ORIGINAL ARTICLES
Biotyping of *Acinetobacter baumannii* Iraqi Isolates

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Abstract
Background: *Acinetobacter baumannii* is an opportunistic bacterium associated with many nosocomial infections as *Acinetobacter calcoaceticus, Acinetobacter nosocomialis,* and *Acinetobacter pittii*.
Aim: To determine the Biotyping of *Acinebactor baumannii* Iraqi Isolates.
Materials and Methods: One hundred *Acinetobacter species* samples were collected from wound and burns in hospitalized patients in Al-yarmook Teaching Hospital in Baghdad province. Twenty isolates of *Acinetobacter baumannii* were identified by conventional and molecular methods, the highest rate of the bacterial isolates were 12%

from wounds and 8% from burns. All isolates were detected phenotypical, microscopical and biochemical tests and confirmed by ViTEK2 device.

Results: Most of bacterial isolates showed resistant to the antibiotic Cephalexin (CL) (100%), Piperacillin (PRL) (65%), Ceftazidime (CAZ) (60%), Ticarcillin-Clavanic acid (TIM) (75%), Ampicillin-Sulbactum (SAM) (65%), Ceftriaxone (CRO) (60%), Imipenem (IMP) (50%). In contrast, all isolates were found sensitive to Cefotaxime (CTX) (100%). About 90% of *Acinetobacter baumannii* isolates were positive to biofilm formation, 70% were own efflux Pump, 100% have ability to adhere to the epithelial cells, 60% could extended spectrum β- Lactamase. Molecular subtyping of ITS region or 16S rRNA gene got through RNA 16 amplified the target gene and sequences of PCR products; sequenced data was blasted in NCBI and analysis to construction of rooted phylogenetic tree.

Conclusion: ITS region or 16S rRNA gene was more discriminative than the other gene especially in closely related bacterial isolates, identification and categorization using PCR and sequence technique for 16S rRNA gene is dependable and provides epidemiological importance of *Acenetobacter baumannii*.

Keywords: ***Acinetobacter baumannii,*** **ITS region, 16S rRNA**

Introduction

The ***Acinetobacter*** species are widespread in environment, they have been found in soil, air, water, and specimens of animal and human. Before 1970s the genus of Acinetobacter was considered safe for humanity. Later, this attitude changed when the scientists were noted that the bacteria of Acinetobacter have become epidemic, and multi-drug resistant (MRD). In addition, the ***Acinetobacter*** species were increasingly isolated from hospitalized patients [1]. Opportunistic infections by *Acinetobacter baumannii* in particular have become collaborate with hospital-acquired infections. It has a clinical notoriety in incidence associated with infected military conflict troops reverting from conflict regions such as desert conflicts, and Operation Iraqi freedom (OIF), the moniker “Iraqi bacter” that was earned it in this period [2].

Acinetobacter baumannii have major factors that able to be pathogenic bacteria include adhesion with epithelial cells, biofilms formation, resistance of antimicrobial agents and acquire genetic

characteristics another genera that have led to difficult control on its [3]. *A. baumannii* is a causative agent to emergence of nosocomial infections particularly in Intensive Care Unit (ICU). For example, infections of primary and secondary meningitis, blood stream infections (bacteremia), infections of urinary tract is associated with antibiotic therapy or catheterization, infective keratitis, peritonitis, endocarditis, infections of traumatic injuries, postsurgical wounds, and infections of burns [4,5].

The main reason of *A. baumannii* is opportunistic bacterium associated with nosocomial phenomena that have ability to multi-drug resistant strains of this species, adhesion and invasion of host epithelial cell. However, this genus is ability to persist in the environmental hospital by spread in the middle of ill patients and it is difficult to eradication [6]. *A. baumannii* bacterium is characterized by their ability to change pathogenic features constantly [7,8]. Generally, the approaches of traditional investigations those known as "culturing, phenotyping and biochemical tests" to identify pathogenic bacteria are unwieldy and time-intensive because of difficult to differentiate the species. Sequencing rely on amplification of ITS region or 16S rRNA gene that the best methods to differentiation and identification among species especially in a taxonomic studies so the amplification of 16S rRNA gene (target sequence) matching with loci 10-1,507 of the numbering system for *Escherichia coli* is compared to database of species in closely related to it. In addition, this region (16S rRNA gene or ITS region) is featured to universality, conserved nature [9, 10].

Materials and methods

Bacterial isolation and cultivation

One hundred wounds and burns samples were collected from hospitalized patients at Al-yarmook Teaching Hospital in Baghdad province during period from15January-15 July 2016. All samples were sub cultured on Congo- red agar, Simmon citrate, Urea agar medium, and blood agar and incubated aerobically at 37°C for 24 h. The isolates were identified by traditional biochemical tests including , Gram stain, Oxidase, Catalase test, Indol test, Triple – Sugar iron, Urease production test, Methyl – Red test, Voges-proskauer test, Motility test, and by VITEK2 system (bio-Mérieux, France), and the growth test at 44° C to confirm this species *A. baumannii* [11-13].

Antimicrobial sensitivity assay

Antimicrobial susceptibility of isolates was estimated using Kirby-Bauer's single disk diffusion technique in Mueller Hinton Agar [17, 19]. The test was performed using eight antibiotics includes Cephalexin (CL 30 mg), Piperacillin (PRL 100 mg), Ceftazidime (CAZ 30 mg), Ticarcillin-Clavanic acid (TIM 5 mg), Ampicillin-Sulbactum (SAM 20 mg), Ceftriaxone (CRO 30 mg), Imipenem (IMP10 Mg), and Cefotaxime (CTX 30%). Suspension of bacterial isolates as a turbidity compared to a 0.5 McFarland standard was inoculated on the media plates, there after Minimum inhibitory concentrations (MIC) were estimated by the E-test strips, and to investigate of metallo-beta lactamase MBL enzymes using the Imipenem (IPM) –EDTA [14].

Virulence factors of *A. baumannii*

Biofilm formation was detected using tissue culture plate method (TCP) as described previously [13]. Bacterial isolates efflux activity was evaluated using Efflux Pump substrates such as Ethidium bromide (EB). The test was performed using EB agar cartwheel method as described before [15], adhesion with epithelial cells [16]. The Extended Spectrum β- Lactamase (ESβLs) activity of the isolates was determined using double-disk synergy test [17].

DNA extraction

Extraction of genomic DNA from twenty bacterial isolates using genomic DNA kit Geneaid (Bioneer Company, Korea) and the test was performed according to manufacturer's instructions. Concentrations and purity of genomic extract determined using Nanodrope Instrument.

16S rRNA amplification and sequencing

Amplification of gene 16S rRNA was performed using primer set: Forward primer F27: AGA GTTTGATCMTGGCTCAG, and reverse primer R 1492: CGGTTACCTTGTTACGACTT, in conventional PCR (Apindroofe, Germany) using the run program (1cycle 95C for 10min, 40 Cycles: 95C 20s, 59C 30s, 72C 40s,I cycle 72 for 10 min). Amplified fragments PCR products were cleanup by (microClean,), and submitted to the sequence using genetic analyzer 3100 (Applied Biosystems, USA) in forensic DNA research and training center. Data were analyzed using Sequencing Analysis 5.2. Evolutionary analyses were conducted in MEGA X [18]. Its inferred using the UPGMA method [19], and the evolutionary distances were

computed using the Maximum Composite Likelihood method (MCL) [20].

Results and discussion

The recent study included isolation, and identification of 20 isolates of *Acinetobacter baumannii* out of 100 samples collected from wound and burns from hospitalized patients. The phenotypic characterization, microscopical, and biochemical tests, confirmed by ViTEK2 device have shown Table1. *A. baumannii* the ability to grow at 44^0C was used in addition to molecular detection and sequencing of 16sRNA was used to confirm that isolates belong to *A. baumannii*. Three isolates were submitted to NCBI GenBank, and published under GenBank accession numbers KY000691.1, KY000691.2, KY000691.3. The phylogenetic analysis involved 20 nucleotide sequences with a total of 682 positions in the final dataset. The optimal tree with the sum of branch length = branch length =22.17501921 (Figure1). ITS region or 16S rRNA gene was more discriminative than other gene especially in closely related bacterial isolates, identification and categorization using PCR and sequence technique for 16S rRNA gene is dependable and provides epidemiological importance of *A. baumannii* [21]. The tree was drawn to scale includes two clusters, as the Iraqi isolates fall within the second cluster including 4 sub cluster ,the second cluster including other Iraqi isolates with the isolates from USA, China, India, Australia and Egypt.

The investigation of virulence factors for *A. baumannii* isolates results showed that 18(90%) of the isolates have the ability to produce biofilm, Table 2. The possession of bacteria biofilm increases the difficulty of penetrating the antibiotic for this membrane, and developing resistance to many antibiotics, resistance palatability, opsonisation, phagocytosis, and different environmental factors [22, 23]. Previous study [24] reported that Biofilm formation and hemolytic activity, may occur significantly, and more frequently in virulent isolates as compared with none virulent.

The present study was higher than that reported by others [25], with a percentage of (80%) of the biofilm production, and did not agree with the findings of others [26] that indicated the percentage of isolates producing biofilm was (17.6%). Perhaps the reason for the biofilm formation varies between studies may be due to several environmental factors that affect the formation of the bacterial biofilm

and give variable results including temperature, humidity, oxygen and others factors [27]. Fourteen (70%) of the isolates had possessed highly efficient efflux pumps which was consistent finding of others [28]. All isolates [20 (100%)] have the ability to adhere to surfaces of epithelial cells of humans. Bacterial cell surface play a crucial role for her pathogenicity in fumble to the environment and host interaction [29, 30]. In addition the process of adhesion is associated with the presence of pilli in the bacterial cells may lead to pelvic infections [31]. This explains the ability of *A. baumannii* to adhere to the surfaces of epithelial cells as an initial and fundamental stage of colonization and infection. Although the overall pathogenicity of these bacteria is low, colonization may cause severe consequences for people with pneumonia when the balance between the host and the bacterium is disturbed, leading to outbreaks of nosocomial infections in the hospital [32]. The results of extended spectrum β- lactamase indicated that 12(60%) of isolates producing extended spectrum β-lactamase enzymes and metallo β- lactamase. The investigation of the sensitivity of the isolates for ten antibiotics is shown in Table 3. All the isolates 20 (100%) were resistant to cephalexin (100%), followed by Tetracycline (75%), the other antibiotics resistance ratios ranged between 50% -65%. In contrast, all the isolates were found sensitive (100%) to Cefotaxime. Fully resistance to Cephalexin was consistent with the findings of other studies [11,25]. The *A. baumannii* resistance to tetracycline was found variable according to different studies finding. Our finding is comparable with those of Iran [19] as they reported that 89% of their bacterial isolates were resistant to tetracycline. However, other study [22] reported resistance rate of 49% to tetracycline. Chiang et al [22] found that MDR of A. baumannii strains with biofilm-formation ability may be more sever in nosocomial infection because of significantly higher desiccation and ethanol resistances than their planktonic type. The results showed that the resistance of A. baumannii isolates to Imipenem was 50%, and this result was consistent with others [13]. Additionally, the present study shows resistance rate of 50% to Meropenem which was agreed to that reported before [33] as their isolates were resistant to Imipenem (49%). However, other studies indicated higher sensitivity rate of A. baumannii to Meropenem (100%) [34, 35] and Imipenem [36].

The ability of *A. baumannii* to induce resistance to antibiotic has evolved over time. The A. baumannii became resistant to

antibiotics through multiple mechanisms of which the production of beta lactamase enzymes such as Carbapenemases, reduce the permeability of the cell membrane of the antibiotics change the proteins of this membrane, reduce the affinity to the enzyme Penicillin-Binding Proteins, and the possession of flow pumps that act to extrude Extracellularly.

Most of *A. baumannii* isolates (75%) were multiple antibiotics resistant for eight antibiotics tested in this study. To prevent further spread of MDR *A. baumannii* strains require antimicrobial stewardship programs that enforce infection control measures in order to achieve a successful therapy against these resistance strains in Iraq.
In conclusion, ITS region or 16S rRNA gene was more discriminative than the other gene especially in closely related bacterial isolates, identification and categorization using PCR and sequence technique for 16S rRNA gene is dependable and provides epidemiological importance of *Acenetobacter baumannii*.

Table.1. Microscopical and biochemical characterization of *A .baumannii* Isolates

No	Tests	Results
1	Gram Stain	-
2	Hemolysis Production	-
3	Growth in 44C°	+
4	Lactose fermentation	-
5	Oxidase	-
6	Catalase	+
7	Urease	+
8	Indol	-
9	Methyl red	-
10	Voges_ proskaure	-
11	Simmon citrate	+
12	TSI	Alkaline/ not change
13	Motility	-
14	H2S Production	-
15	Ability to grow in 44^{0}C	+

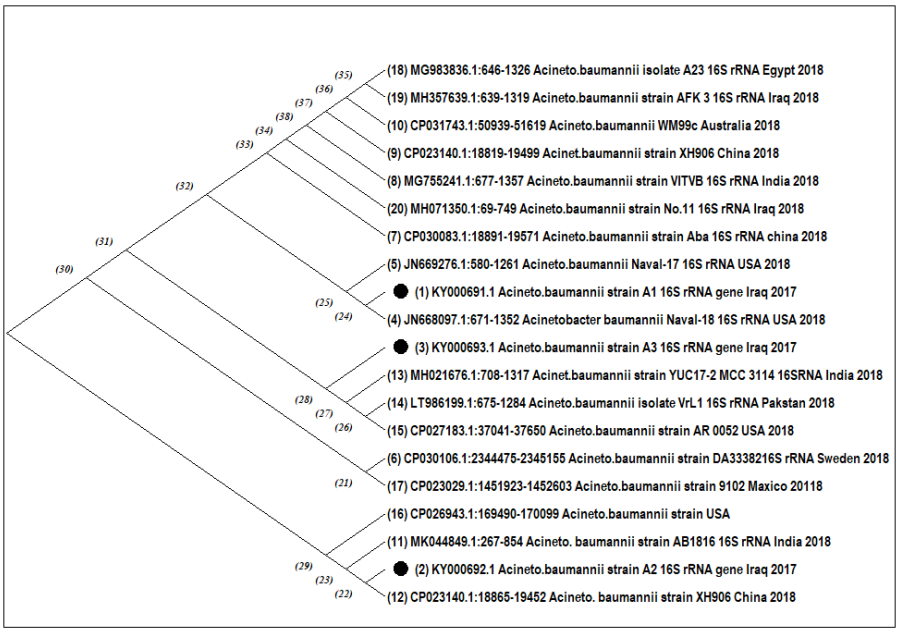

Fig.1. Evolutionary relationships of *A. baumannii* 16sRNA partial gene compared with the related NCBI strains.

Table. 2. The virulence factors of *A. baumannii*

	Biofilm formation	Efflex Pump	Adhesion with Epithelial Cells	Extended Spectrum β-Lactamase
Positive	18(90%)	14(70%)	20(100%)	12(60%)
Negative	2(10%)	6(30%)	0(0%)	8(40%)

Table. 3. The antibiotic susceptibility test results

Antibiotics	Percentage of susceptibility		
	Sensitive	Intermediate	Resistant
CL	0(0%)	0(0%)	20(100%)
PRL	2(10%)	5(25%)	13(65%)
CAZ	2(10%)	6(30%)	12(60%)
TIM	3(15%)	2(10%)	15(75%)
SAM	5(25%)	2(10%)	13(65%)
CRO	6(30%)	2(10%)	12(60%)
IMP	7(35%)	3(15%)	10(50%)
CTX	20(100%)	0(0%)	0(0%)

Antibiotics: CL= Cephalexin, PRL= Piperacillin, TIM = Ticarcillin - Clavanic acid, SAM = Ampicillin-Sulbactum, CTX = Cefotaxime , CAZ = Ceftazidime, CRO = Ceftriaxone, IMP = Imipenem.

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