PUTATIVE VIRULENCE FACTORS AND GENOTYPING OF *ACINETOBACTER BAUMANNII* COLONIZING RESPIRATORY TRACTS OF PATIENTS.

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Authorization Form

I, Noor Khaled Al-Dabaibah, authorize the University of Jordan to supply copies of my Dissertation to libraries or establishments or individuals on request.

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Date: **25/11/2010**

COMMITTEE DECISION

This Thesis (Putative virulence factors and genotyping of *Acinetobacter baumannii* colonizing respiratory tracts of patients) was successfully defended and Approved on 25/11/2010

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DEDICATION

To
my beloved family for their love, support, and encouragement.

To
all my friends who gave me hand and encouraged me.

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LIST OF ABBREVIATIONS

A. baumannii	*Acinetobacter baumannii*
Acb	*A. baumannii-A. calcoaceticus*
AIF	Apoptosis-Inducing Factor
ATCC	American Type Culture Collection
CHDLs	Carbapenem hydrolyzing class D β-lactamases
CICU	Cardiac Intensive Care Unit
CLED	Cysteine lactose electrolyte deficient agar
DHBA	Di-Hydroxybenzoic Acid
DIP	2,2'-Dipyridyl
EDTA	Ethylenediaminetetraacetic acid
ERIC	Enterobacterial repetitive intergenic consensus
E-test	Epsilometer test
HA	Hemagglutination
ICU	Intensive Care Unit
i.v.	intravenous
JUH	Jordan University Hospital
LOS	Length of Stay
LPS	Lipopolysaccharide
MBLs	Metallo-β-Lactamases
MDR	Multi Drug resistant
MICs	Minimum Inhibitory Concentrations
MICU	Medical Intensive Care Unit
MSA	Minimal-salt agar supplemented with 1% acetate
CLSI	Clinical and Laboratory Standards Institute
OMPs	Outer Membrane Proteins
OPD	Outpatient Department

LIST OF ABBREVIATIONS

PCR	Polymerase Chain Reaction
QS	Quorum Sensing
SICU	Surgical Intensive Care Unit
Spp.	Species
SPSS	Statistical Package of Social Science
TBE	Tris-Borate EDTA
TLR	Toll-Like Receptor
UTIs	Urinary Tract Infections
VAP	Ventilator-Associated Pneumonia
VIM	*V*erona *i*ntegron-encoded *M*BL

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PUTATIVE VIRULENCE FACTORS AND GENOTYPING OF *ACINETOBACTER BAUMANNII* COLONIZING RESPIRATORY TRACTS OF PATIENTS.

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ABSTRACT

A. baumannii is an important nosocomial pathogen that is rapidly evolving toward multidrug resistance and is involved in various nosocomial infections that are often severe. However, its pathogenic characteristics are not well defined. This study investigated the rate of *A. baumannii* colonizing the respiratory tract of 185 patients admitted to the Jordan University Hospital over a period of 10 months (May 2009 to February 2010).

The identity of isolates was confirmed by biochemical tests. Risk factors for respiratory colonization by *A. baumannii* were determined. Multivariate analysis showed that the only independent risk factors for the acquisition of *A. baumannii* were long duration of mechanical ventilation and long stay in the ICU. All *A. baumannii* isolates were examined for their antimicrobial susceptibility patterns using the disc diffusion method. MIC was determined by the E-test.

The results demonstrated that (98.4%) of *A. baumannii* were resistant to more than five antimicrobial agents. The highest resistance rates among *A. baumannii* isolates were as follow: meropenem, ertapenem and ceftazidime (100%), followed by piperacillin/tazobactam, and aztreonam (98%), gentamicin (94%), ciprofloxacin (91%), amikacin (73%), and imipenem (63%), whereas, no resistance was detected to tigecycline and polymyxin E. Both amikacin and imipenem have the highest MICs, (13.6 and 12 respectively). None of the strains was positive for hemolysin production, protease or lecithinase activity. All of the strains were positive for lipase activity. Gelatinase activity was detected in 6 (9.4%) of isolates. All of the isolates were able to grow in iron-limiting conditions. Thirty and 23 of the isolates agglutinated human group AB and O erythrocytes in the presence of mannose, respectively. Sixty two isolates (96.9%) showed positive results in the modified Hodge test and only two strains were positive in the imipenem-EDTA-double disk synergy test. All strains of *A. baumannii* possessed $bla_{\text{OXA-51-like}}$ gene, 73% and 19% of the isolates harbored $bla_{\text{OXA-23-like}}$ and $bla_{\text{OXA-24-like}}$ genes, respectively. Only two strains harbored MBL-encoding gene (bla_{VIM}). The genetic similarity of 64 *A. baumannii* isolates using Enterobacterial Repetitive Intergenic Consensus (ERIC)-PCR and dendrogram similarity gave 2 major clusters (genotypes) including genetically related isolates.

In conclusion, high prevalence of multidrug resistant *A. baumannii* colonizing the respiratory tract of hospitalized patients in ICUs is an important risk factor to develop infection later. The circulation of few genotypes of *A. baumannii* isolates at the Jordan University Hospital indicates the importance of implementing extensive infection control measures.

1. Introduction

1.1. Introduction

Members of the genus *Acinetobacter* are non-motile, ubiquitous Gram-negative bacteria that can be recovered from a wide range of sources such as human, soil, water, food products, and medical environments (Peleg et al., 2008). During the last 40 years, *Acinetobacter baumannii* has become an increasingly important cause of nosocomial infections in intensive care units (ICUs), and it occurs either in sporadic cases or as outbreaks of infection (Wadl et al., 2010; Luna and Aruj, 2007). *A. baumannii* has become an increasingly important cause of nosocomial pneumonia, particularly in mechanically ventilated patients. Ventilator-Associated Pneumonia (VAP) caused by *A. baumannii* is emerging as a prominent complication of hospitalization. Prolonged hospitalization, and mechanical ventilation, and prior use of antibiotics are the recognized factors that increase the risk of VAP due to *A. baumannii* (Routsi et al., 2010; Luna and Aruj, 2007). Because of its wide distribution and colonizing capability, *A. baumannii* does not always act as an infecting pathogen (Garcia-Garmendia et al., 2001). Patients in intensive care units are at most risk, particularly those with severe burns, mechanical ventilation, and those who are catheterized, whereas community acquired Acinetobacter infections are rare (Wadl et al., 2010; Luna and Aruj, 2007; Hanlon, 2005). It has been observed that *A. baumannii* is notorious for its capacity to survive for long periods in patients and their environment, which is at least partly due to its remarkable genomic flexibility that enables the organism to acquire resistance to many antibiotics. *A. baumannii* infections are difficult to treat owning to innate and acquired antimicrobial resistance (Higgins et al., 2010). As a result, the treatment of hospital-acquired *A. baumannii* infections is becoming problematic (Huys et al., 2005). Acinetobacter infections have also been reported among military and civilian personnel

injured in combat, possibly due to direct environmental contamination of wounds. For example, during the Vietnam War, *A. baumannii* was the most common Gram-negative bacterium recovered from traumatic injuries to extremities (Ecker et al., 2006), and more recently it has caused serious infections among American military personnel serving in Iraq and Afghanistan (Loehfelm et al., 2008). *A. baumannii* strains responsible for hospital outbreaks are resistant to a wide range of antimicrobials, including broad-spectrum β-lactams, carbapenems, aminoglycosides, and flouroquinolones (Tripodi et al., 2007). An increasing incidence of carbapenem-resistant *A. baumannii* bacteremia and pneumonia has been observed in patients hospitalized in ICUs (Routsi et al., 2010; Valencia et al., 2009). The incidence of carbapenem resistance has risen dramatically and is considered a global sentinel event (Bogiel et al., 2010; Mera et al., 2010). Carbapenem resistance in *A. baumannii* is mediated most often by oxacillinases (OXAs) and less frequently by metallo-β-lactamases (MBLs) (Higgins et al., 2010). Much is known about the processes involved in multidrug resistance, but those factors underlying the pathogenicity and virulence potential of the organism are only beginning to be elucidated (Gordon and Wareham, 2010). Although *A. baumannii* is considered to be a relatively low-grade pathogen, some characteristics of this bacterium may enhance virulence of the strains involved in infection (Joly-Guillou, 2005; Braun and Vidotto, 2004). Among the virulence factors are: adhesion to human epithelial cells in the presence of fimbriae and/or capsular polysaccharide, the high surface hydrophobicity of certain strains, the ability to grow in iron-limited conditions by secreting iron-regulated siderophores (Braun and Vidotto, 2004), the presence of mannose-resistant hemagglutinin (Cevahir et al, 2008; Braun and Vidotto, 2004), and production of extracellular enzymes (Gerischer, 2008). Adherence of bacteria to epithelial cells is considered to be an essential first step in colonization

and subsequent infection of a host. In addition, biofilm formation is thought to be an important pathogenic feature, especially in relation to intravascular line infections and VAP (Lee et al, 2008).

Discrimination between strains within a species is important to monitor epidemiological patterns and a number of typing methods have been developed to achieve this. These include, biotyping, antibiogram typing, ribotyping, polymerase chain reaction (PCR) fingerprinting, and analysis of genomic DNA by pulsed-field gel electrophoresis (PFGE) (Ayan et al., 2003). Enterobacterial repetitive intergenic consensus (ERIC) PCR seems to be useful for tracing epidemic strains during outbreaks (Bergogne-Berenzin and Towner, 1996).

1.2. Objectives

The objectives of this study are to:-

- Investigate the frequency of *A. baumannii* colonizing the respiratory tract of adult patients admitted to ICUs at Jordan University Hospital (JUH) over a 10 months period.
- Investigate the frequency of *A. baumannii* colonizing respiratory tracts of patients referring to respiratory outpatients clinics at JUH over a 5 months period.
- Determine the phenotypes of these *Acinetobacter* isolates.
- Determine susceptibility of *A. baumannii* isolated from those patients to common antimicrobial drugs.
- Detect OXA carbapenemase and MBL encoding genes in these *A. baumannii* isolates.
- Determine genotypes of these *A. baumannii* isolates.
- Evaluate the capacity of these *A. baumannii* isolates colonizing respiratory tracts of patients for the production of putative virulence factors in vitro.

2. Literature Review

2.1. General characteristics of *Acinetobacter* species

The name Acinetobacter, (derived from the Greek word *akinetos*), was proposed by Brisou and Prevot in 1954 which means "unable to move" (Bergogne-Berenzin, 2008). The genus Acinetobacter is classified under the family *Morxellaceae* and comprises strictly aerobic, Gram-negative, non-motile, non lactose-fermenting, oxidase-negative, catalase-positive coccobacilli (Karageorgopoulos and Falagas, 2008).

It is wide spread in nature, being found in soil and water. Acinetobacter, like other non-fermentative Gram-negative bacilli, has been recognized as a nosocomial pathogen since the 1970s. *A. baumannii* is an important cause of nosocomial infections in many hospitals (Cisnerso and Rodriguez-Bano, 2002). Initially regarded as of little clinical significance, it is now being isolated more frequently, particularly in intensive care settings where it is a cause of serious infections (Higgins et al., 2010).

Available data suggest that *A. baumannii* is a remarkable microorganism because of the diversity of its habitat, the way it accumulates mechanisms of antimicrobial resistance, its resistance to desiccation, its propensity to cause outbreaks of infection, and the complexity of its epidemiology (Fournier and Richet, 2006a).

2.2. Taxonomy of the genus Acinetobacter

Bacteria now classified as members of the genus Acinetobacter have been the subject of taxonomic change (Bergogne-Berenzin and Towner, 1996). The genus Acinetobacter, as currently defined, comprises Gram-negative, strictly aerobic, non-fermenting, non-fastidious, non-motile, catalase-positive, oxidase-negative bacteria with a DNA G+C content of 39% to 47%. Based on more recent taxonomic data, it was proposed that members of the genus Acinetobacter should be classified in the new family *Moraxellaceae* within the order *Gammaproteobacteria*, which includes the genera

Moraxella, *Acinetobacter*, *Psychrobacter*, and related organisms (Peleg et al., 2008). Even today, the situation is not settled yet and is still undergoing continuous change. Studies based on DNA/DNA hybridization have resulted in the description of at least of 32 'genomic species', 17 of which have been assigned species names (Giamarellou et al., 2008). Only 10 species have been isolated from human specimens (*A. baumannii, A. calcoaceticus, A. haemolyticus, A. johnsonii, A. junii, A. lowffii, A. parvus, A. radioresistens, A. schindler, and A. ursingii*) and seven newly described species were isolated from activated sludge plants that include; *A. baylyi*, *A. bouvetii*, *A. gerneri*, *A. grimontii*, *A. tandoii*, *A. tjernbergiae*, and *A. towneri*) (Dortet et al., 2006). *A. baumannii, A. calcoaceticus* and the unnamed *A.* genospecies 3 and 13 TU are difficult to distinguish phenotypically and therefore they are often given the name *A. baumannii-A. calcoaceticus* (*Acb*) complex (Giamarellou et al., 2008).

2.3. Identification in the laboratory

2.3.1 Identification to the genus level

Acinetobacters are short, plump, Gram-negative rods, typically 1.0 to 1.5 by 1.5 to 2.5 μm in the logarithmic phase of the growth but often becoming more coccoid in the stationary phase. *Acinetobacter* spp. normally form smooth, sometimes mucoid, pale yellow to grayish-white colonies on solid media, although some environmental strains that produce a diffusible brown pigment have been described. Acinetobacters can be readily grown on common laboratory media such as nutrient agar and tryptic soy agar, although defined media consisting of a mineral base containing ammonium or nitrate salts and one or more carbon sources have been used for specific purposes (Bergogne-Berenzin and Towner, 1996). *Acinetobacter* spp. of human origin grow well on solid media that are routinely used in clinical microbiology laboratory such as sheep blood agar or tryptic soy agar at 37 ^{o}C incubation temperature. Isolates of the species *A.*

hemolyticus and several other currently not well-defined species such as *Acinetobacter* genomic species 6, 13BJ, 14BJ, 16, and 17 show hemolysis on sheep blood agar, a property that is never present in Acinetobacter isolates belonging to the *Acb* complex. (Peleg et al., 2008). To facilitate isolation of strains of the *Acb* complex, Leeds Acinetobacter Medium (LAM) was proposed, which is a selective and differential medium containing antibiotics for the inhibition of growth of accompanying microorganisms (Jawad et al., 1994).

For the recovery of Acinetobacters from environmental and clinical specimens (e.g., skin swabs to detect skin colonization), enrichment culture at low pH in a vigorously aerated liquid mineral medium supplemented with acetate or another suitable carbon source and with nitrate as the nitrogen source has proven useful. Most Acinetobacter strains grow between 20^{o}C and 37^{o}C with most strains showing an optimum growth at 33-35^{o}C. Strains of some species grow at higher temperatures (e.g., growth at 44^{o} C is a characteristic feature of *A.baumannii*) (Peleg et al, 2008)**.**

2.3.2.Identification to the genomic species

For epidemiological and clinical purposes, accurate methods of strain identification are necessary to monitor *A. baumannii* infections (Quelle et al., 2001). Because phenotypic identification of Acinetobacter isolates to the species level has proven to be insufficient (Zarrilli et al., 2009), several genotypic methods have been developed for (genomic) species identification of Acinetobacters, including the *Acb* complex. These methods include restriction analysis of amplified ribosomal DNA (ARDRA), the 16S-23S rRNA gene intergenic spacer (ITS) region, the whole ribosomal operon, and the *recA* gene (Chang et al., 2005). More recent developments include the identification of *A. baumannii* by detection of the *bla*$_{\text{OXA-51}}$-like carbapenemase gene intrinsic to this species (Peleg et al., 2008).

2.4. Reservoirs

A. baumannii is ubiquitous in nature and has been recovered from soil, water, animals, and humans (Fournier and Richet, 2006a). *A. baumannii* may rather infrequently colonize the skin of healthy human beings, typically at low density and for a short-term duration. Colonization of other body sites such as the throat, nares, and the intestinal tract, has been found rarely in healthy individuals (Karageorgopoulos and Falagas, 2008). *Acinetobacter* spp. are frequently isolated from the throat and respiratory tract of hospitalized patients. It has been suggested that human skin could be the source of severe *A. baumannii* infections such as bacteraemia (Fournier and Richet, 2006a). Anstey et al. (2002) found that wet-season throat carriage of *A. baumannii* was found in 2 of 20 (10%) community residents with excess levels of alcohol consumption. Seifert et al. (1997) investigated the colonization with *Acinetobacter* spp. of the skin of 40 hospitalized patients and 40 healthy volunteers, a total of 31 *Acinetobacter* isolates were recovered from 17 nonhospitalized individuals, giving colonization rates of 42.5%. The most frequently isolated species were *A. lwoffii* (58%), *A. johnsonii* (20%), *A. junii* (10%), and *Acinetobacter* genomic species 3 (6%). In patients hospitalized on a regular ward, the carriage rate of *Acinetobacter* spp. was even higher, at 75%.

Berlau et al. (1999a) studied skin carriage of Acinetobacter among healthy volunteers, a carrier rate of 44% was found, with *A. lwoffii* (61%), *Acinetobacter* genomic spp. 15BJ (12%), *A. radioresistens* (8%), and *Acinetobacter* genomic species 3 (5%) being the most prevalent species. Dijkshoorn et al. (2005) studied fecal carriage of Acinetobacter and found a carrier rate of 25% among healthy individuals, with *A. johnsonii* and *Acinetobacter* genomic spp. 11 predominating. In contrast, *A. baumannii* was found only rarely on human skin with a rate of 0.5 % and 3% in Berlau et al. (1999a) and Seifert et al. (1997) studies respectively, and in human feces with a rate of 0.8%

(Dijkshoorn et al., 2005).

In recent studies, *A. baumannii* was isolated in unsuspected sources such as food and arthropods (Giamarellou et al., 2008). Therefore, Berlau et al. (1999b) investigated in the United Kingdom the distribution and frequency of *Acinetobacter* spp. in a variety of purchased or harvested fresh fruits and vegetables. Results showed that 17% of vegetables grew Acinetobacter in small numbers and that *A. baumannii* and genomic spp. 11 were the species most frequently isolated. The *Acb* complex accounted for 56% of all strains isolated from fruits and vegetables. According to the latter study, hospital food could be a potential source for *A. baumannii* infection. *Acinetobacter* spp. have been also identified in small-size living organisms (body lice, fleas and ticks) that are potential vectors for infection transmission (Vallenet et al., 2008). It is of interest to note that *A. baumannii* strains were isolated from body lice collected from homeless persons in Marseille (France), indicating that epizootic *A. baumannii* infections among 22% human body lice could be a source of human infection (Scola and Raoult, 2004). *Acinetobacter* spp. may survive on dry surfaces longer than reported for *Staphylococcus aureus* and *Pseudomonas aeruginosa* and there is no difference between the survival times of sporadic and outbreak strains of *A. baumannii*. Survival is probably due to the minimal nutritional requirements needed by *Acinetobacter* spp. to grow and their ability to grow at different temperatures and pH values (Vila et al., 2007). *A. baumannii* was recovered from the washcloth after 7 days of storage under dry conditions. Other investigators demonstrated that *A. baumannii* can survive for 6 days on dry filter paper, 13 days on formica, more than 7 days on glass, and more than 25 days on cotton (Wendt et al., 1997).

In hospital settings, these bacteria can be recovered from the environment, from medical and non-medical equipment and from the hands of healthcare workers (AitMhand et al.,

2003). *A. baumannii* is distinguished by its propensity to cause outbreaks, which are probably related to its multidrug resistance patterns as well as to its resistance to desiccation (Giamarellou et al., 2008). Many of these outbreaks could be traced to environmental sources, e.g., ventilators, suction equipment, mattresses, pillows, humidifiers, bed rails, bedsides, containers of distilled water, urine collection jugs, intravenous (i.v.) nutrition equipment, potable water, reusable arterial pressure transducers, the knobs of electrocardiographs, wash basins, infusion pumps, sinks, hygroscopic bandages, showers, stainless-steel trolleys, resuscitation equipment and tables, i.v. access devices, portable radiology equipments, bed linen, soap dispensers, spinometers, temperature probes and multidose nebulisers (Paterson, 2006).

2.5. Acinetobacter as a nosocomial pathogen

Members of the genus Acinetobacter first began to be recognized as significant nosocomial pathogens during the early 1970s (Towner, 2009). Currently, *A. baumannii* is considered to be an important and emerging hospital-acquired pathogen worldwide. It is responsible for 2%-10% of all Gram-negative bacterial infections in intensive care units in Europe and the United states (Fournier and Richet, 2006b). The ability of *A. baumannii* to colonize skin and respiratory tract, to acquire multiple antibiotic resistance and to survive on inanimate and dry surfaces for prolonged periods of time, may contribute to the endemic or epidemic behavior of this nosocomial pathogen (Quelle et al., 2001).

Acinetobacter spp. also survive exposure to the commonly used disinfectants like chlorhexidine, gluconate, and phenols, particularly if not used in the appropriate concentrations. Although little is known about the natural reservoirs of Acinetobacter, its unique characteristics favor the persistence of the organisms in the environment that can result in extended outbreaks (Rathinavelu et al., 2003).

Multidrug-resistant strains of *A. baumannii* are notorious for their ability to spread among hospitalized patients. Outbreaks of multidrug-resistant *A. baumannii*, both within and between hospitals, have been reported with increasing frequency worldwide (Dent et al., 2010; Lee et al., 2006). Several studies have identified general characteristics of patients that place them at increased risk for the acquisition of multidrug-resistant outbreak strains. Specific characteristics of affected patients include advanced age, presence of serious underlying diseases, immune suppression, major trauma or burn injuries, having undergone of invasive procedure, as well as presence of indwelling catheters, support with mechanical ventilation, extended hospital stay (Dent et al., 2010; Wadl et al., 2010; Falagas et al., 2006; Robenshtok et al., 2006; Abbo et al., 2005), and previous administration of broad-spectrum antimicrobial agents, particularly third-generation cephalosporins, carbapenems, and fluoroquinolones contribute to increased frequency of Acinetobacter infection (Young et al., 2007; Tomas et al., 2005).

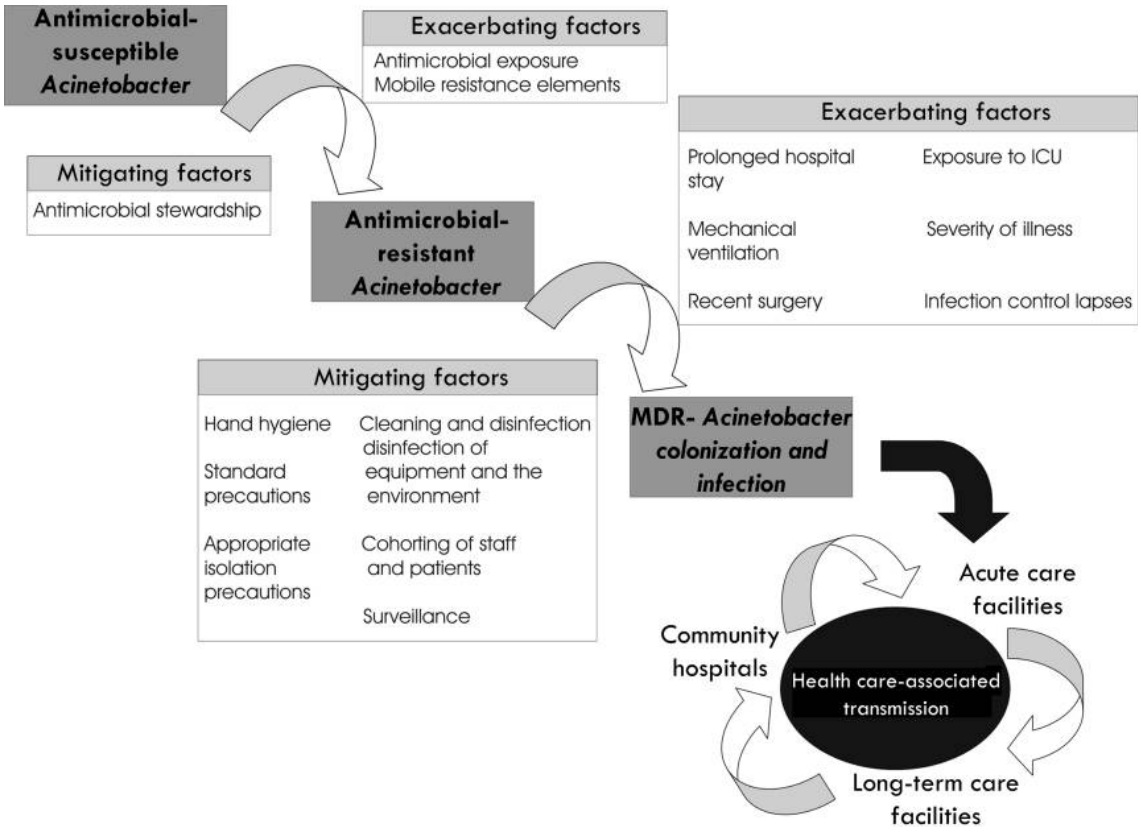


Fig. 1: Factors leading to the emergence and transmission of multidrug-resistant (MDR) *Acinetobacter* spp.. Maragakis and Perl, (2008).

2.6. Acinetobacter diseases

2.6.1. Health care-associated infections

Infections caused by to *A. baumannii* are frequent among patients admitted intensive care units (ICUs), where this opportunistic pathogen is capable of causing severe invasive infections in critically ill and immunocompromised patients. It is implicated as the cause of VAP, urinary tract infections (UTIs), bacteremia, and wound infections (Sung et al., 2008; Perez et al., 2007; Karlowsky et al., 2003; Rathinavelu et al., 2003). In most institutions, the majority of *A. baumannii* isolates are from the respiratory tract of hospitalized patients (Peleg et al., 2008). *A. baumannii* is frequently isolated from the tracheobronchial secretions of mechanically-ventilated patients. A significant proportion of these patients are simply colonized, whereas the rest develop clinically significant infection of the respiratory tract (Falagas and Kopterides, 2006).

In large surveillance studies from the United States, between 5 and 10% of cases of ICU-acquired pneumonia were due to *A. baumannii* (Gaynes and Edwards, 2005). Ventilator associated pneumonia due to *A. baumannii* carries significant morbidity and mortality in the ICU setting. It commonly occurs more than 5-7 days after mechanical ventilation (late-onset VAP) (Betrosian et al., 2008). Acinetobacter pneumonia does not differ clinically from most severe Gram-negative pneumonias. A combination of clinical signs including fever, neutrophilia, purulent sputum production, and appearance of new infiltrates on chest radiographs or computerized tomography (CT) scans must lead to microbiologic investigation (Bergogne-Berezin, 2001). The prognosis associated with this pathogen is worse than that associated with other bacteria, with the exception of *P. aeruginosa*. In a study of patients in whom a diagnosis of VAP was made, mortality associated with Pseudomonas or Acinetobacter was 75%, compared with 55% for VAP caused by other organisms (Luna and Aruj, 2007). In a recent case control study that included 87 ICU patients, the attributable mortality rate for Acinetobacter acquisition was 30% in 48 patients with pneumonia and 53% in patients with other infection. The most severe form was bacteremic pneumonia, often associated with shock and sepsis occurring in the presence of risk factors (Bergogne-Berezin, 2001). Factors increasing significantly the risk of VAP due to *Acinetobacter* spp. include: longer hospital stay, longer time on mechanical ventilation, reintubation and prior antibiotic use (Luna and Aruj, 2007).

A. baumannii bloodstream infection is associated with a high overall and attributable mortality (Chen et al., 2005), but it is difficult to distinguish morbidity and mortality attributable to *A. baumannii* from that attributable to the common and severe co-morbidities in these patients. The clinical manifestations of bacteremia due to *A. baumannii* are not specific. The most common sources of bacteremia are intravascular

catheters and the respiratory tract infections (Cisneros and Rodriguez-Bano., 2002).

In a survey by the Health Protection Agency in England, it was found that patients with Acinetobacter bacteraemia were mostly males aged >50 years, 54% were hospitalized in ICUs and only 5% in general wards. Sepsis and/or septic shock has been reported in 19% of patients with Acinetobacter bacteraemia, highlighting the true pathogenicity of several strains (Giamarellou et al., 2008).

In a typical study of 584 Acinetobacter strains isolated from 420 patients at 12 different hospitals over 12-month period, 426 (72.9%) strains were identified as *A. baumannii*, with 208 (48.8%) *A. baumannii* isolates being recovered from respiratory tracts specimen, 113 (26.5%) from blood cultures and central venous lines, 70 (16.4%) from wound swabs, and 35 (8.5%) from other miscellaneous specimens (Luna and Aruj, 2007). *A. baumannii* also causes, albeit less frequently, peritonitis, endocarditis, complicated skin and soft tissue, abdominal, and central nervous system infections (Fournier and Richet, 2006a; Chen et al., 2005).

Patients at risk for postneurosurgical bacterial meningitis include those with cerebrospinal leakage, concomitant incision infection, prolonged duration of surgery, surgery that enters a sinus, increased severity of illness, prolonged external ventricular drainage, and need for repeat surgery (Kim et al., 2009). Of recent significance is the observation that *A. baumannii* has become a major pathogen found in combat-associated wounds (Aronson et al., 2006). Acinetobacter infections have frequently been reported in trauma victims, but it is not clear whether these infections arose from environmental contamination at the scene or were acquired from the health care facilities in which these patients were subsequently treated (Turton et al., 2006a).

The distribution of the different types of infection varies from one hospital to another and is probably related to the hospital population and the type of procedures and

interventions performed (Fournier and Richet, 2006a). A study performed in Spain on 240 *A. baumannii* infections showed that >90% of infections were nosocomially acquired and that only 4% were community acquired. In this study, respiratory tract infections were the most common (39%), followed by exudates and abscesses (24% each) and UTIs (23%). Of interest is that bacteremia represented only 3% of all infections (Rodriguez-Bano et al., 2004). In a retrospective study performed in a 3956-bed teaching hospital in Marseille, France (from 1 January 2002 to 31 December 2004), 656 *A. baumannii* infections were identified, with exudates and abscesses predominating in 32%, followed by UTIs in 25%, respiratory infections in 20% and bacteraemia in 12%. In the same study, an unexplained seasonality was observed, with a significantly greater number of infections occurring from July through September (Giamarellou et al., 2008; Fournier and Richet, 2006a). Large numbers of *A. baumannii* infections have recently been reported among military casualties repatriated from war zones in Iraq and Afghanistan (Towner, 2009).

2.6.2. Community-acquired infections

A. baumannii can also cause community-acquired infections, which are encountered mainly in southeast Asia and tropical Australia. Pneumonia is the most common clinical syndrome reported in this setting, followed by bacteremia. The disease most typically occurs during the rainy season among people with a history of alcohol abuse and may sometimes require admission to an ICU. Community-acquired *A. baumannii* pneumonia typically affects patients with underlying chronic obstructive pulmonary disease, renal failure, or diabetes mellitus, as well as individuals who are heavy smokers or excessive alcohol consumers (Falagas et al., 2007; Falagas and Karveli, 2006; Leung et al., 2006; Anstey et al., 2002). It has also been suggested that the humid environment of these areas predisposes individuals, especially those with the above mentioned co-

morbidities, to Acinetobacter infections (Falagas and Karveli, 2006).

In the United States, community-acquired infections are rare. In 1979, *A. baumannii* pneumonias occurred in three foundry employees who worked within meters of each other. Postmortem evaluations in two of the patients showed severe underlying pneumoconiosis. *A. baumannii* was isolated from foundry air, but the source was not identified (Munoz-Price et al., 2008).

2.7. Virulence mechanisms of Acinetobacter

In the past, Acinetobacter was considered to be an organism of low virulence. However, the occurrence of fulminant community-acquired Acinetobacter pneumonia indicates that these organisms may sometimes be of high pathogenicity and cause invasive disease. Studies on Acinetobacter virulence factors are still at an elementary stage (Joly-Guillou, 2005). The precise mechanisms involved in the establishment and progression of *A. baumannii* infection are unclear. The organism is not known to produce either diffusible toxins or cytolysins, and few virulence factors have been identified (Gordon and Wareham, 2010).

2.7.1. Lipopolysaccharide (LPS)

The inflammatory potential of *A. baumannii* LPS, a molecule central to the development of Gram-negative sepsis, has also been investigated. LPS derived from *A. baumannii* has been shown to be a potent inducer of pro-inflammatory cytokine expression in human monocytes, via pathways that are dependent on both Toll-like receptor (TLR)-2 and TLR-4 stimulation. Purified LPS stimulates inflammation via the TLR-4 pathway, whereas killed whole cells act via both TLR-2 and TLR-4. This study demonstrated the potent endotoxic potential of *A. baumannii* LPS, which stimulated the proinflammatory cytokines interleukin-8 and tumor necrosis factor alpha (TNF-α) equally to the stimulation by *E. coli* LPS at similar concentrations (Erridge et

al., 2007).

2.7.2. Capsular polysaccharide

Capsular polysaccharide formed of L-rhamnose, D-glucose, D-glucuronic acid, and D-mannose, which probably renders the surface of the strains more hydrophilic, although hydrophobicity may be higher in Acinetobacter strains isolated from catheters or tracheal devices (Towner and Bergogne-Berezin, 1996). Capsular polysaccharide is known to block the access of complement to the microbial cell wall and to prevent the triggering of the alternative pathway of complement activation, as demonstrated in experimental models of Gram-negative infections.

In experimental studies, approximately 30% of Acinetobacter strains produce exopolysaccharides. Exopolysaccharides producing strains of Acinetobacter have been shown to be more pathogenic than non-exopolysaccharide-producing strains, especially in polymicrobial infections with other spp. of higher virulence (Joly-Guillou, 2005).

2.7.3. Outer membrane proteins (OMPs)

The major OMP of *A. baumannii* is a 38 KDa porin (Omp38) (Choi et al, 2005). Jyothisri et al. (1999) structurally defined this OMP, which is a trimeric porin with a pore size of 1.3 nm and which acts as a general diffusion pore. Omp38 localizes to the mitochondria and induces the release of cytochrome *c* and an apoptosis-inducing factor (AIF) into cytosol, which mediates caspase-dependent and AIF- dependent apoptosis in epithelial cells. The cytotoxic effects of *A. baumannii* were investigated in human laryngeal HEp-2 cells, and the findings indicate that this bacterium induces apoptosis of HEp-2 in vitro through both caspase-dependent cascades, which are mediated by cell surface signaling and mitochondrial disintegration, and the AIF-dependent pathway (Choi et al., 2005).

2.7.4. Acinetobacter fimbriae

Adherence of *A. baumannii* to human bronchial epithelial cells and erythrocytes is mediated by fimbrial-like structures (Lee et al., 2006; Gospodarek et al., 1998). The adherence of *A. baumannii* to the bladder tissue is a natural attribute of different strains, a comparable trait found in an uropathogenic *E. coli* strains (Sepulveda et al., 1998). Hemagglutination assay have been used for the *in vitro* assay of the expression of many mannose-resistant hemaglutinins, designed fimbriae type P, S, Dr, and F, frequently found in extra-intestinal *E. coli* (Braun and Vidotto, 2004). Sepulveda et al. (1998) observed the presence of fimbrial structures in *A. baumannii* isolates by transmission electron microscopy, and the hemagglutinating activity of strains was not inhibited by either D-mannose or D-galactose. Braun and Vidotto (2004) verified that *A. baumannii* strains agglutinated human erythrocytes, in the presence of mannose, but the genes *pap*, *afa/dra,* and *sfa* coding for the adhesins P, Dr, and S, respectively, were not detected by means of PCR with specific primers. Gohl et al. (2006) indicated the presence of thin pili (fimbriae) in Acinetobacter; the data showed that these thin pili were not essential for motility on solid surfaces, but for adhesion to hydrophobic surfaces and erythrocytes.

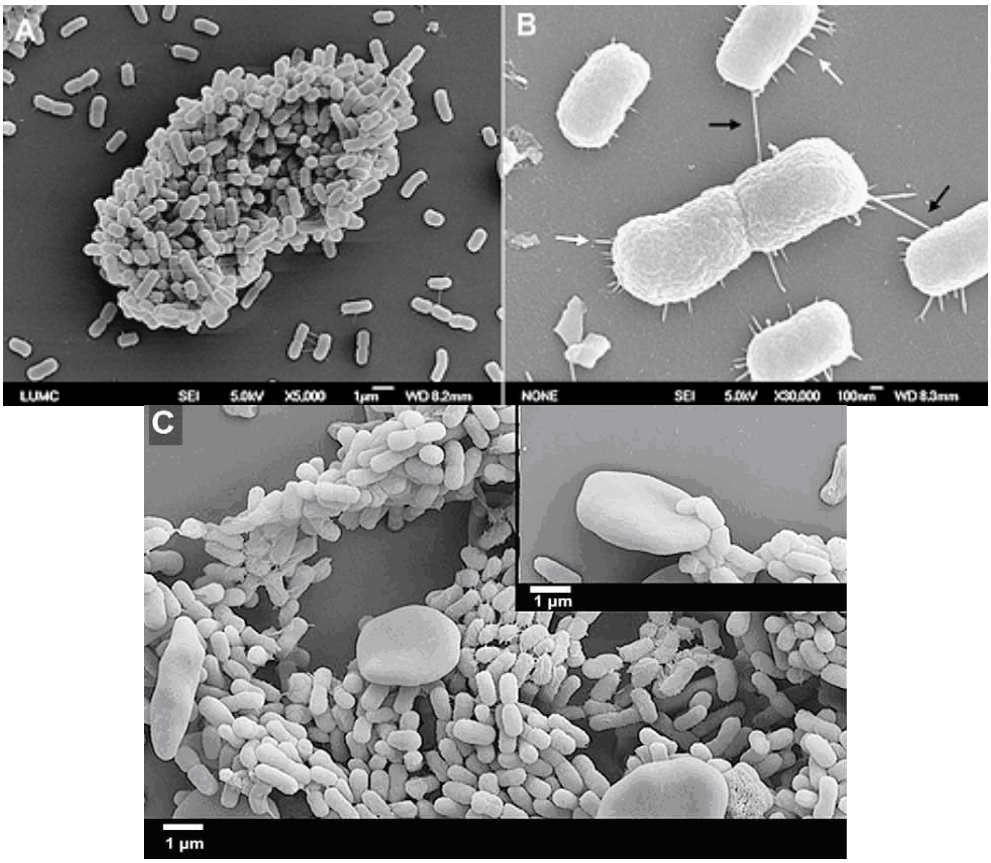


Fig. 2: Scanning electron micrographs (SEM) of *A. baumannii* (A and B). Black arrows indicate long cell extensions; white arrows indicate thin and short pili. (C) SEM of *A. baumannii* strain in the presence of sheep erythrocytes. Bars: 1 µm (A and C) and 100 nm (B). (Breij et al., 2009).

2.7.5. Hydrophobicity

Boujaafar et al. (1990) showed that *A. baumannii* strains obtained from catheter and tracheal devices had a high surface hydrophobicity, in contrast to the strains obtained from healthy carrier skin.

2.7.6. Siderophores

The ability of *A. baumannii* to obtain and utilize resources such as iron is an important factor in its ability to survive both in the host and in the environment. *A. baumannii* secretes a variety of molecules involved in iron binding, including the siderophore acinetobactin, and also produces a hemin utilization system (Gordon and Wareham, 2010; Zimbler et al., 2009). Siderophores are relatively low-molecular weight,

ferric ion specific chelating agents elaborated by bacteria growing under low iron stress (Neilands, 1995). Interestingly, the expression of these elements can vary greatly between clinical strains of *A. baumannii* (Dorsey et al., 2003), and these elements have structural and functional similarities to a siderophore produced by the fish pathogen *Vibrio anguilarum*, a potential origin of this critical virulence mechanism (Dorsey et al., 2004).

All of these observation suggest that *A. baumannii* is a versatile pathogen that could acquire iron to prosper under iron-limiting conditions, such as those found in the human host, by expressing different iron acquisition systems (Zimbler et al., 2009). The ability of bacteria to assimilate iron is known to be related to invasiveness. The lung and systemic infections, where there is iron restriction, suggest the presence of an iron uptake system in Acinetobacter (Actis et al., 1993). Smith et al. (1990) detected the presence of iron chelator 2,3-dihydroxybenzoic acid (DHBA) and iron-repressible outer membrane proteins (IROMPs) in the culture supernatant of *Acinetobacter* spp. Actis et al. (1993) verified that all clinical isolates of *A. baumannii* were able to grow under iron-deficient conditions,where some of them excreted an iron-regulated siderophore into culture supernatants. This iron-scavenging phenotype was associated with the production of iron-repressible catechol. Siderophore utilization bioassays showed the presence of DHBA in the growth medium, and neither enterobactin nor aerobactin was detected in culture supernatants obtained under iron-deficient conditions.

Braun and Vidotto (2004) reported that all *A. baumannii* isolates were able to grow under iron-limiting conditions, showing that *A. baumannii* produces some type of a siderophore. However, the genes *iutA* and *fyuA*, from iron uptake system of *E. coli* and *Yersinia* spp. respectively, were not present in the isolates, suggesting the presence of a

different type of siderophore. Iron-regulated proteins are present in both inner and outer membranes of clinical strains of *A. baumannii*. The presence of two iron-regulated proteins localized in the inner membrane was reported by Echenique et al. (1992). The Fur protein of *A. baumannii*, which regulates the genes involved in iron uptake, was sequenced and demonstrated to be 63% identical to that of *E. coli* (Daniel et al., 1999).

2.7.7. Quorum sensing

Bacteria use small molecule as signals to communicate with each other. Intercellular signalling at high population cell densities is termed quorum sensing (QS) and explains many aspects of bacterial physiology observed in single species cultures entering stationary phase in the laboratory. Quorum sensing relies on the production and release of small molecule signals by the bacterium into its environment. These signals have also been termed “autoinducers” and bacterial “pheromones ”. Once a critical threshold concentration is reached, a target sensor kinase or response regulator is activated, so facilitating the expression of QS-dependent target genes. Several chemically distinct families of QS signal molecules have been described, of which the N-acylhomoserine lactone (AHL) family in Gram-negative bacteria have been the most intensively investigated (William, 2007). Quorum sensing systems are known to be involved in a range of important microbial activities. These include extracellular enzyme biosynthesis, biofilm development, exopolysaccharides synthesis and extracellular virulence factors in Gram-negative bacteria (Simoes et al., 2010). Up to four different QS signal molecules have been identified in Acinetobacter (Gonzalez et al., 2001), indicating that this may be a central mechanism for autoinduction of multiple virulence factors (Joly-Guillou, 2005).

2.7.8. Biofilm formation

A. baumannii readily adheres both to biological and abiotic surfaces, on which it is able

to form biofilms (Cevahir et al., 2008; Lee et al., 2008; Vidal et al., 1996). Sechi et al. (2004) demonstrated that 16 of 20 Acinetobacter strains were able to form a biofilm. Biofilm formation involves a variety of pathways that are regulated by QS and a number of two-component regulatory systems (Gaddy et al., 2009). Fimbriae (pili) are important for initial adhesion, followed by the production of exopolysaccharide, an important constituent of mature biofilm that suppresses the activity of neutrophils and contributes to serum resistance (Gordon and Wareham, 2010).

2.7.9. Enzymatic activities in Acinetobacter

Many bacteria synthesize enzymes that play a role in the group of bacterial virulence factors. Acinetobacter is best known for its capacity for bioremediation of alkanes and aromatic hydrocarbons, as well as production of high molecular weight heteropolysaccharides that act as powerful emulsifiers, many with high commercial potential. Many of Acinetobacter strains have been found to secrete esterolytic enzymes (Snellman and Colwell, 2004). Strong activities of esterases, which are known to hydrolyze short-chain fatty acids at ester linkages may contribute to its ability to damage lipid tissues. Also, Acinetobacter strains have been found to secrete lipase enzymes. Lipases are triacylglycerol hydrolases, which catalyze the hydrolysis of triglycerides to glycerol and free fatty acids (Saisubramanian et al., 2008). Microbial lipases have been studied for their role in virulence and their applications in biotechnology (Snellman and Colwell, 2004). Microbial lipases which may, by hydrolyzing the lipids on the epithelial surface of human beings, enhance the colonization of the skin by organisms that produce lipase enzyme (Lonon et al., 1988). Lipases produced by *Acinetobacter* spp. are highly hydrophobic in nature, even in comparison with other bacterial lipases. Many Acinetobacter lipases show stability and maximum activity at alkaline pH. Lipolytic strains of Acinetobacter have been isolated

from a variety of substrates, including human skin, dairy and other food products, in addition to diverse soil, and water habitats, both pristine and highly polluted. Clinical strains are often lipolytic, causing severe nosocomial infections in neonates and immunocompromised patients (Snellman and Colwell, 2004).

Proteases play a crucial role in numerous pathologic processes. Microbial proteases have been proposed as virulence factors in a variety of diseases caused by microorganisms. Gelatinase is able to hydrolyze gelatine and some other bio-active peptides. The enzyme is a 31.5-kDa, strongly hydrophobic, protein with and a broad pH optimum of 6-8 (Kanemitsu et al., 2001). Sechi et al. (2004) did not detect gelatinase and protease activity in *A. baumannii* strains. In contrast, Cevahir et al. (2008) detected gelatinase activity in 12 of 86 (14%) *A. baumanni* strains.

Hemolysin is a cytolytic protein capable of lysing erythrocytes. Hemolysin producing microbial strains have been shown to be virulent in animal models and human infections and to be associated with increased severity of infection (Vergis et al., 2002). Most hemolytic isolates are identified as *A. haemolyticus* or *Acinetobacter* genomic spp. 6 (Bergogne-Berezin and Towner, 1996). Gospodarek (1993a) detected hemolytic activity in *A. haemolyticus.*

2.8. Antimicrobial resistance in Acinetobacter

One of the main concerns about *A. baumannii* is that it represents a major threat to public health, namely, its remarkable ability to rapidly develop antimicrobial resistance, mostly by acquisition of gene clusters carried by plasmids, transposons, or integrons (Garnacho-Montero and Amaya-Villar, 2010; Vila et al., 2007; Fournier and Richet, 2006a; Kraniotaki et al., 2006).

Antimicrobial resistance among *Acinetobacter* spp. has increased substantially in the past decade (Maragakis and Perl, 2008). This resistance is multiple, causing serious

therapeutic problems. Practices in ICUs contribute to the development of antimicrobial resistance in *A. baumannii* because the use of antimicrobials per patient and per surface area are significantly higher in this part of the hospital.

Definitions of MDR *Acinetobacter* spp. vary, referring to a wide array of genotypes and phenotypes, but several authors consider an isolate to be MDR if it is resistant to three or more classes of antibiotics (Falagas et al., 2006; Cisneros and Rodriguez-Bano, 2002).

2.8.1. Resistance to β-lactams

Described resistance mechanisms include (i) hydrolysis by β-lactamases, (ii) alterations in outer membrane proteins (OMPs) (porins) and penicillin-binding proteins (PBPs) and (iii) increased activity of efflux pumps (Giamarellou et al., 2008; Perez et al., 2007).

2.8.1.1. β-Lactamases

2.8.1.1.1. Class A β-lactamases

There are wide range of class A extended-spectrum β-lactamases (ESBLs), including those of the TEM, SHV, CTX-M, GES, SCO, PER and VEB families (Gordon and Wareham, 2010). *A. baumannii* strains harboring PER-1, an ESBL, demonstrate high-level resistance to penicillins and extended-spectrum cephalosporins, but fortunately, PER-1 β-lactamase does not confer resistance to carbapenems in *A. baumannii* (Perez et al., 2007).

2.8.1.1.2. Class B β-lactamases

Metallo-β-lactamases (MBLs) are class B β-lactamases (IMP, VIM, and SIM families) that are able to hydrolyze carbapenems as well as every other β-lactam antibiotic with exception of aztreonam (Gordon and Wareham, 2010; Perez et al., 2007) and are usually found in association with complex class 1 integrons (Gordon and Wareham, 2010; Zarrilli et al., 2009; Giamarellou et al., 2008). Class B enzymes are unique among

β-lactamases in having a zinc ion (or ions) at their active site, their catalytic activity depends on the zinc ion, and activity is lost if it is sequestered with EDTA (Livermore and Woodford, 2000). Metallo-β-lactamases IMP were first described in a strain of *P. aeruginosa* found in Japan in 1988 (Watanabe et al., 1991). Although MBLs are not the predominant carbapenemases in *A. baumannii*, several have been described, IMP-1, IMP-2, IMP-4, IMP-5, IMP-6, and IMP-11 (Walsh, 2005; Walsh et al., 2005). *V*erona *i*ntegron-encoded *M*BL (VIM-1) was first identified in Italy in 1997 in *P. aeruginosa* isolates (Lauretti et al., 1999). VIM-2 β-lactamases detected in *A. baumannii* isolates from Korea confer significant levels of resistance to carbapenems (Walsh et al., 2005; Lee K. et al., 2004; Yum et al., 2002).

2.8.1.1.3. Class C β-lactamases

Chromosomal cephalosporinases (AmpC enzymes) are common to all strains of *A. baumannii.* To date, there has been no evidence to indicate that the chromosomal cephalosporinase is inducible (Giamarellou et al., 2008; Bonomo and Szabo, 2006). These enzymes hydrolyze penicillins and narrow-spectrum and extended-spectrum cephalosporins, but not cefepime or carbapenems (Giamarellou et al., 2008; Perez et al., 2007).

2.8.1.1.4. Class D β-lactamases

The most widespread carbapenemases in *A. baumannii* are OXA (oxacillinases) β-lactamases (Higgins et al., 2010, Zarrilli et al., 2009; Giamarellou et al., 2008). Carbapenem resistance in *A. baumannii* is mediated most often by oxacillinases (OXAs) and less frequently by MBLs (Higgins et al., 2010). The class D carbapenemases found in *A. baumannii* can be classified into four distinct groups: OXA-23-like (OXA-23, -27 and -49), OXA-24-like (OXA-24/40, -25, -26 and -72), OXA-58-like (OXA-58 and –96), and OXA-51-like enzymes (Zong et al., 2008; Walther-Rasmussen and Høiby,

2006). The first description of such an OXA carbapenemase in *A. baumannii* was OXA-23, which was obtained from a clinical isolate found in Scotland in 1985 before the introduction of carbapenems (Brown and Amyes, 2006; Afzal-Shah et al., 2001). OXA-23 was initially named ARI-1 (*a*cinetobacter *r*esistant to *i*mipenem) (Perez et al., 2007). Acquired carbapenem-hydrolyzing class D oxacillinase (CHDL) gene clusters have been identified either in the chromosome or in plasmids of *A. baumannii* strains (Zarrilli et al., 2009). The ubiquitous nature of *bla*$_{\text{OXA-51- like}}$ genes in *A. baumannii* has led to this gene becoming an important genetic marker in identification of the organism to the spp. level (Turton et al., 2006b). OXA-51-like enzymes are able to hydrolyze penicillins (benzylpenicillin, ampicillin, ticarcillin and piperacillin) and carbapenems (imipenem and meropenem) but they do this only very weakly (Poirel et al., 2005). A significant contribution to β-lactam resistance by OXA-51- like enzymes, therefore, requires the presence of an insertion element IS*Aba 1* upstream of the gene, able to act as a strong transcriptional promoter (Turton et al., 2006c). The commonest enzymatic mode of carbapenem resistance is the production oxacillinases encoded by genes of the *bla*$_{\text{OXA-23,}}$ *bla*$_{\text{OXA-24/40,}}$ and *bla*$_{\text{OXA-58}}$ –like lineage (Gordon and Wareham, 2010; Zarrilli et al., 2009; Lee J. H. et al., 2007). Different insertion sequence (IS) elements at the 5' and/or the 3' end of *bla*$_{\text{OXA-23}}$-, and *bla*$_{\text{OXA-58}}$ –like genes have been demonstrated to regulate their expression (Zarrilli et al., 2009; Zong et al., 2008). *bla*$_{\text{OXA-23}}$ gene has been isolated in Brazil, Polynesia, Singapore, Korea, and China (Perez et al., 2007), *bla*$_{\text{OXA-24}}$ gene was mostly found in the Iberian peninsula and Asia, but also detected in Iran, Belgium, Czech Republic and the United States of America (USA) (Zarrilli et al., 2009), while *bla*$_{\text{OXA-58}}$ gene has been isolated from England, Scotland, Spain, Austria, Greece, Romania, Turkey, and Kuwait (Giamarellou et al., 2008). The spread of carbapenem-resistant *A. baumannii* carrying the *bla*$_{\text{OXA-58}}$ gene might had also been

contributed by international transfer of colonized patients, as recently demonstrated from Greece to Belgium, Greece to Australia, and Iraq to USA military services (Zarrilli et al., 2009).

2.8.1.2. Changes in OMPs and PBPs

Many outbreaks of infection with imipenem-resistant *A. baumannii* were due to porin loss. In isolates from Madrid, the loss of 22-kDa and 33-kDa OMPs combined with the production of OXA-24 resulted in resistance to carbapenems (Bou et al., 2000).

2.8.1.3. Efflux pumps

The natural role of efflux is to remove chemicals that could potentially disorganize the cytoplasmic membranes (Bonomo and Szabo, 2006). The AdeABC efflux pump has been well characterized in *A. baumannii*. It pumps the aminoglycosides, cefotaxime, tetracycline, erythromycin, chloramphenicol, trimethoprim and fluoroquinolones (Magnet et al., 2001), whereas its overexpression in conjunction with carbapenem-hydrolyzing oxacillinases can also confer high-level resistance to carbapenems (Marque et al., 2005).

2.8.2. Resistance to aminoglycosides

In addition to AdeABC efflux pump, resistance to aminoglycosides in *A. baumannii* is mediated by aminoglycoside-modifying enzymes (AMEs). These include aminoglycoside phosphotransferases, aminoglycoside acetyltransferases, and aminoglycoside adenyltransferases (Bonomo and Szabo, 2006).

2.8.3. Resistance to quinolones

Mutations both in *gyrA* and *parC* are responsible for quinolone resistance. However , the plasmid-mediated quinolone resistance gene, *qnrA*, has not yet been detected in *A. baumannii* isolates, although it has been found in other Gram-negative bacteria, such as

Enterobacter and *Klebsiella* spp. (Bonomo and Szabo, 2006).

2.8.4. Resistance to tetracyclines

Two different mechanisms of resistance to tetracyclines, which are specific transposon-mediated efflux pumps, have been widely described in *A. baumannii.* TetA and TetB are specific transposon-mediated efflux pumps; TetB determines the efflux of both tetracycline and minocycline, whereas TetA drives only the efflux of tetracycline. Neither TetA nor TetB affect tigecycline, the new glycylcycline. However, tigecycline is a substrate for a plasmid-borne flavin-dependent monooxygenase, TetX (Giamarellou et al., 2008; Perez et al., 2007). Navon-Venezia et al. (2007) evaluated the in vitro activity of tigecycline against 82 MDR *A. baumannii* isolates. The study found that 66% of the isolates were resistant to tigecycline.

2.8.5. Resistance to polymyxins

As the use of polymyxins increases, resistance to colistin (polymyxin E) will become more common and more widespread. The in vitro and in vivo activities of colistin (polymyxin E) suggested that it would be an effective antimicrobial agent against *A. baumannii.* However, some polymyxin-resistant strains have been reported (Arroyo et al., 2005). Urban et al. (2001) reported a case of polymyxin B-resistant *A. baumannii*. The mechanism of resistance to colistin likely resides in modifications in the LPS of *A. baumannii* (acidification, acylation, or presence of antigens that interfere with binding of the antibiotics to the cell membrane) (Perez et al., 2007).

2.9. Molecular typing of Acinetobacter

To investigate the molecular epidemiology of *A. baumannii*, a variety of typing systems have been developed, including ribotyping, genome analysis by selective amplified fragment length polymorphism (AFLP), PFGE, multilocus sequence typing (MLST) and infrequent-restriction-site PCR (Wisplinghoff et al., 2008).

Several PCR-based DNA fingerprinting techniques have been used to study nosocomial outbreaks of the *A. baumannii*, including randomly amplified polymorphic DNA (RAPD) analysis, repetitive extragenic palindromic (REP) PCR (Bou et al., 2000), and ERIC PCR (Sung et al., 2008).

Among these, PFGE is regarded as the "gold standard" of epidemiological typing (Seifert et al., 2005). However, PFGE technique is associated with expensive laboratory equipments, more complex procedures and manipulation. Thus, it is not always practical for small labs (Zhang et al., 2008). PFGE take about five days to obtain the results. In contrast, the PCR method takes only one day to prepare and perform (Matsumoto et al., 2001). One type of rep-PCR is the amplification of genomic DNA located between ERIC elements (Giovanni et al., 1999).

The ERIC sequences are a set of DNA sequences which have been successfully used for DNA typing. ERIC sequences are 126-bp elements which contain a highly conserved central inverted repeat and are located in extragenic regions of the bacterial genome (Matsumoto et al., 2001; Giovanni et al., 1999; Olive and Bean, 1999). They have been defined primarily based on sequence data obtained from *E. coli* and *Salmonella typhimurium* (Olive and Bean, 1999).

The repetitive elements ERIC1 and ERIC2 were used successfully as single primers in one study for typing *A. baumannii* isolates from tertiary-care hospital (Bergogne-Berezin and Towner, 1996).

ERIC amplification can be performed with a single primer, a single set of primers, or multiple sets of primers. ERIC patterns give good discrimination at the strain level. (Olive and Bean, 1999). ERIC is extremely rapid, simple, inexpensive tool for routine epidemiological investigations, and seems to be useful for tracing epidemic strains during outbreaks on a day-to-day basis (Bergogne-Berezin and Towner, 1996).

All of these methods have contributed to a better understanding of the epidemiology and clinical significance of *Acinetobacter* spp. during recent years (Peleg et al., 2008).

3. Materials and methods

3.1. Materials

3.1.1. Bacterial standard strains

* *Pseudomonas aeruginosa* strain ATCC 27853 is included for quality control of antimicrobial susceptibility test.

* *Escherichia coli* strain ATCC 25922 was used as quality control strain for the modified Hodge test.

* *Klebsiella pneumoniae* strain ATCC BAA-1706 was used as negative control for the modified Hodge test.

* *Klebsiella pneumoniae* strain ATCC BAA-1705 was used as positive control for the modified Hodge test.

* *E. coli* strain K12 was used as positive control for hemagglutination assay.

* *Serratia mercenses* strain ATCC 13880 was used as positive control for protease and gelatinase enzymes.

* Non-O1 *Vibrio cholerae* clinical strain (Shehabi et al. 1984) was used as positive control for lecithinase and lipase enzymes and hemolysin production.

3.1.2. Antimicrobials

* One set of commercially antimicrobial discs (Mast group Ltd., Merseyside, U.K) were used to investigate the susceptibility of *Acinetobacter* isolates in vitro (Table 1).

* Tigecycline (Tgc, 15 µg), Imipenem (IMI, 10 µg), Colistin (Co, 10 µg), and Ertapenem (ETP, 10 µg), were obtained from (Oxoid, Unipath Ltd., Hampshire, England).

* Antimicrobial E test strips were obtained from the AB Biodisk, Solna, Sweden. The strips were stored at -20° C until use.

Table (1): Antibiotic discs used in agar diffusion test

Antimicrobial Name	Concentration/ µg	Abbreviation
Amikacin	30	Ak
Piperacillin/Tazobactam	110	PTZ
Imipenem	10	IMI
Meropenem	10	MEM
Aztreonam	30	ATM
Ceftazidime	30	CAZ
Gentamicin	10	GM
Ciprofloxacin	5	CIP

3.1.3. Reagent kit

Rapid NF plus system, for biochemical identification of medically important glucose-nonfermenting, Gram-negative bacteria, was purchased from Remel, USA.

3.1.4. Equipment and disposables

The following major equipment was used throughout the work:

* PCR Thermocycler (MJ research- IMC, USA).

* Horizontal gel electrophoresis cell and power supply (Bio-Rad, USA).

* Gel documentation system including; UV camera, monitor and printer (UVP, USA).

* Microcentrifuge (Hettich, Germany).

* Shaking incubator (Daiki Scientific Co., Korea).

* Waver shaker (Major science, Taiwan).

* Centrifuge (Thermo electron corporation, USA).

* 96-well round- bottom plates (Greiner bio-one, Germany).

* 1.5 ml Eppendrof tubes, (AB genes House, UK).

* PCR-tubes – (AB gene House, UK).

* Cryotubes (1.8 ml) – (AB gene House, UK).

* Trap cultures (Bicakcilar, Istanbul, Turkey).

* Syringe filters pore size 0.2 µm (Albet, Germany).

* Syringes (Becton Dickinson, Meylan, Spain).

3.1.5. Reagents and Solutions for DNA Extraction

* Ethanol and Isopopanol (E. Merck, Germany).

* Wizard Genomic DNA Purification kit (Promega, USA).

3.1.6. PCR primers and chemicals

* Nuclease free water (Promega, USA).

* PCR 5x qARTA•Taq master mix ready to load (QartaBio, USA): 5x qARTA•Taq buffer, 12.5 mM $MgCl_2$ (1x PCR solution-2.5 mM $MgCl_2$), 1 mM dNTPs of each (1x PCR solutions-200 µM dATP, 200 µM dCTP, 200 µM dTTP and 200 µM dGTP), qARTA•Taq DNA polymerase, and blue and yellow dyes.

* Taq polymerase, buffer, $MgCl_2$, and dNTPs were purchased form (Promega, USA).

* PCR DNA markers (100 bp and1 Kb) (Promega, USA).

* PCR ERIC primers (table 3), OXA-genes primers and MBL genes primers (table 2).

* PCR ERIC primers and MBL genes primers were purchased from (AlphaDNA, Montreal, Canada), and OXA-genes primers were purchased from (Midland, Texas) and all primers were dissolved in nuclease free water.

Table (2): Oligonucleotide primer sequences used in the detection of OXA- and MBL-encoding genes used in this study .

Target gene	Primer sequences	Amplicon size (bp)	Reference
bla$_{\text{OXA-51-like}}$	OXA-51-like 1→5'-TAATGCTTTGATCGGCCTTG-3' 2→5'-TGGATTGCACTTCATCTTGG-3'	353-bp	Woodford et al., 2006
bla$_{\text{OXA-23-like}}$	OXA-23-like 1→5'-GATCGGATTGGAGAACCAGA-3' 2→5'-ATTTCTGACCGCATT TCCAT-3'	501-bp	Woodford et al., 2006
bla$_{\text{OXA-24-like}}$	OXA-24-like 1→5'-GGTTAGTTGGCCCCCTTAAA-3' 2→5'-AGTTGAGCGAAAAGGGGATT-3'	246-bp	Woodford et al., 2006
bla$_{\text{OXA-58-like}}$	OXA-58-like 1→5'-AAGTATTGGGGCTTGTGCTG-3' 2→5'-CCCCTCTGCGCTCTACATAC-3'	599-bp	Woodford et al., 2006
bla$_{\text{IMP}}$	IMP 1→5'-CATGGTTTGGTGGTTCTTGT-3' 2→5'-ATAATTTGGCGGACTTTGGC-3'	488	Sung et al., 2008
bla$_{\text{VIM}}$	VIM 1→5'-ATTGGTCTATTTGACCGCGTC-3' 2→5'-TGCTACTCAACGACTGAGCG-3'	780	Sung et al., 2008

Table (3): ERIC primers used in this study (Sung et al., 2008).

Primers	Sequence (5' to 3')
ERIC1	5'-ATGTAAGCTCCTGGGGATTCAC-3'
ERIC2	5'-AAGTAAGTGACTGGGGTGAGCG-3'

3.1.7. Electrophoresis chemicals

* LE – Agarose, (Promega, USA).

* Boric acid, (Riedl – de Haen , Germany).

* Ethidium bromide, (Sigma, England).

* Di-sodium EDTA, (Biochemical, BDH, England).

* Tris – base, (Promega, USA).

3.2. Methods

3.2.1. Sample Collection.

A total of 234 samples were collected from the respiratory tracts of 185 patients divided into two groups:

Group 1: 93 patients admitted to Medical Intensive Care Unit (MICU), Surgical Intensive Care Unit (SICU), and Cardiac Intensive Care Unit (CICU) at JUH over a period of 10 months, from May 2009 to February 2010.

Group 2: 92 patients referred to respiratory outpatient clinics at JUH over a period of 5 months, from October 2009 to February 2010.

For patients admitted to ICUs, one specimen was collected from patients at the first/or second day of admission and two others were collected after three days of admission.

Bronchoalveolar lavage, tracheal aspirate, or sputum samples were collected from patients admitted to ICUs.

Sputum or throat swab samples were collected from patients referred to respiratory outpatient clinics.

Bronchoalveolar lavage and tracheal aspirate samples were collected using trap culture. Throat swab samples were collected using sterile cotton tipped swabs after moistening them with sterile 0.9% saline.

3.2.2. Collection of clinical data

For all ICUs patients; the name, age, sex, diagnosis, length of ICU stay between admission and date of collection of the specimen, and the type of antimicrobial administered were all recorded. Additional information about mechanical ventilation, using nasogastric tubes, and presence of tracheoctomy were also taken from patient's records. For all outpatients; the name, age, sex, diagnosis, and the type of antibiotic administered were all recorded.

3.2.3 Culture Procedures

3.2.3.1. Media preparation

All the media used were prepared according to manufacturer instructions. Cysteine lactose electrolyte deficient agar (CLED) agar was prepared by suspending 18 g of powder media in 500 ml of distilled water and brought gently to the boiling temperature to dissolve completely and sterilized by autoclaving at 121^{o}C for 15 minutes, allowed to cool to 50^{o}C, then poured into sterile Petri dishes. Plates were then stored in plastic bags at 2 to 8^{o}C up to 2 weeks. Blood agar was prepared by suspending 20 g blood agar base in 500 ml of distilled water and the mixture was brought gently to the boiling temperature so as to dissolve completely, sterilized by autoclaving at 121^{o}C for 15 minutes then allowed to cool to 50^{o}C. Subsequently, 5% of sterile, defibrinated blood

was added to the cooled sterilized blood agar base and mixed well, then poured into sterile Petri dishes. Plates were stored in plastic bags at 2 to 8°C for up to 2 weeks. A minimal-salt agar supplemented with 1% acetate (MSA) was used as selective medium for *Acinetobacter* species. MSA media were prepared according to Berlau et al. (1999a) as follows: 20 g NH_4Cl, 4 g NH_4NO_3, 8 g Na_2SO_4, 12 g K_2HPO_4, 0.4 g $MgSO_4.7H_2O$ per liter of distilled water; the agar base was 20 g/l agar no. 3 (Oxoid)-250 ml of salt solution was added to 750 ml of agar base, 10 ml of 20% filtered glucose solution and sodium acetate to 1% final concentration.

3.2.3.2. Sample processing

All samples were processed within 1 hour of collection. Specimens were inoculated on Blood agar, CLED agar, and MSA media plates and incubated at 37°C for 24 to 48 hrs, and the plates were examined for the presence of *Acinetobacter* growth.

3.2.3.3. Detection of *A. baumannii*

Acinetobacter species isolates were primarily identified using standard biochemical tests including, Kligler iron agar, simons citrate utilization, tryptophan production, motility, urease and oxidase production. *Acintobacter* species pure growth isolates were subcultured on CLED agar (Oxoid, England). Identification as *A. baumannii* was done using commercially available Rapid NF plus system, Remel Kit (USA); biochemical systems for non-fermenter Gram-negative bacilli. Five colonies were inoculated in brain-heart infusion agar plus 15% glycerol and kept frozen at -70 ºC until used for further investigation. The total number of isolates from 185 patients was 64 *A. baumannii*.

3.2.4. Antibiotic susceptibility tests

Antimicrobial susceptibility of *A. baumannii* isolates was determined using the disc diffusion method, where 4-5 colonies of identical morphology were picked from

fresh cultures of *A. baumannii* and suspended in 5 ml of Mueller– Hinton broth and incubated for 15 minutes where the turbidity was adjusted to 0.5 McFarland standard. A sterile cotton swab was dipped in suspension, squeezed against the walls of the tube and then it was streaked on Mueller–Hinton agar plates which were allowed to stand for 15 minutes at room temperature. Using a sterile forceps, and under aseptic conditions, discs containing antimicrobials (Table 1) were placed on the surface of each inoculated agar and pressed gently with the tip of the forceps to ensure close contact. The plates were incubated at 37^{o}C for 18-24 hours before the inhibition zones surrounding the antimicrobial discs were measured using a ruler. Resistance (R) or susceptibility (S) of each isolate to the antimicrobial was determined according the guidelines of CLSI 2009.

3.2.5. Determination of the minimum inhibitory concentration (MIC) for *Acinetobacter baumanii*

The minimum inhibitory concentrations (MICs) were determined by the E test (AB Biodisk, Solna, Sweden) for imipenem, tigecycline , amikacin and colistin. The E test was performed for *A. baumannii* isolates according to the manufactures recommendations. Few colonies (4-5) of each fresh *A. baumannii* culture were suspended in 5 ml of Mueller– Hinton broth, after mixing with a vortex mixer, the turbidity of the suspension was adjusted to match a 0.5 McFarland turbidity standard. A sterile cotton swab was dipped in the suspension and used to streak the Mueller–Hinton agar plate in three directions.

The plates were allowed to dry for around 15 min then the E test strip was applied to the surface of the media and pressed gently with the tip of the forceps to ensure intimate contact. The plates were incubated at 37°C and the MICs were read after 24 hrs. Reading and interpretations of the MICs in the E- test were done according to the manufacture's instructions, generally the MIC was determined by examining elliptical

zones of inhibition around the calibrated E test strip. The MIC was read where inhibition of growth intersected the E-test strip. When small colonies grew within the zone of inhibition or a haze of growth occurred around MIC end points, the higher MIC intersect was recorded**.**

3.2.6. Virulence factors tests

All *A. baumannii* isolates were subcultured twice on CLED and Blood agar plates at 37 °C for 24 hrs, to ensure purity before doing any of the virulence factors tests.

3.2.6.1. Protease test procedure

Extracellular protease activity of *A. baumannii* was analyzed by two methods. Skim milk agar was made according to the modified technique described by Pailin et al. (2001) as follows: 11.25 g of nonfat dry milk (skim milk) (Oxoid) was reconstituted with 250 ml of distilled water. The mixture was stirred thoroughly and autoclaved at 121°C for 15 min. Likewise, 500 ml of 2.5% agar solution was sterilized, then skim milk and agar solutions were allowed to cool to 50^{o}C, and then the skim milk was poured into the agar bottle and mixed thoroughly. The skim milk agar was poured quickly into plates.

Extracellular protease activity was tested by two methods as follows:

- The first method was according to the technique described by sechi et al. (2004): single colony, two colonies, and three colonies of each isolate respectively were inoculated in spot form on the skim milk agar. After incubation for up to 72 h at 37°C, the production of protease was evidenced by the formation of a clear zone caused by casein degradation.

- The second method was according to the technique described by Chantawannakul et al., (2002): 5 colonies of isolated bacteria were cultivated in 50 ml nutrient broth (Oxoid) with vigorous shaking (150 rpm) at 37°C for 24 h. The culture was then

centrifuged at 10,000 rpm for 5 min at 4°C. The supernatant was collected and used as the enzyme solution. For this, 5 µl of crude protease produced from isolated bacteria were spotted on skim milk agar plates. The plates were then incubated at 37°C for 18-24 h. Proteolytic activity of the crude proteins was detected by observing the presence of clear zones. All isolates were tested in duplicate and a reference strain of *Serratia mercenses* ATCC 13880 served as positive control.

3.2.6.2. Gelatinase test procedure

Gelatinase activity of *A. baumannii* was analyzed by two methods. Gelatin agar plates were prepared according to the modified technique described by Kanemitsu et al. (2001). The basic medium consisted of 3.6% (w/v) gelatin and 5% (w/v) agar. The media prepared as follows: 18 g gelatin was reconstituted with 150 ml of distilled water. The mixture was stirred thoroughly and autoclaved at 121°C for 15 min. Likewise, 25 g agar was dissolved in 350 ml distilled water and autoclaved at 121°C for 15 min, then gelatin and agar solutions were allowed to cool to 50°C, and then the gelatin was poured into the agar bottle and mixed thoroughly. The media was poured quickly into plates. Gelatinase activity test was done by two methods as follows:

-The first method: single colony, two colonies, and three colonies of each isolate respectively were inoculated in spot form on the gelatin agar plates. After incubation for up to 48 h at 37°C, the production of gelatinase was evidenced by the formation of a turbid halo around the colonies.

-The second method: five colonies of isolated bacteria were cultivated in 50 ml nutrient broth (Oxoid) with vigorous shaking (150 rpm) at 37°C for 24 h. The culture was then centrifuged at 10,000 rpm for 5 min at 4°C. Cell free supernatant was collected and 5 µl of this was spotted on gelatin agar plates. The plates were then incubated at 37°C for 18-24 h, the production of gelatinase was evidenced by the formation of a

turbid halo. All isolates were tested in duplicate and a reference strain of *Serratia mercenses* ATCC 13880 served as positive control.

3.2.6.3. Lecithinase test procedure

All *A. baumannii* isolates were screened for the production of extracellular lecithinase enzyme by growing them on egg yolk agar according to the modified technique described by Moreno and Landgraf (1998). The egg yolk medium consisted of nutrient agar (Oxoid); 0.11% $CaCl_2$ and 10% (v/v) egg yolk emulsion (Oxoid).

First, the components without the egg yolk were mixed and sterilized then the egg yolk was centrifuged at 500 x g for 10 min at room temperature and 10% (v/v) of the supernatant was added to the sterilized, cooled media, then mixed well and poured into sterile Petri dishes. Then, single colony, two colonies, and three colonies of each isolate respectively were inoculated in spot form on the egg yolk agar plates. After incubation for up to 7 days at 37°C, the production of lecithinase was evidenced by the formation of a precipitation zone around the colonies. All isolates were tested in duplicate and a reference strain of Non-O1 *Vibrio cholerae* clinical strain served as positive control.

3.2.6.4. Lipase test procedure

All *A. baumannii* isolates were screened for the production of extracellular lipase enzyme according to the technique described by Moreno and Landgraf (1998). The basic medium consisted of nutrient agar, 0.11% $CaCl_2$, and 1% (v/v) Tween 80 (polyoxyethylene sorbitan mono-oleate). All components were mixed and sterilized by autoclaving at 121^{o}C for 15 minutes, then allowed to cool to 50^{o}C, and poured into sterile Petri dishes. Then, single colony, two colonies, and three colonies of each isolate respectively were inoculated in spot form on the Tween 80 agar plates. After incubation for up to 7 days at 37°C, the production of lipase was evidenced by the formation of a

precipitation zone around the colonies. All isolates were tested in duplicate and a reference strain of Non-O1 *Vibrio cholerae* clinical strain served as positive control.

3.2.6.5. Hemolysin test procedure

Hemolysin production was screened according to the modified technique described by Izumi et al. (2005) using sheep and human blood agar plates.

Human/ sheep blood agar was prepared by suspending 20 g blood agar base in 500 ml of distilled water and brought gently to the boil to dissolve completely and sterilized by autoclaving at 121^0C for 15 minutes then allowed to cool to 50^0C, 5% (v/v) human blood/sheep blood was added to blood agar base and mixed well and then poured into sterile Petri dishes. Then, single colony, two colonies, and three colonies of each isolate respectively were inoculated in spot form on the sheep and blood agar plates. After incubation for 24-48 hr at 37°C, the production of hemolysin was evidenced by the formation of a clear zone around the colonies. All isolates were tested in duplicate and a reference strain of Non-O1 *Vibrio cholerae* clinical strain and *E. faecalis* strain ATCC 29212 served as positive controls.

3.2.6.6. Growth under iron-limiting conditions

This test was evaluated according to the technique described by Braun and Vidotto (2004). The basic medium consisted of M9 minimal medium containing the iron chelator 2,2-dipyridyl (DIP) (Sigma) in concentrations of 50 µM and 200 µM and 2% agar. 2,2-Dipyridyl was prepared as a 20 mM (0.312 g DIP was dissolved in 100 ml distilled water) stock solution in distilled water and kept at -20°C. To prepare M9 minimal medium according to Fluckinger et al. (2004) add 200 ml of sterile 5x M9 minimal salts [6.8 g of $Na_2HPO_4 \cdot 7H_2O$, 3 g of KH_2PO_4, 0.5 g of NaCl, 1 g of NH_4Cl] were added to 750 ml of sterile agar base, then 2 ml of 1 M $MgSO_4$, 20 ml of filter-sterilized 20% glucose, 0.1 ml of sterile 1M $CaCl_2$, and 50 or 200 µM DIP were added,

mixed well, and the final volume was adjusted to 1L. A single colony was picked from fresh cultures of *A. baumannii* and suspended in 10 ml of nutrient broth and after mixing with a vortex mixer, a calibrated loop (0.001 ml) was dipped in suspension, then it was streaked on M9 minimal media with 50 µM DIP and on M9 minimal media with 200 µM DIP. The plates were incubated at 37°C for 24-48 hours. All isolates were tested in duplicate.

3.2.6.7. Hemagglutination (HA) assay

This test was evaluated according to the technique described by Izumi et al., (2004). Hemagglutination (HA) was determined via a microhemagglutination test using 96-well round-bottom plates and fresh human group O and AB Rhesus-positive erythrocytes. The phosphate buffered saline (PBS) was prepared by dissolving the following: [8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, and 0.24 g KH_2PO_4] in 1 liter of distilled water followed by adjusting to pH 7.4. The washed erythrocytes were suspended in PBS to a final concentration of 3% and stored at 4°C until use. The erythrocyte suspensions were used in the HA assays. Bacteria were grown in Brain Heart infusion broth (Oxoid) at 37°C for 16 h under static conditions, centrifuged at 10,000 rpm in a microcentrifuge at 4°C for 10 min, and suspended in 300 µl of the same broth. Fifty microliters of a bacterial cell suspension were mixed with 50 µl of a 3% erythrocyte suspension for each isolate tested. The microtiter plate was rocked at room temperature for 1 h and then placed at 4°C for 2-4 h. Wells exhibiting a small button of erythrocytes at the bottom of the well after 2 h of incubation were considered HA-negative, while wells showing a carpet of erythrocytes across the well were considered HA-positive. An erythrocyte suspension in PBS was included for each isolate as a negative control. The HA assays were performed at least two times on each of the clinical strains examined. *E. coli* strain k12 was used as positive control for hemagglutination assay.

3.2.6.8. Hemagglutination inhibition testing with mannose

Bacteria were grown in Brain Heart infusion broth according to the method fo the HA assay. A 50 µl aliquot of each suspension was added to the wells and mixed with an equal volume of a suspension of 3% erythrocytes and 1% D-mannose (Sigma). The microtiter plate was rocked at room temperature for 1 h and then placed at 4°C for 2-4 h. Wells exhibiting a small button of erythrocytes at the bottom of the well after 2 h of incubation were considered HA-negative, while wells showing a carpet of erythrocytes across the well were considered HA-positive. An erythrocyte suspension in PBS was included for each isolate as a negative control. The HA assays were performed at least two times on each of the clinical strains examined.

3.2.7. β-Lactamase assays

A **modified Hodge test** was performed to screen carbapenemase production. A suspension of *Escherichia coli* ATCC 25922, which was adjusted to the turbidity of the McFarland standard No. 0.5 tube was inoculated evenly on a Mueller-Hinton agar plate. Then, an imipenem disk was placed at the center of the plate. Test isolates were streaked heavily from the edge of the disk to the periphery of the plate. The presence of a distorted inhibition zone after 16 to 18 hours of incubation at 37°C was interpreted as a positive modified Hodge test (Yang et al., 2009). *Klebsiella pneumoniae* ATCC BAA-1706 was used as negative control and *Klebsiella pneumoniae* ATCC BAA-1705 was used as positive control for the modified Hodge test.

An **imipenem-EDTA double disc synergy test** was performed to screen for the production of metallo-β-lactamases. An overnight culture of the test isolates was inoculated on a Mueller–Hinton agar plate. The imipenem disc and a blank filter paper disc were placed 15 mm apart from edge to edge, and 10 µL of 0.5 M of EDTA solution was applied to the blank disc. After overnight incubation, the presence of even

small synergistic inhibition zone was interpreted as a positive result, which shows the inactivation of class B metallo-β-lactamase activity by EDTA (Lee et al., 2007).

A 0.5 M of EDTA solution was prepared by dissolving 186.1 g of disodium EDTA•$2H_2O$ in 1,000 ml of distilled water and adjusting it to pH 8.0. The mixture was sterilized by autoclaving at 121^{o}C (Yong et al., 2002).

3.2.8. Multiplex PCR for genes encoding prevalent OXA carbapenemases in *A. baumannii*

3.2.8.1. Extraction of *A. baumannii* DNA and PCR reactions.

Genomic DNA was extracted according to the manufacture's instructions of Wizard Genomic DNA purification kit (Promega, USA). All isolates were subjected to the multiplex PCR for the detection of genes encoding for OXA-23-like, OXA-24-like, OXA-58-like and OXA-51-like carbapenemase. PCR was carried out in 25 µl reaction volumes with 2.5 µl of extracted DNA, 20 pmol of each primer in table 2 (Midland, Texas), 1.5 U of Taq DNA polymerase, 5 µl of 5x PCR buffer containing 1.5 mM $MgCl_2$, and 0.5 µl of 10 mM dNTP mix (Promega, USA). The volume was made up to 25 µl using nuclease free water. The amplification conditions according to Woodford et al. (2006) were, initial denaturation at 94°C for 5 min, 30 cycles of 94°C for 25 s, 52°C for 40 s and 72°C for 50 s, and a final elongation at 72°C for 6 min. The PCR assays for the target genes were performed by using programmable thermocycler (MJ research- IMC, USA). Control tubes containing master mix but without template DNA were included in each run as negative control. Tubes were held at 4°C when the cycles were ended.

3.2.8.2. Agarose Gel Electrophoresis

A stock solution of 10x TBE buffer was prepared by dissolving 55 g Boric acid , 108 g Tris base, and 7.4 g Di-sodium EDTA in 0.5 liter of distilled water followed by

adjusting to pH (8.3 ± 0.1). In a final volume of one liter. For usage; one part of stock solution was diluted with nine parts of distilled water.

The 2% (w/v) agarose gel was prepared as follows:

- Agarose (4.0 g) was added to 200 ml of 1X TBE buffer with gentle shaking.
- Agarose was completely dissolved by boiling. After cooling the solution was cooled to about 50 °C, 30µl of diluted ethidium bromide was added.
- The agarose was then poured into an electrophoresis cell and left to solidify at room temperature for about 20 minutes; after removal of combs and gates, 1X TBE buffer was added to the electrophoresis cell.

The amplified products and the PCR DNA marker were separated via electrophoresis on 2% agarose gels containing 15% ethidium bromide, for 30-40 min at 130 volt, and then visualized using Gel documentation system including: UV camera, monitor and printer (UVP, USA).

3.2.9. Multiplex PCR for genes encoding metallo-β-lactamases (MBL) in *A. baumannii*

All isolates were subjected to the multiplex PCR to detect genes encoding for IMP and VIM. PCR was carried out in 25 µl reaction volumes with 2.5 µl of extracted DNA, 20 pmol of each primer in table 2 (Alpha DNA, Montreal, Canada), 5 µl of 5 x qARTA•Taq master mix (Qartabio, USA). The volume was made up to 25 µl using nuclease free water. The amplification conditions according to Sung et al., (2008) were, initial denaturation at 95°C for 5 min, 35 cycles of 95°C for 20 s, 59°C for 40 s and 72°C for 30 s, and a final elongation at 72°C for 5 min. The PCR assays for the target genes were performed by using programmable thermocycler (MJ research- IMC, USA). Control tubes containing master mix but without template DNA were included in each run as negative control. Tubes were held at 4°C when the cycles were ended. The

amplified products and the PCR DNA marker were separated via electrophoresis on 2% agarose gels containing 15% ethidium bromide, for 30-40 min at 130 volt, and then visualized using Gel documentation system including: UV camera, monitor and printer (UVP, USA).

3.2.10. Genotyping of *A.baumannii* by ERIC-PCR

The ERIC-PCR was done for represntative *A. baumannii* isolates, using two primers (Tabel 3) according to Sung et al. (2008).

3.2.10.1. PCR reactions and amplification of ERIC-PCR in *A. baumannii*

ERIC-PCR was conducted with a 25 µl reaction mixture containing 50 ng of chromosomal DNA, 5 µl of 5 x qARTA•Taq master mix, and 25 pmol of each of the primers in table (2) (Promega, USA). The volume was made up to 25 µl using nuclease free water. The cycling conditions were as follows: an initial denaturation step at 95°C for 5 min; followed by 30 cycles of amplification steps at 92°C for 50 sec, 52°C for 55 sec, and 70°C for 7 min; and a final extension step at 70°C for 10 min. Control tubes containing master mix but without template DNA were included in each run as negative control. Tubes were held at 4°C when the cycles ended.

The volume of PCR product loaded on 2% agarose gel for the reaction mixture was 15µl of PCR product, and the PCR DNA marker was loaded too. The gel then ran for 45 min-1 hour at 150 volt, and then photographed using Gel documentation system including: UV camera, monitor and printer (UVP, USA).

3.2.10.2. ERIC analysis

A total of 64 *A. baumannii* isolates were investigated and their PCR products were separated on the same gel electrophoresis. ERIC banding patterns of the 64 isolates were recorded. Bands were scored, with the data coded as a factor of 1 or 0,

representing the presence or absence of bands, respectively. The presence or absence of an amplified fragment was treated as an independent character without consideration of the quantitative aspect of the results, i.e. band intensity. Densely bands were considered as one band except when they tend to separate into two bands on other individuals.

3.2.11. Statistical analysis

Data acquisition and analysis were performed using Statistical Package of Social Science program (SPSS) version 16 which was used to determine the P values.
The student's *t*-test for independent and paired continuous variables and the Chi-square test or Fisher's exact test were used when appropriate to compare proportions. A *P*-value of <0.05 was considered statistically significant. Multivariate analysis using logistic regression method was performed, including all variables with $P<0.05$ or lower in the univariate analysis.

4. Results

4.1. Prevalence of *A. baumannii* colonizing patients.

A total of 185 patients were included in this study, and a total of 64 isolated *A. baumannii* were isolated from 63 patients. One strain was recovered from blood and bronchoalveolar lavage specimens of the same patient. The *A. baumannii* isolates were characterized by phenotypic and genotypic methods. Table (4) shows the sources from which these isolates were recovered.

Table 4: Sources of 64 *A. baumannii* isolates.

Source	No.(%) of positive *A. baumannii* culture	Total No. (%) of samples
Tracheal aspirate	55 (23.5)	122 (52.1)
Bronchoalveolar lavage	5 (2.1)	5 (2.1)
Sputum	3 (1.2)	96 (41.1)
Blood*	1 (0.4)	1 (0.4)
Throat swab**	0 (0.0)	10 (4.3)
Total	64 (27.4)	234 (100.0)

* One strain was recovered from blood and bronchoalveolar lavage specimens of the same patient.
** Throat swab was taken from patient who don't have sputum.

4.2. Description of patients with positive and negative *A. baumannii* cultures

Table (5) describes 185 patients (positive and negative for *A. baumannii*) according to gender, age group, patient group, exposure to antibiotics, type of antibiotics used, use of mechanical ventilation, use of nasogastric tube, presence of tracheostomy, diagnosis, and length of stay in ICUs (LOS).

Table 5: Demographic Characteristics of 185 patients with positive and negative *A. baumannii* isolates

Variables	*A. baumannii* colonized patients No. (%)	*A. baumannii* not colonized patients No. (%)	P-value
Gender			
Male	36 (40.0)	54 (60.0)	0.097
Female	27 (28.4)	68 (71.6)	
Age			
0-64 years	33 (28.7)	82 (71.3)	0.038
≥ 65 years	30 (42.9)	40 (57.1)	
Ward			
ICUs	63 (67.7)	30 (32.3)	<0.001
OPD	0 (0.0)	92 (100.0)	
Length of stay (LOS) in ICU before colonization (mean±std), n=93	7.51 ± 1.595	2.73 ± 2.377	<0.001
LOS in ICU			
LOS (1-3 days)	1 (3.7)	26 (96.3)	< 0.001
LOS (4-8 days)	56 (94.9)	3 (5.1)	
LOS (> 8 days)	6 (85.7)	1 (14.3)	
Antibiotic treatment			
Yes	50 (58.8)	35 (41.2)	< 0.001
No	13 (13.0)	87 (87.0)	
Type of antibiotics			
Carbapenems			
Yes	37 (77.1)	11 (22.9)	< 0.001
No	13 (35.1)	24 (64.9)	
4th Generation cephalosporins			0.371
Yes	11 (68.8)	5 (31.2)	
No	39 (56.5)	30 (43.5)	
Quinolones			
Yes	20 (50.0)	20 (50.0)	0.119
No	30 (66.7)	15 (33.3)	
Aminoglycosides			
Yes	3 (75.0)	1 (25.0)	0.640
No	47 (58.0)	34 (42.0)	

*Continue to the next page

Variables	*A. baumannii* colonized patients No. (%)	*A. baumannii* not colonized patients No. (%)	P-value
Nasogastric tube usage			0.001
Yes	29 (50.9)	28 (49.1)	
No	34 (26.6)	94 (73.4)	
Mechanical ventilation			<0.001
Yes	60 (84.5)	11 (15.5)	
No	3 (2.6)	111 (97.4)	
Length of ventilation before taking specimen (mean±std), n=71	5.02 ± 1.17	1.36 ± 0.67	<0.001
Tracheostomy			0.001
Yes	11 (78.6)	3 (21.4)	
No	52 (30.4)	119 (69.6)	
Diagnosis			
Diabetes mellitus			0.030
Yes	19 (48.7)	20 (51.3)	
No	44 (30.1)	102 (69.9)	
Pulmonary diseases			0.124
Yes	34 (29.8)	80 (70.2)	
No	29 (40.8)	42 (59.2)	
Cardiac diseases			0.631
Yes	11 (37.9)	18 (62.1)	
No	52 (33.3)	104 (66.7)	
Neurologic impairment			<0.001
Yes	18 (66.7)	9 (33.3)	
No	45 (28.5)	113 (71.5)	
Malignancy			0.951
Yes	5 (33.3)	10 (66.7)	
No	58 (34.1)	112 (65.9)	

4.3.1. Demographic characterization of the patients colonized with *A. baumannii*

Table (6) demonstrates that the prevalence of *A. baumannii* colonization among male and female patients was (40.0%) and (28.4%), respectively, with no significant relationship between gender and *A. baumannii* colonization (P>0.05).

Table 6: Distribution of *A. baumannii* colonized patients according to gender.

Gender	Colonized patients No. (%)	Not Colonized patients No. (%)	Total No. (%)	P-value
Male	36 (40.0)	54 (60.0)	90 (100.0)	0.097
Female	27 (28.4)	68 (71.6)	95 (100.0)	
Total	63 (34.1)	122 (65.9)	185 (100.0)	

Table (7) demonstrates that the prevalence of *A. baumannii* colonization among patients aged (0-64 years) was (28.7%), and among patients aged (≥ 65 years) was (42.9%), There was a significant relationship between age and *A. baumannii* colonization (P<0.05).

Table 7: Distribution of *A. baumannii* colonized patients according to age groups

Age Groups	Colonized patients No. (%)	Not Colonized patients No. (%)	Total No. (%)	P-value
0-64 years	33 (28.7)	82 (71.3)	115 (100.0)	0.038
≥ 65 years	30 (42.9)	40 (57.1)	65 (100.0)	
Total	63 (34.1)	122 (65.9)	185 (100.0)	

Table(8) demonstrates that the prevalence of *A. baumannii* colonization among patients from all ICUs was (67.7%); MICU was (65.5%), SICU was (67.5%), and CICU (75.0%), and OPD patients was (0.0 %), and there was a significant relationship between ICUs admission and *A. baumannii* colonization (P<0.05).

Table 8: Distribution of *A. baumannii* colonized patients according to patient's hospital admission.

Hospital admission	Colonized patients No. (%)	Not Colonized patients No. (%)	Total No. (%)	P-value
MICU	27 (65.9)	14 (34.1)	41 (100.0)	< 0.001
SICU	27 (67.5)	13 (32.5)	40 (100.0)	
CICU	9 (75.0)	3 (25.0)	12 (100.0)	
Total ICUs	63 (67.7)	30 (32.3)	93 (100.0)	
OPD	0 (0.0)	92 (100.0)	92 (100.0)	
Total	63 (34.1)	122 (65.9)	185 (100.0)	

Table (9) demonstrates that the prevalence of *A. baumannii* colonization among patients with LOS in the range (1-3 days) was (3.7%), that for patients with LOS between (4-8 days) was (94.9%) and that for patients with LOS (> 8 days) was (85.7%). There was a significant relationship between LOS and *A. baumannii* colonization (P<0.05).

Table 9: Distribution of *A. baumannii* colonized patients according to ICU length of stay (LOS).

Status of Patients	Colonized patients No. (%)	Not Colonized patients No. (%)	Total No. (%)	P-value
LOS (1-3 days)	1 (3.7)	26 (96.3)	27 (100.0)	< 0.001
LOS (4-8 days)	56 (94.9)	3 (5.1)	59 (100.0)	
LOS (> 8 days)	6 (85.7)	1 (14.3)	7 (100.0)	
Total	63 (67.7)	30 (32.3)	93 (100.0)	

Table (10) demonstrates that the prevalence of *A. baumannii* colonization among patients who were treated with antibiotics was (58.8%), patients who were not treated

with antibiotics was (13.0%), pointing to a significant relationship between exposure to antibiotic and *A. baumannii* colonization (P<0.05).

Table 10: Distribution of *A. baumannii* colonized patients according to previous antibiotics treatment.

Treatment	Colonized patients No. (%)	Not Colonized patients No. (%)	Total No. (%)	P-value
Antibiotic	50 (58.8)	35 (41.2)	85 (100.0)	< 0.001
No Antibiotic	13 (13.0)	87 (87.0)	100 (100.0)	
Total	63 (34.1)	122 (65.9)	185 (100.0)	

Table (11) demonstrates that the prevalence of *A. baumannii* colonization among patients who were treated with carbapenems was (77.1%), among those who use 4th generation cephalosporins was (68.8%), among those who were treated quinolones was (50.0%), and among those who were treated aminoglycosides was (75.0%), indicating a significant relationship between carbapenems use and *A. baumannii* colonization (P<0.05), but no significant relationship between 4th generation cephalosporins, quinolones, and aminoglycosides use and *A. baumannii* colonization (P>0.05).

Table 11: Distribution of *A. baumannii* colonized patients according to type of antibiotics used

Antibiotics	Colonized patients No. (%)	Not Colonized patients No. (%)	Total No. (%)	P-value
Carbapenems				
Yes	37 (77.1)	11 (22.9)	48 (100.0)	<0.001
No	13 (35.1)	24 (64.9)	37 (100.0)	
Total	50 (58.8)	35 (41.2)	85 (100.0)	
4th generation cephalosporins				
Yes	11 (68.8)	5 (31.2)	16 (100.0)	0.371
No	39 (56.5)	30 (43.5)	69 (100.0)	
Total	50 (58.8)	35 (41.2)	85 (100.0)	
Quinolones				
Yes	20 (50.0)	20 (50.0)	40 (100.0)	0.119
No	30 (66.7)	15 (33.3)	45 (100.0)	
Total	50 (58.8)	35 (41.2)	85 (100.0)	
Aminoglycosides				
Yes	3 (75.0)	1 (25.0)	4 (100.0)	
No	47 (58.0)	34 (42.0)	81 (100.0)	0.640
Total	50 (58.8)	35 (41.2)	85 (100.0)	

Table (12) demonstrates that the prevalence of *A. baumannii* colonization among patients who used nasogastric tube was (50.9%), patients who did not use nasogastric tube was (26.6%), indicating a significant relationship between nasogastric tube usage and *A. baumannii* colonization (P<0.05).

Table 12: Distribution of *A. baumannii* colonized patients according to using nasogastric tube

Nasogastric tube using	Colonized patients No. (%)	Not Colonized patients No. (%)	Total No. (%)	P-value
Yes	29 (50.9)	28 (49.1)	57 (100.0)	0.001
No	34 (26.6)	94 (73.4)	128 (100.0)	
Total	63 (34.1)	122 (65.9)	185 (100.0)	

Table (13) demonstrates that the prevalence of *A. baumannii* colonization among patients who were on mechanical ventilation was (84.5%), patients who were not on mechanical ventilation was (2.6%), indicating a significant relationship between mechanical ventilation and *A. baumannii* colonization (P<0.05).

Table 13: Distribution of *A. baumannii* colonized patients according to mechanical ventilation

Mechanical ventilation using	Colonized patients No. (%)	Not Colonized patients No. (%)	Total No. (%)	P-value
Yes	60 (84.5)	11 (15.5)	71 (100.0)	<0.001
No	3 (2.6)	111 (97.4)	114 (100.0)	
Total	63 (34.1)	122 (65.9)	185 (100.0)	

Table (14) demonstrates that the prevalence of *A. baumannii* colonization among patients who had tracheostomy was (84.6%), patients who did not have tracheostomy was (30.2%), pointing to a significant relationship between tracheostomy and *A. baumannii* colonization (P<0.05).

Table 14: Distribution of *A. baumannii* colonized patients according to tracheostomy

Tracheostomy	Colonized patients No. (%)	Not Colonized patients No. (%)	Total No. (%)	P-value
Yes	11 (78.6)	3 (21.4)	14 (100.0)	0.001
No	52 (30.4)	119 (69.6)	171 (100.0)	
Total	63 (34.1)	122 (65.9)	185 (100.0)	

Table (15) demonstrates that the prevalence of *A. baumannii* colonization among patients who have diabetes mellitus was (48.7%), among those with pulmonary diseases was (29.8%), among those with cardiac diseases was (37.9%), among those with neurologic impairment was (66.7%), and among those with malignancy was (33.3%), and indicating a significant relationship between co-morbidity with diabetes mellitus and neurologic impairment and *A. baumannii* colonization ($P<0.05$), but no significant relationship between co-morbidity with pulmonary diseases, cardiac diseases and malignancy and *A. baumannii* colonization ($P>0.05$).

Table 15: Distribution of *A. baumannii* colonized patients according to primary diagnosis.

Disease	Colonized patients No. (%)	Not Colonized patients No. (%)	Total No. (%)	P-value
Diabetes mellitus				
Yes	19 (48.7)	20 (51.3)	39 (100.0)	0.030
No	44 (30.1)	102 (69.9)	146 (100.0)	
Total	63 (34.1)	122 (65.9)	185 (100.0)	
Pulmonary diseases*				
Yes	34 (29.8)	80 (70.2)	114 (100.0)	0.124
No	29 (40.8)	42 (59.2)	71 (100.0)	
Total	63 (34.1)	122 (65.9)	185 (100.0)	
Cardiac diseases**				
Yes	11 (37.9)	18 (62.1)	29 (100.0)	0.631
No	52 (33.3)	104 (66.7)	156 (100.0)	
Total	63 (34.1)	122 (65.9)	185 (100.0)	
Neurologic impairment***				
Yes	18 (66.7)	9 (33.3)	27 (100.0)	< 0.001
No	45 (28.5)	113 (71.5)	158 (100.0)	
Total	63 (34.1)	122 (65.9)	185 (100.0)	
Malignancy				
Yes	5 (33.3)	10 (66.7)	15 (100.0)	0.951
No	58 (34.1)	112 (65.9)	170 (100.0)	
Total	63 (34.1)	122 (65.9)	185 (100.0)	

* Chronic Obstructive Pulmonary Disease (COPD), asthma, pneumonia, bronchitis, bronchiactesis, and productive airway disease.
**Congestive heart failure, chronic cardiac failure, peripheral vascular disease, and coronary artery disease.
***Cerebrovascular accident, and traumatic brain injury.

4.3.2. Multivariate analysis of risk factors for the occurrence of *A. baumannii*

Demographic data were incorporated for multivariate analysis table (16), which revealed that the longer duration of mechanical ventilation and longer stay in the ICU ($P<0.05$) were the only significant independent risk factors for infection with *A. baumannii.*

Table 16: Multivariate analysis of risk factors for the occurrence of *A. baumannii*

Risk factor	P-value
Length of ventilation before taking specimen	<0.001
LOS in ICU	0.032
Mechanical ventilation	0.149
Neurologic impairment	0.312
Nasogastric tube using	0.320
Tracheostomy	0.394
Age	0.821
Prior antibiotics treatment	0.998
Carbapenems treatment	0.999
Diabetes mellitus	0.999
Ward	0.999

4.4. Antimicrobial susceptibility testing of *A. baumannii* isolates

4.4.1. Antimicrobial susceptibility by the disc diffusion method

The results of antimicrobial susceptibility as revealed by the disc diffusion method of all 64 *A. baumannii* isolates are shown in Table (17). *A. baumannii* isolates were 100% resistant to meropenem, ertapenem and ceftazidime, 98% resistant to

piperacillin/tazobactam, and aztreonam, 94% resistant to gentamicin, 91 % resistant to ciprofloxacin, 73% resistant to amikacin, and 64% resistant to imipenem but all *A. baumannii* isolates were susceptible to colistin, and tigecycline.

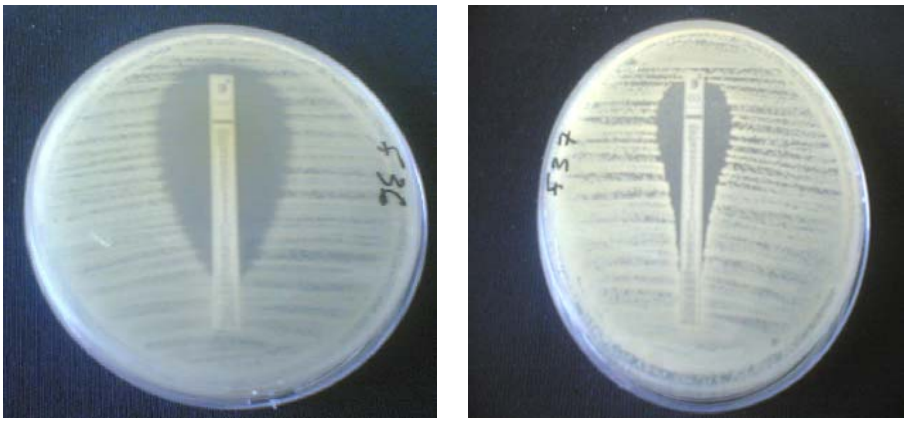
4.4.2. Minimal inhibitory concentrations (MICs) for *A. baumannii*

The results of MICs for 64 *A. baumannii* isolates to amikacin, imipenem, colistin and tigecycline are shown in Table (17). Both amikacin and imipenem have the highest MIC90 (13.6 and 12, respectively), whereas tigecycline and colistin have the lowest MICs (2 and 1 respectively).

Table (17): Antimicrobial resistance patterns of 64 *A. baumannii* isolates from ICUs patients

Antimicrobial	No. (%) Resistant isolates	MIC_{90} (mg/L)	MIC-range (mg/L)
Ceftazidime (Caz)	64 (100)	ND *	ND
Ertapenem (Etp)	64 (100)	ND	ND
Meropenem (Mem)	64 (100)	ND	ND
Piperacillin/Tazobactam (Ptz)	63 (98)	ND	ND
Aztreonam (Atm)	63 (98)	ND	ND
Gentamicin (Gm)	60 (94)	ND	ND
Ciprofloxacin (Cip)	58 (91)	ND	ND
Amikacin (Ak)	47 (73)	13.6	1-16
Imipenem (Imi)	40 (63)	12	0.19-12
Colistin (Co)	0 (0)	1	0.19-1
Tigecycline (Tgc)	0 (0)	2	0.38-4

***ND= Not done**



A B

Figure (3): E test for (A) tigecycline and (B) colistin, eliptical zone of inhibition around the calibrated E test strip.

4.4.3. Distribution of multidrug resistance among 64 *A. baumannii* isolates

Table (18) shows resistance profiles of 64 *A. baumannii* isolates which can be summarized as follows: forty two percent were resistant to Mem, Gm, Ptz, Atm, Cip, Imi, Caz, Ak, Etp, 22% to Mem, Gm, Ptz, Atm, Cip, Caz, Ak, Etp, 13% to Mem, Gm, Ptz, Atm, Cip,Caz, Etp, 8% to Mem, Gm, Ptz, Atm, Cip, Imi, Caz, Etp, 5% to Mem, Gm, Ptz, Atm, Imi, Caz, Etp, and the rest of isolates were associated with 1% - 3% resistance profiles.

Table (18): Distribution of antimicrobial resistance profiles among 64 *A. baumannii* clinical isolates from ICUs patients

Antimicrobial resistance profiles	No. (%) of strains showing the same profile
Mem, Gm, Ptz, Atm, Cip, Imi, Caz, Ak, Etp	27 (42.2)
Mem, Gm, Ptz, Atm, Cip, Caz, Ak, Etp	14 (21.9)
Mem, Gm, Ptz, Atm, Cip,Caz, Etp	8 (12.5)
Mem, Gm, Ptz, Atm, Cip, Imi, Caz, Etp	5 (7.8)
Mem, Gm, Ptz, Atm, Imi, Caz, Etp	3 (4.7)
Mem, Gm, Ptz, Atm, Imi, Caz, Ak, Etp	2 (3.1)
Mem, Cip, Ptz, Atm, Imi, Caz, Ak, Etp	2 (3.1)
Mem, Gm, Ptz, Cip, Imi, Caz, Ak, Etp	1 (1.6)
Mem, ,Ak, Ptz, Atm, Cip,Caz, Etp	1 (1.6)
Mem, Atm, Caz, Etp	1 (1.6)

4.5. Modified Hodge test

Out of 64 strains screened, 62 (96.9%) strains showed a distorted inhibition zone around the imipenem disc (figure 4).

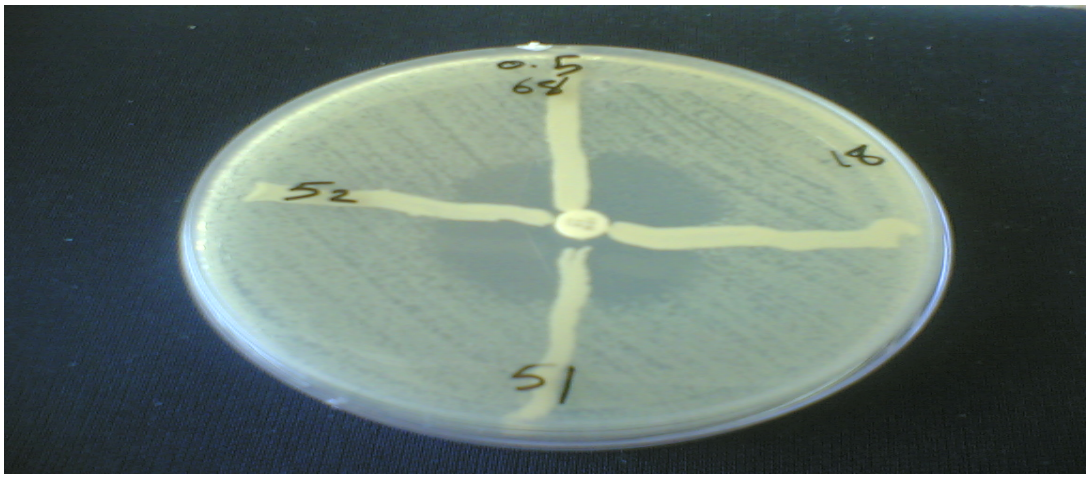


Figure (4): Modified Hodge test. The distorted inhibition zone showed positive test of carbapenem-hydrolyzing strains whereas, carbapenem-non-hydrolyzing strains with no effect on the zone.

4.6. Imipenem-EDTA double disc-synergy test

Out of the 40 imipenem resistant strains screened, only two strains were positive for the presence of metallo-β-lactamases, whereby these enzymes were inhibited by EDTA (figure 5).

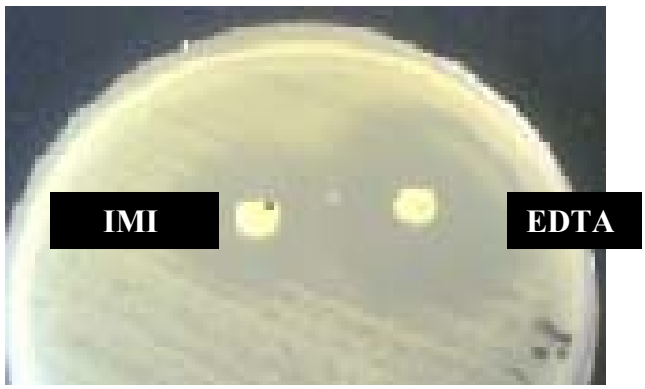


Figure (5):Imipenem-EDTA double disc-synergy test. An imipenem disk and an EDTA impregnated disk produced synergistic inhibition zone for positive isolates.

4.7. Prevalence of OXA carbapenemase and MBL genes in 64 *A. baumannii* isolates

Table (19) shows the prevalence of $bla_{\text{OXA-51}}$-like, $bla_{\text{OXA-23}}$-like, $bla_{\text{OXA-24}}$-like, $bla_{\text{OXA-58}}$-like, bla_{IMP}, and bla_{VIM} genes, among 64 *A. baumannii*, which demonstrates that 100% of the isolates possessed the encoding gene for an intrinsic OXA-51-like carbapenemase, 73% were positive for OXA-23-like, 19% were positive for OXA-24-like, and only two (3%) were positive for VIM β-lactamases, but none of the isolates harbored the encoding gene for IMP and OXA-58-like β-lactamases.

Table (19): Prevalence of OXA carbapenemase and MBLs among 64 *A. baumannii* isolates from ICUs patients

Class of β-lactamase	Type of β-lactamase	Prevalence (%)
Class B	IMP	0 (0)
	VIM	2 (3)
Class D	OXA-51-like	64 (100)
	OXA-23-like	47(73)
	OXA-24-like	12 (19)
	OXA-58-like	0 (0)

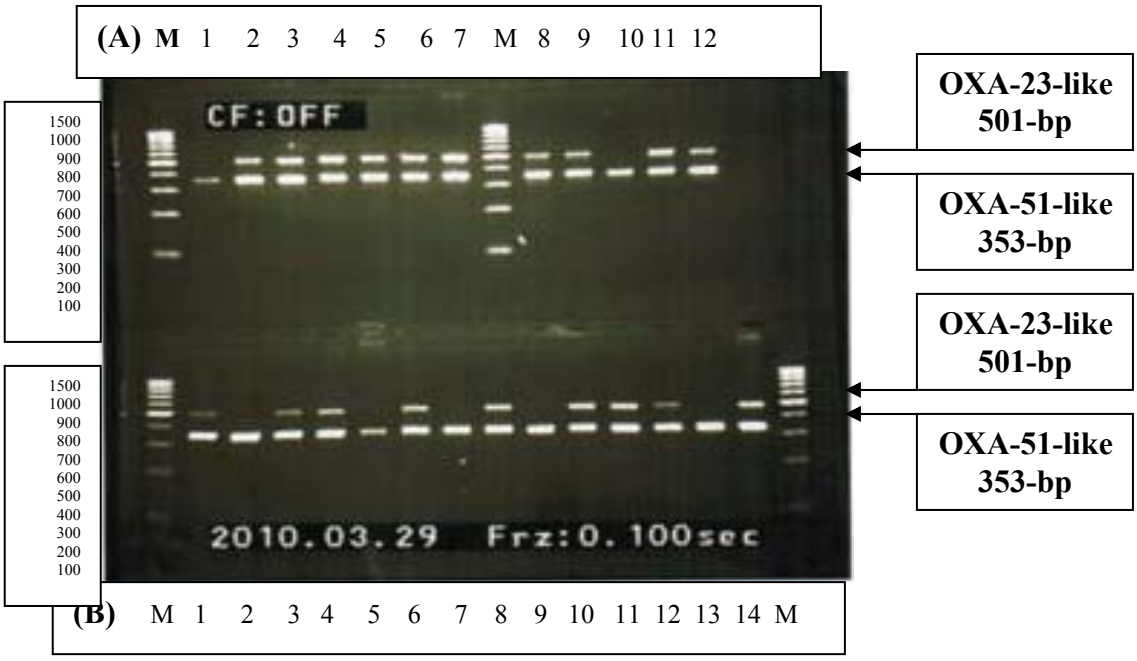


Figure (6): Agarose gel electrophoresis of multiplex PCR for genes encoding OXA-51-like and OXA-23-like carbapenemases. LaneM: 100 bp DNA Ladder marker. (A) Lane 1-12 positive for OXA-51-like. Lane 2-9, and 11-12 positive for OXA-23-like in *A. baumannii* isolates. (B) Lane 1-14 positive for OXA-51-like. Lane 1, 3-4, 6, 8, 10-12, and 14 positive for OXA-23-like.

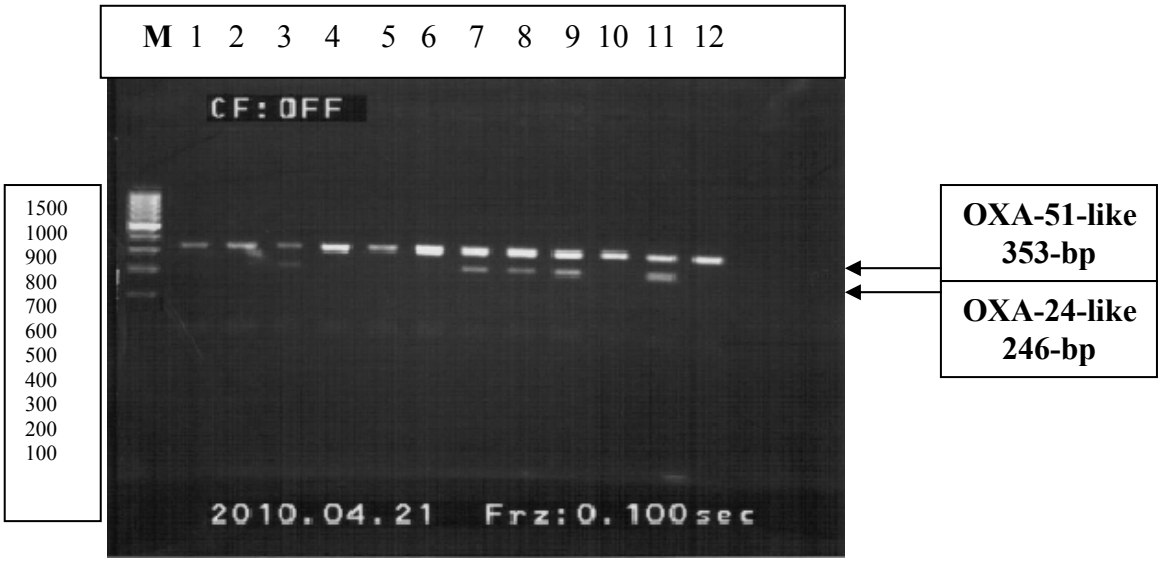


Figure (7): Agarose gel electrophoresis of multiplex PCR for genes encoding OXA-51-like and OXA-24-like carbapenemases. LaneM: 100 bp DNA Ladder marker. Lane 1-12 positive for OXA-51-like. Lane 7-9, and 11 positive for OXA-24-like in *A. baumannii* isolates.

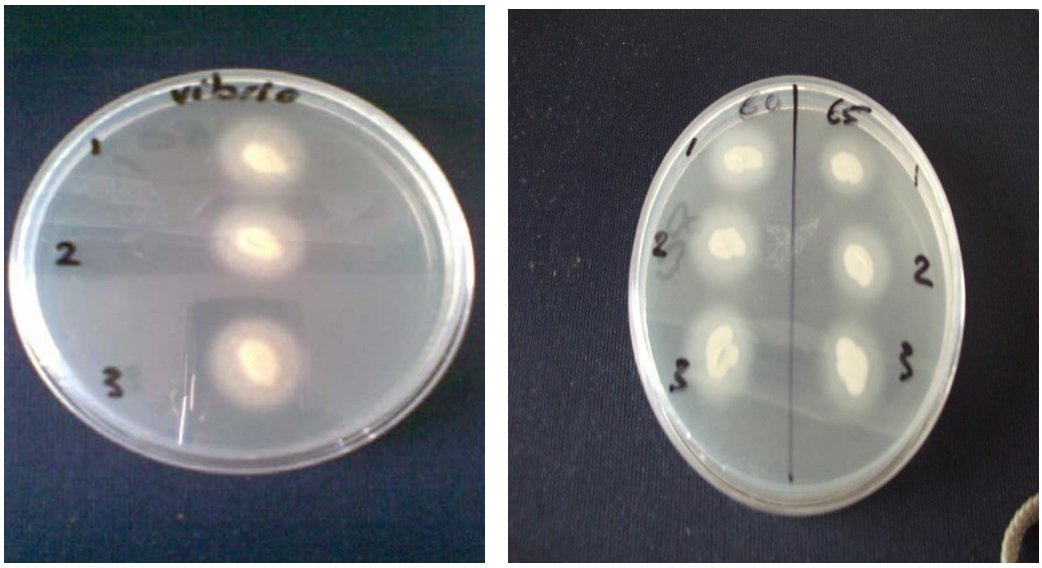
4.8. Virulence factors results for *A. baumannii* isolates

4.8.1. Putative virulence enzymes of 64 *A. baumannii* isolates

The results of putative virulence enzymes tests of *A. baumannii* isolates are shown in table (20). None of the 64 *A. baumannii* isolates produced protease, lecithinase, and hemolysin. All *A. baumannii* isolates produced lipase enzyme. For gelatinase, only 9.4% of *A. baumannii* isolates were producer for this enzyme.

Table (20): Putative virulence enzymes of the 64 *A. baumannii* isolates from ICUs patients

Putative enzyme production	Positive isolates No. (%)
Protease	0 (0)
Lecithinase	0 (0)
Hemolysin factor	0 (0)
Gelatinase	6 (9.4)
Lipase	64 (100)



A **B**

Figure (8): Lipase activity. (A) Non-O1 *Vibrio cholerae* clinical strain. (B) *A. baumannii* isolates.

Figure (9): Protease activity. *Serratia mercenses* strain ATCC 13880**.**

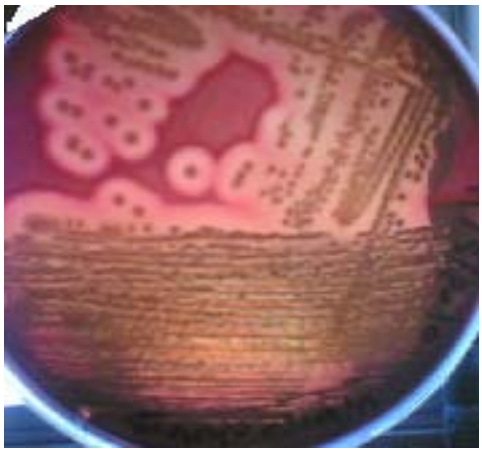


Figure (10): Hemolysin production. Non-O1 *Vibrio cholerae* clinical strain.

4.8.2. Growth of 64 *A. baumannii* isolates under iron-limiting conditions

The results of this test are shown in table (21).All *A. baumannii* strains were able to grow in M9 medium plates containing 50 µM or 200 µM DIP.

Table (21): Growth of 64 *A. baumannii* isolates from ICUs patients on iron-chelated media

No. (%) / Growth	Growth on M9 minimal media + 50 µM DIP	Growth on M9 minimal media + 200 µM DIP
Heavy	64 (100)	58 (90.6)
Moderate	0 (0)	3 (4.7)
Few	0 (0)	3 (4.7)
No growth	0 (0)	0 (0)
Total No. (%)	64 (100)	64 (100)

4.8.3. Hemagglutination activity of 64 *A. baumannii* isolates.

The results of hemagglutination activity with and without 1% D-mannose are shown in table (22). Of 64 isolates of *A. baumannii*, 73% agglutinated human group AB+ erythrocytes, and 52% agglutinated human group O+ erythrocytes in the absence of

mannose. Of sixty four isolates of *A. baumannii*, only 47% of *A. baumannii* isolates agglutinated human group AB+ erythrocytes, and 36% agglutinated human group O+ erythrocytes in the presence of mannose.

Table (22): Hemagglutination activity of 64 *A. baumannii* isolates from ICUs patients against human erythrocytes

No. (%) of strains showing hemagglutinating activities			
Hemagglutination activity		**Mannose-resistant Hemagglutination activity**	
AB+	**O+**	**AB+**	**O+**
47 (73)	33 (52)	30 (47)	23 (36)

4.9. Enterobacterial repetitive intergenic consensus (ERIC)

4.9.1. Enterobacterial repetitive intergenic consensus (ERIC) of 64 isolates of *A. baumannii*

The 64 isolates of *A. baumannii* showed 2 major clusters (genotypes) including genetically related isolates (Table 23), using two primers (ERIC1 and ERIC2), with a DNA fragment range of (200-3000bp) (figure 11). The average of mean similarity is (0.42) indicating that the isolates share approximately (42%) of their ERIC fragments.

4.9.2. Genetic diversity of 64 clinical *A. baumanni* isolates.

A dendrogram (Figure 12) was constructed by Ahmed Haidar, Charité – Universitätsmedizin Berlin, on the basis of similarity index among 64 clinical *A. baumannii* isolates using the two ERIC primers. A dendrogram similarity gave 2 major clusters (genotypes) including genetically related isolates.

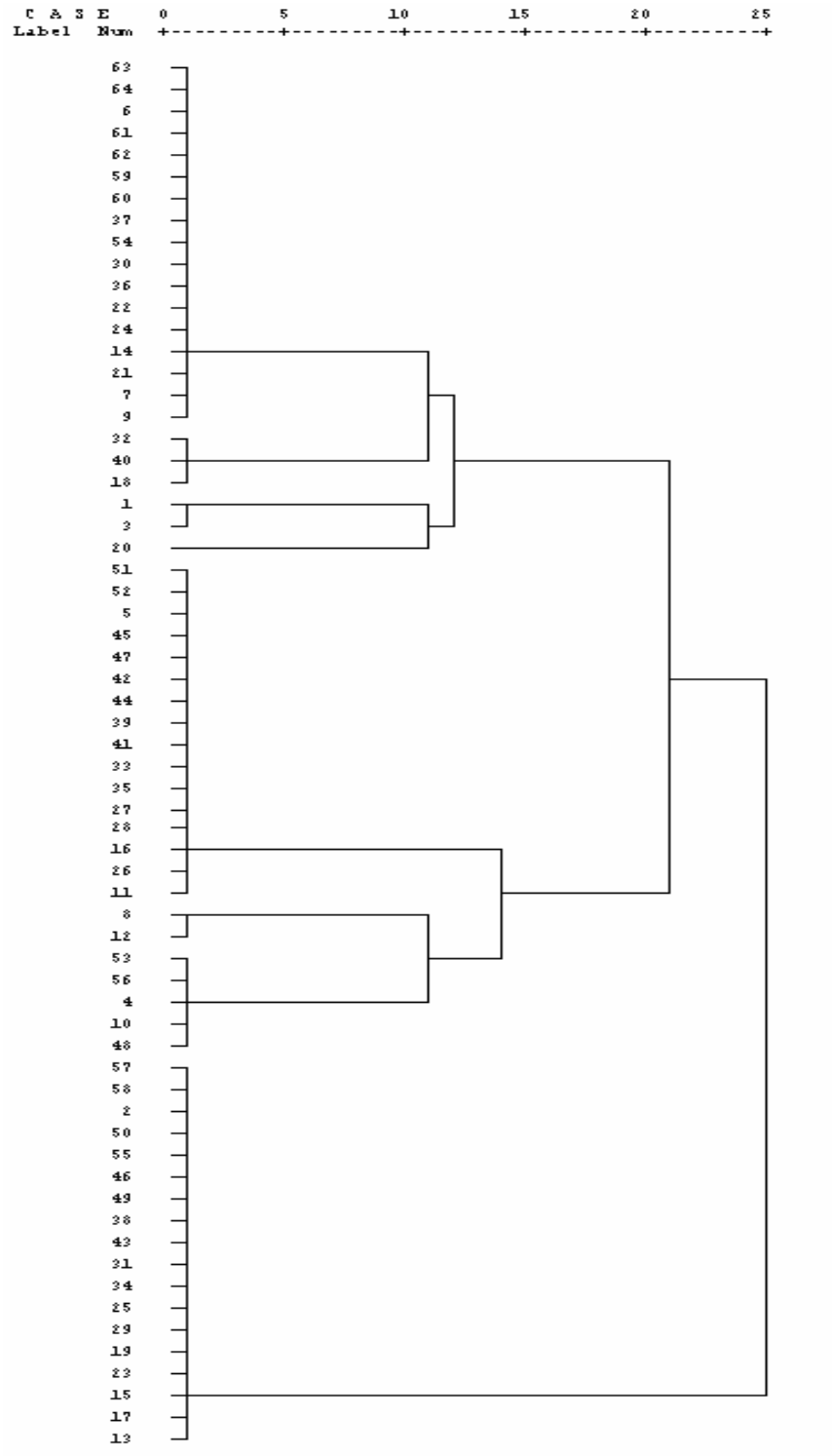
A 50% similarity cut off value for the 1st major group gave one genotype contains 18 identical isolates with a similarity 100%. Moreover, a 50% similarity cut off value for the 2nd group gave 3 clusters (genotypes 1-3) including genetically related isolates. A

total of 23 clinical isolates belonged to the genotype 1 (range of similarity = 0.60-1.00), 16 identical isolates with a similarity 100% belonged to the genotype 2 and 7 clinical isolates belonged to the genotype 3 (range of similarity = 0.60-1.00).

Table (23): Genotyping of 64 *A. baumannii* isolates.

Major cluster	genotype	No. of isolates
1st	1	18
2nd	1	23
	2	16
	3	7

Figure (11): ERIC-PCR patterns generated by *A. baumannii* isolates using 2 primers. Lane M: 100 bp DNA Ladder marker, Lane 1-12, ERIC pattern of *A. baumannii* isolate.



Figure(12): Dendrogram of 64 *A. baumannii* isolates

5. Discussion

5.1. Demographic characterization of the patients colonized with *A. baumannii*

The aim of this prospective study was to investigate the rate of colonization, and to determine phenotypes, genotypes, and potential virulence of *Acinetobacter* spp. in the respiratory tract of adult patients admitted JUH. The results demonstrate that *A. baumannii* colonized 34.1 % of the hospitalized patients in ICUs, but only one positive specimen was obtained from both blood and respiratory tract specimens during the 10-month period of the study (2009/2010). The study was not intended to identify patients with true infections versus those who are colonized.

This study found that risk factors associated with *A. baumannii* colonization/infection were mostly ICU admission, longer ICU stay, older age ≥65 years, prior antibiotic use, treatment with carbapenems, usage of medical equipment including nasogastric tubes, and mechanical ventilators, longer period of mechanical ventilation, presence of tracheostomy, and co-morbidity with diabetes mellitus and neurologic impairment ($P<0.05$). Previous studies from different part of the world have demonstrated that some or most of these variables were risk factors for developing colonization and infection with *A. baumannii.* Also, numerous recent studies have identified longer ICU stay as a high risk factor for colonization or infection with *A. baumannii* (Ye et al., 2010; Lin et al., 2009; Zainy Deris et al., 2009; Baran et al., 2008; Katragkou et al., 2006; Wong et al., 2002). *A . baumannii* colonization/infection often occur in immunocompromised hosts including patient with advanced age (Ye et al., 2010). In particular, findings of this study were similar to those studies which observed that the use of mechanical ventilators was highly associated with *A. baumannii* VAP (Dent et al., 2010; Zainy Deris et al., 2009; Leepethacharat et al., 2007; Katragkou et al., 2006; Smolyakov et al., 2003). However, a study of Carbonne et al. (2005) reported that the use of mechanical

ventilators did not increase the risk of *A. baumannii* VAP. Tracheostomy is also reported as a risk factor for *A. baumannii* nosocomial respiratory tract infection or colonization (Zainy Deris et al., 2009; Young et al., 2007; Mah et al., 2001). Almost all studies have found that invasive devices like nasogastric tube is a risk factor for *A. baumannii* colonization/infection (Zainy Deris et al., 2009; Lin et al., 2009; Leepethacharat et al., 2007; Wong et al., 2002; Mah et al., 2001).

Previous treatment with antimicrobial therapy has also been found to predispose for infection/colonization with *A. baumannii* (Dent et al., 2010; Zainy Deris 2009; Baran et al., 2008; Katsaragakis et al., 2008, Leepethacharat et al., 2007; Smolyakov et al., 2003).

The present study found that treatment with carbapenems out of 4 classes of antibiotics used, was a significant factor ($P< 0.001$) in colonization with *A. baumannii* among ICUs hospitalized patients at JUH (Table 11). Prior carbapenem treatment was also recognized as another risk factor for the emergence MDR *A. baumannii* infection or colonization in previous studies (Ye et al., 2010; Routsi et al., 2010; Lautenbach et al., 2009; Lin et al., 2009; Katragkou et al., 2006; Cisneros et al. 2005; Wong et al., 2002). Data presented in this study support other studies which observed that there is an inducible mechanism for emerge of carbapenem resistant strains under the selective pressure of carbapenem (Ye et al., 2010; Falagas and Kopterides, 2006; Del Mar Tomas et al., 2005; Garnacho-Montero et al., 2005; Lee SO. et al., 2004; Corbella et al., 2000). Moreover, this study has not recognized any statistically significant relationship between rates of *A. baumannii* colonization and certain factors such as gender, use of 4th generation cephalosporin, quinolones, or aminoglycosides, and co-morbidity with pulmonary diseases, cardiac diseases, and malignancy. Also, this study found that underlying diseases including malignancy, respiratory tract diseases, and cardiac

diseases did not increase the risk of colonization/infection similar to the findings of other studies (Ye et al., 2010; Shih et al., 2008).

In a logistic regression analysis as shown in Table (16), *A. baumannii* acquisition was independently associated with the longer stay in the ICU and longer duration of mechanical ventilation. Many studies (Lee SO. et al., 2004; Mah et al., 2001) reported despite many intensive efforts, the nosocomial acquisition of *A. baumannii* remains problematic, especially in ICUs, since *A. baumannii* is capable of rapidly adapting to the hospital environment, and outbreaks may result from intrinsic contamination of the medical equipment or devices, especially respiratory equipment or due to contamination of the environment, and deficient in infection control practices. These factors together explain the difficulty of controlling *A. baumannii* infections which can be endemic or causing outbreaks of hospital infections.

5.2. Multidrug-resistant *A. baumannii* (MDRA) in JUH

The resistance rate of *A. baumannii* isolates was very high (63%-100%) at JUH to many commonly used antimicrobial agents in Jordan, including meropenem, ertapenem, ceftazidime, piperacillin/tazobactam, aztreonam, gentamicin, ciprofloxacin, amikacin, and imipenem (Table 17). There was no resistance, however, to both tigecycline and colistin (0%); which are still not commonly used in Jordan. In addition, this study shows that the majority (98.4%) of *A. baumannii* isolates were resistant to more than five commonly used antimicrobial agents in clinical medicine (Table 18). The most prevalent resistance profile (42.2%) was observed to nine antimicrobial agents; Mem, Gm, Ptz, Atm, Cip, Imi, Caz, Ak, and Etp (Table 18). This result indicated that the majority of *A. baumannii* isolates of this study are multidrug resistant (Table 18). The definition of MDR *A. baumannii* varies in the literature, but several studies consider an isolate to be MDR if it is resistant to three or more classes of

antibiotics (Falagas et al., 2006; Hujer et al., 2006; Adams et al., 2008; Chopra et al., 2008; Huang et al., 2008). A recent study from China found that all *A. baumannii* isolates showed a antimicrobial resistance rates of 100%, but the polymyxin sensitivity was 100% (Sun et al., 2010).

A study from Greece (Pournaras et al., 2006) reported an outbreak caused by multiple clones of imipenem-resistant *A. baumannii* isolates in the ICU of the Red Cross General Hospital. These isolates were multidrug resistant exhibiting resistance to all other β-lactams (ampicillin/sulbactam, aztreonam, ceftazidime, cefepime, piperacillin and piperacillin/tazobactam), aminoglycosides (amikacin, gentamicin, netilmicin and tobramycin) and fluoroquinolones (ciprofloxacin and ofloxacin). The isolates were susceptible only to colistin. A prospective study in a Moroccan university has reported a high-level resistance to β-lactams was noted where resistance rates were 91% to cefotaxime, 50.3% to ceftazidime, and 42.6% to imipenem, whereas aminoglycosides resistance varied from 17.9% for netilmicin to 72.1% for gentamicin. The resistance rate to ciprofloxacine was 65.8%, and to trimethoprime-sulfamethoxazole 75.8%, while in ICUs, the antimicrobial resistance rate of *A. baumannii* was higher to most tested antimicrobials ($p<0.05$), except for tigecycline where these isolates were 100% susceptible (Lahsoune et al., 2007). Another study by Nayman et al. (2010) found that all of the 100 *A.baumannii* isolates (100%) were found susceptible to tigecycline (MIC values ≤ 2 µg/ml; MIC ranges: 0.032-1.5 µg/ml). In contrast, a study by Navon-Venezia et al. (2007) found that 66% of the *A. baumannii* isolates were resistant to tigecycline, and the MIC_{90} of tigecycline was 32 mg/L.

It is concluded from these studies and this current study that patterns of antimicrobial resistance of *A. baumannii* markedly varied among different geographic regions, and especially between nosocomial and other clinical isolates (Gales et al., 2001).

5.3. Prevalence of OXA- and MBL-encoding genes in *A. baumannii* isolates

Carbapenems are among the drugs of choice used to treat nosocomial infections caused by MDR *A. baumannii*, however the emergence of resistance to carbapenems is of serious concern in clinical medicine. These naturally occurring β-lactamases have been identified as a source of carbapenem resistance in *A. baumannii*. These enzymes belong either to the class B (MBLs), or to the class D oxacillinases (Yang et al., 2009). A recent study reported that carbapenem resistance in Europe is mostly due to the presence of OXA type carbapenemases, whereas class B MBLs represented by IMP and VIM MBLs are more prevalent in the Far East. (Kulah et al., 2010).

Regardless of their susceptibility to carbapenems, all of the 64 isolates of *A. baumannii* of this study produced a 353-amplicon, which corresponded to the $bla_{\text{OXA-51-like}}$ gene as detected by PCR analysis. This gene was not detected among any other *Acinetobacter* spp. These results confirm other studies which had suggested that $bla_{\text{OXA-51-like}}$ gene is species-specific to *A. baumannii* (Yang et al., 2010; Feizabadi et al., 2008; Turton et al., 2006b; Brown et al., 2005). The relationship between $bla_{\text{OXA-51-like}}$ gene and the resistance of *A. baumannii* isolates to carbapenem still needs to be investigated. A study by Turton et al. (2006c), reported that IS*Aba1* (Insertion Sequence *A. baumannii*), which is adjacent to $bla_{\text{OXA-51-like}}$, plays a major role in the development of resistance to carbapenems.

The findings of this study indicated that OXA-23-like carbapenemases were predominant in 73% of the clinical *A. baumannii* isolates, whereas OXA-24-like carbapenemase was less common. The presence of MBLs in the form of the bla_{VIM} gene was limited to two isolates and none of isolates harbored $bla_{\text{OXA-58-like}}$ or bla_{IMP} gene. On the other hand, similar results was shown by Mendes et al. (2008) where the distribution of OXA-type and MBL-encoding genes among *A. baumannii* isolates in

Asia-Pacific nations was comprised mainly of $bla_{\text{OXA-23-like}}$, while $bla_{\text{OXA-24-like}}$ and $bla_{\text{OXA-58-like}}$ were less common, and only one *A. baumannii* isolate from Korea harboured $bla_{\text{VIM-2}}$ gene. Also, Zhang et al. (2010), Yang et al. (2009) and Lee et al. (2007) found that all of *A. baumannii* isolates possessed the encoding gene for an intrinsic OXA-51-like carbapenemase and many isolates have an acquired OXA-23-like carbapenemase and none of the isolates had MBL encoding genes. Fu et al. (2010) and Mungnier et al., (2010) found that PCR amplification was positive for $bla_{\text{OXA-23}}$ in most carbapenem resistant isolates.

In Greece, many of *A. baumannii* ICU isolates contained both OXA-58 and OXA-51-type β-lactamase genes (Pournaras et al., 2006), and Coelho et al. (2006) found OXA-58 and OXA-51-type β-lactamase genes co-existing in *A. baumannii* collected from Argentina, Kuwait and Southern England over a 10 year period. A study by Sung et al. (2008) found that 48.4% harbored $bla_{\text{IMP-1}}$, 3.2% harbored $bla_{\text{VIM-2}}$, and 22.6% harbored $bla_{\text{OXA-23-like}}$ gene. In this study, the screening tests (modified Hodge test and imipenem-EDTA double disc-synergy test) showed good correlation with PCR results. These microbiological tests for carbapenemase activity allow for such easy identification and constitute simple methods for the screening of clinical isolates for producing class D carbapenemases and/ class B MBLs. This approach will allow physicians to prescribe the most appropriate antibiotic for treatment of infection, thus reducing selective pressure and antibiotic resistance (Jeong et al., 2006).

5.4. Virulence factors

This study has shown that *A. baumannii* isolates produce certain putative virulence enzymes of gelatinase and lipase in the rate 9.4% and 100%, respectively (Table 20). Gelatinase is able to hydrolyze gelatine and some other bio-active peptides. It may be an advantage for *A. baumannii* to hydrolyze collagen in the subcutaneous tissue during

wound infection. Cevahir et al. (2008) detected gelatinase activity in 12 of 86 isolates (14%). In contrast, Sechi et al. (2004) did not detect gelatinase activity in any of the clinical *A. baumannii* strains.

The most prominent role of extracellular lipases for a micro-organism is the digestion of lipids for nutrient acquisition. Lipolysis might provide a carbon source that the micro-organism could use for growth, where released catalytical end products due to lipolytic activity which could support cell-to-cell and/or cell-to-host tissue adhesion and colonization. Lipases and their catalytical end products may have an effect on different immune cells and might initiate inflammatory processes (Stehr et al., 2003). It becomes more obvious that secreted lipases play a part in the group of bacterial virulence factors. Many studies detected lipase activity in *Acinetobacter* spp. (Li et al., 2004&2001; Chen et al., 1998; Dharmsthiti et al., 1998; Kok et al., 1996&1995). None of the examined *A. baumannii* strains was able to produce hemolysin under the experimental conditions reported, and none showed protease or lecithinase activity (Table 20).

The same observation was reported by Sechi et al. (2004) who did not detect protease and hemolysin activity in *A. baumannii* strains. These enzymes are unlikely to be virulence factors in this organism. In the present study, about half of *A. baumannii* isolates agglutinated human group AB erythrocytes and one third agglutinated human group O erythrocytes in the presence of mannose This interaction with erythrocytes reflects the presence of surface structures (fimbriae) with a role in adherence to epithelial cells that may be essential for the infection process. Hemagglutinating properties are indicative of an ability to adhere to intestinal mucosal surfaces (Mikcha et al., 2004). In general, results of this study are similar to many studies. Gospodarek (1993) tested 309 strains of *A. baumannii* for hemagglutinating activity and obtained a positive result for 75% to 85% of the strains. Another study by Sepulveda et al. (1998)

showed that *A. baumannii* strains also have D-mannose- and D-galactose-resistant hemagglutinating ability. A similar study by Cevahir et al. (2008) 25.6 % and 70.9% of the isolates agglutinated human group O and AB erythrocytes in the presence of mannose respectively. In contrast, a study by Braun and Vidotto (2004) demonstrated that 9 of 13 isolates agglutinated human group AB erythrocytes in the presence of mannose, but none agglutinated human group O erythrocytes.

The present study shows that all *A. baumannii* strains were able to grow in M9 medium plates containing 50 and 200 μM DIP (Table 21). These results conclude that this bacterium produces siderophores by isolates under iron-limiting conditions. The ability of bacteria to assimilate iron is known to be related to invasiveness. This suggests the presence of an iron uptake system in *A. baumannii*. Many studies have observed that *A. baumannii* produces some type of siderophore (Zimbler et al., 2009; Braun and Vidotto, 2004; Dorsey et al., 2004; Daniel et al., 1999; Actis et al., 1993; Echenique et al., 1992; Smith et al., 1990).

Based on the overall results of this study, it is evident that infection by *A. baumannii* is the result of multifactorial events and different compounds that are synthesized and secreted by the bacterial cells may equally contribute to the colonization and persistence of this opportunistic pathogenic micro-organism in humans and the environment. Several other parameters, including host factors, the bacterial burden, and the virulence of the individual strain, may play important roles in causing infection in colonized patients.

5.5. The genetic diversity of clinical *A. baumannii* isolates

Molecular typing of *A. baumannii* isolates was performed by ERIC-PCR generated DNA fragments. This method proved to be a useful method to distinguish between certain Gram-negative bacterial strains (Sung et al., 2008; Zong et al., 2008; Ozgumus

et al., 2007; Erac and Gulay, 2007; Jeong et al., 2006; Silbert et al., 2004; Oh et al., 2003). A dendrogram was constructed and demonstrated the overall genetic relatedness of *A. baumannii* isolates, and gave 2 major clusters (clones) among 64 isolates; of these one cluster accounted for 71.9% of all *A. baumannii* isolates (Figure 12). This cluster was divided into 3 major genotypes assigned as genotype 1, genotype 2, and genotype 3. These findings suggest that *A. baumannii* strains originated from 2 clones and have been successfully circulated between patients at the JUH, and indicating cross transmission within the ICU setting. In a study performed by Jeong et al. (2006), one outbreak involved seven cases of infection by *A. baumannii*, which were to have been caused by a single ERIC-PCR clone, and their results were verified by PFGE analysis. It is concluded that ERIC-PCR is a simple, rapid molecular typing method that can be used to study nosocomial outbreaks of *A. baumannii* with acceptable reproducibility and discrimination index.

6. Conclusions.

Based on the findings of this study, the following conclusions can be drawn:

- Several risk factors are associated with colonization of patients by *A. baumannii*; including ICU admission, treatment with carbapenems, usage of mechanical ventilation equipments, and old age ≥65 years.

- A high prevalence of multidrug resistant *A. baumannii* colonize the respiratory tract of hospitalized patients in the ICU at JUH.

- A high prevalence of class D oxacillinases with activity against carbapenems exists.

- All *A. baumannii* isolates seems to originate from 2 clones which have been successfully circulating between patients at the JUH.

- Infections by *A. baumannii* are multifactorial and different putative enzymes and compounds synthesized by the bacterial cells may contribute to the colonization and infection of this opportunistic pathogen.

7. Recommendations

Based on conclusions drawn, the following recommendations can be made:

- Monitor periodically the patterns of biotypes, genotypes, and antimicrobial susceptibility patterns of all *A. baumannii* isolates from the hospitalized patients with invasive infections.

- Investigate for the presence of extracellular toxins and enzymes secreted by *A. baumannii.*

- Investigate the attachment of *A. baumannii* strains using cell culture lines or animals.

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Appendices

Appendix 1:

Culture media and chemicals used in this study

Culture media	**Company, country**
Brain Heart infusion agar	Oxoid, Unipath Ltd., Hampshire, England
Brain Heart infusion broth	Oxoid, Unipath Ltd., Hampshire, England
CLED agar	Oxoid, Unipath Ltd., Hampshire, England
Blood agar base	Oxoid, Unipath Ltd., Hampshire, England
Mueller Hinton agar	Oxoid, Unipath Ltd., Hampshire, England
Mueller Hinton broth	Oxoid, Unipath Ltd., Hampshire, England
Nutrient agar	Oxoid, Unipath Ltd., Hampshire, England
Nutrient broth	Oxoid, Unipath Ltd., Hampshire, England
Bacteriological Agar No. 3	Oxoid, Unipath Ltd., Hampshire, England
Kligler Iron agar	Oxoid, Unipath Ltd., Hampshire, England
SIM medium	Oxoid, Unipath Ltd., Hampshire, England
Urea agar	Oxoid, Unipath Ltd., Hampshire, England
Simmons Citrate agar	Oxoid, Unipath Ltd., Hampshire, England

Chemicals and Solutions	**Company, country**
D-glucose	Sigma,London,England
D-mannose	Sigma,London,England
Sterile egg yolk	Oxoid, England
Human Blood	Blood Bank in JUH
Sheep Blood	JUH
Sodium acetate	Gibco – BRL, Scotl
Gelatin	Difco, USA
Skim milk	Oxoid, England
Tween 80	Sigma, London, England
Glycerol	Merk, Germany
Sodium chloride	Promega, USA
2,2'-Dipyridyl (DIP)	Sigma, London, England
NH_4Cl	Sigma, London, England
NH_4NO_3	Sigma, London, England
Na_2SO_4	Sigma, London, England
K_2HPO_4	Sigma, London, England
$MgSO_4.7H_2O$	Sigma, London, England
$Na_2HPO_4 \cdot 7H_2O$	Sigma, London, England
KCl	Sigma, London, England
KH_2PO_4	Sigma, London, England
$CaCl_2$	Sigma, London, England

Appendix 2:
The application form used for patients' data collection;

Distribution of *A. baumannii* colonizing respiratory tracts of Jordanian patients/2009

1- Name:----------

2- Sex: 2.1.M 2.2.F 2.3-Age:---------

3- Hospital no.:---------

4- Serial laboratory no:---------

5- Date:----------

6- Outpatient:----------

7- Inpatients:----------

8- Date and duration of hospitalization:----------

9- Type of antibiotic administration:

10- Diagnosis:

11- Patient agreed to be included in this study:-------

12- Culture results:

Clinical comments by examined physicin:

عوامل الفوعة المفترضة و التنوع الجيني للأسينيتوباكتر بوماني التي تستوطن الجهاز التنفسي للمرضى

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ملخص

الأسابنيتوباكتر بوماني أهم مسببات الأمراض الخاصة بالمستشفيات التي تتطور بسرعة نحو متعددةِ مقاومةِ الدواء والمشتركة في عدة إصابات خاصة بالمستشفياتِ التي تكون حادّة في أغلب الأحيان. على أية حال خصائصها المسبّبة المرض ليستْ واضحة المعالمَ.
تَحرّتْ هذه الدراسةِ إنتشارَ الأسابنيتوباكتر بوماني التي تَستعمرُ الجهاز التنفسي ل 185 مريض أدخلو إلى مستشفى الجامعةِ الأردنية على مدى 10 شهور (مايو 2009 إلى فبراير2010). تم التحقق من هوية كل العزلات عن طريق الاختبارات الكيميائية الحيوية. عوامل الخطورة لإستيطان الجهاز التنفسي بالأسابنيتوباكتر بوماني تم تحديدها. التحليل متعدد النوع أظهر أن عوامل الخطورة المستقلة الوحيدة لظهور الأسابنيتوباكتر بوماني هي أطول مدة تهوية ميكانيكية، وأطول مدة بقاء في وحدة العناية المركزة.
جميع عزلات الأسينيتوباكتر فُحِصَتْ حساسيتها للمضادات الميكروبية باستخدام طريقة الانتشار القرصي. النتائج بينت أن 98.4 % من أصناف الأسينيتوباكتر كانت مقاومة لأكثر من خمسة مضادات ميكروبية. أعلى معدل مقاومة من بين عزلات الأسينيتوباكتر بوماني كانت على النحو الاتي :ميروبينام, إيرتابينام، سيفتازيديم 100%، تليها بايبرسيلين\تازوباكتم، وأزتريونام 98%، ,جينتاميسن 94%، سيبروفلوكساسين91%، أميكاسين 73%، اميبينام 64%، بالترتيب. بينما لا مقاومة اكتشفت لتقيسايكلين و البوليميكسين إي. كلا أميكاسين و اميبينام يملكان أعلى قيمة لأقل نسبة تركيز (13.6، 12، بالترتيب) باستخدام شريط أختبار التحسس إي. ولا عزلة تنتج الهيمولايسين، والبروتييز، واليسيثاينيز. جميع العزلات تنتج إنزيم اللاييز. انزيم الجيلاتينيز تم اكتشافه في 6 عزلات (9.4%). جميع العزلات قادرة على العيش في ظروف محدودة لوجود الحديد.
30 و 23 عزلة تلصق كريات الدم الحمراء من النوع البشري AB و O بوجود سكر المانّوز، بالترتيب. اثنتان وستون عزلة (96.9%) أعطت نتائج ايجابية لإختبار modified Hodge و عزلتان فقط أعطت نتائج إيجابية لإختبار imipenem-EDTA-disk synergy. جميع العزلات لديها جين $bla_{\text{OXA-51-like}}$ ، 73% و 19% من العزلات تحوي جين $bla_{\text{OXA-23-like}}$ ، $bla_{\text{OXA-24-like}}$ بالترتيب. فقط عزلتان تحوي جين bla_{VIM}. تم أيضا دراسة التشابه الجيني بين 64 عزلة أسابنيتوباكتر بوماني وتم فحص البصمات عن طريق الحمض النووي من خلال دراسة الشرائط الوراثية و التباين الوراثي، وكشفت النتائج عن وجود سلالتان وراثيتان (عنقودان) رئيسيتان تتضمنان عزلات ذات علاقة جينية (وراثية).

والخلاصة، ارتفاع نسبة انتشار متعددة مقاومة الدواء الأساينيتوباكتر بوماني التي تستوطن الجهاز التنفسي للمرضى المقيمين في المستشفى في وحدة العناية المركزة هو أهم عامل لظهور العدوى فيما بعد. انتشار بضعة انواع جينية من الأساينيتوباكتر بوماني في مستشفى الجامعة الأردنية يدل على أهمية تطبيق مقاييس السيطرة الشاملة على العدوى.