BACTERIOLOGIGAL AND GENETIC STUDY OF *Pseudomonas aeruginosa* ISOLATES

S. M. Tawfiq
Dept. Soil &Water Sci.,Coll. Agric.,Univ. of Sallahaddin- Erbil- Kurdistan Region
e.mail.dr.shamerantawfiq@gmail.com

ABSTRACT

Fifty *P. aeruginosa* isolates were isolated from patients suffered of wound, ear and chronic pulmonary infections to assess the presence of *nan*1 and *nan*2 genes .*P. aeruginosa* isolates were 10(20%) from chronic pulmonary infection ,20(40%) from each of ear and wound infections. isolates exhibted highly sensitivity to (Cefepime ,Meropenem ,Azitheromycin) and version resistance to Cefotaxime, Gentamicin, Ceftazidim ,Clorithromycin, Tobramicin,Ciprofluxacin In percentages (70%),(60%) ,(50%), (40%) , (36%) and(34%) respectively.Seventeen isolates were choosen to searching *nan*1 and *nan*2 genes by PCR technique depending on the resist to four groups of antibiotics (Aminoglycoside,Third generation of beta-lactam ,quinolones ,Macrolides , (29.4%) possess both genes.*nan*1 were distributed in(41.2%) in percentages (50%) for each of wound and ear isolates and (20%)for chronic pulmonary isolates,while *nan*2 was dissemination (70.6%) in percentages100%, 66.6% and 50% for chronic pulmonary, ear and wound isolates respectively In conclusion, these data suggest that the prevalence of *nan*2 in all infections ,that suggest apossible role of this gene in disease evolution especially in pulmonary patients while *nan*1 was low prevalence in isolates may be related to infection site.

Key words: *P. aeruginosa,nan1,nan2* genes, neuraminidase,infections.

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دراسة باكتيرولوجية وجينية لعزلات *P.aeruginosa*

شاميران محمود توفيق
مدرس
قسم التربة والمياه–كلية الزراعة–جامعة صلاح الدين–اربيل– اقليم كردستان

المستخلص

من اصابات الجروح والاذن والتهابات الرئة المزمنة وذلك *P.aeruginosa*عزلت خمسون عزلة من بكتريا ،توزعت العزلات الى 10(20%) من التهابات الرئة المزمنة،20(40%) nan1,nan2 للتحري عن الجينين، Cefepim,Meropenem لكل من اصابات الاذن الوسطى والجروح،اظهرت العزلات حساسية عالية تجاه (Ceftazidim،Clorithromycin ،Gentamicin ،Cefotaxime ومقاومة متباينة تجاه Azitheromycin على الترتيب. (34%)و (36%) (50%),(40%)، (60%)،(70%)بنسب Ciprofluxacin وTobramicin، اعتمادا على مقاومتها لاربعة مجاميع من nan1,nan2 اختيرت سبعة عشرة عزلة للتحري عن الجن الجينين المضادات الحياتية (الامينوكلايكوسايد، الجيل الثالث لمضادات البيتالاكتام، الكينولونات، الماكروليتات)، اظهرتnan1 نتائج فحص تضخم الدنا التضاعفي المتسلسل ان(29.4%) من العزلات كانت موجبة للجينين. توزعت الى (41.2%) ينسب(50%) في كل من عزلات الجروح والاذن و(20%) في عزلات الرئة المزمن، بينما كانت (70.6%)اي بنسبة(100%)،(66.6%) و(50%) لكل من عزلات الرئة المزمن والاذن nan نسبة انتشار 2 في جميع العزلات ربما له دور في تطور الاصابة وخاصة فيnan2والجروح على الترتيب، ان انتشار الجين اظهرت معدل انتشار منخفض ربما يعود ذلك الى موقع الاصابة.nan1 اصابات الرئة المزمن بينما

كلمات مفتاحية: التهاب الرئة، مقاومة، حساسية، عزلات.

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INTRODUCTION

Pseudomonas aeruginosa is a ubiquitous micro-organism that can infect immunocompromised individuals and is responsible for nosocomial infections, *Pseudomonas aeruginosa* has many virulence factors such as formation of biofilm(colonies of *P. aeruginosa* composed of alginates) protects the bacterium from the host's immune response and from antibiotics (13,21). Other virulence factors are Exoenzyme S is an ADP-ribosyltransferase that is secreted by a type-III secretion system directly into the cytosol of epithelial cells (23). Exotoxin A which inhibits protein biosynthesis. LasB elastase has an elastolytic activity,The adhesion of apathogen to epithelial surface is the first step in an infection, *P. aeruginosa* exoproducts, particularly proteinases and neuraminidase (sialidases), are noted in many studies to enhance bacterial adherence and to act as virulence factors and may increase the avaialability of such receptors by cleaving terminal sialic acid residues from cell surface gan gliosides(24) Bacterial adherence to mucosal surfaces is a significant aetiological factor in infections(2),The importance of proteinases is facilitating attachment,Extracellular neuraminidase was secreted in growth medium during the early stationary phase. The enzyme hydrolyzed the alpha 2 leads to 3 glycosidic linkages from N-acetylneuraminlactose and fetuin, *P aeruginosa* strains, with detectable proteinases during adherence assay, have the greatest adherence(2).the contribution of bacterial neuraminidase to the pathogenesis of infection is not as clearly defined. Neuraminidase-producing species such as Hemophilus ***S. pneumoniae*** (9), and ***Pseudomonas aeruginosa*** share a common ecological niche, colonizing the heavily asialylated secretions and surfaces of the upper respiratory tract. Although each can bind to asialylated glycolipids exposed by neuraminidase activity, they differ substantially in their ability to either metabolize or incorporate sialic acid into surface structures, Thus it is likely that bacterial neuraminidases interact with both prokaryotic and eukaryotic glycoconjugates, bacterial adherence to tissue is result of interactions between surface molecules on the bacteria and plasma membrane receptors on host cell(22,18) for exampoles the ability of *P. aeruginosa* to persist in the respiratory tract is correlated with the organism's ability to adhere to buccal epithelial cells (BEC), *nan1* gene initiate colonization and facilitate long-term infection, *P. aeruginosa* cells adhere to BECs *via* pili, which recognize a GalNAcβ1-4 disaccharide exposed in asialylated glycolipids, such as asialo-GM1 (25). Several arguments suggest that the *nan1* gene encodes neuraminidase, an enzyme able to release terminal sialic acid residues from sialylated gangliosides, thus increasing the amount of potential bacterial receptors and consequently enhancing adhesion (11). The removal of sialic acid residues from cell surface proteins also results in higher cell-to-cell contact, presumably by enhancing protein-protein interactions through reduction of the net negative surface charge of one of the distinct features of sialic acid *.P aeruginosa* neuraminidaseis 1000 fold more active than the *Clostridium perfringens* enzyme in realeasig sialic acid this effect correlated with the increasing adherence of *P aeruginosa* to epithelial cells after exposure to *P aeruginosa* neuraminidase(3). The identification of putative neuraminidase genes in *P. aeruginosa* The sequence of the P. aeruginosa PAO1 genome was published recently (accession no. AE004091;. The strategy used to detect putative neuraminidase genes was based on the observation that all described bacterial sialidases have four or five copies of an aspartate box (`Asp-box') motif, Ser–X–Asp–X–Gly–X–Thr–Trp, where X is any amino acid By using this peculiarity and information from the Pseudomonas Genome Project two genes containing four Asp-boxes were identified in the P. aeruginosa PAO1 genome; we named them *nan*1 and *nan*2 (11).

MATERIAL AND METHODS

(I) Bacterial isolation and identification

Pseudomonas aeruginosa isolated from different human infections (Ear, Wond, Sputum) samples activated by culturing in brain heart infusion broth medium and incubation at 37 (18-24) h, After activation inoculated on the MacConKey agar (positive isolates does not ferment lactose), a colonies were selected, for more purification, inoculated on the selective medium cetrimide agar 0.03% cetrimide, suspected isolates identified by growth at 4°C and biochemical tests were [oxidase(+), indol(-), ureas(-,+), citrate(+),catalase(+)] and tested by microscope which showed Gram negative rod shape, stored at -20°C in tubes contained 4ml brain heart infusion broth+1ml Glecerol, till other bacteriological and molecular tests were done(5,6).

Antimicrobial susceptibility test

This test was done according to standard methods by disk diffusion 2009 and 2010 CLSI guidelines. *P. aeruginosa* isolates were tested for their susceptibility: Cefepime FEP 30µg ,Meropenem MEM 10µg, Azitheromycin ATM 30µg, Cefotaxime CTX 10µg, Gentamicin CN 30µg, Ceftazidim CAZ 30µg, Clorithromycin CLR 15µg, Tobramicin ToB 30µg,Ciprofluxacin Cip 5µg).

(II) Molecular Biology Studies

Seventeen isolates of *P. aeruginosa* five from chronic pulmonary infection and six from each of ear and wound infections which had appeared resist aginst four groups of antibiotics were chosen to study the presence of two virulence genes *nan*1,*nan*2 (both genes encoding for neuraminidase).

DNA extraction:

The selected isolates were grown in brain heart infusion broth at 37°C for 24 hr, and DNA was extracted by using extraction and purification kit provided from Promega Company (USA). 3 ml was transferred to ml test tube.The growing cells were deposited by centrifugation at 4000 rpm for 10 min then the pellet was resuspended with 100 µl TB buffer vortex to completely suspended the pellet, 10 µl was added lysozyme, incubated at 37 °C for 10 min., added 100 µl RNaseA was added then incubated in room tempreture for 5 min. centrifuged at 10000rpm/min for 2 min. then supernatedwas transferred to 1.5 ml Eppendrof tube, 220 µl BDL buffer was added, incubated in65°C for 10 min. 220 µl ethanol (96-100%)was added and vortexed for 20 sec.at maximum speed, inserted HiBind DNA mini column into a 2 ml collection tube, transferred. The entire sample was added to the HiBind including any precipitate, centrifuged at 10000 rpm/min for 1 min., discarded the filtrated and the collection tube, inserted the HiBind into new 2ml collection tube, 500 µl HB buffer was added, centrifuged at 10000 rpm for 1 min., discarded the filtrate and reused collection tube. 700 µl DNA wash buffer was added then centrifuged at 10000 rpm/min for 1 min., discarded the filtrate and reused collection tube, centrifuged the empty HiBind at 10000 rpm for 2 min, inserted HiBind DNA mini column into a 2 ml collection tube, added 50-100 µl preheated elution buffer(65°C) to the HiBind into anew nuclease-free 1.5 ml Eppendrof tube,incubated HiBind DNAcolumn for 3-5 min. at room tempreture, centrifuged at 10000 rpm/min for 1 to eluted the DNA, incubated overnight at 4 °C then stored at – 20 °C till use.

Detection of *nan*1 and *nan*2 genes:

Nan1 and nan2 genes determind by PCR (Applied Biosystem, Singapore) using specific primers (Alpha DNA) The frequency of the virulence gene encoding neuraminidase *nan1*and *nan*2 was determined by PCR (11). Amplification was performed with specific primers *nan1* ‑ F(5′-ATGAATACTTATTTTGATAT-3′) and *nan*1R (5′-CTAAATCCATGC TC TGA CCC -3′) yielding a 1316 bp product and primers *nan2* ‑F. (5′- ACAACAACGGGGACGGTAT-3′) and *nan*2R (5′-GTTTTGCTGAT-GCTGGTTCA-3′), yielding a 1161 bp product, PCR was carried out with 2 µl template DNA, 0.25 µM of each primer, 0.2 mM deoxyribonucleoside triphosphates, 1x reaction buffer, 2 mM $MgCl_2$ and 1.5 U Prime Taq DNA polymerase (Promega Company) in a total volume of 25µl shown in Table1. The DNA was amplified using the following protocol: using the following protocol: 94°C for 3 min, 30 cycles of 94°C for 30 s, 55°C for 1 min, and 72°C for 1.5 min, and finally 72°C for5 min. PCR products were separated in 1.5% agarose gel for 50– 110 min at 130 V, stained with ethidium bromide ($0.5 \mu g l^{-1}$) and The electrophoresis products were visualized by UV transilluminator and photographed by Gel Documentation.

Table.1PCR mixture total volume 25

Volume of matter added	Component	Sequence
13.8µl	Distilled water	1
5µl	PCR Buffer1X	2
1.5µl	DNTPs	3
1.2µl	Primer-F	4
1.2µl	Primer-R	5
0.3µl	(1.5U)TaqDNA Polymerase	6
2µl	DNA	7
25µl		Total volum

Table.2 PCR mixture total volume 25

Volume of matter added	Component	Sequence
13.8µl	Distilled water	1
5µl	PCR Buffer1X	2
1.5µl	DNTPs	3
1.2µl	Primer-F	4
1.2µl	Primer-R	5
0.3µl	(1.5U)TaqDNA Polymerase	6
2µl	DNA	7
25µl	Total volum	

RESULTS AND DISCUSSION

Seventeen isolates were selected to evaluate the prevalence of two virulence genes *nan*1 and *nan*2 genes among 50 isolates of *P. aeruginosa* which had appeared resist to four groups of antibiotics (Aminoglycoside, Macrolides,quinolone, Third generation of betalactam) ,(Table 3 shows the antimicrobial resistance patterns) , In current study seventeen isolates we choosed according to (12) whom result that the proportion of multi drug-resistance nosocominal *P.aeruginosa* isolates containing alarg number of virolence genes (38.1%) was higher than the proportion of non multi drug –resistant isolates (17.6%).and according to (10) with result that most virulence factors were produced at higher rate in ESβLs producing than in no ESβLs producing *Pseudomonas aeruginosa* are isolates Table 4, figures1,2,3,4,5,6 illustrate the profiling of the amplification products of *nan*1 and *nan*2 virulence genes in each of the 17 tested isolates),(29.4%) of isolates possess both genes, As shown figures.1,2,3,*nan*1 was detected in 7(41.2%) PCR products of the(17) tested isolates of *Pseudomonas aeruginosa* possess this gene,the result showed that this gene in pulmonary infection was (20%) and (50%) in each of wound and ear infections, since gel electrophoresis results showed DNA bands with molecular size 1316 bp in comparison with DNA marker. While *nan*2 gene was detected in the (70.6%) in all studied isolates, with differences to site of infection, the result showed higher spread this gene among pulmonary isolates(100%) and(66.6%) ,(50%) in each of ear and wound isolates respectively in these isolates,since gel electrophoresis result showed DNA bands with molecular size 1161bp in comparision with DNA marker for all tested isolates as shown in figure4,5,6. Our results were not in agreement with the results reported by (8) reported that the *nan1* gene was detected in 57.9% of *P. aeruginosa* isolates causing bloodstream infections,and with(23) whom detected *nan1* gene in 52 (83.9%) from 62 of hospital *P. aeruginosa* strains and suggested that there was no significant difference in the frequency of this gene among *P. aeruginosa* strains isolated from different sources, they significantly observed higher adhesion of these bacteria to human buccal epithelial cells when the *nan*1 gene was present than when the same gene was absent,they found in their study that the *nan1* gene may play a role in the binding of clinical *P. aeruginosa* strains to buccal cells,and with (15)whom result reported low frequency of this gene(15%) among their study isolates. (12) reported that the frequency of this gene is low(16.9%) in non-cystic fibrosis isolates of *Pseudomonas aeruginosa* which isnot in agreement with our results and the role of this factor is not known in the pathogenicity of this bacterium in non- cystic fibrosis patients. Astudy performed by (10) on 44 MDRPA isolates, 12 isolates representing different resistance profiles and sources of samples were selected for further molecular studies showed that only 4(33.3%) of them possess *nan*1 gene which was relatively low,While (18).detected *nan*1 gene in39(38.2%) among 102 cystic fibrosis *P.aeruginosa* isolates,and were relatively agreed with our results. (11) used PCR to assess the prevalence of eight virulence genes (*algD*, *lasB*, *toxA*, *plcH*, *plcN* and *nan*2), PCR detected *algD*, *lasB*, *toxA*, *plcH*, *plcN* and *nan*2 in all of the 162 isolates used to *nan*1 was detected in isolates (44.4 %), demonstrated that the *nan1* gene was present in 61.7% of CF isolates and 44.4% of non-CF isolates. The frequency of this gene in CF isolates tended to increase as the clinical status worsened. Indeed, *nan1* was detected in 57% of isolates from patients with an excellent or good clinical status, in 63% of isolates from patients with a moderate status, and in 71% of isolates from patients with a poor or weak clinical status. and suggested that the prevalence of this gene in CF isolates tended to increase as the clinical status worsened. Indeed, *nan*1 was detected in 57 % (23/40 isolates) of isolates from patients with an excellent or good clinical status, in 63 % (17/27) of isolates from patients with a moderate status and in 71 % (10/14 isolates) of isolates from patients with a poor or weak clinical status and the distribution of *nan*1 was significantly related to strain origin and the role of this factor is not known in the pathogenicity of this bacterium in non-CF patients, Lanotte results were agreed with present results except for *nan*2,present study showed (100%)pulmonary isolates possess this gene followed (66.6%) and (50%) for ear and woud isolates may be that related to the virulence of *P. aeruginosa* is multifactorial and caused by several extracellular enzymes and other substances. The importance of these virulence factors for the pathogenesis of human *P. aeruginosa* infections is dependent on the type of infection (7). The differences in the distributions of virulence factor genes in the populations strengthen the probability that some *P. aeruginosa* strains are better adapted to the pulmonary conditions found in CF patients (1). (16) reported that the PA2794 neuraminidase locus (*Delta 2794*) mutant of *P. aeruginosa* PAO1 was unable to colonize the respiratory tract. They suggest that inhibition of bacterial neuraminidase could provide a novel mechanism to prevent colonization of the respiratory tract by this important pathogen*(nan1,nan2)* genes is one of many virulence factors that can play an important role in the pathogenesis of *P. aeruginosa* infection (1). *Nan*1,*nan*2The first argument is that the deduced bacterial protein encoded by *nan*1, *nan*2 possesses four Asp-boxes, a characteristic of bacterial sialidases (20). Secondly, the deduced protein contains conserved amino acids at key sites that are probably part of the catalytic site. Although there is a low degree of similarity between *nan*1, *nan*2 and previously identified bacterial sialidases, this is usual for bacterial sialidase genes (20). Nevertheless, part of the deduced protein encoded by *nan*1 has 26 % identity to part of the Clostridium tertium sialidase protein, The G+C content of *nan*1 (48.2 mol%) differs notably from the G+C content of the whole PAO1 genome (66.7 mol%) (17). Thus, the gene was probably acquired by horizontal transfer, as observed previously for some other bacterial sialidase genes. Several arguments suggest that *nan*1, *nan*2 encodes a sialidase, an enzyme theoretically able to release sialic acid from sialylated gangliosides, thus increasing the amount of asialoGM1, a major receptor for adherence to the respiratory tract(22), increased virulence in environmental strains through acquisition of new virulence genes (such as exoU) via horizontal transmission is a feasible explanation (11). *P. aeruginosa* is not an obligate parasite of humans, many factors harmful to humans expressed by this organism will most probably perform a more innocuous function within the organism’s natural habitat. Virulence factors in P. aeruginosa are divided into specific groups dependent upon their mode of action or method of delivery to the host cell. Thus, these virulence factors may be described as belonging to adhesins and secreted toxins and enzymes dependent or independent of the type I secretion system (T1SS), type II secretion system (T2SS) and type III secretion system (T3SS)(2). The GalNAcβ1-4Gal sequence present in asialylated gangliosides can act as areceptor for several pathogens of the respiratory or gastrointestinal tract including *P. aeuroginosa* , Neuraminidase has been implicated as a virulence factor and may serve as a marker for determining virulence of *P. aeuroginosa* strains. This enzyme has a key role in the initial stages of pulmonary, urinary and gastrointestinal tract infections by targeting bacterial glyco-conjugates and contributing to the formation biofilm(19). present study concluded high spread *nan*2 gene among clinical isolates compared with *nan*1 gene, (100% vs.20%, 66.6% vs.50%, 50% vs.50%) in pulmonary, ear, wound infections respectively, the distribution of virulence genes encoding adhesins (neuraminidase) varied in respect to the infection localization in hospitalized patients.

Table 3.sensitivity tests percentages of P.aeruginosa isolates for selected antibiotics

Seq	Antibiotics	symbol	R-Resistant	R%	S-Sensitive	S%
1.	Tobramicin	To	18	36	32	64
2.	Gentamicin	GM	30	60	20	40
3.	Cefepime	CEF	Zero	zero	50	100%
4.	Clorithromycin	CLR	25	50	25	50
5.	Cefotaxime	CFm	35	70	15	30
6.	Ceftazidime	CFX	20	40	30	60
7.	Meropeneme	MPM	Zero	zero	50	100%
8.	Azithromycin	ATM	Zero	zero	50	100%
9.	Ciprofloxacin	CIP	17	34	33	66

Table.4 *nan*1 and *nan*2 genes diversity in isolates from different source

Seq.	Isolate site	*Nan*1	*Nan*2
1-	Sputum(5)	1(20%)	5(100%)
2-	Wound(6)	3(50%)	
3-	Ear(6)	3(50%)	3(50%)
Total	17	7(41.2%)	
			4(2066.6%)
			12(70.6%)

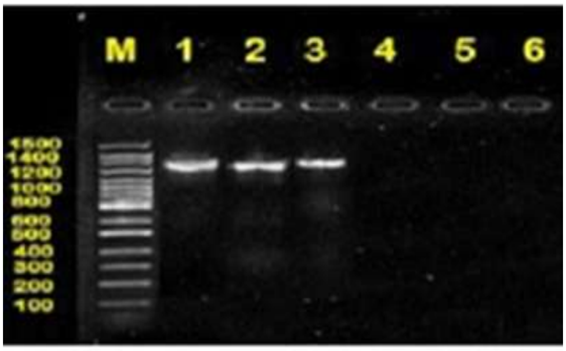

Figure1.result amplification of nan1 of *Pseudomonas aeruginosa* isolated from wound

Agarose gel electrophoresis(1.5% concentration of agarose,60 v voltage,2 h) of specific PCR product (1316bp) by specific primers to *nan1* gene. Lane M represents 100 bp DNA ladder markers. Lanes 1,2,3 represent positive amplification process of the *nan1* gene in DNA of *P. aeruginosa* isolate, Lanes 4,5,6 represent negative amplification process of the *nan1* gene in DNA of *P. aeruginosa* isolate. Lanes from 1 to 6 represents isolates respectively

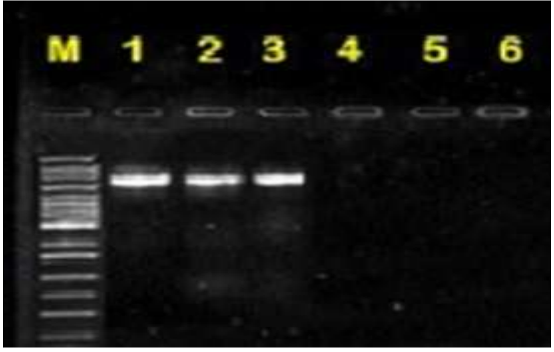

Figure 2. result from amplification of nan1 of *Pseudomonas aeruginosa* isolated from ear

Agarose gel electrophoresis (1.5% concentration of agarose,60 v voltage,2 h) of specific PCR product (1316 bp) by specific primers to *nan1* gene. Lane M represents 100 bp DNA ladder markers. Lanes 1,2,3 represent positive amplification process of the *nan1* gene in DNA of *P. aeruginosa* isolate, Lanes 4,5,6 represent negative amplification process of the *nan1* gene in DNA of *P. aeruginosa* isolate. Lanes from 1 to 6 represents isolates respectively

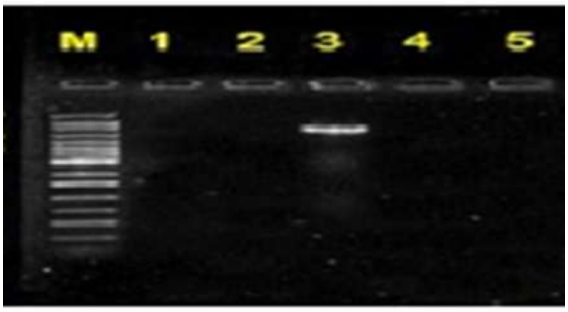

Figure 3. result from amplification of nan1 of *Pseudomonas aeruginosa* isolated from sputum

Agarose gel electrophoresis (1.5% concentration of agarose,60 v voltage,2 h) of specific PCR product (1316 bp) by specific primers to *nan1* gene. Lane M represents 100 bp DNA ladder markers. Lane 3 represent positive amplification process of the *nan1* gene in DNA of *P. aeruginosa* isolate, Lanes 1,2,4,5 represent negative amplification process of the *nan1* gene in DNA of *P. aeruginosa* isolate. Lanes from 1 to 5 represents isolates respectively

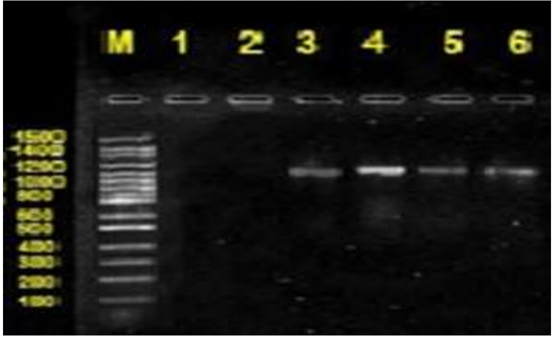

Figure 4. result from amplification of nan2 of *Pseudomonas aeruginosa* isolated from ear

Agarose gel electrophoresis (1.5% concentration of agarose, 60 v voltage, 2 h) of specific PCR product (1161 bp) by specific primers to *nan2* gene. Lane M represents 100 bp DNA ladder markers. Lanes 3, 4, 5, 6 represent positive amplification process of the *nan2* gene in DNA of *P. aeruginosa* isolate, Lanes 1,2 represent negative amplification process of the *nan2* gene in DNA of *P. aeruginosa* isolate Lanes from 1 to 6 represents isolates respectively

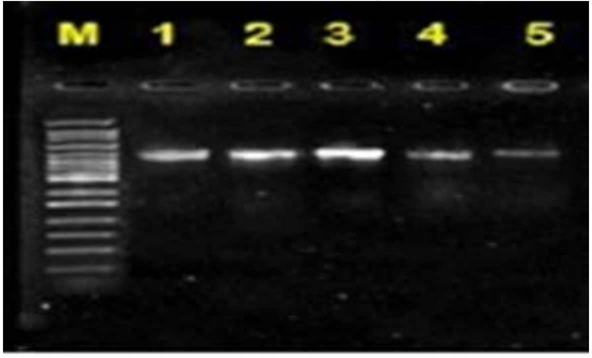

Figure5. Result from amplification of nan2 of *Pseudomonas aeruginosa* isolated from sputum

Agarose gel electrophoresis(1.5% concentration of agarose, 60 v voltage,2 h) of specific PCR product (1160 bp) by specific primers to *nan2* gene. Lane M represents 100 bp DNA ladder markers. Lanes 1, 2, 3, 4, 5 represent positive amplification process of the *nan2* gene in DNA of *P. aeruginosa* isolate, Lanes from 1 to 5 represents isolates respectively

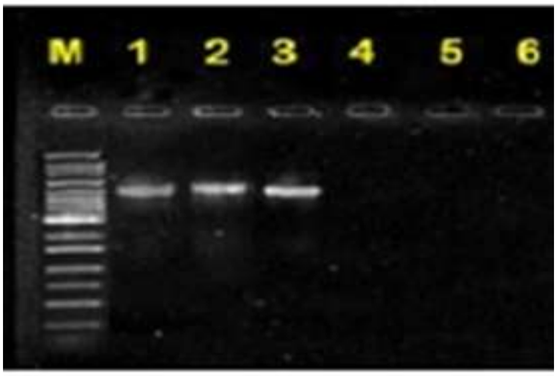

Figure 6. resulted from amplification of nan2 of *Pseudomonas aeruginosa* isolated from wound

Agarose gel electrophoresis(1.5% concentration of agarose, 60 v voltage, 2 h) of specific PCR product (1160 bp) by specific primers to *nan2* gene. Lane M represents 100 bp DNA ladder markers. Lanes 1, 2, 3 represent positive amplification process of the *nan2* gene in DNA of *P. aeruginosa* isolate, Lanes 4,5,6 represent negative amplification process of the *nan2*gene in DNA of *P. aeruginosa* isolate. Lanes from 1 to 6 represents isolates respectively

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