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# Study of Virulence factor of *Acinetobacter baumannii* and detection of *bap* gene

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**Abstract:** A Total of 150 clinical specimens from different clinical sources including burns, wounds, and urinary tract infections in Baquba General Teaching Hospital for the period from September 2016 to May 2017. 14 isolate were identified as *A.baumannii*. Sensitivity of 14 clinical isolates were tested against 14 antibiotics. and the result showed all isolate was MDR. All *Acinetobacter* isolates were investigated for the production of virulence factors which include the ability for the biofilms formation by microtiter plate method, and detection of efflux pumps. 92% of the isolates were biofilms former, and 85.7% of the isolates possess the efflux pumps. also investigate for *bap* gene responsible for biofilm formation.

**Keywords:** *Acinetobacter baumannii*, biofilm, biofilm associated protein (bap) gene, Efflux pumps.

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## Introduction:

*Acinetobacter* is a Gram-negative, stric aerobic cocobacillary non-ferment bacterium which has recently gained prominence as an opportunistic pathogen responsible for many hospital infections (Nosocomial infection).One of main concerns with Acinetobacter is its ability to stay in hospital allowing to contact with patient and causing sever outbreak like urinary tract infection ,wound and burn infections(1).These infections are difficult to treat due to the rapid emerge of multi- drug resistance strain ,therefore limiting drug options with most strain being resistant to clinically useful antibiotic such as; fluoroquinolones, aminoglycosides, β-lactames including carbapenems, trimethoprim-sulfamethoxazole (2).Its resistance mechanisms include the transformation of external membrane proteins (OMP), which is one of the most dangerous types of resistance because it is general by reducing the number and diameters of the channels in the membrane, as well as having Efflux pump systems that exclude antibiotic out the cell , also to have β-lactamase that analyze the penicillins, cephalosepoins and carbapenim (3). Antimicrobial resistance is also associated with its ability to form a biofilm, which is a collection of microbial cells linked to live or non-living surfaces by polysaccharides and proteins. This biofilm increases the difficulty of preventing and controlling infections(4). Some molecular mechanisms associated with phenotype have been studies ; genes associated with biofilm include *bap* gene biofilm associated protein(5). Bap is encoding by a large gene and contein repetitive modules and variable sequence, and play a significant role in intercellular adhesion and accumulation of bacterial cells as well as maintenance of biofilm(6).*Acinetobacter* Bap protein share similar characteristic with other members of Bap protein such as highly molecular weight, modular structure and cell surface location(7).

Efflux-mediated resistance has been found in many bacterial genera (8,9). Overexpression of an efflux system, responsible for reduction in the accumulation of the antibiotic. is an efficient mechanism for drug resistance (8). Five super families of efflux systems are associated with drug resistance: the ATP-binding cassette (ABC)transporters, the small multidrug resistance (SMR) and multidrug and toxic compound extrusion (MATE) families, the major facilitator superfamily (MFS), and the resistance-nodulation- cell division (RND) family. As opposed to single-component efflux systems that confer resistance to a small number of compounds, such as the tetracycline transporters, the RND systems, composed of an inner membrane protein (RND pump) linked by a major fusion protein (MFP) to an outer membrane factor (OMF), are able to extrude a wide range of substrates often unrelated in structure (9, 10,11). They are the most clinically relevant pumps conferring multidrug resistance in Gram-negative bacteria, since they allow crossing of both the inner and outer membranes(12).This study was aimed to study some of virulence factor in *Acinetobacter* isolate and detection of gene was responsible of biofilm formation.

## Material and Methods:

### 1. *Acinetobacter baumannii* isolation and characterization:

This study was conducted in Baquba General Teaching Hospital for the period from September 2016 to May 2017. isolation from wound, burn , urinary tract infections General Teaching Hospital.

### Isolation and identification of *Acinetobacter baumannii* :

In the laboratory under aseptic conditions, the collected specimens were streaked directly on blood agar and MacConkey agar, incubated for 24 hrs at 37ºC. The non hemolytic opaque creamy colonies on blood agar and non lactose fermenting colonies on MacConkey agar were subcultured on MacConkey agar and incubated for another 24 hrs at 37ºC (13). All bacterial isolates were examined for gram stainability and conventional biochemical tests which include: Oxidase test, Catalase test, Kligler iron agar (KIA), Indole production test, Motility test, Urease production test, Citrate utilization test, Lactose fermentation test, Hemolysin production, Growth at 44ºC according to (13), and confirm diagnosis by API-20E and use VITEK2 system.

### Antibiotic susceptibility test:

According to Kirby-Bauer method was dependent in antimicrobials susceptibility test for 14 different antimicrobial: trimethoprime-sulphamethoxazole (1.25/23.75µg), gentamicin (10µg), tetracycline (30µg), chloramphenicol (30µg), ciprofloxacin (5µg), gentamicin (10µg), imipenem (10µg), meropenem (10µg), cefepime (30µg), cefotaxime (30µg), amikacin (30µg), Oxacilin (1 µg), Piperacillin-tazobactam (100/10 µg), Ceftriaxone (30 µg). The result were interted according to the CLSI guidelines(14).

### Biofilm formation:

Biofilm formation is quantifed by the microtiter plate assay method as described by Cheristensen *et al* (15). Briefly,bacterial cell was grown overnight at 37 ° C in 10ml nutrient broth. Each well of 96-well flat

bottomed polystyrene plate (3 well for each isolate) was filled with 20µl of overnight culture. Sterile nutrient broth(180 µl) was add into each wall. Wells inoculated with sterile broth were used as negative control. The plate was covered with lid and incubated aerobically for 24 h at 37 ° C. after incubation the content of each well was removed and followed by washing three time by 0.2ml of phosphate buffer saline to remove free floating bacteria.after washing any remaining biofilm was stained with crystal violate 1%(w/v) for 10min , ecess stain was removed by washing with tap water after plate dried the dye was resolubilzed with 200ml ethanol and optical density (OD) was quantified at 570nm using ELIZA reader.

**Efflux pump:**

The simplest method for the demonstration of an over-expressed Efflux pump system of pathogenic bacteria involves the bacterial strain were grown in 5ml brain –heart broth at 37 °C. Tryyptic soy agar (oxoid) platees containing ethidum bromide (EB) ranging from 0 to 2.5mg/l were prepared on the same day of expreriment day and protected from light. The plates were then divided into sectors by radial lines. Culture were then swapped on EB agar plates starting from the center of the plate toward the edges . The plates are then incubated over-night at 37°C in dark and examined under UV light for evidence of pink fluorescence. The lowest concentration of EB that is associated with the presence of pink fluorescence is recorded (16).

**DNA extraction:**

The method was used in this study described by Chen and Kuo,1993 to isolate total DNA(17).

**Polymerase Chain Reaction (PCR) Techniques**:

Series of PCR reactions was preformed to detect for *bap* gene responsible for an attachment and biofilm maturation and the Forward and reverse primer pair that detecting 1449 bp fragments of bap gene chosen according to the method described by(18), and the sequences of primer pair used were bap

F- ATGCCTGAGATACAAATTAT
R- GTCAATCGTAAAGGTAACG

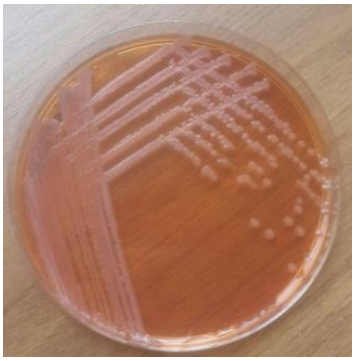
PCR mixture was prepared with 25 µl of Master Mix (promega), 17µl of nuclease free water, 2µl of each primer at 10 pmol/µl, and 4µl of DNA. Thermocycling conditions at which: Initial denaturation at 94°C for 5min followed by30 cycles of (Denaturation at94°C for1 min, Annealing at 55°C for 1 min and Extension at 72°C for 50sec) then Final extension at 72°C for 6min.

**Results and discussion:**

Fourteen isolates (9.3%) of *Acinetobacter* were recovered from 150 clinical different samples (urin, swab of wound and burn). All isolates appeared gram negative, coccbacili, and all isolate show negative results to Indole test, oxidase tset, and urease, and MR-VP and positive results to catalase and for citarate utilization test. And kilglar develop to alkane slant. *Acinetobacter* isolate when culture on MacConky agar appear small pink to lavender color colonies non lactose fermenter as Figure 1, while in Blood agar coloneis appears translucent to opaque, non-hemolytic non pigment. And can growth at $44^0$C This test was used to distinguished *A. baumannii* (which able to grow at this temperature) from other *Acinetobacter* species (19,20). And confirm diagnosis by API20 E system and by VITEK 2 system . The result of biochemical tests in Table:

**Table (1): biochemical test of *A. baumannii***

| ID | Test | Result |
|---|---|---|
| **1** | Lactose fermentation | - |
| **2** | Haemolysin Production | - |
| **3** | Growth in 44C° | + |
| **4** | Gram Stain | - |
| **5** | Oxidase | - |
| **6** | Catalase | + |
| **7** | Urease | - |
| **8** | Indol | - |
| **9** | Methyl red | - |
| **10** | Voges_ proskaure | - |
| **11** | Simmon citrate | + |
| **12** | Motility | - |



**Figure(1): *A.baumannii* on MacConky agar**

## Antibiotics Susceptibility:

Data presented in Figure 2 shows a high level resistance of *A. baumannii* clinical isolates to most of the antibiotics under test. The present study revealed that all *A. baumannii* clinical isolates had 100% resistance to cefotaxime,ceftriaxon, chloramphenicol, piperacillin,oxacillin. 91.7 to Ciproflaxacin and 71.4% to Pipracillin/ tozabactam and Amikacin and Trimetoprim/sulfamide. 85.7% to gentamicin and cefepem. 50% to meropenem. 43% to Imipenem.

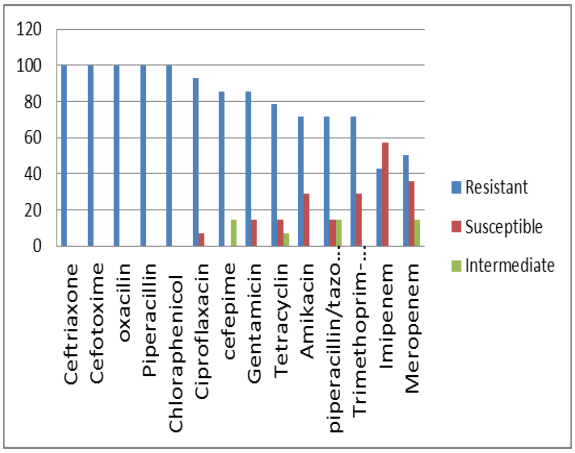



**Figure (2): Antibiotic resistance of 14 *Acinetobacter baumannii* clinical isolates.The resistance of *A.baumannii* .**

This result was very close to study in Iraq Al-Hussein *et al* (22) that found clinical A.baumannii isolate completely resistance to cefotaxime,cefepem a highest resistance to ceftriaxone (97.39%), chloramphenicol (95.65%), piperacillin (91.30%),ceftazidime (89.57%), gentamicin (87.83%), trimethoprime sulphamethoxazole (86.09%), ciprofloxacin (83.48%), amikacin (72.17%).and (58.26%) to both imipenem and meropenem. Results of another study carried out in 2010 found that *A. baumannii* clinical isolates developed 100% resistance to cefotaxime, ceftriaxone, 95.45% to cefepime, chloramphenicol, and 40.90% to imipenem(23). From previous local studies, noticed interestingly the increase of resistance to imipenem and other group of antibiotic in our hospitals. And these resistance return to to ability of this organism to horizontal acquire resistance determents, intrinsic resistance mechanism include: production of enzyme such as β-lactamases which hydrolyzing betalactam group and carbapenem. And aminoglycoside-modifying enzmes responsible for aminoglycosides resistances. And can resist antibiotic by alter pencilin- binding protein and porins, amd by efflux system amd grow as biofilm (24).

**Biofilm formation:**

The result of biofilm formation in micro titer method showed one isolate was cannot produce biofilm, 4 isolate formed weak biofilm, 4 isolate created moderately biofilm, 5 isolate were strong biofilm producer all isolate which formed biofilm were MDR. And this result was very close to this reported by Azize *et al* (25). Biofilm formation enhance persist in hospital environment and promote antibiotic resistance/ tolerance because its act as barrier prevent antibiotic prevention. The *bap* gene was investigated in 10 isolates. The results revealed that 9 isolates were carrying the *bap* gene at 90% as Figure 3. which is responsible for biofilm formation. This is perhaps the first research for detection of *bap* gene of *A.baumannii* in Iraq, and this result is very close with study in Australia Goh *et al* (26).that found 92% of isolated have *bap* gene while different from study in Iran(25) in which 66% of isolate have *bap* gene.and this different may return to type of specimen and the ability of clinical isolate to form biofilm.

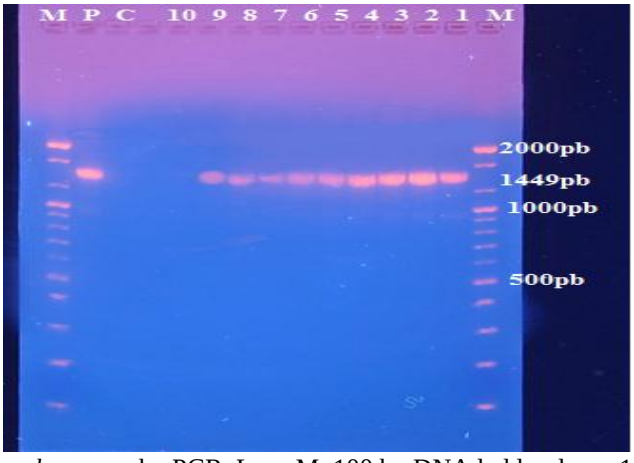



Figure (3): Detection *bap* gene by PCR. Lane M, 100 bp DNA ladder; lanes 1-10, *Acinetobacter baumannii* isolates; lane C, Negative control (had all PCR mixture including water instead of DNA template). Detection was done on agarose gel (1.5%) at 5 V/cm for 1.5 hour, stained with ethidiumbromide and visualized on a UV transiluminator documentation system.

## Efflux pump:

The EB-agar cartwheel method used for the identification of overexpressed efflux system showed efflux activity in 85.7 % of isolate. The minimum concentration of ethidium bromide at which strain with efflux activity showed fluorescence was 1mg/l. at this concentration *A.baumanniii* isolate were found to be fluoresce as Figure(4).

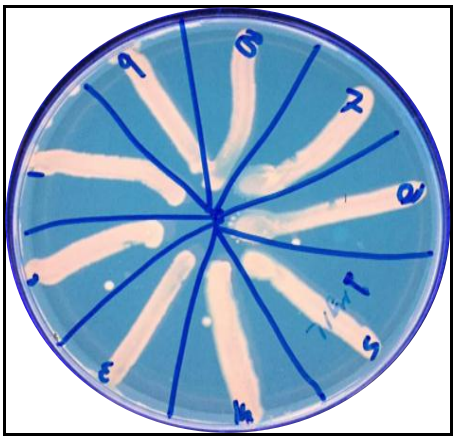


**Figure (4): Accumulation of fluorescent –Tryptic soy agar containing 1mg/l concentration of ethidium bromide,swapped with *A.baumanniii*.**

Efflux system play a key mechanism role in the development of drug resistance in Gram – negative bacteria, these pumps solute out of the cell, therapy allowing the microorgannisms to regulate their internal environment by removing toxic substance such as antimicrobial agent (27,28).there are a close relationship between efflux pump and MDR isolate, in this study found the isolate have efflux pump resistance to most of antibiotic group.

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