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

August 2009

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

August 2009

Major Supervisor Name:	**Signature**
1. Prof. Khalid Matalka	----------
Examination Committee	
1. Dr. Mona Bustami	----------
2. Dr. Elham Al-Kaissi	----------
3. Dr. Rula M. Darwish	----------

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

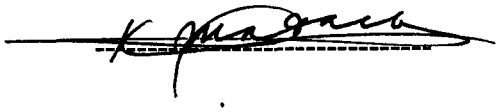
By
Nermen Dakkak
Petra University, 2009
Under the Supervision of Prof. Khalid Matalka

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 characterstics 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-23Sr RNA 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 *Alu*1, *Cfo*1, *Rsa*1, *Msp*1 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 MATALKA



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 Matalka 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.

I offer my sincerest gratitude to Dr. Mona Bostami, who has supported me thoughout my thesis with her patience and knowledge whilst allowing me the room to work in my own way. I attribute the level of my Masters degree to her encouragement and effort and without her this thesis, too, would not have been completed or written. One simply could not wish for a better or friendlier supervisor.

I want also to acknowledge Dr. Elham Al kaissi, for the time she spent revising this dissertation, and for her continous encouragement and support. I have a big sense of gratitude to Rana Afifi for the time the spent with me in the lab and for her help and patient answers to my questions.

I want to acknowledge Dr.Abdul Jalil Farwati for his support and help.

I am very happy and proud of making wonderful friends in Jordan. Especially I am thinking to my best friends: Sereen, Basma, Alaa, Lina, Nour, Salma and Safaa . I will always cherish the wonderful time spent together with them.

Finally, I would like to thank my brothers: Rami, Nabegh, and Tarek, mum and dad who have endured and support me through this period, and forked out the money for my extension, I love you so much.

TABLE OF CONTENTS

List of Tables

List of figures

List of abbreviation

ICU	**Intensive care unit**
IDSA	**Infectious Disease Society of America**
***Acinetobacter* spp**	**Acinetobacter 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**
PBPs	**Penicillin-binding proteins**
BSI	**Bloodstream infection**
MRSA	**Methicillin-resistant *Staphylococcus aureus***
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 chang 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. jujnii, 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

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. gerneri, A. grimontii, A. tandoii, A. tjernbergiae*, and *A. towner.* 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, Zimbler *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 car (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).