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Effect of D-Mannose on Gene Expression of Neuraminidase Produced from Different Clinical Isolates of *Pseudomonas aeruginosa*

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Abstract:

The present work aimed to investigate the neuraminidase (*nan1*) gene expression in 32 different clinical isolates of *Pseudomonas aeruginosa* to explore the role of the enzyme in different types of infection and might give a better understanding of host cell-pathogens interaction. In addition, the effect of monosaccharide D-mannose on neuraminidase gene expression in eight isolates was studied by utilizing a reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The results demonstrated that the highest expression of *nan1* gene was in otitis samples (208,913.81) which were significantly higher than that from other infections ($P < 0.01$). While, the concentrations of gene copies obtained from urine, sputum and burns samples were 93,535.34, 92,254.64 and 74,029.63respectively. While the least expression in wound samples (32,017.06). This suggests that neuraminidase in ear samples might be more virulent and invasive followed by that from urine, sputum, burns and wounds samples. The considerable interest of addition D-mannose significantly reduced the rate of neuraminidase activity reached fivefold in some isolates. This indicates that D-mannose down regulates *nan1* gene expression. Hence, this sugar could be used in the development of potential new antibacterial agents where it acts as a competitive neuraminidase inhibitors.

Key words: D-mannose, Gene expression, Neuraminidase, *Pseudomonas aeruginosa*, RT-qPCR.

Introduction:

Pseudomonas aeruginosa is a Gram-negative rod-shaped bacterium which is ubiquitous in the ordinary environment, animals and humans. This bacterium is able to grow in the moisten positions and it succeeds to use a wide extent of organic compositions (1). Serious infections may be linked with high ratio of mortality in immunocompromised patients e.g. cancer, HIV and cystic fibrosis (CF). These infections often result in significant morbidity and mortality (2, 3). Also, this bacterium can lead to intense life-menacing infections in patients with (CF), endocarditis, skin injuries or artificial implants (4). The pathogenicity of *P. aeruginosa* is linked with different metabolic abilities, multiple mechanisms of resistance, and a large repertoire of virulence factors and adaptation, with gene expression is responsible of tightly organizing of all of these activities (5). Several mechanisms have been suggested for these actions, including increasing of a sialoglycolipid receptor numbers and beyond that is the possible augmentation of exposition to the bacterial enzyme, neuraminidase (6).

This enzyme has ability to cleave terminal sialic acid (neuraminic acid) residues from many glycoconjugates expressed on host cells surface and have been correlated with pathogenesis of infectious diseases, bacterial nutrition and cellular interactions(7). Neuraminidases are widely distributed in nature, from viruses, and microorganisms such as bacteria, protozoa, and fungi to avian and mammalian species among the vertebrates, However, the enzymes are absent in plant, yeast, and insects (8). Neuraminidase is encoded by a gene called *nan1* that is responsible for adherence to the respiratory tract, and plays a significant role in bacterial attachment and subsequent invasion into the host cells, particularly into epithelial cells (9). *P. aeruginosa* neuraminidase (NanPs) production has been related to biofilm formation and lung colonization (10). Group of researchers observed that a catalytic pocket of neuraminidases are structurally analogous to canonical crystallographic analyses of *P. aeruginosa* neuraminidase, which has appeared to be more opened compared to others (11). Some investigators hypothesized that stimulation of neuraminidase production in pathogenic bacteria

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depends on the presence of macromolecular complexes like glycoprotein which may be considered as a way to adaptation and invading the host. That postulate is boosted by the specificity of bacterial neuraminidases toward one or different types of bonds in the substrate, whereas the hydrolysis rate is differed for the different substrates based on their molecular size and hydrophobic or hydrophilic character (12).Until recently there were very limited researches about the production of bacterial neuraminidase being as one of important virulence factors. Therefore, in the present study, we estimated the gene expression of neuraminidase gene *nan1* in different clinical isolates of *P. aeruginosa* by utilizing the real-time PCR based on Stephenson (13).He indicated that this technique is considered as a powerful tool to quantify gene expression genetically at accurate level, depending on the quantitative endpoint for real-time RT-PCR and the threshold cycle (CT). It is the first time in Iraq to display the level of diversity in gene expression of neuraminidase between different isolates sources, which may lead to different result of invasion. Additionally, there is a need for new therapeutic strategies to inhibit of bacterial neuraminidases by preventing bacterial infection through blocking the receptors of adherence by utilizing of D-mannose. This monosaccharide is used here as a new attempt for providing new competitive neuraminidase inhibitors that inhibits the neuraminidase activity *in vitro*. Thus, they represents as a potential new antibacterial agents.

Materials and Methods:

Samples collection, Isolation and Identification:

One hundred and twenty two different isolates were obtained from patients who visited as "out-patients" to Al Yarmouk, Al Kindi and Baghdad teaching hospitals at Baghdad city during March to August 2017. Different samples were streaked out onto MacConkey agar, blood agar and nutrient agar. The samples were further sub cultured onto Pseudomonas agar and Citrimide agar to obtain pure colonies, and 55 isolates suspected as *P. aeruginosa*. Those colonies were then checked with routine microscopic and biochemical tests to confirm identification. The isolates were incubated at 37 ^{o}C for 24 h and stored at-80 ^{o}C in Brain Heart Infusion Broth (BHIB) containing 20% (v/v) of glycerol (14). They were preserved at microbiology lab, Department of Biology, College of Science, University of Baghdad.

RNA extraction of *P. aeruginosa* isolates:

After the existence of *nan1* gene was detected in 55 clinical isolates of *P. aeruginosa* by PCR technique (15). 32 positive isolates of *P. aeruginosa* carrying *nan1* gene were subjected to RNA extraction from the pure broth culture of *P. aeruginosa* using TRIzol™ reagent (Thermo Scientific, USA). Quantus fluorimeter (Promega, USA) was utilized to reveal the concentricity of extracted RNA with a view to determine the suitability of samples for subsequent usage. 1 µl from each RNA sample was mixed with 199 µl of diluted quanty flour dye and incubated for 5 min at room temperature in dark place to detect RNA concentration values.

Neuraminidase gene expression by absolute quantification using the standard curve:

The *nan1* gene expression was determined by the reverse transcription-quantitative polymerase chain reaction (RT-qPCR) technique by absolute quantification using the standard curve. This includes, creating standard curve with genomic DNA (gDNA) templet. The standard curve method employs a dilution series of known template copy numbers in the qPCR assay. Linear regression of log concentration (copy μl^{-1}) versus CT gives the standard curve, and then used to calculate template concentration (copy μl^{-1}) of the sample. Seven of 0.2 ml tubes were prepared, 90 µl of nuclease free water was added to each tube then 10 µl from sample of $46*10^{10}$ copy μl^{-1} was added to the first tube. Serial dilution was performed by transferring 10 µl from first tube to second tube and so on. The standard curve reaction started from the tube of $46*10^{7}$ copy μl^{-1} to the tube of $46*10^{3}$ copy μl^{-1}.After that we detected the samples expression by one step real-time PCR (RT-PCR) that was performed on a Mic qPCR Cycler (Bio Molecular System, Australia) using GoTag qPCR Master Mix GoTaq®1-Step RT-qPCR System, (Promega, USA). Standard curve of neuraminidase was created with five-fold serial dilutions of one isolate of *P. aeruginosa* RNA extract from 46×10^{3} to 46×10^{7}. The slope of the standard curve gives the amplification efficiency of a RT- PCR. According to the manufacturer's instructions, each reaction was composed of 10 µl for each sample included: 5 µl qPCR Master Mix, 0.25µl of RT mix, 0.25µl of MgCl2, 0.5 µl of each primer of virulence gene neuraminidase (*nan*1) using *nan1-f* 5′-GGAGCGTGTTCCTGTGTGTATAG-3′ and *nan1-r* 5′AGAAGA CGTCTCCCTGAATAAGA-3′ (15), 2.5 µl of nuclease free water and 1µl RNA template. The conditions for each cycle were 37 °C for15 minutes to RT enzyme activation, 95 °C for 10 min for initial denaturation, followed by forty cycles of

95 °C though 30 seconds and 60 °C for 30 seconds. Subsequently, a melt on green curve analyses of 72-95 °C at 0.3°C/sec were performed (13, 16).

Effect of D-Mannose on gene expression of neuraminidase:

The effect of D-Mannose (DIFCO laboratories, Detroit, USA) on *P. aeruginosa* neuraminidase gene expression was achieved with eight randomly collected isolates. According to de Assis *et al.* method (17), our experiment was designed to depend only on the sugar concentration. Then, we designed our modification in the type of monosaccharide (D-mannose) and in the method of neuraminidase inhibition detection and as well as the estimation by relative real time PCR technique. Briefly, the experiment was performed by mixing 1 ml of fresh bacterial growth grown in (BHI) broth treated with1 ml of (1mM). Mannose was prepared by dissolving sugar in 80 mM of sterilized PBS and incubated at room temperature for 30 min. Then, incubation was stopped and RNA extraction was started for treated and non-treated D-Mannose samples (as control). Gene expression of neuraminidase was measured by the relative (RT-qPCR) technique, as performed previously by One step real-time PCR using the following primer sets (18): *oprD-f* 5`-TCCGCAGGTAGCACTCAGTTC-3`and *opr*5`AAGCCGGATTCATAGGTGGTG-3` as a housekeeping gene was used to the normalizing gene, and the same set of new designed primer of neuraminidase *nan1* gene was used in gene expression of neuraminidase by absolute RT-qPCR. (18, 19)

Statistical Analyses:

The Statistical Analysis System (SAS) was used to determine the difference of neuraminidase gene expression in different clinical isolates of *P. aeruginosa*. Then, Least significant difference – LSD test and T test were used to clarify the significant differences between treated and untreated isolates with D-mannose under probability levels of < 0.05 and <0.01(19).

Results and Discussion:

Isolation and Identification:

A total of hundred and twenty two different nosocomial infection samples were obtained from patients whom visited several teaching hospitals in Baghdad city. All collected isolates were cultured on MacConkey agar, Blood agar, nutrient agar, Pseudomonas agar and Citrimide agar. In this study only fifty-five positive cultures were identified, which were from burn isolates (n=21) assigned numbers from 1 to 21), bronchial wash isolates (n=3) assigned from numbers (22 to 24), sputum isolates (n=7)from (25 to 31) ,urine mid-stream isolates (n=5) from number (32 to 36), surgical wounds or abscesses isolates (n=8) from number (37 to 44), otitis infection isolates (ear swabs) (n=8) from number (45to 52), other samples in search were isolated; one for blood (n=1) with number (53), vaginal swab isolate (n=1) with number (54) and cerebrospinal cord fluid isolate (CSF) (n=1) with number (55).The colonies appears large flat and colorless on MacConkey agar because this pathogen is not fermenting lactose and with large flat colonies. However, when isolates were streaked on blood agar, they gave positive results that produced zones of beta-hemolysis with a grape like odor mucoid, pigmented colonies with distinguished metallic sheen. Then colonies were sub-cultured onto nutrient agar and most of them produce a diffusing green pigment in the agar (20). Next, these isolates were cultured on Pseudomonas agar on which all colonies showed growth with blue-green or brown pigmentation that is an indication for these bacteria (21). Finally, all isolates were cultured on nutrient agar where most of them gave a distinguished blue-green pigment. Isolation and identification of *P. aeruginosa* from other species of *Pseudomonas* were performed on Citrimide agar containing nalidixic acid, due to their resistance; Colonies were having ability to produce green-blue/yellow pigments when cultured on this agar, because citrimide acts as a detergent and inhibitor to the growth of other microorganisms. Therefore it is regarded as a selective differential medium. Such medium activates the production of pyocyanin and a fluorescent yellow-green pigment due to its iron content (22). A number of biochemical tests were performed in order to confirm the identification of the pure colonies. The tested organisms had a positive result for oxidase, catalase and growth at 42° C (20).

Neuraminidase gene expression by absolute RT-qPCR:

The virulence gene *nan1* expression among various isolates may give a better understanding of interaction between bacteria and host cell. They were examined with absolute real-time PCR by standard curve after overnight of incubation at 37° C in (BHIB). The bacterial growth was compared with a standard suspension of McFarland tube 0.5 to obtain a microbial suspension at a concentration of 1.5×10^8cell per ml to different clinical isolates of *P. aeruginosa*. The results were registered in Tables from 1 to 6 and indicated significant differences in the gene expression of *nan1* in different types of isolates under the probability level of P<0.01.This was due to differences in the areas of infection, number of isolates as well as different physiological

condition of patients. This difference led to a variation in the enzyme concentrations within each type of infection.

Table 1. *Nan1* copy numbers in burn samples

Isolate code	Gene copy no.
1	22,779.9
5	2,340.5
20	196,968.5
LSD value	2667.83 **

**** (P<0.01)**

Table 2. *Nan1* copy numbers in sputum samples

Isolate code	Gene copy no.
22	240,422.5
23	2,508.7
26	4,893.7
27	210,965.6
31	2,482.7
LSD value	4187.72 **

**** (P<0.01)**

Table 3. *Nan1* copy numbers in urine samples

Isolate code	Gene copy no.
32	2,562.7
33	105,375.9
34	3,180.7
35	2,639.8
36	353,917.6
LSD value	4409.17 **

**** (P<0.01)**

Table 4. *Nan1* copy numbers in wound samples

Isolate code	Gene copy no.
37	2,872.5
38	167,023.8
39	2,842.0
40	3,165.5
41	69,955.7
42	2,786.6
43	7,268.9
44	221.5
LSD value	7263.52 **

**** (P<0.01)**

Table 5. *Nan1* copy numbers in otitis samples

Isolate code	Gene copy no.
45	567,763.3
46	23,692.8
47	230,397.4
48	122,569.5
49	113,582.9
50	511,609.6
51	98,291.2
52	3,403.8
LSD value	11846.59 **

**** (P<0.01)**

Table 6. *Nan1* copy numbers in different samples

Isolate code	Gene copy no.
53	295,587.2
54	3,692.5
55	3,608.5
LSD value	4672.91 **

**** (P<0.01)**

Table 7 explained the rate of *nan1* gene copies in different groups of samples. They were correlated with types of infection and indicated significant differences under the probability level (P<0.01). The highest expression of gene was detected in otitis samples at 208,913.81, followed in descending order with the copy numbers 93,535.34 to urine 92,254.64 copy numbers to sputum, while the copy numbers of burns 74,029.63. Finally, a least expression in wounds samples 32,017.06. *Nan1* gene was up-regulated in otitis samples with more than two fold copy numbers in urine and sputum samples and more than three folds gene expression to burns and wounds samples. While, the positive gene expression in the rare other types of isolates, showed high copy numbers of gene expression in blood sample (295,587.2) followed by 3,692.5 and 3,608.5 copy numbers for the vaginal and CSF samples respectively.

Table 7. Rate of *nan1* gene expression for different clinical isolates in different clinical isolates

Samples source	Concentration rate of *nan1* gene expression
Burns	74,029.63
Sputum	92,254.64
Urine	93,535.34
Wound	32,017.06
Ear	208,913.81
LSD value	1962.76 **

**** (P<0.01)**

A variety of glycosylated molecules with terminal sialic acid residues are presented in mucosal surfaces of mammals. Sialoglyco proteins in mucin participate have several important physical, immunological and antibacterial properties through effectively greasing, covering and defending epithelial surfaces. Sialic acid molecules play an important role in molecular mimicry to mammalian cell surface, nutrition as source of sugar and explaining cell to cell interactions through different metabolic mechanisms in great groups of mammalian pathogens and commensals. (23). Additionally , the prominent terminal sites of host sialic acids have made one of more extremely targeted carbohydrate receptors for bacterial adherence for more than a hundred model of sialic acid-binding agglutinins,

adhesins and lectins between mammalian pathogens (24). Bacterial neuraminidases and their mucosal sialoglycan targets contributed to host–microbe interactions at every mammalian mucosal surface as in otitis media, which is a common and often recurrent bacterial infection. Bacterial neuraminidase can unmask implied ligands leading to exposure and adherence of host cells to bacterial toxins. This was identified at neutral pH in the middle ear fluids from patients with otitis media associated with effusion. The researchers observed that the plasma of patient lacked neuraminidase activity, indicating that neuraminidase in the middle ear fluid originated in the middle ear and was not transudate from blood. Since, mammalian neuraminidase has optimal activity near pH 4. So, the authors concluded that microorganisms were the source of neuraminidase (25). As for the *nan1* expression in UTI samples, it may be attributed to the observation that high mortality in hospitalized patients is correlated with *P. aeruginosa*, this expression increases in people with intense comorbidity such as chronic renal failure, advanced liver disease or diabetes mellitus. Inappropriate antibiotic treatment is connected with poor results, which indicates the importance of adjusting experimental antibiotic treatment according to the microbiological susceptibility results (26). The researchers observed oligosaccharide chains in the glycoproteins of the urinary epithelium of pig bladder. These oligosaccharide chains were analogous to those oligosaccharide of human urinary epithelium mucin that has neuraminic acid acting as a substrate to neuraminidase, which is necessary in cell adherence and immunological operations (27). Regarding sputum samples**,** the human air passage encoded multiple neuraminidase-like enzymes. Some evidence suggested that *P. aeruginosa* neuraminidase facilitates bacterial association with the lung epithelium (6). A group of researchers hypothesized that the host cells react with flagellar protein of *P. aeruginosa*, leading to recruitment of the human neuraminidase by increasing liberation of mucin through activated muc1 gene from the epithelial cell surface. This way, it facilitates a cryptic binding site on mucin epidermal cells to flagellin binding and increasing *P. aeruginosa* adhesion to the airway epithelium due to removal of sialic acid residues from the extracellular cell surface protein mucin. On the other hand, human sialidase controls the epidermal cell mucin (muc1) by rising cleaving and shedding mucin from cell surfaces and blocking *P. aeruginosa* adhesion to airway cell associated epidermal cells. It is enticing to suppose that the balance between these opposite processes which include *P. aeruginosa* adherence versus decoy receptor function (28, 29). However, other studies found that *P. aeruginosa* neuraminidase was 1000-fold more active than the *Clostridium perfringens* enzyme in releasing neuraminic acid from respiratory epithelial cells (30). As for the genetic expression of neuraminidase for both wounds and burns, a neuraminic acid commonly was found at the outermost position of glycan chains at the surface of most mammalian cells (31). Commonly, the end of the O-linked oligosaccharide is linked to sialic acid in humans found that N-acetyl neuraminic acid in surface on red blood cells (32). The negative charge of RBC membrane comes from the presence of N-acetyl neuraminic acid which account for 74–94 % (33). These acids control the morphology, membrane deformation, oxidation capacity, RBC aging and even the construction and distribution of hemoglobin molecules in human RBCs (34). Interestingly, vaginal excretions with normal healthy flora generally consist of lactobacilli, do not show neuraminidase activity. Whereas, infected women with bacterial vaginitis have elevated activity of neuraminidase in vaginal fluids that is possible to be bacterial origin (35). Hence, permanence of neuraminidase -producing vaginal bacteria in enough numbers to rise vaginal fluid neuraminidase activity may be a risk factor for subclinical intrauterine infection and preterm delivery.(36) Sadula *et al.* (37) showed higher levels of sialic acid and aspartate transaminase (AST) levels in cerebrospinal fluid have been described in pyogenic meningitis (PM) as compared to tubercular meningitis (TBM). The neuraminic acids are among the most predominant molecules at the host-microbe surfaces. The varied types of neuraminic acids ease cell to molecule interactions through direct or indirect cell signaling including siglec-sialoglycoconjugate reactions (38). Additionally, structural evolution of neuraminic acids is analogous in various types of microorganisms and thus is seen as an arms racing between and within species (23). We can conclude from the above information, that the variations of *nan1* gene expression in different clinical isolates could be associated with the variable amount of sialic acid in host cell surface glycoconjugates, where, host sialic acid up regulates the secretion of neuraminidase from *P. aeruginosa* as a specific substrate to the enzyme ,according to the type of infected tissues.

Effect of D-Mannose on gene expression of neuraminidase:

There is a necessity for alternative curative strategies against *P. aeruginosa* due to the growing problem of prevalence of new resistant strains (39). It is important to find inhibitors targeting the *P.*

aeruginosa neuraminidase to prevent infection because of its role in colonization (10). This enzyme offers an attractive target for therapeutic intervention (40). The data in Table 8 revealed the effect of the monosaccharide D-mannose through inhibition of neuraminidase production between 0.1 in isolate no.41 to 0.9 in isolate no. 20 when compared with the control isolate, which significant differences under the probability level of $P<0.05$. We also found that D-mannose effectively and competitively inhibited neuraminidase expression by occupying the binding site of the enzyme active site's pocket. Therefore, we believe that a neuraminidase inhibitor to the respiratory tract infection can be used as a prophylactic agent against microbial pneumonia after the influenza virus infection and also in populations at risk (41). Our results agreed with de Assis *et al.* (17) who used D-galactose as one of monosaccharides to neuraminidase inhibition by heamagglutination assay, Nevertheless, we used D-mannose instead with manipulation in the procedure to measure the change in neuraminidase production by relative RT-PCR as quantitative assay, with the aim of obtaining new competitive neuraminidase inhibitors. The sugar suppressed bacterial growth and may thus represent a potential new antibacterial agent.

Table 8. Gene expression of *nan1* in different clinical isolates of *P. aeruginosa* in the presence of D-mannose by relative real time PCR.

Isolate code	Fold change without treatment	Fold change with treatment	T-Test
1	1.0	0.5	0.416 *
20	1.0	0.9	NS
31	1.0	0.6	NS
33	1.0	0.3	0.468 *
41	1.0	0.1	0.542 *
48	1.0	0.3	0.468 *
54	1.0	0.5	0.416 *
55	1.0	0.4	0.437 *
Mean	1.0	0.45	0.419 *
LSD value	NS	0.469 *	---

*** (P<0.05)**

In conclusion, an up-regulation of *nan1* gene expression was observed in otitis samples, by approximately more than two fold copy numbers in urine and sputum samples expression and more than three folds to burns and wounds sample compared with control. The impact of D-mannose was more pronounced in down-regulation of *nan1* gene, which implies that it can be used as a neuraminidase inhibitor.

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Conflicts of Interest: None.

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تاثير دي- مانوز على التعبير الجيني للنيورامينيديز المنتج من بعض العزلات السريرية لبكتريا *Pseudomonas aeruginosa*

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الخلاصة:

هدفت الدراسة الحالية الى التحري عن التعبير الجيني لانزيم النيورامينيداز *nan1* في 32 عزلة سريرية مختلفة من بكتريا *Pseudomonas aeruginosa* لفهم دور الإنزيم في أنواع الاصابات المختلفة والذي يمكن أن يعطي فهما أفضل للتفاعل بين الكائن الممرض والخلية المضيفة. وكذلك دراسة تأثيرالسكر الاحادي D-mannoseعلى التعبير الجيني للنيورامينيداز في ثمانية عزلات بطريقة الاستنساخ المعاكس الكمي- تفاعل انزيم البلمرة المتسلسل (RT-qPCR). أظهرت النتائج الحالية أن أعلى تعبير لجين *nan1* زاد معنويا في عينات التهاب الأذن وكان (208,913.81) بينما كانت التراكيزلنسخ الجين 93,535.34 ، 92,254.64 و 74,029.63 التي تم الحصول عليها من عينات الادرار ،القشع والحروق على التوالي وكان أقل تعبيرللجين في عينات الجروح (32,017.06).هذا يشير إلى أن دور النورامينيداز في عينات الأذن قد يكون أكثرضراوة واجتياحا متبوعًا بدوره في عينات الادرار و القشع وغيرهامن العينات. إن الفائدة الكبيرة من إضافة سكر D-mannose كانت معنوية في تقليل معدل فعالية النورامينيداز الذي انخفض الى خمسة أضعاف في بعض العزلات . هذا يشير إلى أن D-mannose قلل من تعبير الجين *nan1* الذي يمكن استخدامه في تطوير عوامل جديدة محتملة مضادة للجراثيم التي تعمل كمثبطات تنافسية للنيورامينيديز.

الكلمات المفتاحية: دي- مانوز، تعبيير جيني، نيورامينيديز، بكتريا الزوائف الزنجارية ، RT-qPCR