Virulence Determinants of Acinetobacter baumannii Isolated from Different Infections in Baghdad Hospitals

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Abstract :

Acinetobacter baumannii is an essential pathogenic bacteria cause nosocomial infections worldwide. The current work aimed to characterize several virulence factors in Acintobacter baumannii isolated from diverse clinical specimens in Baghdad. Clinical samples including sputum, blood, urine, CSF and wound swabs were collected from patients who were admitted to hospitals in Baghdad. Identification of Acinetobacter baumannii isolates was performed using Vitek 2 system. Phospholipase (plcN) and elastase (lasB) genes were identified using Polymerase chain reaction (PCR) technique. Other virulence factors such as motility, biofilm formation,hemolysin production were screened.

Sixty-nine isolates were identified as Acinetobacter baumannii. The isolates were screened for Phospholipase (plcN) and elastase (lasB) production.The results of amplification revealed that out of the total isolates, 18 (26.6%) isolates included both (plcN) and (lasB) genes, 18(26.6%) isolates included only (plcN) and 23(33.3%) isolates have only (lasB).

Sixty (86.9%) isolates were positive for biofilm formation, 8 (11.6%) isolates indicated intermediate biofilm producers and 52(75.4%) isolates were weak biofilm producers. Sixty six A.baumannii isolates were motile on Nutrient agar (0.3%). Sixteen isolates were intermediate motile and 50 were highly motile. Positive association was found between the biofilm formation capacity and surface associated motility.

Fifty-seven isolates showed hemolysis on Blood agar on the second day of the incubation. Of these, 48 (69.56%) isolates show β –hemolysis and 9 (13.04%) isolates show α-hemolysis.

Keywords: Acinetobacter baumannii, antibiotics resistance, virulence factors.

محددات الضراوة لبكتيريا *Acinetobacter baumannii* المعزولة من الإصابات المختلفة في مستشفيات بغداد

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*** قسم التقنيات الاحيائية كلية العلوم المساندة والتقنيات المتقدمة في علوم الحياة – جامعة العلوم والثقافة طهران- إيران*

الخلاصة:

تعد بكتيريا Acinetobacter baumannii من البكتيريا المسببة للأمراض الأساسية التي تسبب عدوى المستشفيات في جميع أنحاء العالم. يهدف العمل الحالي إلى توصيف العديد من عوامل الضراوة في Acintobacter baumannii المعزولة من مختلف العينات السريرية في بغداد.
تم جمع العينات السريرية بما في ذلك البلغم والدم والبول وسائل النخاع الشوكي ومسحات الجروح من المرضى الذين تم إدخالهم إلى مستشفيات بغداد.
شخصت عزلات Acinetobacter baumannii باستخدام نظام Vitek 2. تم التعرف على جينات الفوسفوليبيز (plcN) والايلاستيز (lasB) باستخدام تقنية تفاعل البلمرة المتسلسل (PCR). تم فحص عوامل الضراوة الأخرى مثل الحركة، وتشكيل البيوفيلم ، وإنتاج الهيمولايسين.
تم تشخيص تسعة وستين عزلة على أنها Acinetobacter baumannii. تم فحص العزلات لاحتوائها على جينات الفوسفوليبيز (plcN) والايلاستيز (lasB). أظهرت النتائج أنه من إجمالي العزلات, 18(26.6%) عزلة تضمنت جينات كلا من (plcN) و (lasB) و18(26.6%)عزلة متضمنة جين (plcN) و 23(33.3%)عزلة متضمنة جين (lasB).
ستين عزلة (86.9٪) كانت موجبة لتكوين الأغشية الحيوية، بينما أشارت 8 عزلات (11.6٪) إلى تكوين وسيط للغشاء الحيوي و 52 عزلة (75.4٪) كانت منتجة ضعيفة للغشاء الحيوي، 66 عزلة A.baumannii متحركة على (% Nutrient 0.3) a gar (16) عزلة كانت متوسطة الحركة و 50 عزلة عالية الحركة. تم العثور على ارتباط إيجابي بين قدرة تكوين الأغشية الحيوية والحركة السطحية المرتبطة.
أظهرت 57 عزلة انحلال دم على أجار الدم في اليوم الثاني من الحضانة. من بين هذه العزلات ، أظهرت 48 عزلة (69.56٪) انحلال الدم و 9 عزلات (13.04٪) أظهرت انحلال الدم ألفا.

الكلمات المفتاحية:Acinetobacter baumannii, مقاومة المضادات الحياتية,عوامل الضراوة

Introduction

Acinetobacter baumannii is a significant pathogenic bacteria cause nosocomial infections worldwide (1). This pathogen is responsible for opportunistic infections (2).
A. baumannii have virulence factors that responsible for their survival, pathogenicity and antibiotics resistance (3, 4). Virulence factors induces bacterial attachment, colonization and host cells invasion (5, 6). Enzymes and toxins production, Biofilm formation and motility are essential factors that contribute to A. baumannii pathogenicity and survival in different environments.
It has been reported that mutation of virulence factors related genes displayed a diminished of bacterial virulence.
Several enzymes and toxins are known as a virulence factor of A. baumannii includes: Phospholipases, elastase and hemolysin. Phospholipases (PLs) are a various set of enzymes that cleaves phospholipid ester bonds. They are divided into three categories depend on the cleavage site; phospholipase A (PLA), phospholipase C (PLC), and phospholipase D (PLD). Phospholipase A cleaves fatty acids from the glycerol frame, while phospholipase C hydrolyzes the phosphorylated base group. Phospholipase D remove the head group. Phospholipases (PLs) are produced from different pathogenic bacteria causing various infectious diseases. They are considered as important virulence factors contributing to bacterial persistence or spreading without causing host cells damage. In addition, the potential role of PLs as virulence factors is to change host immune response and effect on host cellular signaling. (7, 8) Phospholipase enzymes are essential virulence features in A. baumannii pathogenesis. A. baumannii has phospholipase C (PLC), and phospholipase D (PLD) (9, 10).
Elastases are another essential virulence factors that can damage elastin and contribute to the damage of host defense or tissue in a variety of means (11) This enzyme expressed by the lasB gene, which was found in A.

baumannii isolates, which can breakdown elastin and lead to tissue damage.

The expression of adhesion factors results in bacterial attachment, a key step towards infection (12,13) and biofilm formation. Biofilms exhibit a good defense against host immune system, antimicrobials, and different un- bearable conditions than the planktonic cells (14). It has been reported that 65–80% of infectious disease are produced via biofilm- producing pathogens (15).

Another virulence factor that may aids in the pathogenicity of A. baumannii is motility. Though, A. baumannii was known as non-motile due to the lack of flagella, it is capable to move in two means: twitching motility and surface-associated motility. It has been revealed that the twitching motility is based on type IV pili (16, 17). For Surface-associated motility in A. baumannii, it has been shown that different factors affect it, such as light (18), quorum sensing (19), iron availability (20,21) production of lipopolysaccharides (22) and at least to five of the six identified efflux pump superfamilies'(23).

In literatures, it has been reported that a rise in bacterial motility is related to an increase in bacterial infection (24). However, A. baumannii mutant's incapable to motile displayed an diminished virulence (25, 23). This study aimed to isolate A. baumannii from diverse clinical samples and detect several virulence factors, which induce pathogenicity and related to bacterial survival.

Methods

1- Isolation and Identification of A. baumannii isolates.

Five hundred of various clinical samples involve sputum, blood, wound swabs, cerebrospinal fluid (CSF) and urine were collected from patients of (ALImamain AL-Kadhimein Medical City /bacteriology laboratory, Baghdad Teaching Hospital, Nu'man Teaching Hospital and Child Central Teaching Hospital) in Baghdad.

Bacterial identification was done using the VITEK-2 compact system (26).

2-Detection of IasB and PlcN genes

Genomic DNA was extracted from A. baumannii isolates using (ZR Fungal/Yeast/Bacterial DNA MiniPrep Kit (ZYMO). The extraction process was done according to the manufacture's instructions.

The phospholipase (plcN) and elastase (lasB) genes were detected using specific primers (Table 1). To amplify IasB and PlcN genes, 25 µl of PCR mixture was used as shown in table 2. The amplification conditions are shown in table 3.

Table1: Primers pairs used for the amplification of IasB and PlcN genes

Primer description	Sequence
IasB gene Forward	5'-GGAATGAACGAAGCGTTCTC - 3'
IasB gene Reverse	5'-GGTCCAGTAGTAGCGGTTGG - 3'
PlcN gene Forward	5'-GTTATCGCAACCAGCCCTAC - 3'
PlcN gene Reverse	5'-AGGTCGAACACCTGGAACAC - 3'

Table 2: Mixture of the specific interaction for diagnosis gene

Components	Concentration
Taq PCR PreMix	5µl
Forward primer	10 picomols/µl (1 µl)

Reverse primer	10 picomols/µl (1 µl)
DNA	1.5µl
Distill water	16.5 µl
Final volume	25µl

Table 3: The optimum conditions of detection gene

No.	Phase	Tm (°C)	Time	No. of cycle
1-	Initial Denaturation	95°C	10 min.	1 cycle
2-	Denaturation -2	95°C	30 sec	35 cycles
3-	Annealing	55°C	30 sec	
4-	Extension-1	72°C	30 sec	
5-	Extension -2	72°C	10 min.	1 cycle

3-Biofilm detection assay (Tissue culture plate method)

A.baumannii was grown at 37º C for 24 hours in 10 mL Trypticase soy broth (TSB). 20 µL of the overnight culture was added to each well of a 96-well flat-bottomed polystyrene tissue culture plate (3 wells for each strain). 180 µL of sterile TSB medium was added in to each well. TSB broth was used as a negative control. The plates were covered and incubated at 37ºC for 24 hours. Then, the content of wells was removed and the wells were washed 3X with sterile phosphate buffer saline (pH 7.2).200 µL of methanol was added to each well for 15 minutes. The plates were poured and left to dry. Then, 200 µL of 2% crystal violet was added for 7 minutes. Plates were washed carefully with tap water and left to dry. To soluble the adherent cells,160 µL of 33% glacial acetic acid was added to each well. The optical density (OD) was measured at 630 nm using ELISA reader.

Average OD value Biofilm production

OD ≤ ODc Non-adherent

ODc < OD ≤ 2 x ODc Weakly adherent

2 x ODc < OD ≤ 4 x ODc Moderately adherent

4 x ODc < OD Strongly adherent

Optical density cut-off value (ODc) = average OD of negative control + 3x standard deviation (SD) of negative control (28).

4-Motility assay

To investigate bacterial motility, a pure colony of A. baumannii was grown in N. broth at 37°C overnight. The bacterial suspension turbidity was adjusted to 0.5 McFarland. Ten microliters of bacterial suspension were inoculated on the center of Nutrient agar plates of 0.3%. The plates were incubated at 37°C for 24 hours. The growth area was measured using ruler. The bacterial motility was characterized depend on the criteria defined previously. These criteria involved (1) negative (-) if the growth area diameter was <5 mm; (2) intermediate (+) if the growth area diameter was 5-20 mm; and (3) highly motile (++) if the growth area diameter was >20 mm (28).

5-Hemolysin production

To investigate hemolytic activity, a pure colony of bacterial isolates was grown in N.broth at 37°C overnight. The suspension turbidity was adjusted to 0.5 McFarland. Ten microliters of bacterial suspension were inoculated on 5% blood agar plates. The plates were then incubated at 37°C for 6 days (28)

Results

Sixty-nine isolates were identified as Acinetobacter baumannii. The isolates were screened for Phospholipase (plcN) and elastase (lasB) production using PCR technique. The PCR products were tested by agarose gel electrophoresis. The results shown the presence of the expected bands. A band size of 466 bp for plcN (Figure 1), and of 300 bp for lasB gene (Figure 2). The results of amplification revealed that out of the total isolates,18 (26.6%) isolates included both (plcN) and (lasB) genes, 18(126.6%) isolates included only (plcN) and 23 (33.3%) isolates have only (lasB).

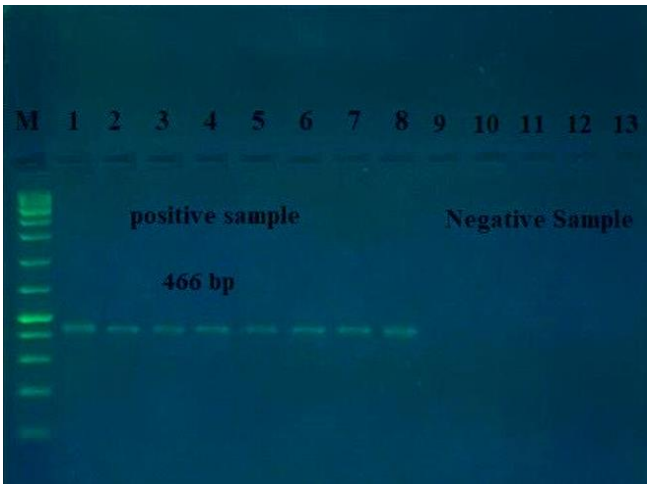

Figure 1: Detection of the phospholipase (plcN) of the A. baumannii isolates. The figure shows results of the gel electrophoresis for detection of the phospholipase (plcN) of the A. baumannii isolates. Lane M: DNA Marker.Lane 1-8: plcN(466 bp).

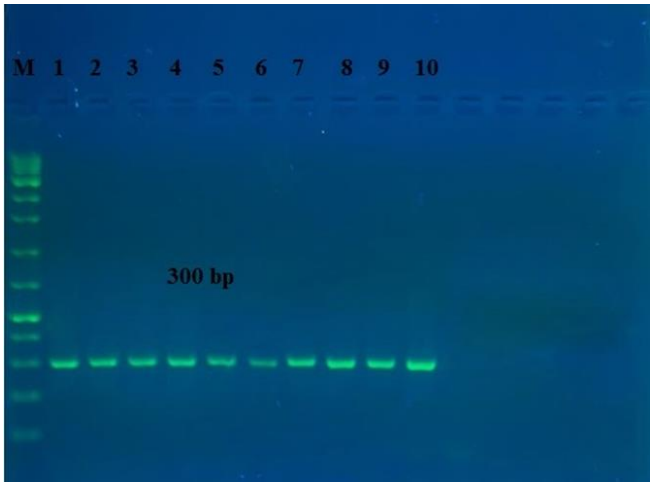

Figure 2: Detection of elastase (lasB) of the A. baumannii isolates. The figure shows the results of the gel electrophoresis for detection of elastase (lasB) of the A. baumannii isolates Lane M: DNA Marker.Lane 1-10: lasB gene (300pb).

Sixty (86.9%) isolates were positive for biofilm formation, 8 (11.6%) isolates indicated intermediate biofilm producers and 52(75.4%) isolates were weak biofilm producers. 66 A.baumannii isolates were motile on Nutrient a gar (0.3%). 50 isolates were highly motile and 16 were intermediate. The data of correlation revealed a significant correlation between motility and biofilm formation among A. baumannii isolates (Correlation coefficient =0.69, P<0.0001).

Fifty-seven isolates showed hemolysis on Blood agar on the second day of the incubation. Of these, 48 (69.56%) isolates show β –hemolysis and 9 (13.04%) isolates show α-hemolysis.

Discussion

Recently, studies have stated that A. baumannii is a nosocomial pathogenic bacterium that spreads in hospitals. The importance of A. baumannii depend on their persistence in clinics and the resistance to the available antibiotics (29, 30,31). In addition to the presence of different virulence factors (32).

The results of amplification shown that out of the total isolates,18 (26.6%) isolates included both (plcN) and (lasB) gene, 18(26.6%) isolates included only (plcN) and 23 (33.3%) isolates have only (lasB). Kareem et al., showed that (23.3%) of the Iraqi A. baumannii isolates were positive by PCR for plcN and (53.3%) for lasB (33). Aliramezani et.al. showed that of 100 A. baumannii isolates, 20 isolates included plcN and four isolates included lasB (29) .

Biofilm is a critical virulence factor contributes to A. baumannii infections. The capability of A. baumannii to produce biofilm facilitates its survival in different environments (33, 34), and reduced susceptibility to antibiotics (36,37, 26).

In A. baumannii, several determinants regulate the biofilm-forming ability. Among them, the biofilm-associated protein expressed by the bap gene (38, 39). The outer membrane protein A (OmpA) also play a significant role in biofilm formation (39). In addition to blaPER-1 gene. It has been reported that blaPER-1 gene contributes in A. baumannii adherence and biofilm forming (41, 42). Pili also contributes to A. baumannii biofilms formation and bacterial attachment—the chaperon/usher system required for the assembly and production of pili. The genes are clustered together in the form of a csu operon, they encode for a pilus-like bundle structure in A. baumannii (43). Therefore, the csuE gene has showed to be an essential factor in A. baumannii biofilm formation (44).

In the present study, 60 (86.9%) isolates were positive for biofilm formation, 8 (11.6%) isolates indicated intermediate biofilm producers and 52(75.4%) isolates were weak biofilm producers. A study reported that 60% A. baumannii isolates produced biofilm (45). The results of the current study demonstrated that 66 A.baumannii isolates were motile (surface associated motility). Motility ability was detected by measuring the diameter of bacterial movement across the nutrient a gar (0.3%) plates. We found only three isolates isolated from blood were none motile. However, all other isolates were motile. Various studies have revealed the presence of the surface associated motility in A. baumannii (46, 47). It has been shown that surface-associated motility of A. baumannii ATCC 17978] was related to rec A protein (48), the CheA/Y-like protein A1S_2811(49) and CheW-like protein, constituents of a hypothetical chemotactic signal transduction system(48).

Surface associated motility is affected by different factors such as experimental conditions, origin of clinical isolates and environmental factors. In clinical A. baumannii isolates, the surface-associated

motility is common and depend on 1,3-diaminopropane (DAP)(46). Another study revealed that A. baumannii motility based on the source of clinical isolates. Vijayakumar et al., shown that the bacterial isolates isolated from blood samples were more motile than the bacteria isolated from sputum (49).

Based on our results, the data of correlation shown a significant correlation between motility and biofilm formation among A. baumannii isolates. Previous studies provide evidence of the association between the biofilm formation capacity and surface associated motility in A. baumannii (50, 51).

Conclusions

Multiple virulence factors might be expressed by A. baumannii isolates. In the current work different virulence factors, such as motility, biofilm, hemolysin, Elastase, and phospholipase were detected among A. baumannii isolates. A significant correlation between biofilm formation and motility has been found.

The findings of the current study showed that multivirulent A. baumannii isolates might cause serious infections.

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