Study *ndvB* gene expression in *Pseudomonas aeruginosa* Producing Biofilm

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Abstract

Pseudomonas aeruginosa have ability to form biofilm, this biofilm responsible for wide infections and antibiotic resistance development. A novel mechanism to antibiotic resistance mechanisms utilized by biofilm represented by the presence of *ndvB* geneencodes a glucosyltransferase enzyme involved in the formation of this glucans, thisglucan mediated sequestration antibiotic away from their cellular targets. In this study was collected 145 samples from burns patients and identification by biochwmicaltsets and Vitek-2 compact. The studied the biofilm formation was measured using micro titer plate method we found that 49 isolates (100%) of *P.aeruginosa* were biofilm positive and measured minimal biofilm inhibitory concentration(MBIC) of biofilm *P.aeruginosa* positive biofilmused cefepime as antibiotic was tested using microtiter plate microdilution method (MBIC) values (1gm/ml).Molecular detection of *ndvB* gene was measured using PCR 100% of isolates were found to contain *ndv B* gene. Expression of *ndvB* gene was significantly high in biofilm isolates using sub- MBIC values of cefepime. Expression of *ndvB* gene was significantly high in biofilm isolates using glucose with concentration 1% and 2%. This study we concluded biofilm formation is an important trait of *P.aeruginosa* that isa cause of antibiotic resistance. *ndvB* gene expression responsible for the biofilm resistance mechanism in *P. aeruginosa* biofilms.

Keywords: *Pseudomonas aeruginosa, ndvB, biofilm.*

Introduction

*Pseudomonas aeruginosa*is as an opportunistic pathogen that give rise to a high rate of mortality and morbidity in hospitalized patients with compromised immune systems and infections are being very hard to be treated, because of the increasing number of antibiotic resistant strains, becoming one of the main problems in hospitals(1). *P.aeruginosa* is capable to compose biofilms in numerous surfaces, biofilms promote the bacterium to adhere and survive on the surfaces and medical devices(2). Biofilms are micro colonies composed of multiple microbial species like as community organization leading to stay survival in harsh conditions, bacterial cells of biofilm are more resistant than planktonic cells(3). The insistence these microorganisms in the environment can provide as a source of transmission in the hospital environment(4). The biofilm contain matrix has been contain a mixture of polymers, including: nucleic acids extracellular DNA (eDNA),proteins and polysaccharides. Exopolysaccharide matrix that borders the cells in the biofilm prevents spread of the

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antimicrobial agents through the biofilm and keep the cells of the biofilm in community together[5]. Biofilm contains many genes *ndvB* geneis one of these genes protect biofilmcells from antibiotics. The *ndvB* gene, encodes theglucosyltransferase enzyme that responsible for the formation of cyclic glucans, the glucans are cyclic polymers of 12 to 15 β- (1→3) linked glucose molecules with phospho glycerol substitutions[6]. This study aimed to detection of the biofilmformation, antibiotic susceptibility pattern of *P.aeruginosa* isolated from clinical samples and measure the gene expression of *ndvB*gene among isolates forming biofilms.

Materials and Method

Bacterial Isolates: In this study, a total of (145) samples (swab) were collected from patients suffering from burns. A total of samples was collected from (Medical City Specialist and Al-Yarmook General Teaching Hospital), The isolates were collected during the study period from initial November/2019 finished in the end of February/2020.

Sample collection and bacterial identification: All samples were cultured onmultiple media (Blood agar, MacConkey agar and Cetrimide agar) were used to identify the bacteria, pure colonies with appropriate of the phenotypic characterization based on physiological, morphological, and further tested by conventional biochemical tests including catalase test, oxidase test, Lactose fermentation test, Hemolysin production test, Pigment production test and growth at 42°Cleading to identification as *P.aeruginosa*.

Antibiotic susceptibility testing

Antibiotic susceptibility testAST of all isolates was determined by Vitek-2 automated susceptibility testing system using GN-AST cardsto all 49 *P.aerugenosa*isolates against twelve antimicrobial agents : amikacin, tobramycin, gentamicin, cefepime, ceftazidime, imipenem, meropenem, ciprofloxacin, ticarcillin–clavulanic acid, piperacillin and colistin. These isolates showed different susceptibility**.**

Biofilm susceptibility assay: *Pseudomonas aeruginosa* biofilmwere measure by microtitre plate method to determine biofilm production. In this method, the *P.aeruginosa* isolates were grown overnight at 37C in Mueller-Hinton Broth (MHB) containing 1% glucose. Then, microtitre plates were inoculated with 125 µl bacterial suspension adjusted to 0.5 McFarland**.** Microtiter plate were incubated at 24 hrs at 37°C. After biofilms formed on the walls of micro titer plate are stained with 150 µl of 0.1% crystal violet for 10 min. Then, plate washing twice with phosphate-buffered saline (PBS) (pH 7.2) to discharge crystal violet stain. After air drying process of wells of microplate. The microplate is re-solubilized by 150µl of 95% ethanol. Then, plate was measured at 570 nm by a microtiter plate reader[7]. According the optical density of the samples were classified Mean OD value (> 0.240) as strong biofilm,(0.120 - 0.240) as moderate biofilm and (< 0.120) as weak biofilm[8].

Determination of minimum biofilm inhibitory concentration (MBIC): After forming biofilms on 96-well plates previously described[7]. The minimum biofilm inhibitory concentration (MBIC) were measured. Preparednine folds dilutioninthe different range (100mg/ml, 10mg/ml, 1mg/ml, 0.1mg/ml, 0.01mg/ml, 0.001mg/ml, 0.0001mg/ml, 0.00001mg/ml, 0.000001mg/ml). The antimicrobial agent cefepime, were diluted using Mueller-Hinton broth (MHB). Then, added 100µl of each serial fold dilutions of antibiotics concentration into the each wells of the plates. Negative control contained only as sterility MHB.All the plates were incubated at 37°C for 24 h. After incubated the microtiter plate was washed with sterile water. Then, themeasured optical density at 650 nm was measured after incubation. TheMBIC was determined as the concentration at which the absorbance is equalto or less than that of the negative control[9].

Polymerase chain reaction (PCR): The amplified *ndvB* gene were by PCR using a specific primer Forward (5′-GGCCTGAACATCTTCTTCACC-3′) and (5′GATCTTGCCGACCTTGAAGAC-3′) Revers[10]. Amplification of *ndvB*gene were performed the amplification of *ndvB* gene was performed as follows: initial denaturation step at 95˚C for 5min (one cycle), followed by 30 cycles consisting of denaturation at 95˚C for 30s, annealing at 56˚C for 30s and extension at 72C for 30s, and finalextension at 72 C for 10 min. The PCR products were visualized following electrophoresis on 1% agarose gels and staining with ethidium bromide.

quantitative real-time PCR (RT-qPCR): It was used to measure the expression of *ndv B* gene to antibiotic resistance after treated with it has been used the concentration (0.0001) as sub-inhibitor MBIC. After that, RNA extraction was done using general RNA extraction kit (Promega, USA) according to manufacture instructions. Tomeasure the *ndvB* gene

expression, a pair ofprimers specific for *ndvB*gene Forward (5′-GGCCTGAACATCTTCTTCACC-3′) and (5′GATCTTGCCGACCTTGAAGAC-3′) Reverse[10]. For a control the primers Forward(5′CCTGACCCGTCGCCACAAC-3(′and Re verse(5′CGCAGCAGGATGCCGACGCC-3′)were used to amplify and quantify mRNA of the expressed bacterial *gyrB* gene[11]. The qPCR real-time PCR) was programmed as following; 95°C for 15 min and 40 cycles each cycle consists of 95°C for 60sec, 56°C for 30sec and 72°C for 30sec. After adding glucose in concentration (1% and 2%). The measure expression of *ndv B* gene after treated with glucos at concentrations of 1% and 2%, the same *ndvB*, control primer *gyrB* gene and the programs were used.

Findings: The study period, 49 *P.aeruginosa* isolates were identified from 145 clinical specimens from burns patients. The antibiotic susceptibility patterns of the *P. aeruginosa* isolates by Vitek-2 compact that (57.14%) of the isolates were Multi-Drug Resistant (MDR). It was found that, among 49 isolates of *P.aerugenosa* isolates.

The results of resistance to antibiotics percentage among isolates show in (table 1).

Table 1: These isolates showed different susceptibility towards these antibiotics

Type of antibiotic	No & percentage (%)
Ticarcillin	40 (81.65%)
Ticarcillin-clavulanic acid	40 (81.65%)
Piperacillin	40 (81.65%)
Cefepime	38 (77.55%)
Amikacin	36 (73.50%)
Tobramycin	36 (73.50%)
Gentamicin	36 (73.50%)
Meropenem	36 (73.50%)
Ciprofloxacin	32 (65.30%)
Imipenem	28 (57.14%)
Ceftazidime	24 (48.96%)
Colistin	49 (100%)
Chi-square (χ^2)	9.891 **
P-value	0.0001

** (P≤0.01).

After measuring 49(100%) from 49 isolates were biofilmpositive were used microtiter tissue culture plate method. Where it is found 4 moderate biofilm and 45 strong biofilmas shown in (table 2). The minimum biofilm inhibitory concentration (MBIC) (0,0001mg/ml) of cefepime against 49 positive biofilm measured by using microtiter plates serial dilutions method.

Table 2: *Pseudomonas aeruginosa* isolates ability to produce biofimby using (MTP) method

Biofilm produce	No	Percentage
Strong (+++)	45	91.8 %
Moderate (++)	4	8.8 %
Total	49	100 %
Chi-square (χ^2)	---	14.286 **
P-value	---	0.0001

** (P≤0.01).

According to PCR *ndvB* gene were found in 49 (100%) from the 49 *P.aeruginosa* isolates. Then, study of gene expression was conducted by select 5 *P.aerugenosa* isolates. The study showed that increase gene expression of *ndvB* gene to the highest biofilm and moderate biofilm according to method (MTP), after treated with the cefepime using a concentration (0.0001gm/ml)the dose of sub-MBIC. Furthermore,increase gene expression of *ndvB* gene to the highest and moderate biofilm which measured the to5isolates were tested after treated with glucose using a concentration (1% and 2%).

Discussion

The present study showed(2) that *P.aeruginosa* isolates responsible for burnsinfenctions that occur in hospital and the emergence of resistant *P.aeruginosa* infections and resistance to antimicrobial agents make it considered is Multi-Drug Resistant (MDR)this is agreement with Emamiet al.[12].

The results of resistance percentage among isolates, to sub-catogeries, the highest prevalence of resistance to antibiotic was detected with Ticarcillin 40(81.65%), Ticarcillin-clavulanic acid 40(81.65%), Piperacillin 40(81.65%), Cefepime 38(77.55%), Amikacin 36(73.5%), Tobramycin 36(73.5%), Gentamicin 36(73.5%), Meropenem 36(73.5%), Ciprofloxacin 32(65.3%) were respectively, but less with Imipenem 28(57.14%), and Ceftazidime 24(48.96%) respectively and all *P. aeruginosa* 49(100%) of isolates sensitive with colistinthis is agreement with

Memar et al[13] because of *P.aeruginosa* have high intrinsic resistance to toxic molecules surrounded

toxic molecules by a poorly permeable of the outer membrane that allows extrusion mechanisms, such as efflux systems are able to export antibiotics and thus, to impair the interaction of drugs with their cellular targets. At least four efflux pumps of the resistance-nodulation-cell division (RND) family can significantly increase the resistance of *P.aeruginosa* to antibiotics when overproduced upon mutations[14].

The ability all isolates of *P. aerugenosa* to produce biofilm was detected by using standard microtiter plates. All *P.aeruginosa* isolates had the ability of biofilm production. The association between biofilm formation and antibiotic resistance revealed that biofilm production was significantly higher among MDR *P.aeruginosa* isolates this agreement with Abidiet al[15]. The results showed determine the minimum biofilm inhibitory concentration MBIC value ofcefepiemto the 49 *P. aerugenosa*isolates(1gm/ml). The highest antibacterial effect against biofilm of *P. aerugenosa* isolates by an absorbance values were able to kill 100% of all biofilm isolates in a 24 h after the treatment with cefepiem were compared with the absorbance values of the control, cefepime demonstrated excellent activity against *P.aeruginosa* this result was in agreement with Khan et al[16].

The PCR tests of *ndvB* gene confirms the occurrence of *P. aerugenosa* gene in mainly in all clinical isolates this agreement with Hall et al[17]. The gene expression of *ndvB* gene increasedafter the treatment with the cefepiem because of*ndvB* gene has a new resistance mechanism throughformation of periplasmic glucans. These glucans function in biofilm-specific antibiotic resistance prevents cell death by sequestering antibiotic molecules in the periplasm and away from their cellular (cytoplasmic) targets[5]. While the gene expression of *ndvB* gene increased after the treatment with the glucose 1% and 2%. The glucose stimulate the expression of *ndvb*gene therefore, the expression increased of*ndvB* gene when used of glucose after treatment, this study is compatible with we investigated[18]. The glucose increase biofilm formation ability of *P.aeruginosa*, this is because of the *ndvB* gene encodes theglucosyltransferase enzyme that responsiblefor the formation of cyclic glucans. The glucans are cyclic polymers of 12 to 15 β- (1 → 3) linked glucose molecules with phospho glycerol substitutions[6].

Conclusion

The ability of *P.aeruginosa* to form biofilm that cause of antibiotic resistance, sub-inhabitor MBIC to cefepime of biofilm positive *P.aeruginosa* isolates to was associated with increased *ndvB* gene expression and increased glucose molecules in biofilm leads to increasesd ability of *P.aeruginosa* to form biofilm therefore,increased *ndvB* gene expression.

Conflict of Interest: Nil

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Ethical Clearance: The principles and the experimental protocol in this study was approved by the Medico-Legal Directorate, Ministry of health, Baghdad, Iraq.

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