

**Genotyping of endemic strains of *Acinetobacter* spp.
isolated from two Jordanian Hospitals**

By

Nermen Dakkak

**A Thesis Submitted in
Partial Fulfillment of the
Requirements for the Degree of
Master of Science
in Pharmaceutical Sciences**

at

**Petra University,
Amman-Jordan**

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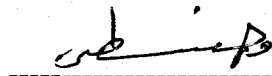
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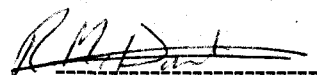
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Genotyping of endemic strains of *Acinetobacter* spp. isolated from two Jordanian Hospitals

By

Nermen Dakkak

Petra University, 2009

Under the Supervision of Prof. Khalid Matalka

Abstract

The emergence of multidrug-resistant (MDR) *Acinetobacter* species as one of the most important nosocomial pathogens in intensive care unit (ICU) patients has been observed world wide. Historically, it has been demonstrated that the increase in nosocomial infections caused by *Acinetobacter baumannii* mainly in the respiratory tract, has paralleled with development of resistance rate. Therefore the understanding of the phenotypic characteristics of *Acinetobacter baumannii* and molecular basis of its pathogenesis is necessary. A total of twelve *Acinetobacter* species were isolated from different clinical specimens at Jordan University Hospital (JUH) and King Hussein Cancer Center (KHCC) at two different time periods (2006 and 2008). In this study all isolates were confirmed for their identity by biochemical tests and all isolates were characterized as *Acinetobacter baumannii*. All *Acinetobacter* isolates were examined for their antimicrobial susceptibility patterns using disk diffusion method, and the results demonstrated that all isolates were resistant to all antimicrobial agents used. Phenotypic identification of *Acinetobacter* isolates to the species level has proven to be insufficient. Therefore the 16S-23S rRNA gene intergenic spacer (ITS) region was used in this study for genomic species identification and the isolates were sequenced. The ITS length was 607 and sequences were highly conserved and all isolates were identified as *Acinetobacter baumannii*. The accuracy of the method was confirmed by amplified ribosomal DNA gene restriction analysis (ARDRA); ARDRA proved to be rapid and reliable method for identification of most of the *Acinetobacter* genomic

species. Restriction analysis was performed with the enzymes *Alu1*, *Cfo1*, *Rsa1*, *Msp1* of the enzymatically amplified 16SrRNA gene allowed us to identify all isolates as *Acinetobacter baumannii*. These results indicated that the amplified ITS gene gave the same genus identification as full sequence data from all 12 clinical isolates evaluated. Sequence analysis of this region needed only one amplification step and two sequence reactions; therefore, the price of reagents approaches the costs of the reagents and labor for many phenotypic methods. In addition, this study demonstrated the high frequency of multidrug resistance *Acinetobacter baumannii* to all antimicrobial agents used at JUH and KHC.

KHALID MATAKA

A handwritten signature in black ink, appearing to read 'K Mataka', is written over a horizontal line. The signature is stylized and somewhat cursive.

To

My Father and Mother

AKNOWLEDGEMENTS

This work would not have been completed without help and support of many individuals. I would like to thank everyone who has helped me along the way. Particularly, Prof. Khalid Matalaka for providing me an opportunity to conduct my master's research under his supervision and for his guidance and support over the course of it.

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List of abbreviation

ICU	Intensive care unit
IDSA	Infectious Disease Society of America
<i>Acinetobacter</i> spp	<i>Acinetobacter</i> species
MLST	Multilocus sequence typing
MLEE	Multilocus enzyme electrophoresis
PFGE	Pulse field gel electrophoresis
RFLP	Restriction fragment length polymorphism
REP-PCR	Repetitive sequence based PCR
JUH	Jordan University Hospital
KHCC	King Hussien Cancer Center
MBLs	Metallo- β -lactamases
PBP _s	Penicillin-binding proteins
BSI	Bloodstream infection
MRSA	Methicillin-resistant <i>Staphylococcus aureus</i>
PFGE	Pulsed-field gel electrophoresis
Kb	Kilo base pairs

5'-CS	The 5' conserved segments
3'-CS	The 3' conserved segments
ORF	Open reading frame
ARDRA	Amplified ribosomal DNA restriction analysis
BFGE	Pulsed-field gel electrophoresis
MLST	Multilocus Sequence Typing
ITS	Intergenic spacer
ATCC	American Type Culture Collection
TSI	Triple sugar iron
TBE	Tris-borate –EDTA
NA	Nutrient agar
MHA	Muller Hinton Agar
MHB	Muller Hinton broth
NCCLS	National Committee for Clinical Laboratory Standards
EtBr	Ethidiumbromide

Chapter 1

Thesis Proposal

**Genotyping of endemic strains of *Acinetobacter*
spp. isolated from two Jordanian hospitals**

Chapter 1

Thesis Proposal

It was only thirty years ago when interest has started in *Acinetobacter*. It was considered as commensal of low grade pathogenicity, and was frequently ignored whenever isolated in clinical specimen (Giamarellou *et al.*, 2008). A change in the interests of *Acinetobacter* spp. emerged world wide since it was the major cause of high morbidity and mortality, especially among intensive care unit (ICU) patients (Bergogne-Berezin and Towner, 1996). Accordingly, *Acinetobacter* spp. was described as an important opportunistic pathogen responsible for severe nosocomial infections. In addition to its increasing occurrence and frequent incidence as nosocomial infection, *Acinetobacter* spp. became as a nosocomial pathogen on a global scale. The Infectious Disease Society of America (IDSA) identified *Acinetobacter baumannii* among the most common seven pathogens threatening the health-care delivery system (Talbot *et al.*, 2006). In other statistical studies of European hospitals, *Acinetobacter baumannii* was among 2% - 10% of all gram-negative bacterial infections in intensive care units (Euzeby, 2006). The clinical impacts of *Acinetobacter* infections relay on the various risk factors. First factor, infections are related to the use of medical devices (such as endotracheal tubes, intravascular and urinary catheters). Second factor, threatened patients are exposed to broad-spectrum of antibiotics. Third factor, it is responsible for a number of systemic infections in critically ill and immunocompromised patients, especially among those in ICU (Joshi *et al.*, 2003, Jones *et al.*, 2004, Van Looveren *et al.*, 2004, Guducuoglu *et al.*, 2005).

The majority of outbreaks caused by *Acinetobacter* have involved respiratory tract infections. There are at least 30 different *Acinetobacter* species which are commonly associated with human infections. Including *A. baumannii*, *A. calcoaceticus*, *A. haemolyticus*, *A. johnsonii*, *A. junii*, *A. lowffii*, and *A. radioresistens*. However, *A. baumannii* is now recognized as the most clinical isolate from nosocomial infections with epidemic potential and identified as a major cause of outbreaks or sporadic cases with high mortality rates accounting for about 80% of reported infections worldwide (Prashanth and Badrinath, 2006, Richet and Fournier, 2006, Falagas *et al.*, 2007, Sunenshine *et al.*, 2007). Threats and hazards of *Acinetobacter baumannii* infections had been intensively raised world wide since treatment of *Acinetobacter baumannii* infection has become difficult (Giamarellou *et al.*, 2008). Many strains are resistant to a wide range of antimicrobials, including broad-spectrum beta lactams (Ramires *et al.*, 2000), aminoglycosides, fluoroquinolones, carbapenems and third-generation cephalosporins, and thus recognized as the most important risk factor for multi-resistant bacteria (Boo *et al.*, 2009).

Generally, *Acinetobacter* spp. have intrinsic resistance to antimicrobials and are explain multi-resistant with exposure to certain antibiotics. Both resistant and multi-resistant strains have emerged as a serious problem in many hospitals worldwide. The studies involving the mechanisms of multi-resistant *Acinetobacter baumannii* have demonstrated the presence of specific genes located on transferable plasmids and transposons (Gallego and Towner, 2001, Ruiz *et al.*, 2003, Giamarellou *et al.*, 2008). Several studies have reported that more than 80% of *Acinetobacter* isolates carry multiple indigenous plasmids of various molecular sizes (Van Looveren *et al.*, 2004). The plasmid profiling has been proposed as a method of epidemiological typing for *Acinetobacter* spp. (Joshi *et al.*, 2003). The presence of integrons in *Acinetobacter* has

been well-established (Peleg *et al.*, 2008). It is relatively of high frequency of carriage in epidemic strains. Integrons were demonstrated in 50% of the strains by an integrase gene PCR (Koeleman *et al.*, 2001). Epidemic strains of *A. baumannii* were found to contain significantly more integrons than non-epidemic strains and the presence of integrons was significantly correlated with simultaneous resistance to several antibiotics (Gallego and Towner, 2001, Koeleman *et al.*, 2001, Ruiz *et al.*, 2003, Mak *et al.*, 2009).

Investigating the mechanisms underlying *Acinetobacter* infections are essential for treatment and developing control measures. The investigations should determine the original sources of the infection and the endemic profile that includes the genotypes involved and their geographical spread (Ecker *et al.*, 2006). Genotyping has been introduced into epidemiology as an important tool for nosocomial pathogen grouping and in coping with epidemic spread (Seifert *et al.*, 2005). The ideal typing method should be rapid, easy to use, have a high throughput, and be applicable to a wide range of microorganisms. It is very important to confirm or exclude the genetic relationship among the isolates in a short term in order to trace the source of infection as well as investigate epidemiological pattern of serial or overlapping outbreaks (Munoz-Price *et al.*, 2008). Therefore, there are several molecular typing systems used with this aim. Such as PCR multilocus enzyme electrophoresis (MLEE), multilocus sequence typing (MLST), pulse field gel electrophoresis (PFGE), restriction fragment length polymorphism (RFLP), DNA sequencing ribotyping restriction fragment length polymorphism (AFLP) and repetitive sequence based PCR (REP-PCR) (Fontana *et al.*, 2008). The choice of the proper typing method is difficult and limitations are described for each (Maquelin *et al.*, 2006).

Using proper methods for identification of *Acinetobacter* spp., including those within the *A. baumannii* group, are mandatory to increase our knowledge of the epidemiology, pathogenicity, and clinical impact of the various species of this diverse genus. In Jordanian hospitals, studies implementing such protocols are scanty and may not be available. Therefore, the objectives of this study were: firstly, to identify and characterize *Acinetobacter* spp from clinical isolates from Jordan University Hospital and KHCC. Secondly, to determine the antimicrobial susceptibility patterns among *Acinetobacter* spp. isolates in patients admitted to the Jordan University Hospital and King Hussien Cancer Center (KHCC), and thirdly, to analyze the genotyping of these clinical isolates that were collected from two different hospitals at two different time periods (2006 and 2008).

Chapter 2
Literature Review

Chapter 2

Literature Review

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2.1 General characteristics of *Acinetobacter* species

Acinetobacter was first described in 1911 as *Micrococcus calco-aceticus*. Since then, it has had several names, becoming known as *Acinetobacter* in the 1950s (*Akinetos* Greek adjective, unable to move, *Bakterion*, Greek noun, rod). It belongs to the family of Moraxellaceae (Towner *et al.*, 1991). It comprises heterogeneous collection of Gram – negative coccobacillus, nonmotile, non fermentative, oxidase – negative and catalase positive bacteria. The cell wall ultrastructure is typical of Gram-negative bacteria in general, but the cells are occasionally difficult to destain, (Bergogne-Bérézin and Towner, 1996). Cells commonly occur in pairs, but also in chains of variable length. No spores are formed and flagellae are absent. Although generally considered to be nonmotile, “twitching” or “gliding” motility has been reported to occur, particularly on semisolid media (Towner, 2006). Many strains are encapsulated, and the capsule may be readily seen in India ink wet mounts. Upon growing on nutrient agar, colonies are 1–2 mm in diameter, mucoid, usually non-pigmented, but some strains form white to cream - colored colonies, which vary in consistency from butyrous (buttery) to smooth surface. All members of the genus *Acinetobacter* are strict aerobes and can grow at a wide range of temperatures. Most strains will grow at 33°C, but some environmental isolates prefer incubation temperatures from 20°C–30°C (Bergogne-Bérézin *et al.*, 1996). Clinical isolates of *Acinetobacter* will normally grow at 37°C and some strains can grow at 42°C. Most strains of *Acinetobacter* can grow in a simple mineral medium containing a single carbon and energy source (Juni, 1972). A wide variety of organic compounds can be

used as carbon sources by particular strains, although relatively few strains can use glucose. Most strains are unable to reduce nitrate to nitrite in the conventional nitrate reduction assay (Bergogne-Berezin and Towner, 1996). Few clinical strains may show hemolysis on sheep blood agar plates owing to the production of phospholipase C (Warskow and Juni 1972). There are number of distinctive physiological features that support the versatile lifestyle of this genus. It is worth emphasizing that many strains of *Acinetobacter* used in physiological studies were originally isolated in the 1970s or earlier and have never been properly identified to the genomic species level. In addition, the detailed physiological studies have been based on a very limited number of strains. Although rare strains of *Acinetobacter* showing growth factor requirements have been isolated, the vast majority of strains resemble saprophytic pseudomonad in utilizing a large range of organic compounds as a carbon and energy source in an otherwise mineral medium (Towner, 2006). Although the utilization of carbohydrates is relatively uncommon, the major biochemical feature of the genus is that many strains are able to metabolize a range of compounds including aliphatic alcohols, some amino acids, decarboxylic and fatty acids, unbranched hydrocarbons, sugars, and many relatively recalcitrant aromatic compounds such as benzoate, mandelate, n-hexadecane, cyclohexanol and 2,3-butanediol (Juni, 1978). Members of the genus are therefore particularly suitable organisms for studying a variety of unusual biochemical pathways, and may have a role in degrading a range of pollutants and industrial products (Lamb *et al.*, 2000, Arunachalam *et al.*, 2003).

2.2 Occurrence and habitats of *Acinetobacter* spp.

Acinetobacter spp. natural habitats are water and soil, and have been isolated from foods, arthropods, and the environment (Towner, 2006). It has been estimated that *Acinetobacter* may constitute as much as 0.001% of the total heterotrophic aerobic population of soil and water (Baumann, 1968) and have been found at densities exceeding 10^4 organisms per 100 ml in freshwater ecosystems and 10^6 organisms per 100 ml in raw sewage (LaCroix and Cabelli, 1982). *Acinetobacter* spp. can be isolated from heavily polluted water, such as that found in wastewater treatment plants, but are found more frequently near the surface of fresh water and where fresh water flows into the sea (Towner, 2006). In humans, *Acinetobacter* can colonize on or within skin, wounds, respiratory and gastrointestinal tracts and are also isolated from clinical environment as commensals, such as the skin of hospital staff and patients (Towner, 2006), under nails of nurses, medical equipments and tools used medical ICU, surgical ICU, shock-Trauma ICU, medical wards, nursery, burn and plastic surgery wards (Villegas and Hartstein, 2003) (Table 1.1). It was observed that remarkable ability of *Acinetobacter* spp. to survive under a wide range of environmental conditions for prolonged periods of time (Jawad *et al.*, 1996, Founier and Richet, 2006). These unique characters potentiate *Acinetobacter* to be a frequent cause of outbreaks of infection and an endemic, health care setting pathogen (Munoz-Price and Weinstein, 2008).

Table 1.1 common source of *Acinetobacter* causing out breaks in hospitals (Villegas and Hartstein, 2003)

Bedside humidifiers
Warming bath water
Hospital prepared distilled water
Heparinized saline solution
Patient mattresses
Feather pillows
Water taps in staff room with mesh aerators
Cardiac Catheterization
Respirometers
Bronchoscopes
Lotion dispenser
Air supply
Jugs
Bowls
Soap
Hand cream
Plastic screens
Bed linen
Service ducts /dust
Bedside charts
Computer keyboards
Blood pressure cuffs
Cell phones

2.3 Epidemiology of *Acinetobacter*:

As stated earlier, *Acinetobacter* is primarily a pathogen in the health care setting. It is increasingly reported as the cause of outbreaks and nosocomial infections such as blood-stream infections, ventilator-associated pneumonia, urinary tract infections and wound infections. Outbreaks that have been traced were a cross -infection by the hands of health care workers, infected patients or touched contaminated fomites, and to the occasional health care worker who carries an epidemic strain (Villegas and Hartstein, 2003, Maragakis *et al.*, 2004). Once introduced into a hospital, *Acinetobacter* often has an epidemiologic pattern of serial or overlapping outbreaks caused by various multidrug-resistant strains. Endemics may be related to multiple strains and a single endemic strain predominating at any time (Villegas and Hartstein, 2003). The occurrence of monoclonal outbreaks in multiple hospitals suggests inter-institutional spread, presumably by movement of patients or personnel, or exposure to common-source contamination of food or equipment. Such outbreaks highlight the importance of ongoing surveillance, inter-facility communication, and addressing-measures to prevent the introduction of *Acinetobacter* into, and the spread from nursing homes.

Numerous studies have now supported the observation that *A. baumannii* and its close relatives are the main genomic species associated with outbreaks of hospital infection. This ubiquitous occurrence of *Acinetobacter* in the environment, and as commensals on human skin, means that such isolates in clinical specimens are often considered to be contaminants. Other reservoirs of these organisms may include a range of both moist and dry surfaces and equipment within the hospital environment as well as desiccated environments. Such characteristics may promote the ability of such

remarkable genus to be easily transmitted through a fomite contamination in hospitals (Munoz-Price and Weinstein, 2008).

2.4 Taxonomy of *Acinetobacter*:

Over the last 30 years, *Acinetobacter* has undergone significant taxonomic modification. There are 31 described (genomic) species, that have been recognized by DNA-DNA hybridization and defined numerically, and 17 of which have validated names (Peleg *et al.*, 2008). Only 10 species have been isolated from human specimens these are: *A. baumannii*, *A. calcoaceticus*, *A. haemolyticus*, *A. johnsonii*, *A. junii*, *A. lwoffii*, *A. parvus*, *A. radioresistens*, *A. schindleri*, and *A. ursingii*. Seven described species were isolated from activated sludge plants that include: *A. baylyi*, *A. bouvetii*, *A. gernerii*, *A. grimontii*, *A. tandoii*, *A. tjernbergiae*, and *A. townner*. Moreover, the association of some yet unnamed species with human clinical samples has also been reported, especially genomic species 3, 13TU, 10, and 11 (Dortet *et al.*, 2006).

The four species: *A. calcoaceticus*, *A. baumannii*, *Acinetobacter* genomic species 3, and *Acinetobacter* genomic species 13TU were very closely related and difficult to be distinguished from each other by phenotypic properties. It has therefore been proposed to be referred to as the *A. calcoaceticus* - *A. baumannii* complex (Gerner-Smidt *et al.*, 1991). However, this group of organisms comprises the three most clinically relevant species that have been implicated in the vast majority of both community-acquired and nosocomial infections, i.e. *A. baumannii*, *Acinetobacter* genomic species 3, and *Acinetobacter* genomic species 13TU.

2.5 *Acinetobacter* pathogenicity:

There has been no toxin or toxin-like product detected in *A. baumannii* strains. Yet, their pathogenicity relies essentially on its cell surface components, and hydrolytic enzymes. These two virulent factors are very much related to antimicrobial resistance of *Acinetobacter* spp.

Virulent factors that have been reported so far include a novel pilus assembly system involved in biofilm formation (Tomaras *et al.*, 2003, Wroblewska *et al.*, 2008), an outer membrane protein (Omp38) that stimulate apoptosis in human epithelial cells (Vila *et al.*, 2007), and a polycistronic siderophore-mediated iron-acquisition system conserved between *A. baumannii* and *Vibrio anguillarum* (Dorsey *et al.*, 2003, Zimblet *et al.*, 2009). These presumably comprise a small fraction of elements involved in *Acinetobacter* pathogenesis. Accordingly, novel global approaches are essential to comprehensively understand the basic features of this organism in order to ultimately control the spread of *Acinetobacter* spp. infections and to develop effective countermeasures against this harmful pathogen.

In addition to its pathogenesis, the genus *Acinetobacter* is particularly interesting for other reasons. First, *Acinetobacter* spp. are capable of catabolizing a wide range of carbon sources and metabolites and, as such, were briefly classified as *Pseudomonads* (Stanier *et al.*, 1966). In fact, *Acinetobacter* spp. are among the most widely used microbes for petroleum remediation. Second, representatives of *Acinetobacter baylyi* have an extraordinary ability to acquire foreign DNA, thus described as a remarkable strain with high competence for natural DNA transformation (Young *et al.*, 2005, Wang *et al.*, 2007). It is currently unknown how pervasive natural competence, which is deafened as acquisition of novel, advantageous alleles such as antibiotic resistance in nosocomial environments, is among *Acinetobacter* spp. Since this trait is

considered an important mechanism by which *Acinetobacter* spp. achieve genetic diversity (Juni, 1972, Vries and Wackernagel, 2002, Mendes *et al.*, 2009). Recently researchers are focusing on pathogens that can rapidly acquire drug resistance and pathogenicity islands (PAIs). PAIs encode various virulence factors in pathogenic strains that are normally absent in non-pathogenic strains of the same or closely related species, since they have the advantage of identifying novel virulence factors unique among those strains. In addition this knowledge has added a lot to the understanding of the evolution of bacterial virulence (Gal-Mor and Finlay, 2006).

2.6 Antimicrobial resistance:

Antimicrobial resistance is a natural biological phenomenon of bacterial response to the selective pressure associated with the use of antimicrobials. This phenomenon has been recognized since the last decade of the 19th century. As antimicrobial are frequently misused and overused in many developed and developing countries, resistance to antimicrobials has led to an increase in the morbidity, the mortality and the cost of health care (Rashmi *et al.*, 2005, Falagas *et al.*, 2007). Scientists are now racing to develop methods and therapies to reverse the trend. However, until other therapeutic options and strategies become available, the key to reversing the trend in the next decade will entail careful and appropriate antibiotic selection, surveillance, and infection-control procedures. There are two major types of antimicrobial resistance:

1. *Inherent (natural) resistance* in which bacteria may be naturally resistant to an antimicrobial such as an organism lacks a transport system for an antimicrobial; lacks the target of the antimicrobial molecule; or as in the case of Gram-negative bacteria, the cell wall is covered with an outer membrane that establishes a permeability barrier against the antimicrobial (Rashmi *et al.*, 2005).

2. Acquired resistance can develop due to mutation or gene transfer, where antimicrobial resistance genes are often carried on mobile genetic elements. Bacteria exchange genetic information by the horizontal transfer of conjugative plasmid, transposon and integron (Redfield, 2001). Gene transfer can occur through transformation, transduction and conjugation. Mutation may occur in the gene encoding a target protein, a transport protein, a protein for drug activation or a promoter or regulatory gene affecting expression of the target transport protein or an inactivating enzyme (Kraniotaki *et al.*, 2006, Haft *et al.*, 2006). Horizontal gene transfer among bacteria, directed by strong antimicrobial selective pressure, has resulted in widespread multidrug resistance genes on plasmids and transposons within Gram-negative bacteria (Gaur *et al.*, 2006, Mendes *et al.*, 2009).

Over the last two decades, multiple antimicrobial resistance to useful classes of antibiotics, including the penicillins, cephalosporins, aminoglycosides, and fluoroquinolones, has gradually increased among most of Gram-negative hospital pathogens, especially *Klebsiella pneumoniae*, *Enterobacter* spp, *Pseudomonas aeruginosa*, and *A. baumannii*. Clinical trials have revealed that about 4% of infecting microorganisms became resistant upon antimicrobial treatment (Marinez and Baquero, 2000). In addition, a correlation has been discovered between the amount of antibiotics used and the level of resistance (Rivera *et al.*, 2003). In addition, the intensive use of antibiotics over the past 50 years has resulted in an increase in the number of commensal and pathogenic bacteria that are resistant to antimicrobial drugs (Rivera *et al.*, 2003, Mendes *et al.*, 2009). Human bacterial flora does play a potential role in the transfer of antibiotic resistance, and there is increasing evidence that the normal flora represents a pool for selection of resistance genes, which may

disseminate to other species and genera by horizontal transfer via conjugation, transduction or transformation (Smeets and Vandenbroucke-Grauls, 2007).

2.7 Antimicrobial resistance in *Acinetobacter*:

Antimicrobial resistance among *Acinetobacter* spp has increased substantially in the past decade (Maragakis and Perl 2008). The capacity of *Acinetobacter* spp. for extensive antimicrobial resistance may be due in part to the organism's relatively impermeable outer membrane and its environmental exposure to a large reservoir of resistance genes (Bonomo and Szabo, 2006). Mechanisms of its resistance are impressive and rival those of other nonfermentative Gram-negative pathogens (Peleg *et al.*, 2008). The intensive studies were driven by the wide spread of the multidrug resistant strains of *Acinetobacter* that was mostly found belonging to *A. calcoaceticus* – *A. baumannii* complex (Valenzuela *et al.* 2007). The *A. calcoaceticus* – *A. baumannii* complex is commonly resistant to multiple antibiotics including β -lactams, aminoglycosides, carbapemems and quinolones. These bacterial species are generally susceptible to tigecycline; however, a few clinical strains with decreased tigecycline susceptibility have been isolated (Ruzin *et al.*, 2007).

2.7.1 Mechanisms of resistance of *Acinetobacter*:

The mechanisms of antimicrobial agents resistance generally fall into 3 categories: (1) antimicrobial-inactivating enzymes, (2) reduced access to bacterial targets, or (3) mutations that change targets or cellular functions (Maragakis and Perl 2008). Regarding the first mechanism, *Acinetobacter* spp. was found to express a wide array of beta-lactamases which allow *A. baumannii* to be resistance to penicillins, cephalosporins, and carbapenems. For instance, AmpC cephalosporinases are chromosomally encoded and confer resistance to broad-spectrum cephalosporins (Thomson and Bonomo, 2005), and Carbapenems resistance were found to be due to class D OXA-type enzymes or due to class B metallo- β -lactamases (MBLs) both with activity against carbapenems (Thomson and Bonomo, 2005). Activity against carbapenems was recently reported in locations that include Scotland, Spain, France, Japan, Singapore, China, Brazil, Cuba, and Kuwait (Brown and Amyes, 2006). In addition to β -lactamases, it was also discovered that carbapenems resistance is due to changes in penicillin-binding proteins (PBPs) that prevent their action as well as an alterations in the structure and number of porin proteins that result in decreased permeability to antibiotics through the outer membrane of the bacterial cell (Perez *et al.*, 2007). Aminoglycoside resistance, which is common in *Acinetobacter*, results primarily from inactivation of the antibiotic by specific modifying enzymes. Three classes of aminoglycoside-inactivating enzymes (acetyltransferases, phosphotransferases, and adenyltransferases) have been identified in *Acinetobacter* (Magnet *et al.*, 2001). A recent, 16S rRNA methylation has been described for *A. baumannii* strains from Japan, Korea, and the United States. This emerging resistance mechanism impairs aminoglycoside binding to its target site and confers high-level

resistance to all clinically useful aminoglycosides, including gentamicin, tobramycin, and amikacin (Peleg *et al.*, 2008).

The third category of resistance mechanisms involves point mutations that alter bacterial targets or functions, decreasing the affinity for antimicrobial agents or up-regulating cellular functions, such as the production of efflux pumps or other proteins. Such type of resistance is illustrated in colistin resistance. It is thought that such resistance is mediated by changes in the bacterial cell membrane that interfere with the agent's ability to bind bacterial targets (li *et al.*, 2005). This type of mechanism is also seen in *Acinetobacter* resistance to quinolone agents from mutations in the bacterial targets gyrA and parC topoisomerase enzymes (Bronomo and Szabo, 2006).

In addition to the above mechanisms, *Acinetobacter* spp. can acquire resistance genes from other organisms. Mutations leading to resistance can develop over time in *Acinetobacter* strains; or subpopulations with preexisting resistance may emerge and become dominant under antimicrobial selective pressure (Fzal- Shah *et al.*, 2001). This may explain the emerging resistance of *Acinetobacter* spp. A recent study in France revealed that multidrug-resistant *Acinetobacter* strain had a large genomic "resistance island" containing 45 resistance genes that appeared to have been acquired from *Pseudomonas*, *Salmonella*, or *Escherichia* genera (Fournier *et al.*, 2006). In conclusion, *Acinetobacter* multi drug resistance may be explained by both selective pressure exerted by the use of broad-spectrum antimicrobials and transmission of *Acinetobacter baumannii* strains among patients, although the relative contributions of these mechanisms are not yet known (Maragakis and Perl, 2008).

2.7.2 Molecular epidemiology of antibiotic-resistant in *Acinetobacter*:

In the past decades, there has been an increasing interest in the molecular epidemiology of bacteria. Data generated by a variety of phenotypic and genotypic methods can be used to identify the routes of transmission, both in a localized outbreak situation as well as in inter-hospital or cross-country spread, (Bergogene-Bérézin *et al.*, 2001).

To date, there are three clinically important *Acinetobacter* species: *Acinetobacter baumannii* and the unnamed *Acinetobacter* genomic species 3 and 13TU. Among those, *A. baumannii* is the most significant nosocomial pathogen. It has been detected in patients with impaired host defenses, especially in the intensive care unit (Chang *et al.*, 2005). It has been implicated in nearly all kinds of infections including severe nosocomial infections such as bloodstream infection (BSI), pneumonia, and meningitis (Munoz-Price *et al.*, 2008). Mortality rates in those infections were as high as 64%. Similar to methicillin-resistant *Staphylococcus aureus* (MRSA), major epidemiologic features of these organisms include their propensity for clonal spread, their involvement in hospital outbreaks as well as resistance to multiple antimicrobial agents (Bergogene-Bérézin and Towner, 1996).

At 1986 onwards, when the taxonomy of the genus *Acinetobacter* was revised, molecular methods provided the necessary tools to identify *Acinetobacter* at the species level. Detailed studies of the epidemiology of the different members of this genus became possible (Ecker, *et al.*, 2006). Among the variety of molecular methods developed, plasmid profile analysis and pulsed-field gel electrophoresis (PFGE) were used for typing purposes only, while others – such as ribotyping and AFLP – were primarily developed for species identification. To the present day, PFGE remains the

gold standard for epidemiological strain typing not only for *Acinetobacter* spp. but also for bacteria in general. PCR-based methods – such as randomly amplified polymorphic DNA-PCR (RAPD-PCR) and repetitive extragenic palindromic (REP) PCR – are generally not only easier to perform and less expensive, but they also tend to be less discriminative and less reproducible (Chang, *et al.*, 2005). In order to discuss in more detail method used in this study for epidemiological typing the coming paragraphs will in sight the tools that have been used widely and been involved in the spread of MDR *A. baumannii*.

2.7.2.1 Plasmids:

Plasmids are extrachromosomal circular double stranded DNA molecules that usually present in Gram negative bacteria. Their size varies from 1 to over 400 kilo base pairs (kb), and their copy number varies from one copy, for large plasmids, to hundreds of copies for smaller ones. Antimicrobial resistance is commonly encoded by genes carried on plasmid DNA. It can be acquired through its horizontal transfer of resistance genes carried on plasmids or transposons, and it may be associated with heavy metal resistance (Rivera, 2003, Shakibaie, *et al.*, 1998). Such resistant plasmids (R-plasmids) are also observed among Gram-negative isolates from clinical and environmental sources in Jordan (Shehabi *et al.*, 2004, Shehabi *et al.*, 2006). Plasmid-mediated gene exchange between bacteria plays an important role in their pathogenesis, and has greatly contributed to the rapid spread of antimicrobial resistance among bacterial populations (Smalla *et al.*, 2006).

The presence of R-plasmids is considered as significant feature of *Acinetobacter* spp., and plasmid profiling has been proposed as a method for their epidemiological typing. More than 80% of *Acinetobacter* strains carry multiple indigenous plasmids of various

sizes, and studies have found that clinical and environmental isolates of *Acinetobacter spp.* harbor plasmids of different molecular sizes ranging from 15-56 kb (Joshi *et al.*, 2003). A recent study found that *Acinetobacter spp.* on healthy human skin harbor very few plasmids as compared to clinical or environmental isolates (Pardesi *et al.*, 2007). A study performed in the Netherlands has shown that a transferable plasmid encoding SHV-12 extended-spectrum β -lactamase, TEM-116, and aminoglycoside resistance was responsible for two sequential clonal outbreaks of *Enterobacter cloacae* and *A. baumannii* (AL-Naiemi *et al.*, 2005). The ease with the spreading of *Acinetobacter spp.*, antimicrobial resistance is a major concern which is mainly attributed to the presence of plasmids (Pardesi *et al.*, 2007).

2.7.2.2 Integrons:

The spread of antimicrobial resistance genes among bacterial strains is an increasing problem in infectious diseases (Koeleman *et al.*, 2001). Antimicrobial resistance genes are mostly located on plasmids and on transposons, enabling their transfer among a variety of bacterial species. In recent years, a third mechanism of resistance gene dissemination has been discovered which was coined as integrons (Koeleman *et al.*, 2001). Integrons are transposon-like DNA segments that have the ability to capture and mobilize gene cassettes (Koeleman *et al.*, 2001, Dillon *et al.*, 2005, Turton *et al.*, 2006), notably containing antibiotic resistance gene by site specific recombination (Oh *et al.*, 2002). Insertion and excision of these cassettes occur via a site-specific recombinase that belongs to the integrase family (Koeleman *et al.*, 2001). A distinguishing feature of an integron is the presence of three components within the conserved 5' which are separated by a variable region which includes integrated antimicrobial resistance genes or cassettes of unknown function.

The 5' conserved segments (5'-CS) contains three components (Fig. 1.1): First component is an *intI* gene which encodes an integrase enzyme which is a polypeptide of 337 amino acids and acts as a site-specific recombinase. A common promoter region located in the opposite strand, directed toward the site of integration is the second component. It is responsible for the expression of gene cassettes. Since most genes inserted into integrons lack their own promoter, they are expressed from a common promoter site region as a resistance operon. The third component is an *attI* gene, which encodes the cassette integration site. The 3' conserved segments (3'-CS) contain the *qacE1*, *sulI* genes and an open reading frame (ORF), *orf5* (Koeleman *et al.*, 2001, Le'vesque *et al.*, 1995). The *qacE1* and *sulI* genes determine resistance to ethidium bromide and quaternary ammonium compounds, and to sulfonamide, respectively (Le'vesque *et al.*, 1995), whereas the open reading frame (ORF5) function is unknown (Rebecca *et al.*, 1999). The downstream end of each resistance gene cassette, inserted in variable region of integrons, there is a short imperfect inverted repeat element called the 59-base element. Each of the inserted genes has its own version of this element. These 59-base elements are believed to be important in the recombination events (Le'vesque *et al.*, 1995).

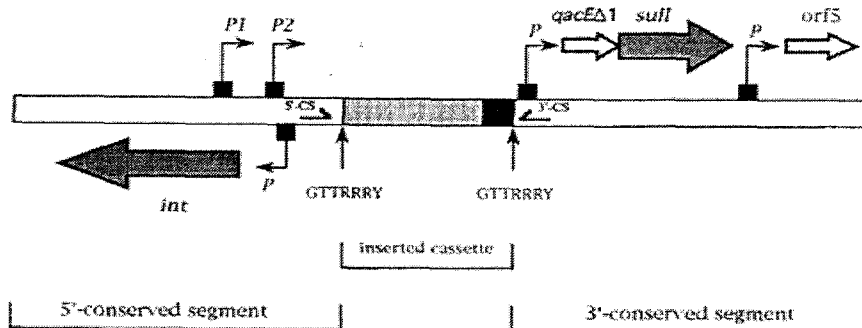


Fig. 1.1 Scheme for PCR detection of integron structure. The grey arrow shows the direction of transcription. 5'CS and 3'CS are specific to the 5'- and 3'-conserved segments of integrons, and were used to amplify the variable regions of class 1 integrons (integron PCR). (Koeleman et al., 2001)

The presence of integrons is well investigated in *Acinetobacter* spp., as has their relatively high frequency of carriage in epidemic strains (Prashanth *et al.*, 2004). Based on the sequence of their *intI* genes, four classes of integrons have been described, three of which (classes 1 to 3) contain antibiotic resistance gene cassettes and each of which code for a distinct but related integrase enzyme (Rosser and Young, 1999). At present, approximately 60 different gene cassettes have been identified, most of which encode resistance to antibiotics (Hall *et al.*, 1994). Class 1 integrons are predominantly associated with a *sulf* gene as part of a 3'-conserved segment (Schmitz and Fluit, 1999).

It is the most prevalent in clinical isolates of Gram-negative bacteria, and over 60 gene cassettes have been identified, most of which encode different resistance to antimicrobial (Jones *et al.*, 1997, Koeleman *et al.*, 2001, Oh *et al.*, 2002). Such integrons have been found in many genetic locations including R46, R388 and R751 as well as transposons Tn21 and Tn1696 (Rebecca *et al.*, 1999). Class 2 integrons (*intI2* gene) whose products are 40% identical to that of the *intI1* (Rebecca *et al.*, 1999, Oh *et al.*, 2002), and they include transposon Tn7 and relatives (Oh *et al.*, 2002,

Dillon *et al.*, 2005), where the integrase appears to be defective (Dillon *et al.*, 2005), and the variable region contains two or more integrated gene cassettes encoding resistance to trimethoprim (dhfrIa or dhfrIb), streptothricin (sat) and streptomycin (ant(3)-Ia) (Rebecca *et al.*, 1999). In class 3 only one integron has been described. It encodes the intI3 integrase showing 61% homology with amino acid sequences of the intI1 integrase. It contains a metallo- β -lactamase gene (blaIMP) (Rebecca *et al.*, 1999, Oh *et al.*, 2002).

Many studies have revealed that integrons are widely distributed in multi-resistant isolates of *Acinetobacter* species (Koeleman *et al.*, 2001). Accordingly, it was assumed that integrons are important for the dissemination of antimicrobial-resistant genes and for nosocomial spread in *Acinetobacter* species (Oh *et al.*, 2002). The prevalence of integrons in clinically significant bacterial isolates appears to be around 50% (Dillon, *et al.*, 2005). A recent Korean study showed that the presence of integrons was significantly ($p < 0.01$) associated with multiple antimicrobial resistance and nosocomial spread in *Acinetobacters* strains (Oh *et al.*, 2002).

Several outbreaks of *A. baumannii* have been documented in ICUs worldwide in recent years due to multidrug-resistant strains, (Rebecca *et al.*, 1999, Oh *et al.*, 2002, Dillon *et al.*, 2005, Kraniotaki *et al.*, 2006, Peleg *et al.*, 2008). Moreover, these studies have shown that common cassettes may be found in class1 integrons of *A. baumannii*, for example the 2.2kb and 2.5kb cassette arrays were found in class1 integrons of *A. baumannii* isolates in Italy, France and Spain (Kraniotaki *et al.*, 2006). The fact that non-related isolates from different geographic areas are able to acquire common integrons also suggests that *A. baumannii* might have a clear affinity for a specific type of integrons (Kraniotaki *et al.*, 2006). It was approved that common cassettes

may be found in class1 integrons of *A. baumannii*. In addition non-related isolates from different geographic areas were able to acquire common integrons which may suggest that *A. baumannii* might have a clear affinity for a specific type of integrons (Kraniotaki *et al.*, 2006).

2.7.3 Methods of detecting genotypes of *Acinetobacter*:

In the last decade there was a tremendous increase in number of patients with *A. baumannii*, which suggests a serious case of nosocomial transmission (Ecker *et al.*, 2006). This condition has endorsed the potential of *Acinetobacter* to become a serious problem in hospitals, and clinical centers which are reservoirs of epidemic strains. On the other hand, the intricacy of *Acinetobacter* infections rises from the difficulty in correct identification and differentiation among *Acinetobacter* species (Van Looveren *et al.*, 2004).

Increasing cases related to *Acinetobacter* infections have widen the understanding the fundamental investigations for controlling *Acinetobacter* infections. Investigations usually include the knowledge of the original sources of the infection, the clonality, and geographical spread, which are all considered as an important requirement for the development of appropriate infection control measures. Genotyping allows investigation of clonal spread and can be used to identify the source of the original infection. Traditional *Acinetobacter* strain typing methods include serotyping (Traub , 1989), multilocus enzyme electrophoresis (Seltmann *et al.*, 1995), and DNA-based methods, including repetitive extragenic palindromic sequence-based PCR (Bou *et al.*, 2000, Huys *et al.*, 2005), amplified ribosomal DNA restriction analysis (ARDRA),

pulsed-field gel electrophoresis (PFGE), amplified fragment length polymorphism (AFLP) (Vanechoutte *et al.*, 1995), ribotyping (Ibrahim *et al.*, 1997, Ibrahim *et al.*, 1997, Misbaha 2005) and Multilocus Sequence Typing (MLST) (Urwin and Maiden, 2003). Conversely, because of increasing availability of sequencing facilities, sequences of specific genes may be useful for identification of *Acinetobacter* spp. Either partial (Tang *et al.*, 1998) or nearly complete (Ibrahim *et al.* 1997) sequence analyses of the 16S rRNA gene for *Acinetobacter* classification have been reported. In addition, the ITS region separating the 16S and 23S rRNA genes has been suggested to be a good candidate for bacterial species identification (Barry *et al.*, 1991), since these regions have degrees of low intraspecies variation and high degrees of interspecies divergence (Chen *et al.*, 2004). In the current study we have applied 16S-23S rRNA gene intergenic spacer (ITS) sequencing technique to analyze the genotyping of clinical isolates of *Acinetobacter* spp that were collected from two different hospitals of Jordan at two different time periods (2006 and 2008).

2.7.3.1 Impact of 16S-23S rRNA gene intergenic spacer (ITS) sequence analysis:

The historical method for performing identifications to clinical isolates, used to be dependent on the comparison of an accurate morphologic and phenotypic description of type strains or typical strains with the accurate morphologic and phenotypic description of the isolate to be identified. To complete the task of identification, microbiologists authoring standard aid references such as Bergey's Manual of Systematic Bacteriology or the Manual of Clinical Microbiology or compiling results from well-characterized strains such as those found at the Centers for Disease Control and Prevention or the American Type Culture Collection (ATCC) that contain published tables summarizing the characteristics of each species of

bacteria (Krieg and Holt, 1948, Funke *et al.*, 1997, Murray *et al.*, 1999). Microbiologist would try to match the results for their unknown clinical strain with a group in these tables. Not infrequently, there would be no perfect match and a judgment would have to be made about the most probable identification. Although different schema and computer programs were also devised to be applied in the assurance of their judgments' identification variations are still present among laboratories (Sussman *et al.*, 1986). In addition, taking into consideration what we have discussed earlier that the traditional phenotypic identification of bacteria is generally not as accurate, identification based on genotypic methods became an alternative identification methodology. A newly emerged genotyping method is the comparison of the bacterial rRNA gene sequence. The ribosomal entity comprises three distinct types: the 16S rRNA, the 23S rRNA and the 5S rRNA. The genes coding for these genes composed of organization operon that is consistent from one bacterium to another (Barry *et al.*, 1991).

The operon organization consist of: a promoter region followed by a sequence of 16S rRNA, a spacer of intergenic sequence, the 23S rRNA sequence, another short spacer sequence, and lastly the 5S rRNA sequence (Srivastava and Schlessinger, 1990). Upon examination of base spacer regions wide variations were observed among different groups of bacteria. Accordingly, researchers have suggested applying this polymorphism to differentiate bacteria to the genus, species as well as to the strain level (Gulter and Stanisich, 1996).

One of the most promising sequence analysis of the 16S-23S rRNA genes spacers was the intergenic spacer (ITS) genes in *Acinetobacter* spp. It has led to the discovery of short sequences very specific for *Acinetobacter* spp., more precisely for *A. baumannii*

(Lagatolla *et al.*, 1998). For that reason the ITS represents an important element in strains typing for environmental (Garcia-Martinez *et al.*, 2001) or clinical purposes (Baudart *et al.*, 2000, Clementino *et al.*, 2001). ITS sequences have multiple functional roles such as (1) presence of secondary structure at the beginning and the end of the spacer that pairs with sequences upstream of the 16S rRNA gene and downstream from the 23S rRNA respectively to allow its excising, (2) presence of antiterminator boxes to avoid the premature termination of transcription (Iteaman *et al.*, 2000) and (3) presence of tRNA genes. This landscape of high-moderate-low conservation makes ITS a potentially useful model for the study of functional motifs in other spacer regions of prokaryotic genomes as well as a powerful identification marker for various bacteria (Carr *et al.*, 2004, Ciantar *et al.*, 2005, Moreira *et al.*, 2005). Amplification of the ITS was performed using universal primers targeted to conserved sites in the 16S and 23S rRNA genes (Jensen *et al.*, 1993). Thus, ITS sequence analysis can be performed on all organisms and yet has the ability to discriminate between species and strains (Anton *et al.*, 1998).

In addition to sequence analysis, restriction digest analysis was applied to be used as a useful tool to genotype strains of *Acinetobacter* spp. for the PCR amplified 16S-23S rRNA intergenic spacer ITS genes (Dolzani *et al.*, 1995, Chang *et al.*, 2005, Ko *et al.*, 2008). Although restriction pattern analysis using number of restriction enzyme endonucleases is not yet suitable for routine analysis in clinical laboratories, yet it is fast and simpler than any other genotyping techniques and lots of attempts in developing more rapid, less labor-intensive typing schemes, have appeared in the literature (Bouchet *et al.*, 2008).

Chapter 3
Materials and Methods

Chapter 3

Material and Method

3.1 Materials:

The media used for cell cultures as well as biochemical testing were supplied by HiMedia, India. Types of media used include Nutrient agar, Nutrient broth, Triple sugar iron (TSI), Muller Hinton Agar and Muller Hinton broth. The Gram stain was supplied from Technopharmachem, India. The API-20E test kit for Enterobacteriaceae that was recently broadened to include the identification of nonfermentative gram-negative bacteria and its reagents were supplied from BioMerieux, France. The API 20E system was used for the biochemical identification of all clinical isolates of *Acinetobacter baumannii*.

The antibiotic discs used in antimicrobial susceptibility test were purchased from HiMedia, India. Genomic DNA purifications were performed using DNA extraction kit supplied by Wizard (Promega, USA). For PCR reactions “GoTaq Green Master Mix” was used From (Promega, USA) which is a premixed ready-to-use solution containing bacterially derived Taq DNA polymerase, dNTPs, MgCl₂ and reaction buffers at optimal concentrations for efficient amplification of DNA templates by PCR. The PCR products were purified with a Wizard SV gel and PCR clean- up kit (Promega, USA).

Agarose electrophoresis grade was supplied from Life Technologies Company, USA. The power supply and an electrophoresis chambers were both supplied from Spectroline. Electrophoresis buffer Tris-borate-EDTA (TBE) was supplied from Promega, USA. Ethidium bromide and screening dye bromophenol blue for DNA electrophoresis were supplied from Sigma, USA.

Two different DNA ladders were used to verify chromosomal DNA isolates as well as amplified DNA fragments. The two DNA ladders were 100 bp plus DNA ladder and 100 bp DNA ladder (Promega, USA).

3.2 Methods:

3.2.1 Bacterial strains:

Twelve isolates of *Acinetobacter baumannii* were recovered from twelve clinical samples were used in this study. Seven isolates were recovered from ICU patients of the King Hussein Cancer Center (KHCC) in 2006. The rest of were from in patients of Jordan University hospital in 2008. The clinical samples were isolated from twelve different patients with different types of infections at two different periods of time. Pure clinical isolates, identified as *Acinetobacter baumannii* in the microbiology laboratory of the above two mentioned hospitals were supplied for conducting this research.

Standard control strains of *Pseudomonas aeruginosa* (ATCC 27853) and *Escherichia coli* (ATCC 25922 strains) were used simultaneously with tested clinical isolates, for the purposes of justifying results obtained in several tests.

3.2.2 Bacterial growth and identification media:

Nutrient agar (NA) medium (peptide digest of animal 5 g/L, tissue 1.5 g/L, beef extract 1.5g/L, yeast extract 1.5 g/L, sodium chloride 5 g/L and agar 15g/L) was prepared by dissolving 11.2 g nutrient agar powder in 1L distilled water and autoclaving it for 15 minutes at 121°C. Clinical isolates were routinely sub cultured on Nutrient agar plates and incubated at 37°C for 18-24 hour. For bacterial culture maintenance nutrient agar slants were prepared by single straight line inoculation on

the surface of the slope in universal bottles and then incubated at 37 °C for 18-24 hours.

Triple sugar iron agar (TSI) was made by dissolving 65 g TSI agar powder in 1L distilled water, dispensed into tubes, autoclaved at 121°C for 15 minutes dispensed in sterile test tube and cooled in a slanted position so that deep butts are formed. Using a sterile needle, an isolated colony on plated media was inoculated by stabbing into the medium in the butt of the tube, and then streak back and forth along the surface of the slant. Several colonies from each primary plate were studied separately, since mixed infections may occur. Inoculated TSI tubes were incubated with caps loosened at 37°C and examined after 18-24 hour for carbohydrate fermentation, gas production and hydrogen sulfide production.

Muller Hinton Agar (MHA) medium was prepared by dissolving 38 g of MHA powder in 1L distilled water and autoclaving for 15 min at 121°C. Muller Hinton broth (MHB) made by dissolving 21g of MHB powder in 1L distilled water and autoclaving it for 15 min at 121°C.

Gelatin agar media was prepared by the mixing 20g of gelatin powder with 28g of nutrient agar powder in 1L of distilled water and autoclaving for 15min at 121°C. Melted gelatin agar was poured in sterile Petri dishes and stored at 4°C. For the hydrolyzed gelatin, mercuric chloride (HgCl₂) was prepared by dissolving 15 g in 20 ml concentrated hydrochloric acid, and distilled water added to make 100 ml.

3.2.3 Bacterial cultures maintenance:

Short-duration working stocks (few weeks) were maintained on nutrient agar slants at room temperature (≈25°C). For long- term preservation, heavy suspension in nutrient broth media with 20% v/v glycerol was stored at -70°C until use. Bacterial