Molecular Evaluation of *Acinetobacter Baumannii* in A Sample of Iraqi Patients: Clinical and Environmental Study

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Abstract

The current study included molecular evaluation of infection with *Acinetobacter baumannii*. During the period from (November 2020) to (June 2021), 200 different samples were collected from some hospitals in the city of Baghdad. (100) samples were from the hospital environment: 36% of them are isolated from patient beds, 14% from floors, 7% from tables, 5% from incubators, 18% from hospital equipment and 20% from standing water, In addition to (100) clinical samples from infected patients, 32% were isolated from sputum, 24% from urine, 16% from wounds, 10% from blood, 3% from burns and 6% fluids such as (spinal cord, pleural and peritoneum). Infections with *Acinetobacter baumannii*. Microscopic examination and culture diagnosis of bacteria were carried out, and the diagnosis was confirmed by complete biochemical tests, using the diagnostic kit API 20E, as well as using the VITEK2 Compact System, in addition to studying the genetic sequence of bacteria 16S rRNA to find out the diversity and genetic difference between bacterial isolates collected from environmental and clinical sources and knowing the ability of bacterial isolates to resist different antibiotics used in the current study.

Keywords: Acinetobacter baumannii , sequence of A. baumannii 16S rRNA, Molecular evaluation of Acinetobacter baumannii.

1. Introduction

Acinetobacter baumannii, is Gram negative bacteria that are (Non-lactose fermenting, characterized by a spherical rod shape (Coccobacilli, Aerobic bacteria), belonging to the family of Moraxellacea [1]. A. baumannii is a non-motile bacterium that does not produce the enzyme cytochrome oxidase, urease citrate, and indole, In addition to produces the enzyme catalase, Many environmental bacterial isolates grow at a temperature ranging from 20C° to 30C°, while A. baumannii bacteria grow at a temperature of 44 C° [2, 3]. They are short, typically (1.0–1.5) µm by (1.5–2.5) µm in size as measured during the rapid phase of their growth but often develop into more coccoid in the stationary phase, usually present in pairs or long chains of different in length [4]. Bacteria of the genus Acinetobacter can survive a low pH=3.37 but the best pH for A. baumannii is 6.5. [5].

A. baumannii is a pathogenic, opportunistic organism that infects humans in society and hospitals, [6]. Patients with immune system defects, in particular, are at risk, especially those with burn infections and patients hospitalized in intensive care (ICU). It plays an important role in many illnesses, including septicemia, pneumonia, meningitis, soft tissues, skin infection, endocarditis. and urinary tract infection (UTI) [7].

A. baumannii can survive for a long time (more than a month) in a dry environment, and this allows it to survive for a long time in hospital environments and cause infections [8]. The natural habitat of this bacteria is water, soil, animals, human tissues, pets, and arthropods [9, 10]. Acinetobacter baumannii is widely distributed in tropical environments, during wars and natural disasters, and in hospitals, [11]. Bacteria can colonize different sites of the body, including the skin, wounds, and the gastro-intestinal ducts [11]. (Mouth biofilm), which leads to the development of pneumonia if the infection develops to the lower respiratory tract [12]. A. baumannii possesses several virulence factors that increase its pathogenicity, including biofilm formation that increases the ability of bacteria to adhere to both living and non-living surfaces (biotic and abiotic), [13]. producing capsular polysaccharides that increases the resistance of bacteria to antibiotics [14], as well as "the production of the enzyme phospholipase," which degrades the host cell wall and increases the virulence of bacteria [15] as well as outer membrane proteins that contribute to the host cell apoptosis process [16].

A. baumannii possesses several mechanisms of antibiotic resistance including the efflux pump mechanism, modifying enzymes mechanism, alteration of outer membrane permeability defect mechanism, and alteration of antibiotic target sites [17], rendering it resistant to many drugs, including a group of beta-lactam antibiotics (β-lactam, an aminoglycoside group) and a group of fluoroquinolones [18, 19].

Studies have shown that phenotyping of Acinetobacter isolates at the species level is insufficient [20]. Therefore, several genetic methods have been developed to identify the species (genetic) of microbes, including A. baumannii. species identification is made possible using advanced molecular techniques including RDRA amplified RNA restriction analysis, 16S-23S rRNA, and the entire ribosomal operon Acinetobacter [21]. The ITS region separating the 16S and 23S rRNA genes, on the other hand, has been recommended as a good option for bacterial species identification since it has minimal intraspecific variability and substantial interspecific divergence [22].

A. baumannii possesses many antibiotic resistance genes and of different groups, including the aacC1 resistance

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gene which makes it resistant to a group of anti-Aminoglycosides [23]. The bla-OXA 23 like gene and the bla-OXA 51 like the gene for the β-lactam against group Carbapinims [24], and the gene (pmrA) that is resistant to the antibiotic Colistin [25].

2. Materials and Methods

During a period between (November 2020) and (June 2021), 200 bacterial samples of Acinetobacter baumannii were collected, 100 of which were collected from the hospital's environment (beds, floors, tables, incubators, equipment, and stagnant water). The other 100 samples were clinical specimens and including blood, sputum, fluids (cerebrospinal, pleural, and peritoneal), urine, wounds, and burns) which were collected from different hospitals in Baghdad.

2.1 Identification of *Acinetobacter baumannii* via API 20E

According to the procedure suggested by the manufacturing company. This system was designed for the performance of 20 standard biochemical tests from a single colony of purified isolate. The bacterial suspension was prepared for all the isolates from well-isolated colonies using API suspension medium and the turbidity adjusted to 0.5 McFarland (1.5x108 CFU/ml), with sterile a Pasteur pipette, the twenty microtubes were inoculated according to the manufacturer instruction.

2.2 Identification of Acinetobacter baumannii via VITEK2 Compact System

The bacterial isolate was identified using the VITEK2 technology. On MacConkey agar dishes, the bacterial isolates were subcultured. Bacterial suspensions in 0.45 percent sterile NaCl solution were employed, which were similar to MacFarland 0.5x 108 standards.

2.3 Methods and Workflow of DNA Extraction

Bacterial DNA was extracted based on the manufacturer's instructions and through a (Genomic DNA extraction) kit, which was prepared by the American company and used to extract DNA (for gram-negative bacteria, the gene (16Sr RNA was used) For the final diagnosis of bacterial isolates for the study using the primers for this gene that were prepared according to the recommendations of the manufacturer.

Table (1) Primer of 16S rRNA

Primer Name	Seq.	Annealing Temp (˚C)	Product size-(bp)
27F	5`-AGAGTTTGATCCTGGCTCAG-3`	60	1500
1492R	5`-TACGGTTACCTTGTTACGACTT-3`		

2.4 Standard Sequencing

PCR products were provided by Macrogen Corporation – Korea for Sanger sequencing utilizing an ABI3730XL automated DNA sequences.

2.5 Antibiotic Susceptibility Test: VITEK® 2 Gram-Negative Susceptibility cards.

Pure isolates of organisms to be tested were taken from Maconkey agar, VITEK 2 Card AST Gram-Negative, age of culture 8-24 hours, McFarland Standard (0.50 to 0.63) x108 CFU/ml, and age of suspension before loading on instrument ≤ 30 minutes. VITEK cards ID-GN for detection susceptibility results of (gram-negative identification), [26].

2.6 Statistical analysis

The mean ± SE of mean was calculated by using the IBM SPSS version 26.0 [27]. The probability was also examined by using student T-test and at the level of probability ≥ (0.05, 0.01).

The WinPepi application version 11.65 was used to calculate the probability for non-parametric data using Pearson's chi-square test [28].

3. Results

3.1 Identification using the API 20 E system.

The API 20 E system was utilized as a sensitive and delicate for Acinetobacter baumannii identification.

3.2 Identification of Acinetobacter baumannii by VITEK 2 system

Most of the samples that were isolated as A. baumannii by using VITEK 2 system with its identification card for Gram negative strains (ID-GNB).

3.3 Distribution of Acinetobacter baumannii based on the source

The isolates from patients were regained and verified as A. baumannii these distributed in 32% isolates for sputum followed by 24% for urine, then 16% for wound, 10% for blood specimens, 6% for ceribro spinal fluid (C.S.F) besides 3% for burns. On the other side, 100 isolates of A. baumannii bacteria were collected and isolated from the environment in the current study, and they were distributed as 36% isolate from beds, 14% floors, 7% tables, 5% incubators, 18% hospital equipment and 20% stagnant water.

3.4 Identification of A. baumannii by detection of 16S rRNA genes.

All the isolates were grown on Chrom Agar for A. baumannii and were incubated for 48 hours at 37°C under aerobic conditions After obtaining the results of the Vitek device, which confirms the identity of the isolated bacteria A. baumannii 20 bacterial isolates were selected, which were isolated from different places from the patients and the environment, and the bacterial colonies were then used for DNA extraction according to the protocol of ABIOpure Extraction.

Amplification of the 16S rRNA gene (1500 bp) was performed by using universal primers 27F(5`-AGAGTTTGATCCTGGCTCAG-3`) and 1492R (5`-TACGGTTACCTTGTTACGACTT-3`) These primers were supplied by (Macrogen Company) in a lyophilized form, After PCR amplification, the existence of amplification was confirmed using agarose gel electrophoresis. The extracted DNA criteria were fully reliant on PCR. These products were submitted to Macrogen Corporation in Korea for Sanger sequencing using an ABI3730XL automated DNA sequences, with the results confirming the identify of A. baumannii being sent isolates for study, 11 selected isolates were recorded in the GeneBank and they were in the following sequences: (OK035267.1 from sputum, OK035269.1 from urine, OK035329.1 from urine, OK035445.1 from urine, OK036427.1 from wound, OK040149.1 from environment of the Pediatric Teaching Hospital, neonate unit, OK036437.1 from environment of the Pediatric Teaching Hospital, OK036439.1 from environment of the Baghdad Teaching Hospital, neonate unit, OK036440.1from environment of Pediatric Teaching Hospital, dialysis unit, OK036452.1 from Environment of stagnant water near of Baghdad Teaching Hospital garden, OK354325.1 from stagnant water).

a. Antibiotic susceptibility test

The sensitivity of the isolates was tested to (19) commonly used anti-biotics to reveal the pattern of their resistance to the treatments used. The study included a group of β-lactams, a group of anti-aminoglycosides, quinolones and other isolated antibiotics. The results were recorded by measuring the diameters of the inhibition zones formed around the discs and comparing them with what was mentioned in the international tables, [29].

Table (2): Antimicrobial types from patient specimens

Antimicrobial types	S. No. (%)	I. No. (%)	R. No. (%)
Ticarcillin	0	10	90
Ticarcillin/ clavulanic acid	11	4	85
Piperacillin	28	4	68
Ceftazidime	22	2	76
cefepime	24	3	73
Imipenem	35	52	13
Meropenem	32	20	48
Gentamicin	41	20	39
Tobramycin	35	5	60
Ciprofloxacin	26	10	64
Minocycline	70	12	18
Piperacillin/ Tazobactam	28	25	47
Trimethoprim/ sulfamethoxazole	12	10	78
Ampicillin	8	0	92
Augmentin	27	2	71
Levofloxacin	30	28	42
Amikacin	31	14	55
Tetracycline	32	7	61
Colistin	75	15	15

S: Sensitive I: Intermediate R: Resistant

4. Dissection

The current study showed the environmental and clinical diversity of the spread of Acinetobacter baumannii, after diagnosing it by several methods such as API E20, as primary diagnosing, then by VITEK 2, and 16S rRNA, in addition to use of 19 antibiotics to determine the sensitivity of the bacteria towards it. A. baumannii are one of the most significant opportunistic pathogens, It might be largely related to their capability of adhering to the instruments or hands of healthcare staff, which promotes the transfer of bacteria from the skin or contaminated areas towards the blood, which progresses to bacteremia [30].

Most of the samples that were isolated as A. baumannii by using VITEK 2 system with its identification card for Gram negative strains (ID-GNB) This system has been employed in a number of previous research and has produced positive findings in terms of biochemical test identification and confirmation. this automated technique could determine the antibiotics of A. baumannii isolates [31]. In this study, the accuracy of this instrument was shown to be 99 percent to 100 percent for identifying A. baumannii, which agrees with Bagudo et al. [32] and Ganda et al. [33].

To evaluate the phylogenetic relationships between A. baumannii isolated from the environment and patients, a phylogenetic tree was generated MEGA 6.0 using the neighbor-joining method with 500 bootstraps and standard settings. 16S rRNA gene sequence from Acinetobacter spp. derived from NCBI GenBank. Phylogenetic organisms of A. baumannii isolates were shown in a tree based on the 16S rRNA gene sequence as shown in Figure (3).

In the current study, for bacterial isolates from patients, a match was observed in 16S rRNA series (OK035267.1 from sputum, OK035269.1 from urine, OK035329.1 from urine, OK035445.1 from urine, OK036427.1 from wound), as shown in Figure (1) shown in patient dendrogram tree, It was resistant to most of the antibiotics used in this study [34].

On the other hand, it was observed from the phylogenetic tree of the environmental isolates OK040149.1 from environment of the Pediatric Teaching Hospital, neonate unit, OK036437.1 from environment of the Pediatric Teaching Hospital, OK036439.1 from environment of the Baghdad Teaching Hospital, neonate unit, OK036440.1 from environment of Pediatric Teaching Hospital, dialysis unit, OK036452.1 from environment of stagnant water near of Baghdad Teaching Hospital garden, OK354325.1 from stagnant water). It has been noticed a great convergence in A. baumannii genes of the hospital environment isolates and the isolates of the stagnant aquatic environment near the hospitals in which the study was conducted, as shown in the figure (2), It was observed that there is a convergence between isolate OK036452.1 isolates of environment of stagnant water near of Baghdad Teaching Hospital garden and isolate OK036440.1 from environment of Pediatric Teaching Hospital, dialysis unit, and isolation OK040149.1 from environment of the Pediatric Teaching Hospital, neonate unit, shares with them Likewise, a convergence was observed between the two isolates OK354325.1 from stagnant water and OK036437.1 from environment of the Pediatric Teaching Hospital, These two isolates also overlap with the three aforementioned isolates, Then isolate OK036439.1 from environment of the Baghdad Teaching Hospital, neonate unit shares the 16S rRNA genetic sequence with them, This overlap in genetic traits between the aforementioned isolates indicates the existence of a very large similarity between those adjacent isolates despite the multiplicity of types of environment isolated from them except OK036437.1 and OK036437.1, The reason for this slight difference may be due to the growth conditions, temperatures, and the use of different disinfectants, according to the nature of work in the hospital [35].

Perhaps the reason is that the environment in which these similar isolates were collected in the 16S rRNA gene is the environment of the neighboring hospitals, even the environment outside the hospital, which is from stagnant water and is very close to those hospitals, and this result is consistent with the result obtained by researchers [36] where they concluded Rapid and reliable availability of molecular technologies Genetic characterization by using of 16S rRNA will increase our knowledge of Ecology, distribution and diversity of microbial communities. They note that A. baumannii is the most common species with high resistance compared to other types.

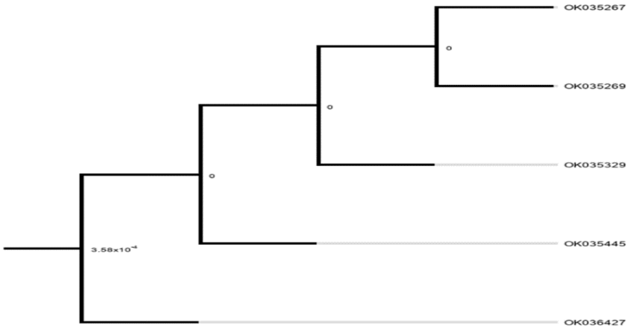

Figure (1) Clinical Dendrogram of genetic relationship among 5 isolates based on 16S rRNA genes.

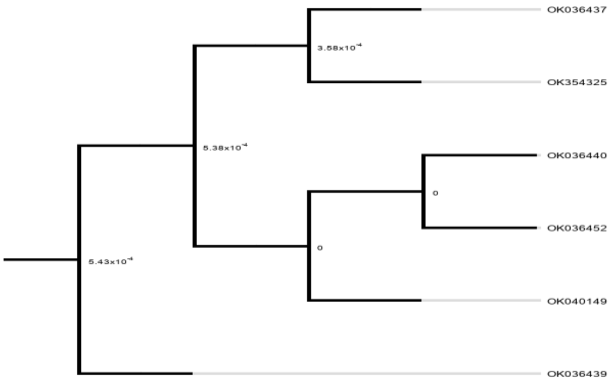

Figure (2): Environmental Dendrogram tree of genetic relationship among 6 isolates based on 16S rRNA genes.

The dendrogram tree of A. baumannii isolated from patient samples was joined with that of bacteria recovered from the environment in figure (3). Except for isolates (OK036427.1 from wound), (OK036437.1 from the Pediatric Teaching Hospital's environment), and (OK036427.1 from wound), the 16S rRNA genetic sequences of the aforementioned isolates show a great deal of similarity despite their variety.

A significant affinity was observed between the two isolates: OK035267.1 from sputum, and OK035269.1 from urine, likewise between the two isolates OK354325.1 from stagnant water and OK036427.1 from wound despite the difference in the environment from which they were collected, while there is a spacing between the isolates OK035267.1 from sputum, and OK036439.1 from environment of the Baghdad Teaching Hospital, neonate unit, and as shown Dendrogram tree figure (3).

The reasons for this diversity may be between the affinity of isolates greatly despite the environmental difference from which they were isolated, and the slight divergence of isolates despite their presence in close environments. The bacteria A. baumannii has the ability to spread widely in different environments due to its possession of multiple resistance genes It is resistant to disinfectants, sterilizers and antibiotics [34, 37, 38].

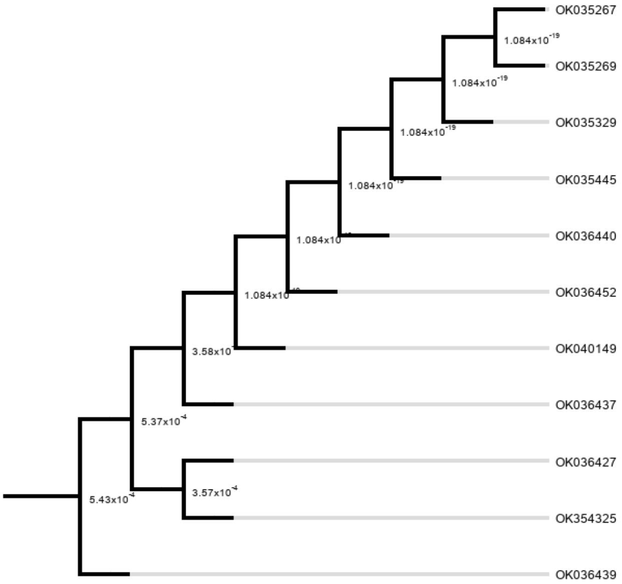

Figure (3): Dendrogram tree of genetic relationship among 11 isolates based on 16S rRNA genes from patients and environment.

It was observed in the current study that the widespread resistance of A. baumannii isolated from patients hospitalized in Baghdad city hospitals to most of the antibiotics used in this study which was ampicillin/sulbactam (92%), ticarcillin (90%), ticarcillin/ clavulanic acid (85%), Trimethoprim/ sulfamethoxazole (78%), ceftazidime (76%), cefepime (73%), Augmentin (71%), piperacillin (68%), ciprofloxacin (64%), tetracycllin (61%), Tobramycin (60%), amikacin(55%), then meropenem (48%), piperacillin/tazobactam (47%), after that was resistance to levofloxacin (42%), Gentamicin (39%), but the results observed the lowest resistance to Minocycline and colistin that was (18%), and (15%) respectively . Thus, A. baumannii was sensitive to the antibiotics colistin then Minocycline was (75%) and (70%) respectively but the bacteria showed little resistance to the antibiotic Imipenem (13%), but moderate resistance to it (52%) As shown in the table (2).

It was noted from the percentages obtained for the bacterial resistance to antibiotics isolated from patients hospitalized that the bacterial resistance to most antibiotics converges in its ratios, and this may be due to the acquisition of bacteria by hereditary factors that carry a trait resistance to antibiotics as well as thousands or more Irregularity in taking antibiotics without consulting a doctor[39].

5. Conclusion

1. This study has attended the following conclusions:
2. Acinetobacter baumannii could be isolated from different sources (clinical and environmental), where the highest percentage of isolates was from sputum and the lowest percentage was from burns for clinical isolates, while environmental isolates had the highest percentage of beds and the lowest percentage of incubators.
3. It was noted that the use of 16S rRNA method in diagnosing A. baumannii as a genotype is considered one of the best and most accurate methods for diagnosing bacteria.
4. It was observed that there were no significant differences in the DNA sequences between A.

baumannii that isolates from the environment and clinical sources by using the Sanger technique, which is an easy and accurate way to know the DNA sequence of the isolated bacteria.

5. Antibiotic susceptibility showed the highest resistance of A. baumannii to ampicillin/sulbactam and ceftazidime comparison the lowest resistance against Tobramycin, colistin then Minocycline, for bacterial isolated.

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