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Acinetobacter baumannii virulence is enhanced by the combined presence of virulence factors genes phospholipase C (*plc*N) and elastase (*las*B)

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ABSTRACT

The ability of multidrug resistance *Acinetobacter baumannii* to persist in any circumstances regard to the acquisition of many virulence factors genes and antibiotic resistance genes is major concern in the hospitals environments. In this study, thirty *A. baumannii* isolates were collected from blood infections from hospitalized patients were subjected to screening for virulence factors genes *plcN* and *lasB* by conventional PCR. The pathogenicity of representative isolates bearing these gene were tested using *galleria mellonella* infection assay and adhesion-invasion assay on A549 cell line, and compared with other strain without this gene. Phylogenetic tree revealed that isolates were sorted in two major groups one of them contained two clusters (Group II and III), and another had the other group (Group I). All the 30 *A. baumannii* isolates were investigated for the presence of virulence factors genes (*plc-N* and *lasB* genes) and results showed that, 16 (53.33%) were harboring *lasB* genes while 7 (23.3%) isolates were harboring *plcN* gene The presence of any of these gene enhance the killing ability of *A. baumannii* strain and increased invasiveness in A549 cell line. Increase nosocomial infection with *A. baumannii* isolates is serious problem especially because of its potency to gain virulence factors genes and its ability to persist in hospital environments. So the shed light in finding which virulence factors these isolates which have is necessary to discover new antimicrobials that targeting the virulence factor of these powerful pathogens.

1. Introduction

Acinetobacter baumannii consider the most versatile nosocomial pathogen easily adapt to any situation and threat the life of hospitalized patients with its enhanced capacity to gain resistant determinants through gene transfer [1]. This bacterial species is now considered most important and problematic human pathogen which is responsible of several types of infections including meningitis pneumonia, septicemia, and urinary tract infections [2], and considered one of the six of the most important multidrug-resistant microorganisms in hospitals worldwide. The major concern to infections caused by *A. baumanni* is due to its rapid development of resistance power towards a wide range of antimicrobials, rapid profundity in transformation, surviving desiccation and persisting in the environment for a very long time [3]. Though it is emerged as most problematic pathogen of considerable health care interest, there is a little knowledge about the virulence's factors that responsible of the organism's ability to cause disease [4]. In comparison with other Gram-negative pathogens, The relatively few virulence factors have been recognized in *A. baumannii* [2], including *OmpA*, Lipopolysaccharide (*lpsB*), Capsular polysaccharide (*ptk* and *epsA*), Phospholipase D and C Penicillin-binding protein, Acinetobactin-mediated iron acquisition system [4,5].

Phospholipases (PLs) with the ability to hydrolyze phospholipid [6], was thought, to contribute to the pathogenesis of Gram negative bacteria by facilitating the lysis host cell membrane [5]. The most significant classes of phospholipases that have been so far shown to play a significant role in bacterial pathogenesis are the phospholipase C (PLC) and phospholipase D (PLD) types [7], these Two types of phospholipases have been reported in *A. baumannii*

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[2,8]. Earlier two homologous extracellular PLCs, a haemolytic PLC (*PlcH*) and a non-haemolytic PLC (*PlcN*) was reported in opportunistic pathogen *Pseudomonas aeruginosa* produces [9]. Later it was also found to be produced by an array of nosocomial pathogen where they were likely to play distinct role in virulence [10]. *PlcH* has been demonstrated to be a virulence determinant of *P. aeruginosa* in a variety of infection models in mammals [11,12]. Another important virulence factor Elastase which has ability to degrade elastin and capable of contributing to destruction of host tissue or defense in numerous ways [13]. It's was first found in *P. aeruginosa* where it plays an important role in the pathogenicity during host infection [14,15].

The aimed of this study was investigate the presence of the important virulence factors enzymes Phospholipases (*plc-N*) and Elastase (*lasB*) genes in *A. baumannii* and establishes its role in pathogenicity. Data from *Galleria mellonella* infection assay and adhesion and invasion assays indicated that virulence of *A. baumannii* is enhanced with combined presence of phospholipase C (*plc*N) and elastase (*las*B).

2. Materials and methods

In a prospective study, a total of 30 clinical relevant non repeat isolates of *A. baumannii* was collected from blood sample of patients attending different hospitals in Baghdad during 2014–2015. All the laboratory procedures were performed at central public health laboratories of Al-kindy teaching hospital and Ibn Al-Baladi Hospital during September 2015 till January 2016. The phenotypic identification was confirmed using morphological testes and by Vitek-2 system (Biomeurix, France).

2.1. Genetic identification of A. baumannii isolates

Convention PCR was performed for genotypic identification of *A. baumannii* species using specific primers for *recA* gene. The PCR reaction mixture composed of 12.5 of 2× GoTaq®Green Master Mix (Promega, UAS), 3 μl template DNA, 1.5 μl primers for each forward and reverse primers with final concentration (0.6 pmol/μl), and nuclease free water up to 25 μl (6.5 μl). The amplified PCR product was run in agarose gel electrophoresis, the amplicon size 425 bp as compared with 100 bp DNA ladder that is that of the *recA* gene was visualized under UV trans illuminator. The sequence of the primers and PCR cycling conditions listed in Table 1.

To confirm the identity of the amplified *recA* gene detected in the PCR, the amplified product were sequenced using the ABI 3730XL DNA analyzer (Applied Biosystems, Carlsbad, CA), analyzed with SeqScape v7.0 (Applied Biosystems, Carlsbad, CA), and compared to known *recA* gene sequences available at the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov) through multiple-sequence alignments using the BLAST program. The sequences of *recA* gene for all the isolates were used to generate dendogram and phylogenetic tree using UPGMA Euclidean to calculate the relatedness among isolates.

2.2. Molecular identification of phospholipases (plcN) and elastase (lasB) genes

Conventional PCR was performed to identify isolates bearing the presence of *plcN* and *lasB* using primers as given in Table 1. The amplified PCR product of each sample was run in agarose gel electrophoresis to look for the amplicon size of 466 and 300 bp respectively for *plcN* and *lasB* which was visualized under UV trans illuminator.

2.3. Expression profile of phospholipases (plc-N) and elastase (lasB) genes by real time PCR

To check the expression of *plcN* and *lasB* in positive sample were identified with real time PCR. Total RNA of actively grown bacteria was extracted using the TRI reagent (Sigma Aldrich) and quality was determined by nano-drop (Thermo Scientific). To normalize all the samples, total RNA for each isolates were diluted to the final concentration of 20 ng/ml. The real time reactions were carried out using Bio-Rad CFX 96 instrument (Bio-Rad, USA), Sybr Green master mix (Promega, UAS)., and all the reactions were performed in triplicates. *c*T value was considered as expression of gene. Each result is the mean value of three independent results with their standard deviation. All the primers used for qPCR are listed in Table 1.

2.4. Galleria mellonella infection assay

The virulence capability of different strains of *A. baumannii* strain were evaluated in an in vivo infection model, *Galleria mellonella*, the greater wax moth. Seeing the melanization, size and their movement in response to touch, we selected GM for further test. Strains of *A. baumannii* were grown on LB medium till we obtained exponential growth phase, washed with 0.9% NaCl and adjusted to an OD_{600} of 2. For the test, 20 GM caterpillars were used to test each bacterial strain in one test. Caterpillars were whipped in 70% EtOH for external disinfection and 10 μl of the 1000 fold diluted cultures of each strain were injected into the left proleg of the larvae. As a control, a set of untreated caterpillars as well as a set of caterpillars in which 10 μl of 0.9% NaCl were injected. The larvae were maintained at 37 °C in the dark for 8 days after injection. Caterpillars were considered as dead if they did not respond to gentle probing. All experiments were repeated for 3 times and experiments were not considered if 2 or more caterpillars in any of the control groups died. The survival rates were plotted by using Kaplan-Meier method. The differences in the virulence capability was assayed based on the survival rates and the statistical significance was analyzed by unpaired 2-tailed student's t-test.

2.5. Adhesion and invasion assays

Human lung epithelial A549 (ATCC CCL-185) cells were used for this assay. Cells were grown to confluence in six well plates in RPMI 1640 medium (Merck Millipore, Darmstadt, Germany)

Table 1
The primers used in the current study for PCR amplification.

Reference	No. Cycle	Size product	Primer sequence (5′→3′)	Target gene
7	35	452	F- CCTGAATCTTCYGGTAAAAC R- TTTCTGGGCTGCCAAACATTAC	*recA*
22	35	466	F-GTTATCGCAACCAGCCCTAC R-AGGTCGAACACCTGGAACAC	*plcN*
22	35	300	F- GGAATGAACGAAGCGTTCTC R- GGTCCAGTAGTAGCGGTTGG	*LasB*