Bulletin of Environment, Pharmacology and Life Sciences
Bull. Env. Pharmacol. Life Sci., Vol 3 [8] July 2014: 37- 42

Online ISSN 2277-1808
Journal's URL:http://www.bepls.com
CODEN: BEPLAD
Global Impact Factor 0.533
Universal Impact Factor 0.9804

ORIGINAL ARTICLE

Molecular investigation of the factors Involved in the Biofilm Formation in clinical Isolates of *Acinetobacter*: *CsuA/BABCDE* Gene cluster

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ABSTRACT
Acinetobacter causes severe infections in compromised patients, survives on abiotic surfaces in hospital environments and colonizes different medical devices. One major reason for the development of the antibiotic resistant classes and the formation of the MDR strains is their high potential to carry and transmit genetic antibiotic resistance factors. In this research, 80 strains were isolated from blood, injury, burn wounds, urine interact infection & identified using standard biochemical test. Antibiogram was performed, using disk diffusion method. The present of CsuA/B-CsuA, CsuA-CsuB ,CsuB-CsuC, CsuD-CsuE, Csuc-CsuD genes were investigated using PCR method. The strains were selected for biofilm formation in microtiter plates. Strains were then cultured in LB broth medium with 1% galactose, glucose , sodium citrate and 10^{-4} *gr/100ml concentration of FeCl3 in polystiren surfaces for biofilm formation. Results show that 93.75% of the isolated strains belonged to Acinetobacter that were all resistance. We used P-value using the SPSS statistics software and ANOVA analysis. Frequency distribution of the genes were measured as follows: CsuA/B-CsuA 86.66% , CsuA-CsuB 88% , CsuB-CsuC 100%, CsuD-CsuE 97.3%, Csuc-CsuD 98.66%. This study showed that chaperone- usher that has a significant role in biofilm formation, is present in Acinetobacter with high frequencies and mediums containing mentioned compounds, have repressing effects on biofilm formation.*
Key words: *Acinetobacter, CsuA/BABCDE* gene, *Biofilm, Multi drug resistance.*

Received 20.02.2014 **Revised 04.04.2014** **Accepted 01.06. 2014**

INTRODUCTION

Members of the genus *Acinetobacter* are non-motile, ubiquitous Gram-negative bacteria that can be recovered from a wide range of sources such as soil, water, food products and medical environments [1]. The capacity of this pathogen to persist in these setting could be due to its ability to form biofilms on inanimate surfaces [2]. *Acinetobacter baumannii* has the ability to survive for long periods of time under extreme environmental conditions such as dryness and also against antimicrobial agents on abiotic surfaces; therefore it has been considered as an opportunistic pathogen with the capability to form biofilms on glass and polystyrene surfaces [3,4]. As it has been shown, *Acinetobacter* has high tendency to develop resistance to different classes of antibiotics in short periods of time. Fast emergence and spreading of the multidrug resistant species of *A. baumannii* and its genetic potency to carry the resistance factors that are serious threats for health care facilities around the world [5]. Monitoring process of the biofilm formation depends of factors such as bacterial cell concentration, presence of different nutrients and concentration of the attainable free cations by bacterial cells. Some of these out of cell signals can be sensed by two regulatory systems such as *BfmRS*. This regulatory system activates the transcription of the Usher chaperons that are responsible for the production of pili required for cell attachment and biofilm formation on polystyrene surfaces, although on the contrary to other systems, it is not essential for the cultivation of the cells on chemical mediums. *BfmRS* also controls the morphology of the cells in minimum essential mediums [5]. Analysis of the sequences has proven that *csu* polycistronic operon consists of 5 genes that encode the proteins related to chaperons and the accumulation of pilus in other gram negative bacteria. Attachment of *A. baumannii* to human bronchial epithelial cells and red blood cells is due to semi-pilus structures in

this microorganism [1]. The inactivation of *bfmR* leads to the prevention of *csu* operon expression and cancellation of the production of pili [6]. Although inactivation of sensor kinase for *bfmS* codon may result in disability, but it doesn't stop the biofilm formation [7]. Genes involved in the biosynthesis of fimbria, belong to the complex class of Usher chaperon which are usually made of one operonic cluster [8]. These operons encode at least 3 different proteins:

1- One fimbriatic unit related to the main structure
2- One chaperon
3- One Usher protein (guide)

Subunit complex/periplasmic Usher chaperon reaction, is through an outer membrane integral protein which facilitates the release of fimbriatic subunits and those with the most end proteins are released the first [9,10,11,12,13,14,15,16 and 17] . In this study the analysis of the processes involved in surface e attachment and biofilm formation associated with the *csuA/BABCDE* gene cluster were investigated [1].

MATERIAL AND METHODS

Bacterial strains and culture condition

This study was performed at the Motahari burn hospital in Tehran city . Approximately 80 clinical microbiological cultures are processed annually. Biochemical tests such as catalase, oxidase, OF, MR-VP, urease test, citrate degradation, TSI , SIM and growth on cetrimide agar (to distinguish between *cintobacter* and *Pseudomonas*) and other related tests [18,19] according to Bergey's manual of systematic bacteriology [20].

We were used a matched case-control study to identify the individual risk factors for having MDR *A. baumannii.* We also performed genetic typing of the organisms to clarify the spread of this nosocomial pathogen. Case-patients were defined as patients from whom MDR *A. baumannii* was isolated from any clinical culture [21]. In order antibiotic discs (Kirby-Bauer disc diffusion method) of ticarcillin 75µg, carbenicillin 100µg, methicillin 5µg, ceftriaxone 30µg, ceftazidime 30µg, cefotaxime 30µg, cefixime 5µg, Colistin, azteronam 30µg, amikacin 30µg, Tetracycline 30µg, cefazoline 30µg, nalidixic acid 30µg, imipenem 10µg, erythromycin 15µg were used on Muller Hinton agar medium and the resistant species were selected.

General DNA procedures

Plasmid DNA was isolated using commercial kits (Qiagen). Primers for the following genes were applied: *csuA/B-csuA, csuA-csuB, csuB-csuC, csuC-csuD, csuD-csuE.* First nucleotide sequences were blasted using NCBI website. PCR-amplified with primers according to table 1. *E.coli Dh5α* was used as Negative control [2].

All of the species of *Acinetobacter* that contained all the genes of *CsuA/BABCDE* were selected and cultivated in the nutrient agar medium (Difco) containing 1% concentration of galactose, glucose and carbon source of sodium citrate (Nutrient agar medium as Control). The temperature profile for all the genes were the same (Table 2). Number of the cycles for all the programs were 30. Extracted genome were run on agarose gel 1%.

Biofilm assays

One milliliter of fresh medium in LB broth cultures containing 1% galactose, glucose and sodium citrate with concentrations of 10^{-2} - 10^{-5} gr/100ml of $FeCl_3$ were prepared. 200 µl of each of these cultures were added in, 96 well micro titer plates. 2 µl of cultures (McFarland Standard No. 0.5) were added to each well. Then with normal salin plank tonic cells were completely washed. The cells attached to the walls were visualized and quantified by staining with crystal violet and solubilization with ethanol–acetone as described by O'Toole et al. (1999). The OD580/OD600 ratio was used to normalize the amount of biofilm formed to the total cell content of each sample tested to avoid variations due to differences in bacterial growth under different experimental conditions. All assays were done at least twice using fresh samples each time [1].

Calculating P-value using the SPSS statistics software and ANOVA analysis, 1% of carbon sources and mineral Iron ($FeCl_3$) were selected as between factors and time of 24 hours were selected as Within factor. In this term all the numbers below 0.05 are meaningful.

RESULTS

The results of biochemical tests showed that 93.75% (75 species) belonged to the *Acinetobacter* genus and 84.85% of the strains were multidrug resistance. All clinical isolates were 100% resistant to antibiotics such as ticarcillin, carbenicillin, methicillin, ceftriaxone, cefotaxime, cefixime, amikacin, cefazoline and nalidixic acid. Results for the resistance of strains towards the drugs were as follows: colistin (46.8% vs. 29%, P =0.005), azteronam (93.4% 11%, P =0.039) , tetracycline (75.9% vs.

13.6%, P < 0.001), imipenem (93.2% vs. 17.6%, P < 0.001) and erythromycin (67.3% vs. 11%, P P < 0.001). The findings of culture of bacterial cells in the different carbon source and iron Chloride concentrations were shown in figures 1-3.

The prevalence of amplified *csuA/BABCDE* gene cluster was shown in Table3 During the process of biofilm formation in the 24 hours period, in mediums containing were observed that presence of different carbon sources to reduced surface adhesion of bacterial cells (Figure 4).

Table 1. Nucleotide sequences and the products sizes [2]

Primer	*Nucleotide sequence*	*Product size(bp)*
csuA/B-csuA F	5'-ACCAGCACACTCGATCTG-3'	802 bp
csuA/B-csuA R	5'-TTACTGGTCAGGTTGACG-3'	802 bp
csuA-csuB F	5'-AAATGCGGGTGAAATCGG-3'	906 bp
csuA-csuB R	5'-TGTAGGTGTTGTAGCAGG-3'	906 bp
csuB-csuC F	5'- CTCATCTACAATCAGACG-3'	842 bp
csuB-csuC R	5'-TATGCAGCAGATCCTCAG-3'	842 bp
csuC-csuD F	5'-TTGAACCGCCTTGATAGG-3'	1029 bp
csuC-csuD R	5'-GAGCAGTCATATCGTCTG-3'	1029 bp
csuD-csuE F	5'- CGTAAAGCTACTCATGTC-3'	835 bp
csuD-csuE R	5'- AAGTGCCTGATGTTCTGG-3'	835 bp

Table 2. PCR temperature profile

PCR program	***Temprature***	***Time***
Initial denaturation	94℃	*5 minutes*
Denaturation	94℃	*45 seconds*
Annealing	53.5℃	*45 seconds*
Extension	72℃	*1 minute*
Final extension	72℃	*10 minutes*
Hold	4℃	

Table 3. Prevalence of PCR amplified genes

Gene name	**Number and the percentage of positive samples**	**Percentage of negative samples**
csuA/B- csuA	(%86.6) 65 species	(%13.3)
csuA-csuB	(%88) 66 species	(%12)
csuB-csuC	(%100) 75 species	_
csuD-csuE	(%97.3) 73 species	(%2.66)
csuC-csuD	(%98.6) 74 species	(%1.3)

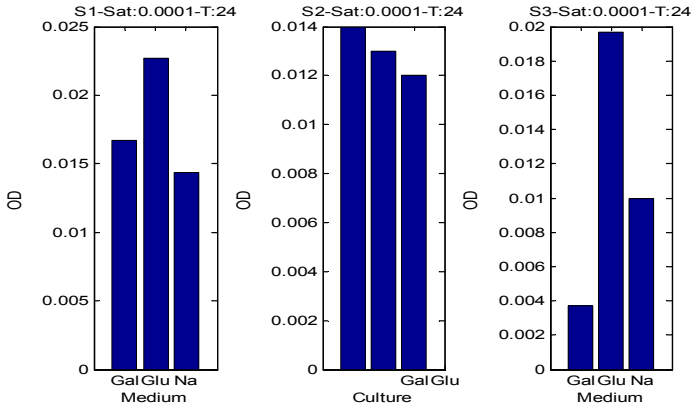

Fig 1. Biofilm formation in 10^{-4} concentration of $FeCl_3$, glucose, galactose and sodium citrate in 24 hours.

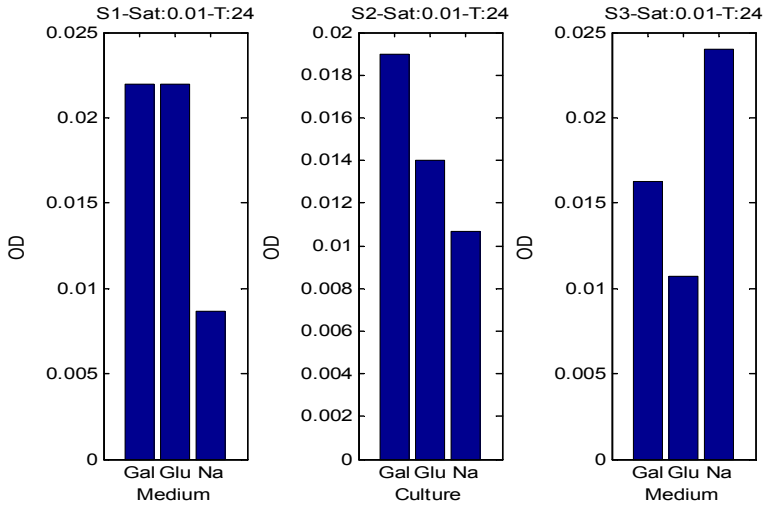

Fig 2. Biofilm formation in 10^{-2} concentration of $FeCl_3$, glucose, galactose and sodium citrate in 24 hours.

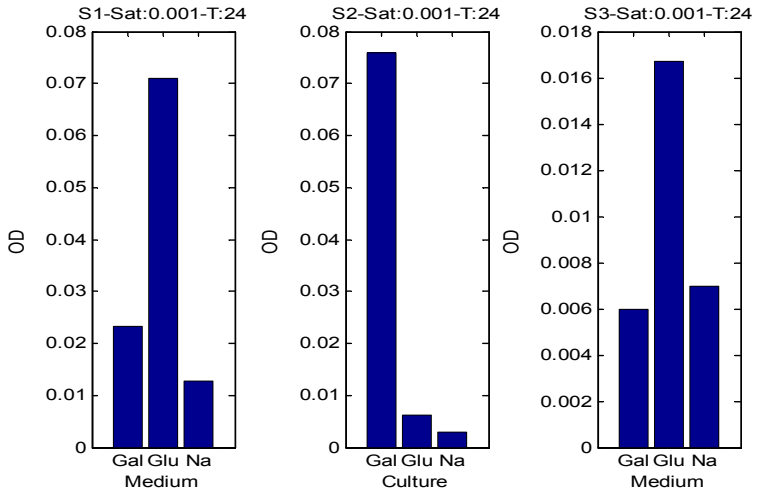

Fig 3. Biofilm formation in 10^{-3} concentration of $FeCl_3$, glucose, galactose and sodium citrate in 24 hours.

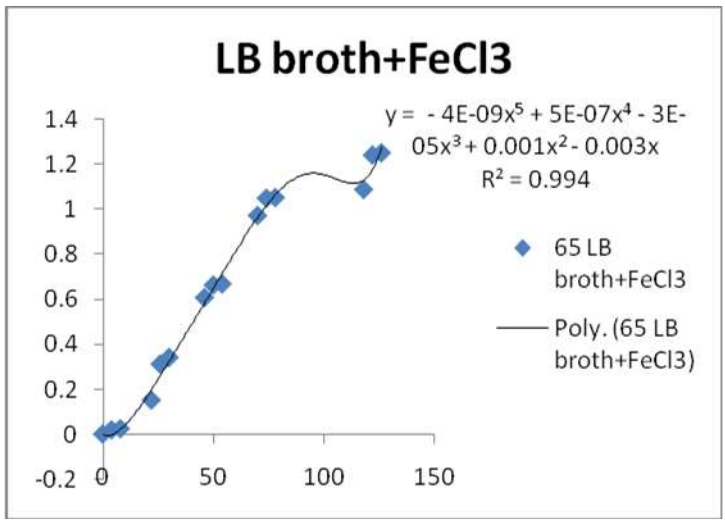

Figure 4. Growth in $FeCl_3$ containing culture

In the process of calculating P-value, it was shown that some of the data had meaningful relation with each other but since their scale of difference was under 1%, clinically it was unimportant and insignificant, because it must have been more than 0.5 in order for bio-film to form. Therefore in LB

broth mediums containing different carbon sources and FeCl3 concentrations, biofilm is either formed very small or not at all. T test was performed for 45 data in which each group was studied with its control and only 7 data had meaningful relation. Although there were relation, but since the differences were less than 0.1, clinical biofilm formation was insignificant. So the fact that these materials can be used as biofilm formation inhibitor can be concluded.

DISCUSSION

Since *Acinetobacter baumannii* is considered a bacterium with high antibiotic resistance that form biofilm on biotic and abiotic surfaces, therefore it has increased resistance to antimicrobial agents and causes widespread untreatable nosocomial infections and increased mortality [21]. Antimicrobial resistance among *Acinetobacter* species has increased substantially in the past decade [22]. Two of the most common definitions of multidrug resistance are carbapenem resistance or resistance to ⩾3 classes of antimicrobials [23]. There are case reports of Rao and et al about correlation between biofilm production and multiple drug resistance in imipenem resistant clinical isolates of *Acinetobacter baumannii* that have compatibility to our research approximately [24].

Hence the necessity to molecular investigation of the responsible genes for biofilm formation in wild strains was sensed. Breij and et al focused on the involvement of CsuA/BABCDE-dependent pili in the interactions between *A. baumannii* 19606 (T) and human bronchial epithelial cells and sheep erythrocytes [25]. We conclude that *csuA/BABCDE* genes are not involved in adherence of *Acinetobacter* strains to polysterene biofilm. The structure of the short pili and their possible role in adherence to human cells requires further investigation. Obtained results of gene frequencies in this survey were completely congruous with Mc Query et.al results (1).

The potential ability of *A. baumannii* to form biofilms could explain its outstanding antibiotic resistance and survival properties. This possibility is supported by a very limited number of publications which showed that a clinical isolate of this bacterium is able to attach to and form biofilm structures on glass surfaces [26]. Furthermore, the adhesion and biofilm phenotypes of some clinical isolates seem to be related to the presence of broad-spectrum antibiotic resistance. In this study biofilm was formed on polystyrene micro-plates and data obtained from biofilm formation were congruous with Tomaras et. al and Mc Query et. al [1,2]. Biofilm formation in presence of carbon sources and $FeCl_3$ due to reduced adhesion of cells to surfaces. Tomaras et. al concluded that adding mineral iron to M9 minimum culture in 24 hours and 37° C would result in decreased adhesion of the cells to the surface and therefore reduced biofilm formation, which is completely aligned with the present research [22]. They also effect of various carbon sources with added 10 mmol sodium succinate, 50mmol ethanol, 0.5 % pyruvate, 0.5 % acetate or lactate based on Simmon salt in 24 hours and 37 °C. They concluded that these materials had little or no effect on the cell adhesion to surfaces [1]. This property could explain the ability of this pathogen to persist successfully in medical environments

where cells could be subjected to the shearing forces of a liquid stream, such as those found with catheters and respiratory tubes, or allowed to persist on less disturbed surfaces such as those of hospital furniture and bed linen [27]. The genetic approach used in this work proved that the presence of pili-like structures on the surface of *Acinetobacter* cells is essential in the early steps of the process that leads to the formation of biofilm structures on plastic surfaces. The disruption of the *csuC* and *csuE* ORFs resulted in non-piliated cells and abolished cell attachment and biofilm formation [1]. In summary, the results presented in this study demonstrate the ability of the opportunistic pathogen *Acinetobacter* to attach to abiotic surfaces under different experimental conditions. In addition, the genetic approach used in this work proved that the expression of CsuA/BABCDE-dependent pili , which belong to a gene cluster related to bacterial loci encoding secretion and pili assembly functions and the production of pili are required in the early steps of the process that leads to biofilm formation.

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Citation of This Article

Zahra B, Farzaneh H, Mitra S. Molecular investigation of the factors Involved in the Biofilm Formation in clinical Isolates of *Acinetobacter*: *CsuA/BABCDE* Gene cluster. Bull. Env. Pharmacol. Life Sci., Vol 3 [8] July 2014: 37-42