Study of Virulence Genes in *Acintobacter baumannii* Isolated from Pediatric Patients with Hospital Acquired Bacteremia

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Abstract

Background:*Acintobacter baumannii* (*A. baumannii*) associated with hospital acquired sepsis in pediatric patients represent health care problem. The multidrug resistance of *A. baumanni* is a risk factor associated with this organism.

Objective: The present study aims were to i. study the presence of virulence factors of *A. baumannii* isolated from bacteremia from children in ICUs, ii. Study antibiotics resistance of isolated *A.baumannii* regarding ESBL and carbapenemase, iii. Study the association between the virulence factors, extended-spectrum-*β*-lactamase(ESBL) and crabapenemase activity.

Method: The study was a retrograde cross-sectional study that included one hundred children with hospital acquired bacteremia admitted to intensive care units. Theisolates were subjected to polymerase chain reaction (PCR) to detect virulence genes bap (biofilm-associated protein), phospholipase (plcN), elastase (lasB), biofilm-associated protein**(bap) and**outer membrane protein A**(Omp) genes. The sensitivity to antibiotics was determined by discs diffusion method, ESBL and crabapenemase activity was determined by double discs diffusion and combined disc diffusion method respectively.**

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Results: The isolated *A. baumanni* were ESBL producers (40%) and carbapenemase producers (96%). The isolates of *Acintobacter baumannii* had high frequency of bap and OmpA genes (95%, 70% respectively) and lower frequency of picN and laspB genes (26%, 8% respectively).The *A. baumannii* isolates with ESBL activity had significant association with OmpA genes (90%, P=0.001) and 57.5% of the isolates had significant association with picN gene (P=0.001). There was significant association between MDR of *A. baumannii* and the presence of OmpA gene and ***plcN*** gene (P=0.048, P=0.001 respectively**.**

The present study highlight the presence of virulence genes bap, OmpA, picN and laspB in clinical isolates of *A. baumannii* isolated from pediatric patients with sepsis. Almost all the isolates had bap gene and more than half isolates have OmpA gene. The ESBL resistance and multi- drug resistance of *A. baumannii* was significantly associated with the presence of OmpA and picN genes.

 Keywords: ***Acintobacter baumannii*, ESBL, pediatric patients, sepsis, virulence genes.**

Introduction

Acinetobacter baumannii (A.baumannii) is a Gram negative bacilli associated with hospital acquired infections. There are several infections linked to *Acinetobacter baumannii* such as pneumonia (1), bacteremia (2), cystitis (3), and meningitis (4). Risk factors for invasive infection with *A. baumannii* include prior hospitalization, underlying etiology of the disease, previous infection an antimicrobial therapy, and the presence of central venous or urinary catheters (5).

The major threat associated with *Acinetobacter baumannii* is marked antibiotics resistance pattern due to broad spectrum antibiotics misuse (6, 7).The common antibiotics resistance of *Acintobacter baumannii* is due to horizontal gene transfer that leads to resistance of β-lactams antibiotics (8). Moreover, the presence of

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adaptive genes in the genome of *Acintobacter baumannii* leads to its prolonged persistence in the hospital environment (9, 10).

The mechanisms of antibiotics resistance of *Acinetobacter* spp. are mediated through different mechanisms such as the production of β-lactamases, alterations in outer membrane proteins, production of penicillin-binding proteins, and enhanced activity of efflux pumps (11). The β-lactamases in*Acintobacter* spp. includeextended-spectrum-β-lactamases (ESBLs), metallo-β-lactamases (MBLs), and oxacillinases (12).

The pathogenesis of *Acintobacter baumannii* depends upon the expression of various virulence factors that play a vital role in adherence of the organism to the host cells. These virulence factors are bap (biofilm-associated protein), OmpA (outer membrane protein A), phospholipaseD, the Csu (chaperone-usher type pilus), the Acinetobacter trimeric autotransporter (Ata), and the acinetobactin-mediated iron acquisition system (13-15).

The virulence genes mediate their actions principally through adherence to the host cells. The bacterial cell surface expressing OmpA leads to cytotoxicity by binding to cell surface death receptors (16).The translocation of OmpA in the mitochondria leads proapoptotic signals, through the activation of Bcl-2 family proteins, the release of cytochrome C and apoptosis-inducing factor (17). OmpA can also be translocated to the nucleus courtesy of its self-encoded nuclear localization signal (KTKEGRAMNRR) leading to the degradation of the host DNA in a DNase I-like manner (18-20). In addition to its cytotoxic properties, OmpA is associated with other virulence attributes, including resistance to alternate complement-mediated killing through factor H binding and promoting adhesion to extracellular matrix proteins, including fibronectin, which is important for lung epithelial colonization (21, 22).

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The Bap virulence factor promotes cell adhesion, biofilm formation (23, 24). Csu Type 1 chaperone-usher pili also play a vital role in adhesion and biofilm formation which establish persistence of *Acintobacter baumannii* in hospital environment and leads to difficulty in eradication of the infections (23, 24).

The phospholipases aids in theinvasion of*Acintobacter baumanni*. The trimeric autotransporter adhesin Ata are known as important virulence factor that regulate adhesion, the formation of biofilm, immune evasion, angiogenesis or cell death (25-28).

There are limited studies about infections of pediatric patients in intensive care units (ICUs) with *Acintobacter baumannii* and the risk factors associated with this infection, antibiotics resistance and virulence genes.

Therefore, the present study aims were to i. study the presence of virulence factors of *A. baumannii* isolated from bacteremia from children in ICUs, ii. Study antibiotics resistance of isolated *A.baumannii* regarding ESBL and carbapenemase, iii. Study the association between the virulence factors, ESBL and crabapenemase activity.

Material and Method

The study was a retrograde cross-sectional study that included children with hospital acquired bacteremia admitted to ICUs at Mansoura University Hospital from January 2020 till January 2022. The diagnosis of hospital acquired bacteremia was based upon the criteria of center of diseases control (CDC) criteria (29). The included children were with age below 18 years admitted to hospital with duration $\geq$ 48 hours with positive blood culture for *Actinobacter baumannii*. The study was approved by Mansoura Faculty of Medicine ethical committee-(R.22.09.1851) and the study was performed according to Helsinki guidelines.

Medical history was taken for each child and clinical examination was performed.

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Blood Culture

Eight militer blood samples were obtained from each child under complete sterile conditions. Blood samples were subjected to automated blood culture by Bact/alert system (Biomerieux-USA). The positive blood culture bottles were subjected to subculture on blood agar, and chocolate agar and incubated for 24 hours at 37ºC. The sub cultured colonies were identified by Gram stain and Gram negative bacilli were further identified by biochemical reaction by API 20 E. The final confirmation was performed by detection of bla OXA-51 carbapenemase gene by PCR (29).

Antibiotics susceptibility by Disc Diffusion Method

The sensitivity of*Acintobacter baumannii*to antibiotics was determined by the use of antibiotics discs diffusion method according to Clinical and laboratory Standard Institute (CLSI) guidelines (30). The antibiotics discs were ceftazidime (30 μg), cefepime (30 μg), imipenem (10 μg), meropenem (10 μg), gentamicin (10 μg), amikacin (30 μg), sulfamethoxazole/trimethoprim (1.25/23.75 μg), and piperacillin/tazobactam (100 μg/10 μg) and cefotaxime (30).

Combined Discs Test for Detection of carbapenemase

Isolates resistant to imipenem and/ or meropenem were further studied by double disc containing imipenem and EDTA. *Acintobacter baumannii* isolates were diluted with Muller-Hinton broth to obtain optical density equal to 0.5 McFarland opacity standard and sub cultured on Mueller-Hinton agar and two 10 μg imipenem discs were placed on the culture plate with 20 mm distance from center to center of the discs and 10 μl of 0.5 Methylenediaminetetraacetic acid(EDTA)was added to one of the imipenem discs. The plate was incubated at 37ºC for 18 hours. The increase in the sensitivity diameter $\geq$7 mm around the disc of imipenem-EDTA disc compared to the imipenem disc were considered as carbapenemase

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producers (30).

Detection of ESBL Producing Isolates

The detection of ESBLs production was performed according to CSLI guidelines by double discs diffusion method. Isolates resistant to ceftazidime and / or cefotaxime were diluted with Muller-Hinton broth to obtain optical density equal to 0.5 McFarland opacity standard and sub cultured on two Mueller-Hinton agar plates and on one plate double discs were added one cetazidime disc and the other disc was cetazidime with clavulinic acid(30/10 μg) on the other plate two discs were added one disc of cefotaxime acid and the other disc was cefotaxime (30 μg) + clavulanic (10 μg) , then the plates were incubated at 37ºC for 18 hours. The increase of the inhibition zone t ≥5 mm around the ceftazidime/clavulanic acid disc and cefotaxime/clavulanic disc compared to ceftazidime and cefotaxime was defined asa ESBLs producing isolate (30).

Polymerase Chain Reaction for Detection of Virulence Genes

DNA Extraction

Colonies from *Acintobacter baumannii* were used for DNA extraction by ready to use QIAamp DNA Micro Kit (Qiagen –Germany). The extraction was performed according to the manufacturer instruction. The extracted DNA was kept frozen at -20ºC till amplification.

Molecular detection of bap (biofilm-associated protein), phospholipase (*plc*N) and elastase (*las*B) and*bap* and Omp genes

The used primers for the amplification were listed in table 1. The amplification was performed by the use of Qiagen amplification mixture with 20 µL of master mix containing with added 2 microns of extracted DNA and 10 pM reverse and forward primers (1 µL each). The amplification procedures included denaturation at 95ºC for 10 minutes, followed by 35 cycles' 0f heating at 95ºC for 30 seconds then 55ºC

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for 30 seconds and 72ºC for 30 seconds. The final step was elongation at 72°C for 10 minutes. The amplified products were subjected to electrophoresis at 1.5 gel with ethidium bromide for 20 minutes (31).

Table (1): The sequences of the used primers and the amplified products base pair(bp)

Gene	Primers	bp
$bla_{oxa-51-like}$	5'-TAATGCTTTGATCGGCCTTG-3' 5'-TGGATTGCACTTCATCTTGG-3'	353
bap	5'-ATGCCTGAGATACAAATTATTGCCAAGGATAATC-3' 5'-AGGTGCTGAAGAATCATCATCATTAC-3'	**560**
plcN	5'-GTTATCGCAACCAGCCCTAC-3' 5'-AGGTCGAACACCTGGAACAC-3'	466
lasB	**5'-GGAATGAACGAAGCGTTCTC-3'** **5'-GGTCCAGTAGTAGCGGTTGG-3'**	300
OmpA	**TGGTCACTTGAAGCCTGCTG** **TGCAGTAGCGTTAGGGTATTCAG**	**208**

Statistical Analysis

 The data of the study was analyzed by SPPS22. The qualitative data was expressed as number and percentages. The comparison was performed by Chi-square test and P was considered significant if <0.05. The numerical data was expressed as mean and standard deviation for parametric data and expressed as minimum, maximum and median if it was non parametric.

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Results

The study included 100 pediatric patients with hospital acquired bacteremia associated with *A. baumannii*. *Acintobacter baumannii* was identified by biochemical reactions and PCR. The age of the patients were from 1.00 month up to 192.00 months with median age 24 months. The patients were 51% males and 49% females.The hospital used devices 'were urinary tract catheter, peripheral venous catheter and central venous catheter in 84%, 78% and 27% of the patients respectively. The common signs were fever, bradycardia and hypothermia in 40%, 48% and 26% respectively, table 2.

The isolated *A. baumanni* were ESBL producers (40%) and carbapenemase producers (96%). The isolates had high resistance to sulfamethoxazole/trimethoprim (46%), imipenem (96%), meropenem (95%), amikacin (52%), gentamicin (55%) cefotaxime (42%), ceftazidime (38%) and cefepime (34%). MDR resistance *A. baumannii* with resistance≥ three antibiotics classes, were 55%, table 3

The isolates of *Acintobacter baumannii* had high frequency of bap and OmpA genes (95%, 70% respectively) and lower frequency of picN and laspB genes (26%, 8% respectively), table 4.

The *A. baumannii* isolates with ESBL activity had significant association with OmpA genes (90%, P=0.001) and 57.5% of the isolates had significant association with picN gene (P=0.001).), table 5.

There was significant association between MDR of A. baumannii and the presence of OmpA gene and ***plcN gene* (P=0.048, P=0.001 respectively, table 6.**

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Table (2): Demographic and clinical data of the studied patients

Parameter	
Sex	
Male	51%
Female	49%
Age –months	
Minimum	1.00
Maximum	192.00
Median	24.00
Central venous catheter (CVC)	27%
Urinary catheter	84%
Peripheral venous cannula	78%
Fever	40%
Hypothermia	26%
Apnea	25%
Bradycardia	46%
Oliguria	13%

Table (3): Antibiotics resistance of isolated *A. baumannii*

Antibiotics	%
ceftazidime	38
cefepime	34
imipenem	96
meropenem	95
gentamicin	55

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amikacin	52
sulfamethoxazole/trimethoprim	46
piperacillin/tazobactam	28
Cefotaxime	42
Multidrug resistance	55
ESBL	40
Carbapenemase	96

Table (4): Virulence genes detected in isolated *A. baumannii*

Gene	%
bap	95
OmpA	70
plcN	26
lasB	8

Table (5): Association of virulence genes, ESBLs and carabamanse in *A. baumannii*

	ESBL		Carbapenemase	
	Positive (n=40) No. %	**Negative (n=60)** No. %	**Positive (n=96)** No. %	**Negative (n=4)** No. %
Bap **Positive** **Negative** **P**	40 100% 0 0% 0.06	55 91.7% 5 8.3%	91 5 0.64	4 0
OmpA **Positive** **Negative** **P**	36 90% 4 10% 0.001	34 56.7% 26 43.3 %	67 29 0.82	3 1

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plcN						
Positive	**23**	**57.5%**	**3**	**5%**	**24**	**2**
Negative	**17**	**42.5%**	**57**	**95 %**	**72**	**2**
P	**=0.001**				**0.25**	
lasB						
Positive	**3**	**7.5%**	**5**	**8.3%**	**8**	**0**
Negative	**37**	**92.5%**	**55**	**91.7%**	**88**	**4**
P	**0.9**				**0.55**	

Table (6): Association of virulence genes and MDR resistance in A. baumannii

	MDR (n=55)		Non MDR (n=45)	
	No.	%	No.	%
Bap				
Positive	53	96.4%	42	93.3%
Negative	2	3.6%	3	6.7%
P	0.5			
OmpA				
Positive	43	78.2%	27	60%
Negative	12	21.8%	18	40%
P	0.048			
plcN				
Positive	23	41.8%	3	6.7%
Negative	32	58.2%	42	93.3%
P	0.001			
lasB				
Positive	4	7.3%	4	8.9%
Negative	51	92.7%	41	91.1%
P	0.8			

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Discussion

Health care associated infections represent major health problem with safety risk of hospitalized children. These infections increase morbidity mortality and prolonged hospitalization with higher health costs (32, 33).

In the present study among 100 pediatric patients with bacteremia, the urinary catheter and CVC were common devices used in pediatric patients. This finding was online with previous reports, these devices were predisposing factors for HAIs among pediatric patients (34, 35). Previous study from United states of America revealed improvement in infections associated with urinary catheter while the improvement was not reported for central venous catheter associated infections, reflecting the need for novel approach for prevention measures (36). There is a need for adherence to the central catheter insertion and maintenance bundles (37). Moreover, there may be a need for different approach in maintenance of central venous catheter in children such as the use of ethanol locks, inclusion of oral care to reduce the risk of translocation from mucositis and inclusion of new criteria for surveillance of central venous catheter bacteremia after 48 hours of admission (38).For prevention of urinary catheter associated infections strict adherence infection prevention bundle is needed with extending beyond aseptic technique and emphasizing appropriate indications for placement and timely removal of indwelling urinary catheters (39).

In the present study, the use of peripheral venous cannula was detected in 78% of the infected pediatrics. There are well known complications associated with peripheral venous cannula such as hematoma, phlebitis, extravasation and bruising (40). Systemic review claimed that the blood stream infections might be a possible complication for prolonged use of peripheral cannula (41). Previous study determined blood stream infections association with insertion of peripheral cannula and this infection may be attributed to catheter-dressing disruptions and proximal insertion of the cannula as predisposing factors to these infections (43).

Infections by *Acinobacter baumanii* had increased due to use of medical devices and improper use of antibiotics a (43).

The isolates of *A. baumannii*had high resistance tosulfamethoxazole/trimethoprim

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(46%), imipenem, meropenem, cefotaxime and cefepime amikacin,gentamicin and multi drug resistance was detected in 45% of isolated *A.baumannii*.

The resistance rates of *A. baumannii* varied between different studies from 31.8 to 92.1% to ceftazidime; 8.8 to 89.9% vs imipenem, from 12.2 to 89.9% vs piperacillin / tazobactam, from 28.8 to 91.6% vs fluoroquinolones and 30 to 90.3% vs aminoglycosides (44-46). This variation of resistance rates can be attributed to the difference in the antibiotics prescription policy.

The isolated *A. baumanni* were ESBL producers (40%) and carbapenemase producers (96%). The rates of ESBL production in *A.baumannii* ranged from 22% up to 59% % (47, 48). Similar rate of carbapenemase production was reported previously from Egypt (97.5%) (49).

The differences in the reporting frequency may be attributed to the difficulty of ESBL identification in *Acinetobacter* spp. due to the lack of the standard protocols (50).

Other hazard associated with *A. baumannii* is the presence of virulence genessuch as bap and omp (51).

In the present study, more than half isolates of *Acintobacter baumannii* had high frequency of bap and OmpA genes and lower frequency was reported for picN and laspB genes. These rates were similar to previous studies (52. 53).

In general, the increasing number of *A. baumannii* infections due to the bacterial persistence in hospital environments and acquiring of virulence factors by the bacteria are major health concerns (53).

In the present study, there was significant association between OmpA and picN genes and ESBL activity. Moreover, there was significant association between MDR of isolated *A. baumannii* and both genes. The exact mechanism of association between OmpA gene and antibiotics resistance may be attributed to extrusion of antibiotics from the bacterial cells through outer membrane and couples with inner membrane efflux systems, such as major facilitator superfamily efflux pumps or RND systems lacking the OMP component (54). The other mechanism that may be associated with virulence genes leading to antibiotics resistance is biofilm formation. The biofilm formation leads to decrease of antibiotics diffusion, slow rate of bacterial growth and adaptive mechanisms to

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antibiotics by the formed biofilm (55).

 The finding of such association between ESBL and virulence genes may suggest the use of anti-virulence agents for management of antibiotics resistant infections associated with A. baumannii (56).

Conclusion

The present study highlight the presence of virulence genes bap, OmpA, picN and laspB in clinical isolates of *A. baumannii* isolated from pediatric patients with sepsis. Almost all the isolates had bap gene and more than half isolates have OmpA gene. The ESBL resistance and multi- drug resistance of *A. baumannii* was significantly associated with the presence of OmpA and picN genes.

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