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Research Article

Biofilm formation and beta lactamase resistance of *Acinetobacter* isolates obtained from CCU patients

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ABSTRACT

Acinetobacter is clinically important pathogen with extensive resistance to various antibiotics This study aimed to antimicrobial resistance pattern in Acinetobacter species isolated from venous catheters. Extended-spectrum beta-lactamases (ESBLs) isolates, Cell Surface Hydrophobicity (CSH) index and biofilm formation was assayed. Sampling was performed from intravenous catheters people hospitalized in Kerman hospitals. Antibiogram tests were performed by disks ciprofloxacin (5 µg), ceftazidime (30 µg), ceftriaxone (30 µg), cefotaxime (30 µg), gentamicin (10 µg), ampicillin (10 µg), colistin (10 µg) Imipenem (10 µg), oxacillin (1 µg). Disks of ceftazidime and cefotaxime, both alone and combined with clavulanic acid were used to identify beta lactamase producing isolates. CSH was determined by the affinity test to xylene. Biofilm formation was analyzed in glass and polypropylene under shaking and non-shaking conditions test tubes. Twenty isolates of Acinetobacter were identified, of which 75% to ciprofloxacin, 100% to ceftazidime, 100% to ceftriaxone, 85% to cefotaxime, 75% to gentamicin, 80% to amikacin, 100% to oxacillin, 95% to imipenem, 85% were resistant to colistin and cefixime. In phenotypic screening of ESBLs positive isolates, 4 (20%) of the isolates were beta-lactamase positive. Five isolate of Acinetobacter showed above 70 % hydrophobicity. All five isolates with CSH Index above 70 % formed moderate biofilm. Analysis of biofilms formed on glass and polypropylene surfaces showed shaking conditions were suitable for biofilm formation and all five isolates formed strong biofilm in shaking condition. Adhesion of Acinetobacter on polypropylene was higher than on glass surfaces.

1. Introduction

Acinetobacter baumannii is an aerobic, nonfermenting, gram-negative, rod-shaped bacterial species (Shi., 2024). It was named in honor of the American bacteriologists Paul and Linda Baumann (Saha, 2024). *A. baumannii* belongs to the ESKAPE group (containing: *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *A. baumannii*, *Pseudomonas aeruginosa*, *Enterobacter* spp.), which includes the most pathogenic bacteria duo to their resistance to antibiotics (Bagińska, 2024). This bacteria can colonize the skin, conjunctiva, oral cavity, respiratory tract, gastrointestinal tract, and genitourinary tract of healthy individuals (Shi et al., 2024). *A. baumannii* is an opportunistic pathogenic bacterium with high patient mortality. Due to its ability to evade antimicrobial treatment,

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emphasizing the importance of expanding usable therapeutics to treat *A. baumannii* infections (shein, 2024). Genomic and phenotypic examination of *A. baumannii* strains identified that the pathogenicity and toxicity among these strains has expanded. The virulence factors in *A. baumannii* strains are capsular polysaccharides, iron chelating systems, lipopolysaccharides, outer membrane porins, phospholipases, proteases, protein secretion systems (Bagińska, 2024), oxacillinases and outer membrane protein A (OmpA) (Pourhajibagher, 2024).

An important factor determining the strong expansion of *A. baumannii* strains in hospital environments is their ability to acquire resistance to currently used antibiotics (Bagińska, 2024) and its capacity form biofilms (Saha, 2024). Common antimicrobial drugs often fail due to resistance developed by inherent and acquired mechanisms, leading to the emergence of extensively drug-resistant (XDR) bacteria (Rastegar et al., 2024). *A. baumannii* exhibits a high degree of resistance to existing antimicrobial classes, such as carbapenems and colistin, which are considered last-resort treatment (SAyem khan,2024). *A. baumannii* employs multiple strategies to counteract the effects of antibiotics. Among these strategies, antimicrobial inactivating enzymes such as β-lactamases, efflux pump overexpression and makes chang in antibiotic target location and outer membrane protein penetrance can be pointed (Shi t al., 2024).

Extended-spectrum β-lactamases (ESBLs) are enzymes which hydrolyse the beta-lactam ring of β-lactam antibiotics. The main resistance determinants extended-spectrum β-lactamases (ESBLs), can make a various range of β-lactam antibiotics ineffective, including penicillins, cephalosporins, and monobactams (Worku, 2024). Enterobacteriaceae, *Pseudomonas aeruginosa*, and *A. baumannii* obtain and disseminate ESBL-encoding genes horizontally, mainly through plasmids (Ejaz et al, 2021). In general, all beta-lactamase variants are classified into four classes, A (serine penicillinases), B (metallo-beta-lactamases), C (cephalosporinases (acinetobacter-derived cephalosporinase or ADC) and D (oxacillinases), which give resistance to penicillins, most β-lactams, cephalosporins and cloxacillin, respectively (Hussain , 2021). Among diverse resistance mechanisms, carbapenemase production, particularly class D β-lactamases (oxacillinases), is one of the most common mechanisms for carbapenem resistance in CRAB. Oxacillinases such as OXA-23, OXA-24, and OXA-58 are frequently found among *A. baumannii* strains. These enzymes are encoded by various alleles with differing hydrolytic capacitie (SAyem khan, 2024).

The potential of this bacterium to product biofilms and persevere in dry environments, along with the expansion of drug resistance, presents a serious challenge in treating *A. baumannii* infections (Rastegar., 2024). Biofilm formation is a complex process in which microorganism cells transition from planktonic cells to unstable growth, and it is influenced by various environmental conditions such as surface porosity, fluid flow, and nutrient availability (Saha, 2024). Biofilm formation is a multiplex process that is affected by several factors, such as the production of aggregation substances, adhesion of collagen, pili expression, and iron acquisition. Whence, several genes and proteins are involved in the various developmental stages. In particular, *A. baumannii* biofilm-related protein (bap) is crucial for complete biofilm production on surfaces such as polypropylene, polystyrene. Furthermore, the presence of the outer membrane protein A (ompA) increase its attachment to both abiotic and biotic substance, while contributing to the intrinsic resistance of this species to a lot of antibiotic (Lysitsas et al., 2024). The purpose of this investigation was to explore the beta-lactamase and biofilm production clinical Acinetobacter isolated from intravenous catheters of people hospitalized in hospitals.

2. Materials and Methods

2.1. Collection of Samples

210 intravenous catheters were collected from hospitalized patients in Shafa Hospital, Kerman. According the aim of the research, we only focused on the isolated bacterial traits without any use of patient information as a primary item for ethical scrutiny and other patients' information were not also addressed such as gender, age, and type of disease. After transporting samples to laboratory of microbiology by ice box, Sterile swabs were drawn well over intravenous catheters and

placed inside sterile physiological serum. The collected samples were streak cultured on blood agar medium. Different colonies according their morphologies were selected and purified.

2.2. Morphological and biochemical identification of bacteria

Isolated bacteria were examined by gram staining test. Identification of bacteria was made by cultivation (Chromagar Acinetobacter media, MacConkey agar, TSI, Simon's citrate, SIM and Blood agar), as well as Catalase and Oxidase tests. A total of 20 *A. baumannii* isolates were obtained (Sehree et al., 2023).

2.3. Antibiotic resistance pattern

The commercially antibiotic discs (PadtanTeb, IRAN) were used for investigating the antibiotic resistance pattern of *A. baumannii* isolates. ciprofloxacin (5µg), ceftazidime (30µg), ceftriaxone (30µg), cefotaxime (30µg), gentamicin (10 µg), ampicillin (10 µg), colistin (10 µg), cefixime (5 µg), imipenem (10 µg) and oxacillin (1µg) were used as antimicrobial agents. For this purpose, bacterial colonies were inoculated into the nutrient broth (Merk, Germany) and kept in an incubator at 37 °C for 24 hours. After incubation 0.5 McFarland turbidity standard was prepared to get a total count of 1.5×10^8 CFU/ml. After being inoculated into sterilized Muller Hinton agar (Merk, Germany). All plates were upturned and incubated at 37 °C for 24 h. The Diameter of the Inhibitory Zone (DIZ) or areas showing no growth were calculated and documented in mm with the help of a scale following CLSI criteria. Antibiotics with in- creased susceptibility have reduced growth of the culture and have larger DIZ whereas, antibiotics with less susceptibility towards the bacteria have smaller inhibitory zones. The complete absence of the inhibitory zone indicated that the bacteria were resistant to a specific antibiotic (bashir, 2023).

2.4. Screening of phenotypic ESBL producing isolates

Extended-spectrum Beta-lactamase (ESBL) production was confirmed both in the Combination Disk Test (CDT) and by the decreased susceptibility to one of ceftazidime and cefotaxime according to the Clinical and Laboratory Standard Institute (CLSI) recommendations (Humphries, 2021). In this test, a disk containing cefotaxime (30 µg) alone and ceftazidime (30 µg) alone was placed in the opposite direction to a disk containing cefotaxime and ceftazidime plus clavulanic acid (20/10µg) with 15 mm distance on Muller Hinton Agar medium (worku, 2024).

2.5. Cell surface hydrophobicity (CSH) of isolates

CSH was determined by the affinity test to xylene. The hydrophobicity index (HI) was calculated using the following equation:

$$HI: (A_{600}nm - B_{600}nm) / A_{600}\ nm \times 100$$

Where A600nm denotes the initial absorbance and B600nm represents the absorbance after vortex mixing. The isolates were considered as strongly hydrophobic when the HI was >70% and with hydrophilic character when the HI was <30% (Kazemi Pour, 2011).

2.6. Biofilm formation on abiotic surfaces

Biofilm formation was analyzed in glass and polypropylene test tubes. The biofilms were formed by adding 0.1mL of the culture to 10mL LB dispensed in glass test tubes. The experiment was performed in duplicates and the cultures were incubated at 37 1C for 72 h under two sets of different conditions: (1) shaking at 200 r.p.m. and (2) stationary. After incubation, the medium was removed, the tubes were washed with distilled water, air dried and biofilms were assayed using the crystal violet method (Kazemi-Pour, 2011).

2.7. Identification of Acinetobacter

The reaction mix for the PCR assay had a total volume of 25 µL, and it was composed of: forward primer 1 µL, revearse primes 1 µL, Genomic DNA 1 µL, Master mix (Sina colon company, Iran) 5 µL and distilled water 17 µL. After PCR amplification, 5 µL of amplified DNA fragment was separated by electrophoresis on 1.5% agarose gel (Sigma-Aldrich,Merck) stained with ethidium bromide (0.5 mg/mL). The products were subjected to electrophoresis for 2 min at 130 V and for 40 min at 110 V in 1xTBE buffer (Hubeny et al., 2022). Universal primers sequences used in this study, are shown in table 1.

Table 1. primers sequences

primers 27 F	(5-AGAGTTTGATCCTGGCTCAG-3)
primers 1492 R	(5-TACGGTTACCTTGTTACGACTT-3)

To determine the sequence, it was done by South Korea's Macrogen Company company (volume: 10 microliters, concentration 30 ng/microliter). The results were evaluated with Finch TV software and the phylogenic tree was drawn using Neighbor-joining method with the use of Mega version 7 software after blasting the sequence by NCBI site.

3. Results

The prevalance of antibiotic resistance of the *Acinobacter* spp. isolated from intravenous catheters is presented in figure 1. The highest percentages (100 %) of resistance have been observed against the antibiotics Ceftazidime, Ceftriaxone and Oxacilline. (Figure 2). Since the isolated bacteria showed more than 75% antibiotic resistance, all of them were multidrug resistanse bacteria.

Among 20 isolates, four (20%) were phenotypically confirmed to be ESBL producers by the CLSI screening method (Figure 3).

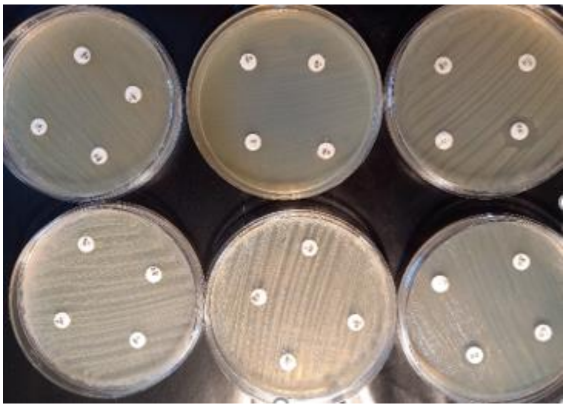

Figure 1. Antibiotic resistance of the *Acinobacter* spp

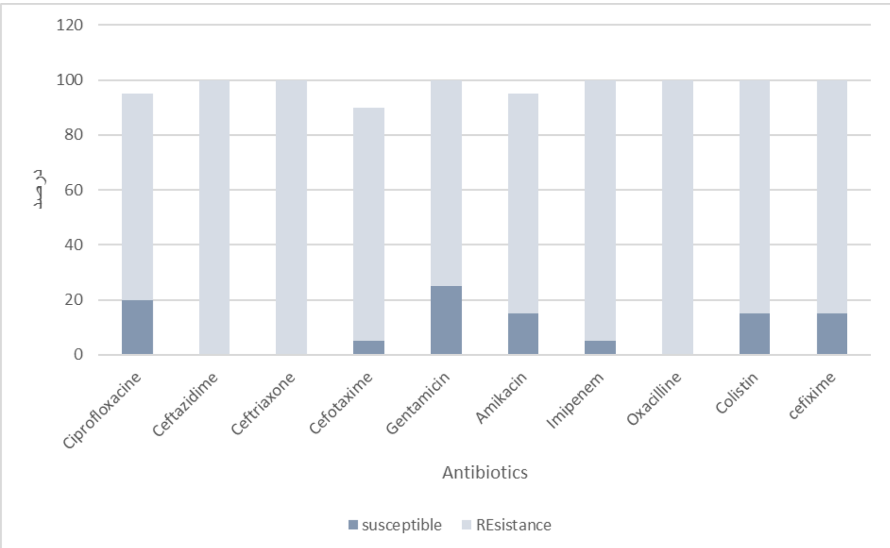

Figure 2. Prevalance of antibiotic resistance of the *Acinobacter* spp

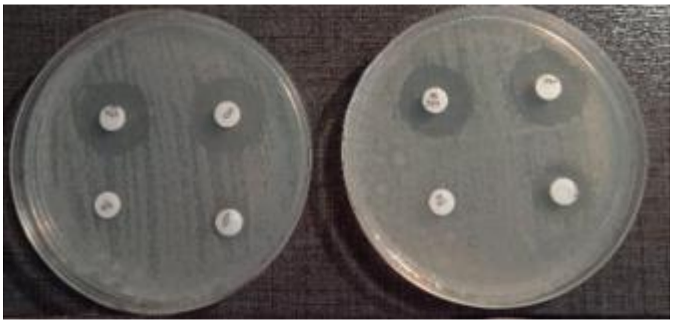

Figure 3. Beta-lactamase positive screening isolates

CSH indices , were determined for all the 20 isolates of *Acinetobacter*. The results varied from 0.5 % to 92.02 %. The five isolates of *Acinetobacter* (A10, A14, A17, A18 and A20) were showed the highest hydrophobicity (above 70 %) indices and listed in Table 2.

Table 2. HI of the cellular surface determined by the microbial adhesion method to xylene

Isolates	HI (%) *
Acinetobacter A10	82.40
Acinetobacter A14	92.02
Acinetobacter A17	74.23
Acinetobacter A18	78.73
Acinetobacter A20	84.87

*HI= HI: (A_{600}nm _ B_{600}nm) / A_{600} nm × 100

In the following, biofilm formation abilities of these five isolates on abiotic glass and polypropylene surfaces under two sets of shaking and stationary condition were assayed. Results showed all five isolates, formed moderate biofilm. Analysis of biofilms formed by *Acinetobacter* showed shaking conditions were suitable for biofilm formation and all five isolates formed strong biofilm in shaking condition. Adhesion of *Acinetobacter* on polypropylene was higher than on glass surfaces. *Acinetobacter* A18 was selected to molecular identification due to its beta-lactamase positivie, its hydrophobicity index above 70% and the formation of a strong biofilm. The phylogenic tree is given in Figure 4 and indicated the species of *Acinetobacter* 18. The Genebank accession number of nucleotide sequence is OR272271.

4. Discussion

The prevalence of resistant strains of pathogens is increasing day by day, while the antibiotics commonly prescribed against them are losing their efficacy, which is pushing the world to the era of pre-antibiotics (Bashir,2023). Infection due to *Acinetobacter* species is a major challenge within the health care facilities and the community in general due to their high drug resistance even to the high potent drugs. In our study, 20 *Acinetobacter* isolates were collected from 210 venous catheters of patients in Shafa Hospital, Kerman, Iran. 100 % of isolates were resistance to Ceftazidime, *Ceftriaxone and* Oxacilline and above 75% of isolates were resistance to Ciprofloxacine, Cefotaxime, Gentamicin, Amikacin, Imipenem, Colistin and cefixime. In the report of Ayenew et al (2021) mentioned more than 70% of patients had taken antibiotics empirically before getting confirmed culture and antimicrobial susceptibility testing (AST) result showing *Acinetobacter* infection. Therefore, *The spread of multidrug-resistant Acinetobacter in hospitals and nursing homes poses serious healthcare challenges* (Gupta et al., 2015).

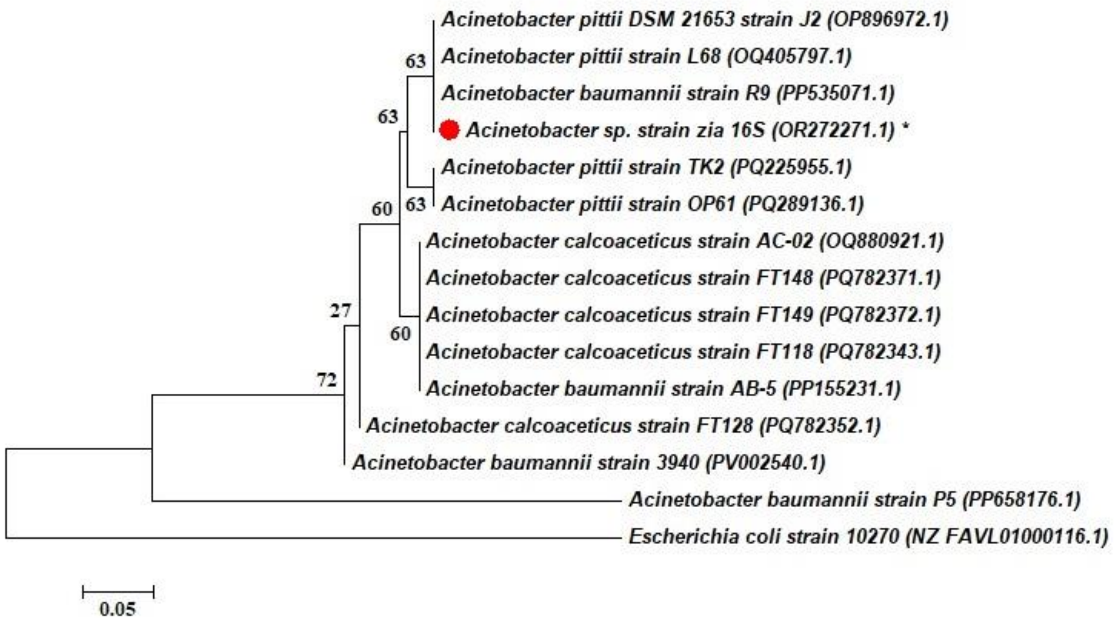

Figure 4. Phylogenetic tree of Acinetobacter 18

Not long ago, ESBL have been classified into three main groups: Ambler class A ESBL ($ESBL_A$), miscellaneous ESBL ($ESBL_M$), and ESBL that degrade carbapenems ($ESBL_{CARBA}$). Most ESBL in the world belong to the $ESBL_A$ group, which includes several types of sulfhydryl reagent variable (SHV) β-lactamases, Temoniera (TEM) β-lactamases, and cefotaxime-M (CTX-M) β-lactamases. More recent outbreaks involving ESBL have been mediated by the CTX-M type rather than the TEM type or the SHV type. CTX-M-type ESBL preferentially hydrolyze cefotaxime over ceftazidime and are inhibited by tazobactam. In present study, four isolates (20%, n = 20) were cefotaxime-M (CTX-M) β-lactamases (Husna, 2023). More than 80 CTX-M types have been reported in both hospitals and communities as well as in food animals, fresh vegetables, water, and the environment. Mobile genetic elements (MGEs) such as ISEcp1 and ISCR1 play an important role in transferring blaCTX-M genes from the chromosomes into the plasmids (Amelia et al. ,2016). Oxacillinases are a type of beta-lactamase enzyme produced by *A. baumannii* that confers resistance to oxacillin and other beta-lactam antibiotics, including carbapenems. Strains of *A. baumannii* that produce oxacillinase enzymes are frequently linked to nosocomial infections (Pourhajibagher, 2024).

Bacterial CSH of Acinetobacter strains is known to be associated with pathogenicity, bacterial adhesion and biofilm formation (Absolon, 1988). In present study, the hydrophobicity of isolates were examined by determining the affinity of cells to xylene (Kazemi pour,2011). Acinetobacter isolates A10, A14, A17, A18 and A20 showed the highest CSH values as compared with the other isolates. Attachment and biofilm formation on glass by clinical isolates of Acinetobacter is the property that is most likely to be associated with the capacity of this pathogen to survive in hospital environments, medical devices, and subsequently causes infections in compromised patients (Tomaras et al., 2003).

In our study, Biofilm formation by Acinetobacter isolates were observed on different abiotic surfaces such as glass and polypropylene. It is important to pay attention that some of these substances are used widely in the fabrication of medical tools. There is a positive relationship between the degree of bacterial hydrophobicity and adhesion to the abiotic surfaces (Costa, 2006 and Kazemi, 2011).

In present study, Biofilm formation of five isolates with CSH index above 70 % showed formed moderate biofilm, shaking condition caused the formation of a strong biofilm, the same result was obtained in Kazemipour's (2011) study. In the report of Wang et al., (2020) Motility of bacteria regulates the unstable adhesion process. Acinetobacter is a non-motile bacterium, therefore shaking of bacteria due to forming strong biofilm. Hence, we should pay attention to this point. In this research, Adhesion of isolates on polypropylene was higher than on glass surfaces

Refereces

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