibiotics, and desiccation (Doi et al., 2015), and are classified to reflect adherence strength into: non-adherent, weakly adherent, moderately adherent, or strongly adherent (Bardbari et al., 2017). Among the 46 isolates used in this study, all except five (ABS44-ABS48) were biofilm formers, with the majority showing moderate adherence, and was in line with other studies (Obeidat et al., 2014; Vijayakumar et al., 2016; Bardbari et al., 2017).

The pellicle is bacterial aggregation or clustering that occurs on liquid suspensions at the air-liquid interface, and allows for bacterial transmission (Chabane et al., 2014; Krasauskas et al., 2019). The formation of a pellicle could be observed as an opaque layer at the air-liquid surface and classified as thick (++), moderate (+) or absent (0) (Chabane et al., 2014). We observed thick and moderate pellicle formers among the studied isolates, and we could not detect any significant correlation between biofilm and pellicle formation.

McQueary and Actis (2011) also had similar findings while Chabane et al. (2014) revealed that temperature is an important determinant for pellicle formation.

Virulence factors help pathogens such as *A. baumannii* to successfully thrive in the healthcare environment and the host. These mechanisms can be attributed to several different factors and mechanisms, including: adherence, biofilm formation, enzymes, iron uptake mechanisms, regulatory mechanisms, and immune evasion. The outer membrane protein A (OmpA) in *A. baumannii* was linked to a decrease in membrane permeability (Sugawara and Nikaido, 2012). OmpA, located on outer membrane vesicles or the cell surface of *A. baumannii*, was found to confer cytotoxicity when interacting with eukaryotic cells by adhering and binding to surface death receptors (Ahmad et al., 2016). Moreover, biofilm associated proteins (BAPs) are found on the cell surface and allow the formation of the biofilm matrix by attaching to abiotic surfaces and mediating intercellular adhesion in response to harsh environmental conditions (Loehfelm et al., 2008; Fattahian et al., 2011). We identified BAPs in all the sequenced isolates. On the other hand, enzymes involved in the hydrolytic activities against human cell membrane phospholipids were among the detected virulence determinants. The two main phospholipases that were studied in *A. baumannii* are Phospholipase C and D. Phospholipase D helps the pathogen to survive in the serum while phospholipase C induces toxicity to epithelial cells (Camarena et al., 2010). We detected the aforementioned virulence determinants in all the ST2 isolates. Interestingly, isolates with the same ST type and virulence determinants exhibited different pathogenicity profiles when tested *in vivo* in a *Galleria mellonella* model, implying that there could be also other virulence factors expressed during the early stages of infection (Zhou et al., 2018).

Capsule formation is an important factor enhancing pathogenesis in *A. baumannii* (Singh et al., 2019). The K locus on the chromosome is conserved among *A. baumannii* strains with some genetic determinants being directly linked to capsular polysaccharide synthesis such as the *wzy* gene (Kenyon and Hall, 2013). Accordingly, *A. baumannii* could be further classified based on structural differences and capsular type into KL1 – KL9 (Kenyon and Hall, 2013). This scheme, however, was further developed with the appearance of new types (up to 130 capsules) (Arbatsky et al., 2021). Variations among *A. baumannii* strains include differences in the length (Shashkov et al., 2019), glycosidic bond locations (Kasimova et al., 2017), sugar composition and linkages between K-units (Arbatsky et al., 2019; Kenyon et al., 2017; Shashkov et al., 2019). We detected KL9 and KL39 among the studied isolates, with KL39 being the only non-chromosomal type linked to a small genetic island (Kenyon et al., 2016).

Lipid-A is part of the lipopolysaccharide (LPS) layer found in most Gram-negative bacteria (Raetz and Whitfield, 2002). *A. baumannii*, does not contain the O-antigen polysaccharide and accordingly the LPS is designated as lipooligosaccharide (LOS) (Kenyon and Hall, 2013). The outer core component (OC) of the LOS in *A. baumannii* is a second locus with variable gene content. The OC locus (OCL) is chromosomal (Kenyon et al., 2014), and to date, 14 OCL gene loci were reported (Wyres et al., 2020). In this study, the predominant OC locus detected in the 22 sequenced isolates was OCL1. The K and OCL, were also used for the molecular typing of *A. baumannii* (Kostyanev et al., 2021).

A. baumannii succeeds in hospitals and healthcare settings due to its genome plasticity allowing it to persist and thrive under high stress and environmental changes.

This occurs either through gaining new genetic elements such as plasmids or by exhibiting modifications in the genome and eventually accumulating SNPs (Bogaty et al., 2018). Studies confirmed that wgSNP-typing could be better at revealing the genotypic relatedness (Alshahni et al., 2015; Kanamori et al., 2016). We used wgSNP-based analysis to better elucidate the relatedness between the isolates undertaken in this study. Our results revealed high relatedness with the differences at the level of SNPs in the ST2 isolates less than 200. During outbreaks, SNP variations could be linked to the dissemination time span (Kanamori et al., 2016).

We also checked for recombination hotspots in the sequenced isolates and detected high recombination frequency regions in the core genome. Seven recombination hotspots of significance were found and designated as A to G. Region A was linked to capsular synthesis, G to the type IV pilus biogenesis, while the the other parts mainly had hypothetical proteins. Recombination events involved in the capsular synthesis and type IV pilus biogenesis detected in this study are of prime importance as they could play a role involved in protecting the organism against harsh conditions including dryness, disinfectants, phagocytosis, and antimicrobial agents (Singh et al., 2019; Geisinger and Isberg, 2015).

We characterized in this study using WGS, plasmid analysis, and PFGE, CRAB recovered from a hospital in Lebanon. Phenotypic antimicrobial susceptibility testing revealed that the isolates were XDR. Intrinsic β-lactam resistance genes, including bla_{ADC25} (*Acinetobacter*-Derived Cephalosporinase) and bla_{OXA66} (a variant of bla_{OXA51}), were among the common resistance determinants. AB-PBRT showed that the isolates were mainly positive for GR2 and GR6. *In silico* analysis of several of the isolates allowed

the detection of multiple virulence factors. The findings obtained through MLST and PFGE depicted that the *A. baumannii* isolates that were MDR or XDR circulating in the hospital were most likely associated with an outbreak linked to ST2.

A. baumannii is a nosocomial pathogen that is difficult to control. CRAB outbreaks are now known to be a threat to hospitalized patients and the data presented in this study further confirms that the best available tool to date to rapidly detect outbreaks in nosocomial settings is whole-genome sequencing. WGS and interventional trials are needed to identify and prevent the spread of XDR microbial pathogens. As a future work we propose studying the pathogenicity islands and performing an in-depth plasmid analysis. Finally, since capsule typing was newly introduced as a typing approach using it should be used to test its validity and correlation with MLST and IC lineages.

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