Available online freely at www.isisn.org

Bioscience Research

Print ISSN: 1811-9506 Online ISSN: 2218-3973

Journal by Innovative Scientific Information & Services Network

RESEARCH ARTICLE BIOSCIENCE RESEARCH, 2018 15(3):1729-1738 **OPEN ACCESS**

Correlation of nan1 (Neuraminidase) and production of some type III secretion system in clinical isolates of *Pseudomonas aeruginosa*

Zina Hashem Shehab[1] and Bahaa Abdullah Laftah[2]

[1]Biology Department, College of Science for Women, University of Baghdad, **Iraq**
[2]Biology Department, College of Science, University of Baghdad, **Iraq**

***Correspondence:** zinahashem4@gmail.com **Accepted:** 09Jun.2018 **Published online:** 04Aug. 2018

In this study a total of 122 different samples included burns, sputum, urine samples, wounds, ear swabs, blood sample, vaginal swab and CSF sample were obtained from patients whom visited as “out-patients” to three Educational hospitals at Baghdad city in 2017, All the samples were identified by biochemical tests, selective media and confirmed diagnosis with genetic identification by using 16SrRNA as a house keeping gene was used for genotypic diagnosis of P. aeruginosa. Fifty five isolates were Pseudomonas aeruginosa .(Polymerase Chain Reaction) PCR was used also for screening the prevalence of some virulence factor genes nan1 (neuraminidase) , exoS (exoenzyme S) and exoU (exoenzyme U).The results showed that 54 (98.18%) isolates were positive for exoS while nan1 and exoU genes 32 (58.18%) and 26(47.27)% respectively were positive, The prevalence of nan1 gene was (100%) in urine ,wound, ear , blood, vaginal and CSF samples followed with sputum (50%) and (14.28%) for burns samples , the frequencies of exoU gene was (100%) for urine , vaginal and CSF samples followed with wound, sputum (75%) and (60%) respectively , but the lowest prevalence in ear and burns infection (25%), (23.80%) respectively. We conclude from our results nan1 gene not essential for virulence compared with T3SS exotoxins exoS and exoU may be enhances ability of production of these effector proteins, because did not appear any isolate have *nan*1 gene alone.

Keywords: *Pseudomonas aeruginosa*, *nan1*, *exoS* and *exoU* genes, Polymerase Chain Reaction.

INTRODUCTION

Pseudomonas aeruginosa is an opportunistic pathogen and one of the most frequent causative agents of nosocomial infections (Cacalano et al., 1992).Which causes a wide range of infections including septicemia, pneumonia, endocarditis, burns ,wounds, otitis , keratitis, urinary-tract infections in catheterized patients, immuno deficient and immuno compromised patients (Berthelot et al., 2003 & Lavenir et al., 2007) and causes persistent respiratory infections in individuals suffering from cystic fibrosis (CF) (Feltman et al., 2001) during last few decades, this pathogen associated with high mortality rate due to the presence of virulence factors in the bacterium, especially people with (CF) or chronic obstructive pulmonary diseases (Lavenir et al., 2007). *P. aeruginosa* is capable of elaborating an impressive array of virulence factors, which are divided into specific groups dependent upon their mode of action or method of delivery to the host cell. Its virulence factors are described as belonging to adhesions' and other secreted toxins. (Bradbury et al., 2010) such extracellular Neuraminidases encoded by *nan*1 gene , Sialidases also referred to as, are key enzymes that hydrolyze the linkage of terminal sialic acids on various sialoglycoconjugates to generate free

sialic acid (Vimr et al.,2004 and Severi et al., 2007) Sialic acids are important components of the serum and mucus and represent the terminal sugar residue of many glycan chains on host cell surfaces, where they are involved in cell- cell recognition (Lewis and Lewis2012).Additionally, type III secretion system (T3SS) Exoenzyme S, encoded by the *exoS* gene, is an ADP ribosyltransferase that is secreted by a type III secretion system directly into the cytosol of epithelial cells (Riese et al.,2002)and *exo*U, a phospholipase activity (Sato and Frank , 2004).In recent study, we used molecular diagnosis such as PCR, which have the potential for identifying microbial species rapidly by amplification of sequences unique to a particular gene in whole genome thus could be used for screening virulence gene (Khan and Cerniglia, 1994).The aim of our study was to evaluate the prevalence of some virulence genes encoding putative Neuraminidase *nan*1 and its role in production of some type III effector proteins *exoS* and *exoU* in clinical isolates of *P. aeruginosa* and their relationship with site of infection.

MATERIALS AND METHODS

Samples collection

A total of 55 different clinical isolates of *P. aeruginosa* were isolated from 122 clinical samples collected from patients whom visited as "out-patients" to Educational Al-Yarmouk, AL Kindi and Baghdad hospitals at Baghdad city during March to August 2017,They were obtained from burns(n:21), bronchial wash (n:3), sputum (n:7),urine mid-stream (n:5), surgical wounds or abscesses (n:8), ear swabs (n:8), blood (n=1),vaginal swab (n:1) and cerebrospinal cord(CSF) (n:1).The samples transferred immediately into the microbiology laboratory for further experiments. Isolates were grown on Brain Heart Infusion Agar (BHIA) at 37 ^{o}C for 24 h and stored at -80 ^{o}C in Brain Heart Infusion Broth (BHIB) containing 30% (v/v) of glycerol.

Isolation and Identification

The specimens were cultured on MacConkey agar (Himedia-India) and incubated at 37℃ for 24 hours under aerobic conditions in order to differentiate the lactose fermented bacteria from the non-lactose fermented bacteria. Well isolated colonies were selected and cultured on Citrimde agar (Himedia-India) to detect the *P. aeruginosa* isolates, which produce a blue green or yellow green pigments. The isolates were identified depending on morphological properties (for cells and colonies) and biochemical tests as described by Brown (2005), followed by Polymerase chain reaction (PCR) amplification of *16SrRNA* gene and investigate prevalence of some virulence genes. Which included, encoding Neuraminidase (*nan*1), exoenzyme S (*exoS*) and exoenzyme U (*exoU*), was determined by PCR. All research genes were amplified with specific primers (Macrogen co. USA) listed in Table (1).

Genotyping detection for isolates

16S rRNA gene a house keeping gene was used for genotypic diagnosis of *P. aeruginosa*, Specific primers listed in table (1) were employed and the amplified size was 144bp. A *16S rRNA* gene PCR assay has the potential to detect pathogens due to this gene being highly conserved in prokaryotes.(Janda and Abbott, 2007) .The genomic DNA was extracted by using Wizard genomic DNA purification kit (Promega, Madison,USA).Briefly,1ml of overnight culture centrifuged for 2min at 13000 rpm. Supernatant then discarded, for getting template DNA from pellet cells. PCR mixture was composed from 10 µl of Green Master Mix, 1µl of forward primer and 1µl of Reverse primer with 6 µl of nuclease free water. Finally, added 2 µl from each sample. PCR was run under the following conditions starting with a primary denaturation step at 95°C for 5min then 35 repeated cycles started with a denaturation step at 95°C for 30sec, then annealing at 60°C for 30sec and 1min at 72°C as extension step followed by final extension step at 72°C for 7min .The concentrations of all DNA samples were determined using nano-drop instrument (Quantus USA). The general ratio (A 260/280) equal to (1.8) perform better for PCR reaction before start with PCR assay. Amplification of Virulence genes Neuraminidase genes (*nan1*), exoenzyme S (*exoS*), and exoenzyme U (*exoU*) were determined by PCR. The genes were amplified with primers selected on the basis of the published PAO1 sequence PCRassay was run by the same method as previously described (Table 1). Then, products were separated in 1% agarose gel for 65 min at 1wat, stained with ethidium bromide and detected by UV transilluminator.(Sambrook and Russel, 2001)

Statistical Analysis

The Statistical Analysis System- SAS (2012) program was used to detect the relation difference between genes different isolates.

Table (1) Primers used for PCR amplification of virulence genes.

Genes	Primer sequence (5`→3`)	Product Size (bp)	Annealing temperature	Reference
16S rRNA-f **16S rRNA-r**	GCA CTT TAA GTT GGG AGG AA CTT TAC GCC CAR TRA WTC CG	**144**	**60**	**(**Hillenbrand et al., 2011)
nan1-f **nan1-r**	GGAGCGTGTTCCTGTGTGTATAG AGAAGACGTCTCCCTGAATAAGA	**100**	**60**	New designed in this study
exoS-f **exoS-r**	CTT GAA GGG ACT CGA CAA GG TTC AGG TCC GCG TAG TGA AT	**504**	**60**	(Mitov et al., 2010)
exoU-f **exoU-r**	GGG AAT ACT TTC CGG GAA GTT CGA TCT CGC TGC TAA TGT GTT	**428**	**60**	(Mitov et al., 2010)

Abbreviations: f, forward primer; r, Reverse primer.

Chi-square test was used to significant compare between percentages in this study

RESULTSAND DISCUSSION

In this study55 isolates of *P. aeruginosa* were collected from different nosocomial infections. Because standard phenotypic methods require several days and most have inherent limitations, genotypic detection depending on certain housekeeping gene was used as confirmatory test which provide a rapid diagnostic identification of bacteria. Al_Jabiri and Al_Jubori (2013) used *16S rRNA* gene for detection the same bacteria and reported positive result for all isolates. Figure (1) shows positive agarose gel electrophoresis results for *16SrRNA gene* products (amplified).

Prevalence of virulence genes

Genomic DNA was used as a template for PCR screening virulence genes of *P. aeruginosa* which are tightly regulated by cell-to-cell signaling systems(Van Delden,&Iglewski,1998).As well as study correlating virulence patterns and infection clinical outcome could be useful for setting up efficient preventive and therapeutic procedures in hospitalized patients with positive *P. aeruginosa* cultures (Holban *et al.,* 2013) . The frequencies of occurrence of virulence genes in all studied strains (n=55) were as follows Table (1) and in Figure (2, 3, 4) shows positive agarose gel electrophoresis results forNeuraminidase (*nan1*), exoenzyme S (*exoS*), and exoenzyme U (*exoU*) genes products (amplified).The results of isolates showed production of T3SS effector protein *exoS* gene with 98,08% , Our results differ from several results of researches such as Lanotte *et al.* (2004) *exoS* was only detected in (84.5%) of isolates; Mitov et al.(2010) reported *exoS* in (62.4%) among 202 strains while Thamir and Al-Jubori (2014) reached to 21 isolate positive for *exoS* gene about (80.7%) and Yousefi-Avarvand et al., (2015) mentioned the frequency of *toxA*, *exoU* and *exoS* genes were 90.4%, 66.7% and 65.4%, respectively. The majority of *P. aeruginosa* strains secrete ExoS, a bifunctional toxin with GTPase-activating protein and ADP ribosyltransferase activities. Numerous *in vitro* studies have investigated the targets and cellular effects of ExoS, linking both its enzymatic activities with inhibition of bacterial internalization. However, little is known about how this toxin facilitates the progression of infection *in vivo* and inhibits phagocytosis during Pneumonia (Rangel et al., 2014).Results of *nan1* and *exoU* genes prevalence revealed that total 29(55.77) and 24(46.15) respectively of the isolates were harbored these genes with high Significant at ($P<0.01$) for *nan1* gene was (11.52) followed by (10.74) for *exoU* gene, respectively. The cytotoxic type III effector ExoU is induced early during acute *P. aeruginosa* Pneumonia, Which causes rapid cell death in a mouse model. (Howellet al.,2013) ,As for the gene called *nan1* encodes a sialidase that is responsible for adherence to the respiratory tract (Bradbury et al., 2010)Which plays an important role in bacterial attachment and subsequent invasion into host cells, and particularly into epithelial cells. (Engel and Balachandran, 2009), Our results disagreed with Lanotte et al.,(2004) *nan1* was detected in 86 isolates (53 %) and (100%) for *exoU* they illustrated that the distribution of *nan1* was significantly related to isolates origin since they reported that the prevalence of *nan1* was higher in CF isolates (61.7 %) as compared with non-CF isolates (44.4%); Ra'oof(2011) reported *nan1* gene were low disseminated (15%) only .While Thamir and Al-Jubori (2014) showed *nan1* gene were detected in 1\26 (3.8%).

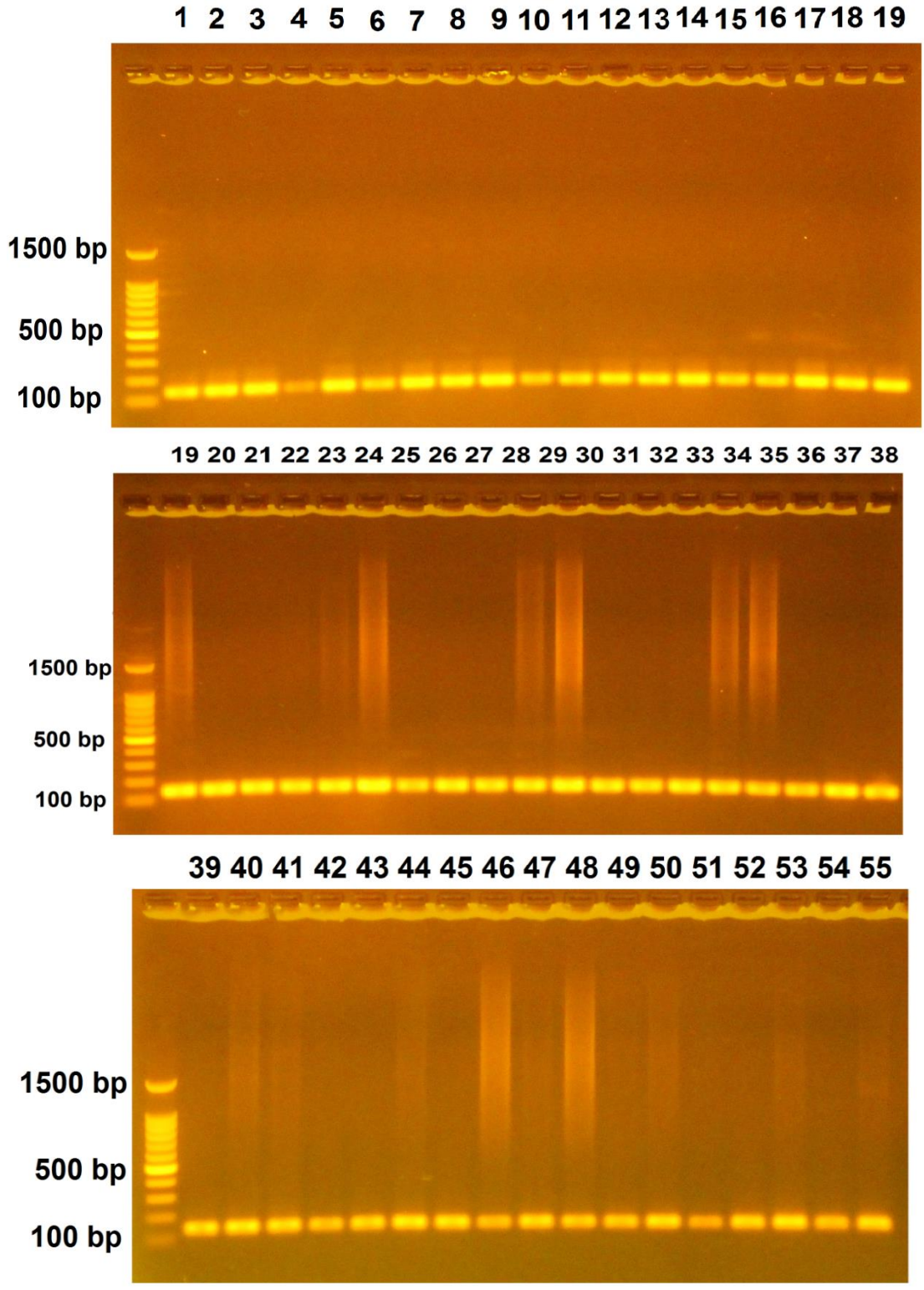

Figure (1): Agarose gel electrophoresis of *16SrRNA* gene PCR product (144bp amplicon), DNA Ladder (100bp), all the lanes were positive for 55 isolates.

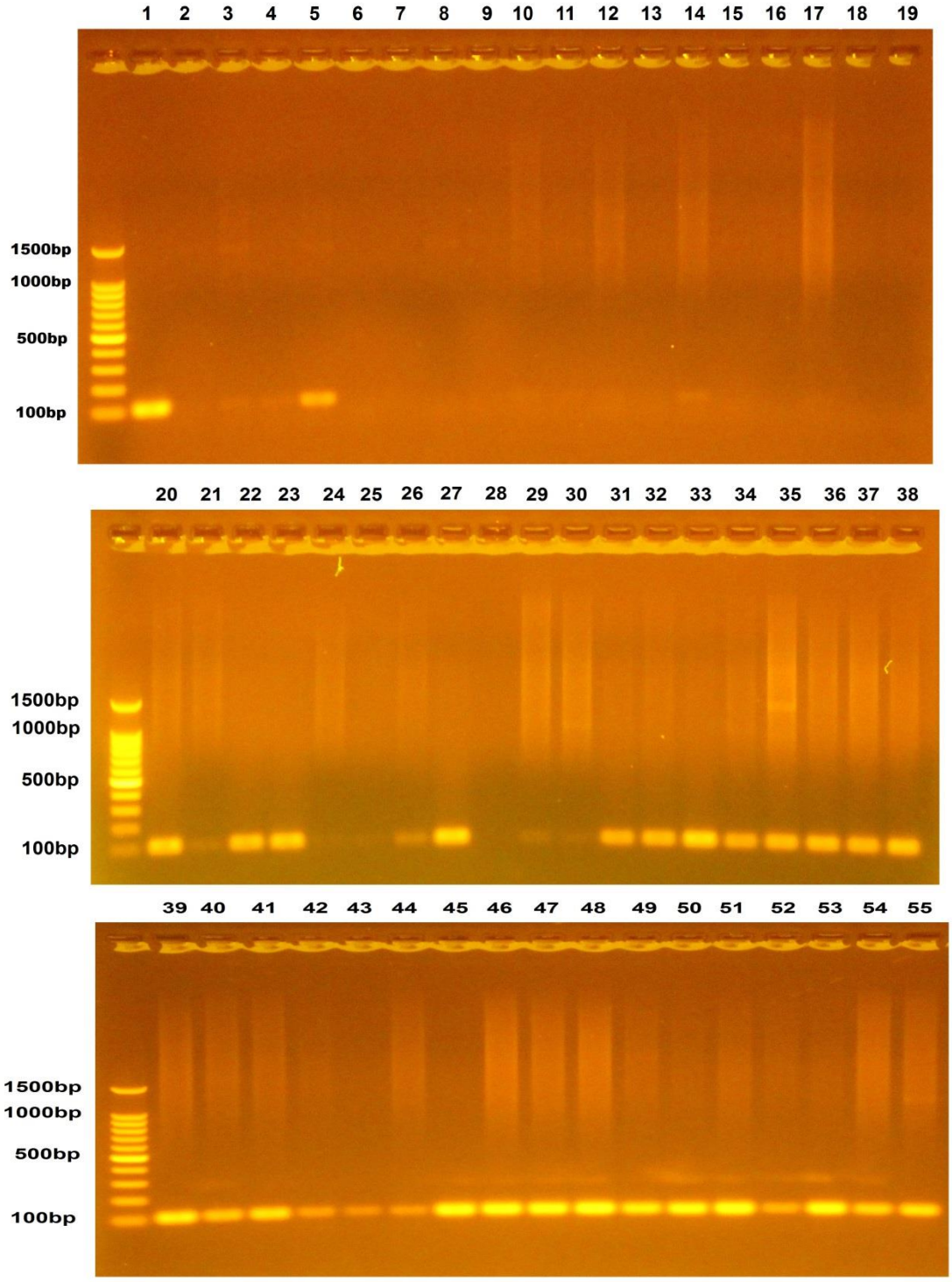

Figure (2): Agarose gel electrophoresis of *nan1* gene PCR product (100 bp amplicon) , DNA ladder (100bp). all the lanes were positive for the *nan1* , except the lanes (2,3,4,6,7,8,9,10,11,12,13,14,15,16,17,18,19,21,24,25,28,29,30) were negative

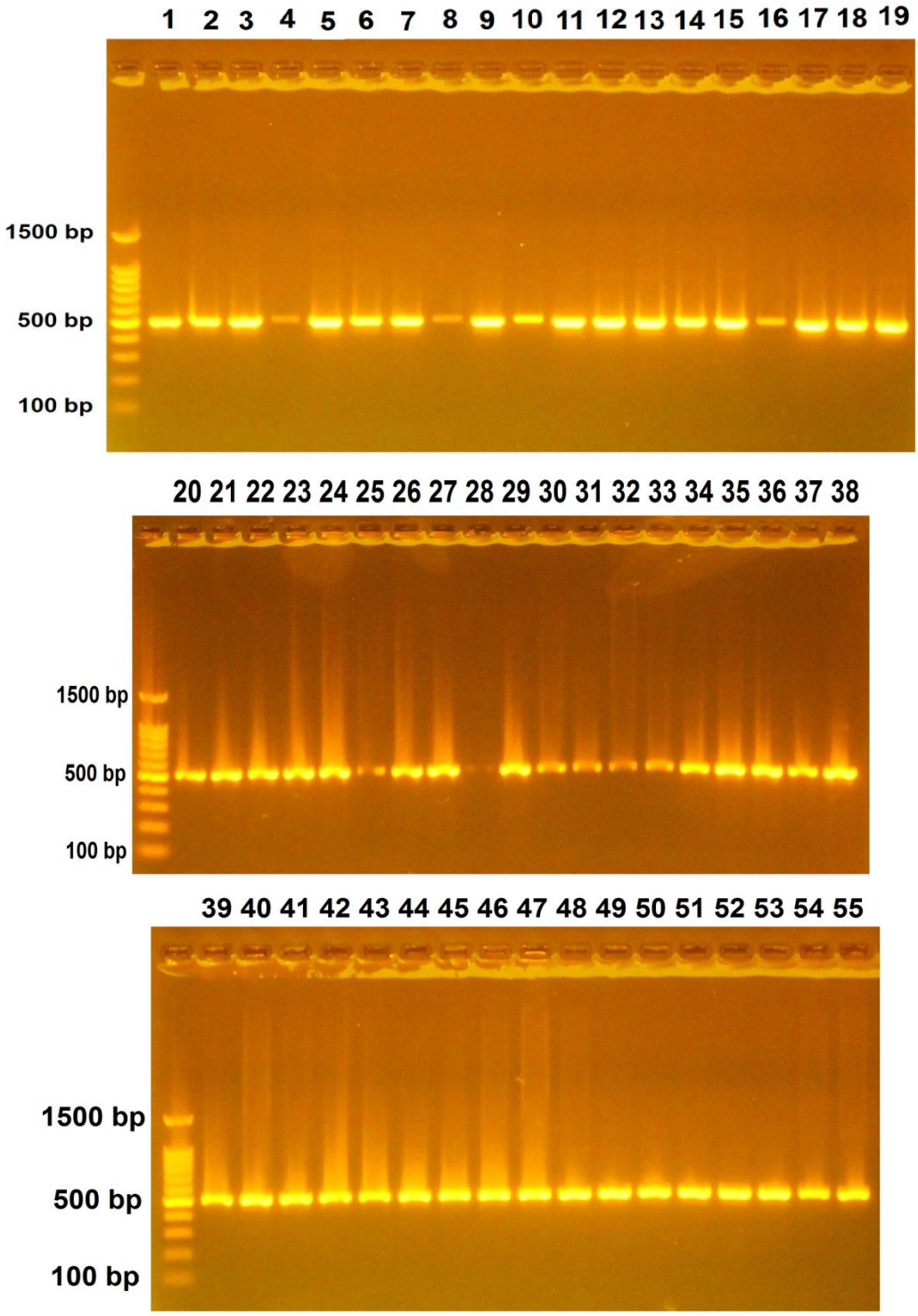

Figure (3): Agarose gel electrophoresis of *exoS* gene PCR product (504 bp amplicon). DNA ladder (500bp), all the lanes were positive for 55 isolates except isolate (28).

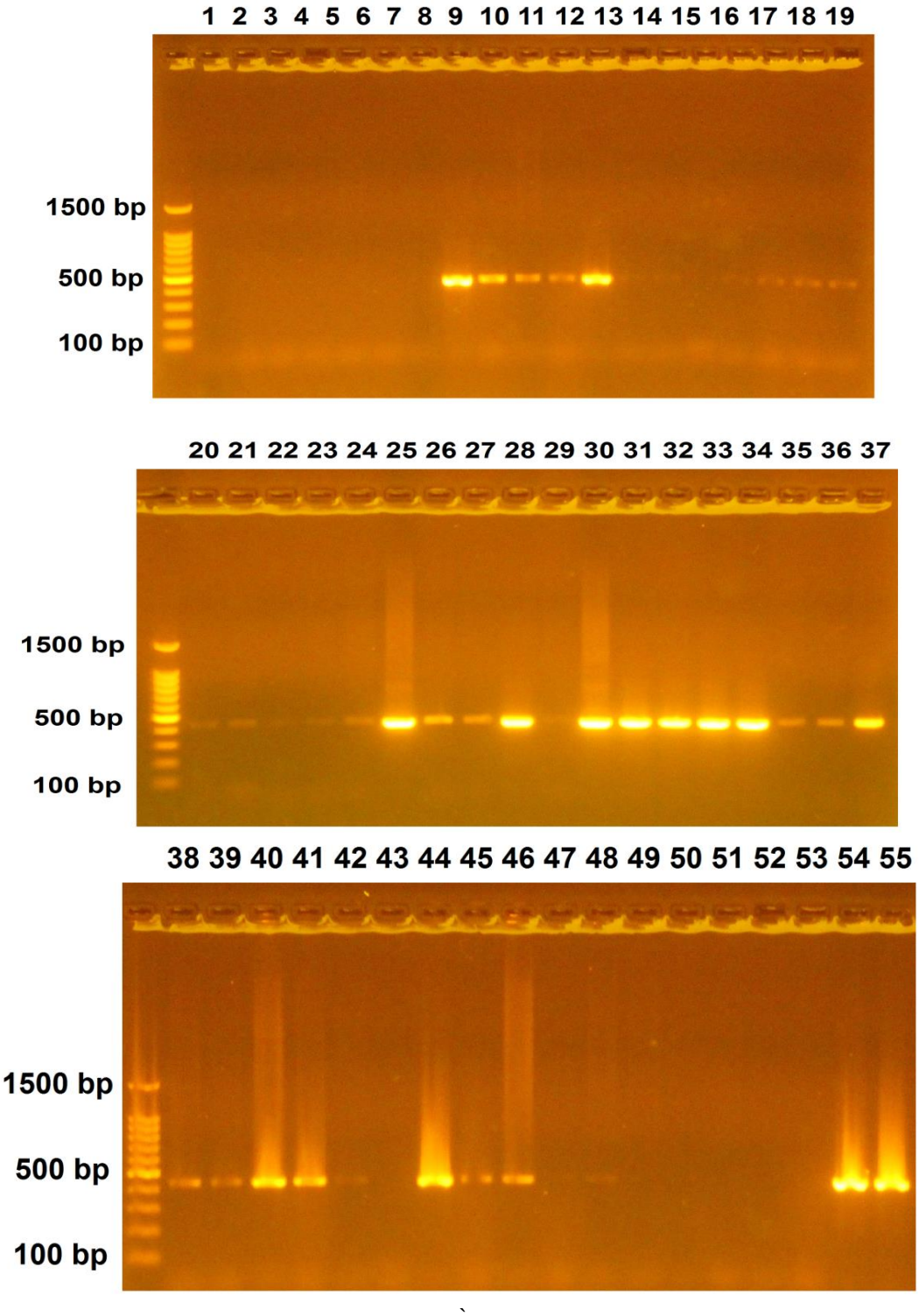

Figure (4): Agarose gel electrophoresis of *exoU* gene PCR product (428 bp amplicon). DNA ladder (500bp), all the lanes were positive exoU, except the lanes (1, 2, 3,4,5,6 ,7,8,14,15,16,17,18,19,20,21,22,23,24,29,42,43,47,48,49,50 ,51,52,53) were negative

Table (2): Prevalence and percentage of some virulence genes among *P. aeruginosa* obtained from various sources.

Samples Genes	burns no.(21)	Sputum no.(10)	urine no.(5)	wounds no.(8)	ear no.(8)	Total% 52	Chi-Square (χ^2)
nan 1	3 (9.68%)	5 (50%)	5 (100%)	8 (100%)	8 (100%)	29 (55.77%)	11.52 **
exoS	21 (`100%)	9 (90%)	5 (100%)	8 (100%)	8 (100%)	51 (98.08%)	4.37 *
exoU	5 (23.81%)	6 (60%)	5 (100%)	6 (75%)	2 (25%)	24 (46.15%)	10.74 **
Chi-Square	14.84 **	10.39 **	0.00 NS	8.25 **	13.62 **	10..86 **	---
*** (P<0.05), ** (P<0.01).**							

Table (3): Prevalence and percentage of some virulence gene groups present at same isolates among *P. aeruginosa* different clinical sources.

Samples Groups	burns no.(21)	sputum no.(10)	urine no.(5)	wounds no.(8)	Ear no.(8)	Total% 52	Chi-Square (χ^2)
G1	3 (9.68%)	5 (50.0%)	5 (100%)	8 (100%)	8 (100%)	29 (55.77%)	14.89 **
G2	0 (0.00%)	3 (30.0%)	5 (100%)	6 (75%)	2 (25%)	16 (30.77%)	15.37 **
G3	5 (23.81%)	5 (10.00%)	5 (100%)	6 (75%)	2 (25%)	23 (44.23%)	14.32 **
G4	0 (0.00%)	3 (30.00%)	5 (100%)	6 (75%)	2 (25%)	16 (30.77%)	14.96 **
Chi-Square	8.25 **	10.63 **	0.00 NS	8.25 **	10.73 **	9.45 **	---
** (P<0.01).							

Abbreviations : G1:(*nan*1+*exoS*)genes ; G2(*nan1*+*exoU*)genes ; G3:(*exoS*+*exoU*)genes ; G4: (*nan*1 +*exoS*+*exoU*) genes

In a recent study disagreed with Mitov et al., (2010) they found that the frequencies of *exoU* and *nan1* were significantly higher among CF and non-CF isolates *exoU* (30.2%) and *nan1* (21.3%) from 202 strains of *P. aeruginosa*. The molecular-genetic detection of the *nan1* gene may be used as an indirect measure of CF pulmonary disease evolution. (Mitov et al.,2010) .In our study difference between virulence genes *exoS*, *exoU* and *nan1* prevalence in the isolates origins was statistically high significant under (P < 0.01) as explained in (Table 1) burns (14.84), ear infections (13.62) and sputum (10.39) and finally wounds (8.25) with non-significant for urine samples .As well as we got other samples in search one for blood; vaginal and CSF samples showed positive results for *nan1* and *exoS* genes and negative for *exoU* gene in blood sample only. The conflicting results of these studies may be due to differences in the number of clinical isolates from different sites or due to the isolates from patients with different clinical and physiological conditions (Feltmanet al., 2001.The *nan1* geneandother virulence factors studied in this research, were found in Table (3) with high percentage for G1 and G3 gene groups with (55.77 and 44.23)% for (*nan*1+*exoS*) and (*exoS*+*exoU*)genes respectively followed with G2 and G4 (30.77%) for (*nan1*+*exoU*) and (*nan*1+*exoS*+*exoU*) genes. Whereas, and did not appear any isolate have *nan1* gene only. The virulence genes appeared significantly higher in isolates of ,G2(*nan1*+*exoU*) group about (15.37) while which equal to prevalence of G1; G3 and G4 groups approximately (14)% .We conclude from our results *nan1* gene not essential for virulence compared with T3SS exotoxinsexoS and exoU may be enhances ability of production effector proteins exoS and exoU, especially, exoU. Many researchers indicated that Sialidase-mediated virulence mechanisms vary and include the enhancement of biofilm formation increased bacterial adherence to host cells (Barker et al., 2009).The PAO1 neuraminidase was 1000-fold more active than the *Clostridium perfringens* enzyme in releasing sialic acid from respiratory epithelial cells.

CONCLUSION

Our results recorded high *nan1* gene percentage compared with only two previous Iraqi studies but not essential for virulence compared with T3SS exotoxins (exoS and exoU) may be enhances ability of production these proteins especially, exoU.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest".

ACKNOWLEGEMENT

This work was supported by Biology department, College of Science and Institute of Genetic Engineering and Biotechnology for Postgraduate Studies, University of Baghdad.

AUTHOR CONTRIBUTIONS

All authors contributed to the design of the experiments. BAL performed the experimental work, ZHS and BAL carried out laboratory tests and wrote the manuscript, all authors revised and approval the final version.

REFERENCES

Al-Jabiri HAK and Al-Jubori SS, 2013 . Prevalence of mexxy gene mediated efflux pumps resistance towards aminoglycosides group among clinical isolates of *Pseudomonasaeruginosa*. Mintage J. Pharma. Med. Sci., 2(1): 7-10.

Berthelot P ,Attree I , Plesiat P , Chabert J , de Bentzmann S , Pozzetto B, B Grattard F and Groupe d'Etudes des Septicémies à Pseudomonas aeruginosa , 2003 .Genotypic and phenotypic analysis of type III secretion system in a cohort of *Pseudomonas aeruginosa* bacteremia isolates: evidence for a possible association between O serotypes and exo genes. J. Infect Dis.,15(188):512-8.

Bradbury RS, Roddam LF, Merritt A, Reid DW and Champion AC, 2010 . Virulence gene distribution in clinical, nosocomial and environmental isolates of *Pseudomonas aeruginosa*. J. Med. Microbiol., 59(8):881-90.

Brown, AE, 2005. Bensons microbiological applications.9th (ed.), Complete version, laboratory manual in general Microbiology, McGraw Hill .

Cacalano G, KaysM ,Saiman L and Prince A ,1992 . Production of *Pseudomonas aeruginosa* neuraminidase is increased under hyperosmolar conditions and is regulated by genes involved in alginate expression. J. Clin. Invest., 89:1866-1874.

Engel J and Balachandran P,2009 . Role of *Pseudomonas aeruginosa* type III effectors in disease. Curr.Opin. Microbiol. , 12:61-6.

Feltman H , Schulert G , Khan S.; , Jain M ,Peterson L and Hauser A ,

2001 . Prevalence of type III secretion genes in clinical and environmental Isolates of *Pseudomonas aeruginosa*. Microbiology, 147(10):2659-69.

Galen JE ,Ketley JM, Fasano A, Richardson SH, Wasserman SS and Kaper J B, 1992 . Role of *Vibrio cholera* neuraminidase in the function of cholera toxin. Infect. Immun., 60:406–415.

Hillenbrand ME , Thompson PP , Shanks RM and Kowalski RP , 2011. Validation of PCR for the detection of *Pseudomonas aeruginosa* from corneal samples. Int. J. Opthalmol. 4(3):262-268.

Holban A , Chifiriuc MC , Cotar A , Bleotu C , Grumezescu AM , Banu O and Lazar V , 2013 .Virulence markers in *Pseudomonas aeruginosa* isolates from hospital acquired infections occurred in patients with underlying cardiovascular disease . Romanian Biotechnological Letters, 18 (6): 8843-54.

Howell HA, Logan LK and Hauser AR, 2013. Type III Secretion of ExoU Is Critical during early *Pseudomonas aeruginosa* Pneumonia. mbio.asm.org, 4(2):1-9.

Janda JM and Abbott SL, 2007. 16S rRNA Gene Sequencing for Bacterial Identification in the Diagnostic Laboratory: Pluses, Perils, and Pitfalls (minireview).JOURNAL OF CLINICAL MICROBIOLOGY, 45(9): 2761-64

Khan AA and Cerniglia CE ,1994. Detection of *Pseudomonas aeruginosa* from clinical and environmental samples by amplification of the exotoxin A gene using PCR. Appl. Environ. Microbiol. 60: 3739-3745.

Lanotte P , Watt S , Mereghetti L , Dartiguelongue N , Rastegar-Lari A ,Goudeau A and Quenn R , 2004 . Gene

features of *Pseudomonas aeruginosa* isolates from cystic fibrosis patients compared with those of isolates from other origins. J. Med. Microbiol. 53(1): 73-81.

Lavenir R , Jocktane D , Laurent F , Nazareth S and Cournoyer B ,2007. Improved reliability of *Pseudomonas aeruginosa* PCR detection by the use of the species-specific ecfX gene target. J. Microbiol. Methods, 70(1):20-9.

Lewis AL and Lewis WG , 2012 . Host sialoglycans and bacterial sialidases: a mucosal perspective. Cellular Microbiology, 14(8):1174–1182.

Mitov IM , Tanya S and Boyka M , 2010 .Prevalence of virulence genes among Bulgarian nosocomial and cystic fibrosis isolates of *Pseudomonas aeruginosa*. Braz. J. Microbiol.,41(3): 588–595.

Parker D, Soong G, Planet P, Brower J, Ratner AJ and Prince A,2009 .The nana neuraminidase of Streptococcus pneumonia is involved in biofilm formation. Infect. Immun., 77: 3722–3730.

Rangel SM, Logan KK and Hauser LK, 2014 . The ADP-Ribosyltransferase Domain of the Effector Protein ExoS Inhibits Phagocytosis of *Pseudomonas aeruginosa* during Pneumonia. mbio.asm.org, 5(3):1-10.

Ra'oof W M , 2011 .Distribution of algD, lasB, pilB and nan1 genes among MDR clinical isolates of Pseudomonas aeruginosa in respect to site of infection. Tikrit Medical Journal, 17(2): 148- 160.

Riese MJ , Goehring UM , Ehrmantraut ME, Moss J , Barbieri JT , Aktories K and Schmidt G , 2002 . Auto-ADP-ribosylation of *Pseudomonas aeruginosa* ExoS. J. Biol. Chem., 277(14): 12082-12088

Sambrook J and Russell DW , 2001 . Molecular cloning: In: A Laboratory manual cold spring harbor. New York, USA, Cold spring harbor laboratory press.

SAS , 2012 . Statistical Analysis System, User's Guide. Statistical. Version 9. 1st (ed.) SAS.Inst. Inc. Cary. N.C. USA.

Sato H and Frank WD, 2004. ExoU is a potent intracellular phospholipase. Mol.Microbiol. 53:1279–1290.

Severi E , Hood DW and Thomas GH , 2007. Sialic acid utilization by bacterial pathogens. Microbiology, 153: 2817–2822.

Shimamoto S, Tamai E, Matsushita O, Minami J, Okabe A, Miyata S, 2005 Changes in ganglioside content affect the binding of *Clostridium perfringens* epsilon-toxin to detergent-resistant membranes of madin-darby canine kidney cells. Microbiol. Immunol., 49: 245–253.

Thamir E and Al-Jubori SS, 2014 .Genetic detection of some virulence genes in *Pseudomonas aeruginosa* isolated from cystic fibrosis and no-cystic fibrosis

patients in IraqJournal ofGene c and Environmental Resources Conservation, 2(3):380-387.

Van Delden, C. and Iglewski BH ,1998 . Cell-to-cell signaling and *Pseudomonas aeruginosa* infections. Emerg. Infect. Dis., 4: 551–560.

Vimr ER , Kalivada KA , Deszo EL , Steenbergen SM , 2004 Diversity of microbial sialic acid metabolism. Microbiol. Mole. Biol. Rev., 68; 132–153

Yousefi-Avarvand A, Khashei R , Ebrahim-Saraie HS , Emami A , Zomorodian K and Motamedifar M , 2015 .The Frequency of Exotoxin A and Exoenzymes S and U Genes Among Clinical Isolates of *Pseudomonas aeruginosa* in Shiraz, Iran. IJMCM, 4(3):167-173.