Evaluation of some Virulence Factors, Hemagglutination and Agglutination of Antigens of *Acinetobacter Baumannii* Isolated From Clinical Samples

Fatima Rammadan Abdul

*Department of Biology; College of Science; Mustansiriya University-Baghdad –Iraq.*

**Abstract**

A total of 50 clinical samples, isolated from various sources include blood septicemia, urine (UTI), wound and burn swabs and swabs of ear (otitis media). The isolates were identified according to the microscopical, morphological, cultural, and biochemical tests, in addition to using VITEK-2 compact system. Results showed that (12) isolates were characterized as *A. baumannii*. Frequency of positive *A. baumannii* clinical isolates was higher in blood (50%), followed urine (25%). The sensitivity of isolates to eight antibiotics, the result showed highly level of resistance for most antibiotics and multi-resistance antibiotics. It was found that all (100%) of bacteria isolates were resistant to Ampicillin and Cefazolin, while Imipenem and Tobramycin were the most effective antibiotics. The isolates showed the ability of biofilm production by Congo red agar method (CRA), 7 (58.3%) isolates positive biofilm production, while 5 (41.7%) isolates are non-biofilm production. Consequences of phenotype were demonstrated 4 (33.3%) of the detaches were protease production, while (8) isolates (66.7%) were not producers of this enzyme, while Sera proteins of patients’ infected by *A. baumannii* showed reactivity with *A. baumannii* antigens (whole cells) as: two *A. baumannii* antigens agglutinated in titer 1:64 of patients sera (antibodies) while 6, 3, 2, 5 of *A. baumannii* antigens agglutinated in the patients sera in titer (1:8, 1:16, 1:128, 1:256) respectively. The hemagglutinin of *A. baumannii* may be a virulence factor in the pathogenesis of infection; However, results founded a good correlation between ability of biofilm formed and the hemagglutination.

**Keywords:** *Acinetobacter baumannii*, Virulence factors, Biofilm, Hemagglutination.

**Introduction**

*Acinetobacter baumannii* (*A. baumannii*) is a Gram negative, a typically short, almost round, rod-shaped (coccobacillus), has turned into an inexorably common reason for nosocomial diseases particularly in immunocompromised and Intensive Care Units (ICUs) patients over the most recent couple of years [1].

It is one of the most frequently isolated pathogen in critically ill patients. It is a major cause of nosocomial infections. Its ability to survive in a range of environments has been a key feature for its persistence and success as an opportunistic pathogen [2]. *A. baumannii* was the third highest mortality of blood stream infection after *P. aeruginosa* and *Candida spp.* Infections [3].

It is a ubiquitous multidrug-resistant bacterium that is found on a variety of surfaces, including skin, hair and soil [4]. The previous studies concerned with *A. baumannii* infections were almost focusing on antibiotic resistance. The protection of *A. baumannii* to antimicrobial operators is interceded by the greater part of the significant protection systems known to occur in microbes, including change of target locales, enzymatic inactivation, dynamic efflux and diminished deluge of medications. Additionally, NaCl a monovalent cation largely found in our skin has recently been associated with enhanced *A. baumannii* multidrug resistance [5].

*A. baumannii* is a frequent cause of nosocomial pneumonias, mainly ventilator-associated, and of bloodstream infections in immunocompromised and debilitated patients with serious underlying diseases [6, 7]. It has also been implicated in complicated
skin and soft tissue infections, nosocomial cases of endocarditis, abdominal and urinary tract infections, and central nervous system infections in neurosurgical patients [7]. The virulence factors of A. baumannii involve Lipopolysaccharide, pili, capsule, biofilm formation, siderophore production, outer membrane protein 38, and protease and lipase production [8].

In the clinical setting A. baumannii is to a great degree risky, because of it is capacity to colonize and contaminate extremely sick patients in Intensive care units (ICUs) [9]. The capability to produce biofilm made the bacteria to form biofilm is a standout amongst the most essential harmfulness factor which allows bacterial survival in a brutal situation and reductions antimicrobial fixation [10].

Biofilm delivered by this bacterium is exceedingly tolerant to natural components and distinctive anti-infection agents. Biofilm-related protein (Bap) assumes a critical part in the biofilm development by A. baumannii and found in the extra cellular matrix of the biofilm [11].

Aims of the Study

The aim of this study to determine the antimicrobial sensitivity, study of some virulence factors produced by A. baumannii. Using A. baumannii as antigens against sera of patients’ infected by this bacteria. Determine the correlation between hem agglutination assays and biofilm formed by A. baumannii.

Materials and Methods

Collection of Samples

Fifty clinical samples were collected from patients, who suffering from blood septicemia samples (Diagnostic by physicians), Urinary tract infection, wounds, burns, and ear (otitis media), in sterilized containers from three hospitals in Baghdad (AL-Kindy, Imam Ali, Ibn Al- Balade ). This study performed during the period from December 2016 to April 2017.

Culturing of the Samples

All study samples subjected to cultured methods and antimicrobial sensitivity test according to standard bacteriological procedures. Under aseptic conditions, MacConkey agar and blood agar, incubated aerobically at 37°C for a maximum of 48hrs. Initial identify of A. baumannii depending on Gram reaction and morphological characteristic of the colonies based on bacterial growth on MacConkey agar and blood agar [12], as well as by biochemical examinations and the VITEK 2 compact system (Bio Merieux, France).

Antibiotic Susceptibility Test

Disk agar diffusion according to Kirby Baur standrized antimicrobial susceptibility single disk method was carried out toward eight antibiotics (Ampicillin, Ampicillin/sulbactam, Amoxicillin/Clavulanic acid, Cefazolin, Impeneme, Tobramycin, Ciprofloxacin and Levofloxacin), Bioanalyse/Turkey [13].

Bio film Formation

Culturing the strains on Congo red agar plates of 18 hrs to 24hrs of incubation at 37°C, dark ends of growing determined the bio film production [14].

Protease Activity

Protease activity were done by measuring the diameter of lysis area after growing for 18-36 hrs of incubation at 37°C on skim milk agar media for all isolates[14].

Immune Assay

Bacterial Antigen Preparation (Whole Cell Killed by Heat)

For agglutination tests, cells were prepared as described by [15].

- Nutrient broth media were inoculated with bacterial isolates and incubated at 37°C for 24 hrs.
- Centrifuged culture at 4000 rpm for 15min, then discarded the supernatant and washed the sediment three times phosphate buffer saline (PBS).
- Re-suspended the sediment in 5ml of PBS by vortex and put the suspension in water bath at 70°C for 1hrs.
- To make sure all bacteria was destroyed, cultured bacterial suspension on nutrient agar media at 37°C for 24 hrs.
- Centrifuged killed bacterial suspension at 4000 rpm for five min. Then sediment was suspension in three ml of normal saline and utilized or stored in - 20°C until utilize.

Antibodies Production

Blood was collected from patients and serum was used [16].
• Collected blood and permitted it clot in an upright position for at least 30 min.
• Centrifuge for 15 min at 1500 rpm.
• Exchange the serum to a plastic screw-cap vial for carrying to the laboratory. Furthermore, serum was stored (frozen at −20°C) until required.

Agglutination Antigens with Antibodies
• Coating all wells by 50 µl (1.5 × 10^8 CFU/mL) of A. baumannii antigen.
• Add serial dilutions of patient's serum antibodies (1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256) to all wells.
• Noted the macroscopically agglutination titers, also evaluated microscopically agglutination. All experiments were done in duplicate with 3 replications [17].

Hem Agglutination Test
• The hem agglutination was executed as described [18]. With a few alterations.
• Hem agglutinating action was determined by micro-hem agglutination test using 96-well round-bottom plates and fresh human blood
• A. baumannii cells from culture in 37°C in trypticase soy broth were grown in fresh TSB supplemented with 0.25% glucose for 18 hr were harvested by centrifugation and furthermore, washed once with PBS containing 0.1% bovine serum albumin.
• The bacterial re-suspended in saline, were adjusted to a McFarland standard of 3.0 in PBS with 0.1% BSA, which correlated with ~10^8 bacteria/ml.
• Every cell suspension were made (100 µl) in well microtiter plates, then 100 µl of the 1% erythrocytes solution (in PBS with 0.1% BSA) was added to each well and to guarantee thorough mixing of the bacteria and erythrocytes.
• Wells containing just the suspension of erythrocytes were utilized as negative control.
• A small pellet of erythrocytes at the bottom after 1 hr. incubation at 4°C were considered negative, and those containing an even sheet of erythrocytes across the well were considered positive.

Results and Discussion

Isolation and Identification of Bacteria
A total of (12) bacterial isolates were identified as A. baumannii in clinical samples of this study, table (1) showed blood samples from patients with septicemia occupied the first place in isolation of A. baumannii forming 6(50%), followed by urine samples 4(33.3%). Only one isolate (8.3%) in wound and burns swabs, as well as 1(8.3%) from swab of otitis media.

Previous study, [19] that the highest number of A. baumannii is isolates with percentage (73.58) was from respiratory tract followed by blood (16.98%), urine (3.77%) and other clinical specimens (5.66%). Other studies [20] found from 33 A. baumannii isolates (n=26) from lower respiratory tract, (4) from urinary tract, (2) from blood, and (1) from pus, whereas [21] found the highest percentage of A. baumannii isolation (48%) was from urine followed by blood (38%), while the lowest percentages of isolation (9%) and (5%) were from wounds and sputum respectively.

Table 1: Prevalence of A. baumannii in study samples

<table>
<thead>
<tr>
<th>Sources of samples</th>
<th>Total number samples</th>
<th>No. of bacterial isolates</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>19</td>
<td>6</td>
<td>50</td>
</tr>
<tr>
<td>Urine (UTI)</td>
<td>14</td>
<td>3</td>
<td>25</td>
</tr>
<tr>
<td>Burns and wounds swab</td>
<td>7</td>
<td>2</td>
<td>16.7</td>
</tr>
<tr>
<td>Ear swab(Otitis media)</td>
<td>10</td>
<td>1</td>
<td>8.3</td>
</tr>
<tr>
<td>Total</td>
<td>50</td>
<td>12</td>
<td>100</td>
</tr>
</tbody>
</table>

A. baumannii isolates were identified according to their microscopically, morphological and biochemical. All isolates appeared as Gram-negative coccobacilli and occasionally arranged in diplococci. Bacterial isolates were culture on MacConkey agar they appeared as small, pale and lactose non-fermenter colonies, while on blood agar they appeared as opaque creamy and non-hemolytic colonies Figure (1).
All isolates showed negative results for oxidase test, motility test, indole production test and urease production test, while the isolates gave positive results to catalase test and citrate utilization test. Kligler iron agar developed an alkaline slant, no change bottom, H2S negative without gas production. The results of biochemical tests were listed in Table (2) [12, 22, 23].

<table>
<thead>
<tr>
<th>ID</th>
<th>Biochemical test</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Catalase production</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>Hemolysin production</td>
<td>- (γ hemolysis)</td>
</tr>
<tr>
<td>3</td>
<td>Indole production</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>Lactose fermentation</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>Motility</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>Oxidase production</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Kliglar iron agar (KIA)</td>
<td>Alkaline slant / No change bottom, No gas , No H2S</td>
</tr>
<tr>
<td>8</td>
<td>Urease production</td>
<td>-</td>
</tr>
</tbody>
</table>

+ : positive result, - : negative result

**Antibiotics Susceptibility**

The susceptibility tests toward eight antibiotics were investigated and data presented in Figure (2) showed that all A.baumannii isolates (100%) were considered as multi-drug resistant (MDR) pathogens because they were irresponsible to multiple antibiotic therapies. MDR was defined as acquired antibiotic insensitivity at least one drug within three or more antimicrobial classes. It was found that all (100%) of bacteria isolates were resistant to Ampicillin and Cefazolin. While Imipenem and Tobramycin were the most effective antibiotics and their resistance percentage were 50%, 40% respectively and Ampicillin/sulbactam, Ciprofloxacin and Norfloxacin were less effective and their resistance percentage were 60% of them. The antibiotic resistance in A.baumanniiis inherent and naturally acquired especially against Penicillin, Chloramphenicol and Gentamicin, some reports recorded Ciprofloxacin resistance in these pathogens which have also developed resistance to other antibiotic classes [24].
Previous study done by [21] showed that isolates were completely resistance to Ticarcillin-Clavulanate, Aztreonam, Cefotaxime, Ceftazidime and Ceftriaxone, while the resistance percentages to Tobramycin and Amikacin were 50% and 56%, separately. Results of another study done by [25], found that A. baumannii clinical isolates developed 100% resistance to Cefotaxime, Ceftazidime and Ceftriaxone, 95.45% to Cefepime, Chloramphenicol, Aztronam and 40.90% to Imipenem. The resistance of A. baumannii to antibiotics agents is interceded by all of the main resistance mechanisms known to arise in bacteria containing modification of target sites, enzymatic inactivation, active efflux and diminished influx of drugs [8].

A. baumannii carries many resistance mechanisms to β –lactam antibiotics, these mechanisms can be categorised into two major groups [20]. The first group contains non-enzymatic mechanisms which contain target change at PBPs, impaired penetration, and efflux pumps. The other group contains an enzymatic mechanism, which is the most important resistance mechanism.

**Virulence Factors**

**Detection of Biofilm-Production**

Capacity of A.baumannii to arrangement biofilm can be incidental by phenotypic characteristic when developed on Congo red agar. Colonies of isolates that products slime form dark colonies, while the colonies that do not yield slime are pink in color, as those appreciated in Figure (3), which exhibited phenotypic production and non-produced slime by all examined isolates.

For the biofilm creation by Congo red agar medium, black color colonies were detected, 7 (58.3%) isolates, while 5(41.7%) isolates gave pink color showing as non-biofilm production. A study done by [26] in which they explained that 56 (63%) of the A. baumannii isolates produced black colonies, while 33 (36%) non-biofilm produce. Another done by [27] found that, 249 (91%) isolates were slime producers as opposed to only 63 non-adherent bacteria.

![Figure 3: Biofilm production by A.baumannii isolates on Congo-red agar](image)

**Production of Protease Enzyme**

The virulence factors created by A. baumannii that add to the pathogenesis of diseases caused by these microorganisms, the pathogenicity has different metabolites are delivered by these bacteria, including enzymes, for example, protease, the results of this study showed that A. baumannii (4) isolates were producer of the enzyme in percent of (%33.3), while (8) isolate in percent of (%66.7) were non producers of this enzyme as shown in Figure (4). Aprotease (or proteimase) is any enzyme that creates proteolysis, that is begins protein catabolism by hydrolysis of the peptide bands that connection amino acids together in a polypeptide chain. Proteases have developed multiple times and diverse classes of protease can make the same response by completely altered catalytic mechanisms. Proteases can be found in plants, animals, bacteria, viruses and archaea. They can attack the immunoglobulin and immune cells [28]. Results of [29] showed protease production by A. baumannii which clearly functions as important virulence factor.
Antigen - Antibodies Titration

Agglutination depends on the presence of antibodies in patient sera that can respond with particular antigens and form unmistakable clumps. The positive reaction between surface antigens of bacteria and the antibodies, which consider as a decent tool utilized for diagnose infection and distinguish bacterial isolates by recognition of bacterial - specific antibodies in tests.

Table 3: agglutination of antigens (whole cells of A. baumannii) against antibodies in sera of patients

<table>
<thead>
<tr>
<th>Concentration of A. baumannii antigens whole cells</th>
<th>No. of patients</th>
<th>Titer of antibodies</th>
<th>control (serum)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1.5×10^8 CFU/mL)</td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>64</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>64</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>128</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>256</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The outcomes were shown in Table (3) represent that sera from patients reactivity with A. baumannii antigens (whole cells) as: two A. baumannii antigens agglutinated in titer 1:64 of patients sera (antibodies) while 6, 3, 2, 5 of A. baumannii antigens agglutinated in the patients sera in titer (1:8, 1:16, 1:128, 1:256) individually. Generally A. baumannii have a pronounced capacity to tie non- specifically to bare polymer surface, this coupling can be blocked by covering the surface with different proteins, vaccine or as a target in passive immunotherapy or prophylaxis. An antibody response to a specified antigen is revealing of its expression in vivo and of its possible for use in a subunit vaccine or as a target in passive immunotherapy or prophylaxis [30]. So some of antigenic A. baumannii proteins are impending targets for immunotherapy, which make available a novel strategy for control of A. baumannii infection and could potentially decrease infection and have a significant effect on human health.

Hemagglutinin Assay

Hemagglutinin of A. baumannii may be a destructiveness factor in the pathogenesis of infection. In this manner, hemagglutinin of A. baumannii may assume a noteworthy part in the adherence of this organism to polymer containing biomaterials.
Figure (5) indicated capacity of A. baumannii to hemagglutination of erythrocytes was appeared to be related with the capacity to adhere to plastic and to produce biofilm. 7 biofilm-positives (micro-hemagglutination-positive strains) and 5 biofilm-negative (micro-hemagglutination-negative strains).

Table 4: Relationship between biofilm formation, Hemagglutination assay in A. baumannii

<table>
<thead>
<tr>
<th>Source of A. baumannii</th>
<th>CRA Method (+)</th>
<th>Hemagglutination assay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Macro-hemagglutination</td>
</tr>
<tr>
<td>Blood</td>
<td>4</td>
<td>+</td>
</tr>
<tr>
<td>Urine (UTI)</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>Wound and burns swab</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>Total No.</td>
<td>7</td>
<td>4</td>
</tr>
</tbody>
</table>

In Table (4), 4, 7 of the all isolates, were the connection between positive both (Macro and Micro hemagglutination) hemagglutination test respectively and biofilm production. So in this studies it was shown that the biofilm which formed by A. baumannii which isolated from blood was 3 out of 4 isolates have positive macroscopically hemagglutination assay and 4 isolates have positive micro-hemagglutination assay, while (1 and 2) isolates have positive macroscopically and micro-hemagglutination assay respectively of biofilm which formed by A. baumannii which isolated from wound and burns swab. Furthermore, only one isolate isolated from urine (UTI) has positive micro-hemagglutination assay but negative macro-hemagglutination these results indicate relationship was founded between the of biofilm production and positive hemagglutination. Furthermore, this results are confirm with [31] who showed (9 of 13) A. baumannii isolates agglutinated human AB blood groups; while [16] depicted that every one of the A. baumannii isolates agglutinated human group O erythrocytes. In this study appearance the A. baumannii isolates has capable to cause haemagglutination, as well as hemagglutination of erythrocytes is a communal property of A. baumannii isolates, which is associated to adherence and biofilm development and may be important for pathogenesis of biomaterial-associated infections caused by A. baumannii.

References


26. Rodríguez-Baño J, Martí S, Soto S, Fernández-Cuenca F, Cisneros JM,


