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Genetic detection of some virulence genes in *Pseudomonase aeruginosa* isolated from cystic fibrosis and no-cystic fibrosis patients in Iraq

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Abstract

Twenty six clinical isolates of *P. aeruginosa* were obtained from patients submitted to Baghdad hospitals/Iraq (22 isolates were from different nosocomial infections and 4 isolates from cystic fibrosis[CF] patients). These isolates were molecularly diagnosed using *rpsl* gene (housekeeping gene). The prevalence of virulence genes, exoenzyme S (*exoS*), exotoxin A (*toxA*) and putative silidase (Neuraminidase-*nan1*) were determined by PCR. The most frequently gene was *exoS* when it was detected in 21/26 (80.7%) and distributed between 19 in different clinical source and 4 in CF samples. *toxA* and *nan1* genes were detected in 19/26 (73%) and 1\26 (3.8%) respectively. Results of DNA sequencing for *exoS and toxA* genes revealed that most isolates display different point mutation as compared with the NCBI data. Point mutation type transversion occur in residue no.17 base 52 in *P. aeruginosa exoS* BW1 isolated from bronchial washer causes alteration T to A and conversion Leucine residue to Glutamine .Point mutation type transition occur in *exoS* B1, *exoS* U1 and *exoS* CyF1 in residue no.18 base 55 when G convert to A cause conversion of Serine residue to Asparagine. *P. aeruginosa exoS* B3 and *exoS* CyF2 isolated from blood and cystic fibrosis respectively showed high similarity made them segregate within the same group or clone while *exoS*U 2 and U3 were within the same clone but not with *exoS*U 1 nor U4. Another point mutation was detected in *P. aeruginosa toxA* CyF2, nitrogen base G no.36 residue 12 was changed to a cause the conversion of arginine to histidin. Also mutation occur in *toxA* CyF1,2,3,3, *toxA* U1,2,3,4 and *toxA* BI, BW1 when nitrogen base A no.134 residue 44 was converted to G causes the conversion of threonine to alanine. Another point mutation occurs with the same above isolates when nitrogen base A no.233 residue 78 changed to G causes conversion of Asparagine to Serine. Such difference may explain the highly virulence in some isolates.

Keywords: *Pseudomonas aeruginosa,* Cystic fibrosis isolate, Virulence factors.

Introduction

Pseudomonas aeruginosa is an opportunistic pathogen capable of causing a wide variety of human infections including severe burn and wounds infections, urinary tract infections, chronic obstructive pulmonar disease (Gellatly and Hancock 2013). It often colonizes immune compromised patients, like those with cystic fibrosis (CF), cancer and AIDS (Botzenhardt and Doring, 1993). In the early stage of CF infection the bacteria could be eradicated, while reduction of bacterial density is desirable during chronic colonization or exacerbations (Canton, 2005). *P. aeruginosa* harbored a large number of virulence factors such as exotoxin A, exoenzyme S, elastase and sialidase beside many other extra cellular products (VanDelden and Iglewski, 1998). Exotoxin A encoded by the *toxA* gene which has ability to inhibit protein biosynthesis just like diphtheria toxin (Hamood, 2004). 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 human epithelial cell (Riese *et al.,* 2002; Anthonv *et al.,* 2007). Extracellular neuraminidase encodes by *nan1*gene (also called sialidase) is response for spreading *P.aeruginosa* within host cells (Daniel *et al.,* 2008). Extracellular virulence factors have been shown to be controlled by a complex regulatory circuit involving cell-to-cell signaling systems (quorum sensing) that allow the bacteria to produce these factors in a coordinated, cell-density–dependent manner (VanDelden and Iglewski, 1998). Conventional microbiological methods for identifying *P. aeruginosa* from clinical and environmental samples are reliable, they require several days to be completed. Molecular diagnosis such as PCR has 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 aims of this study were to investigate the prevalence of some virulence genes in clinical isolates of *P. aeruginosa* obtained from different infections and including *exoS*, *toxA* and *nan1* compared them with those in CF isolates. Analyzing PCR products using Geneious software then Phylogenic analysis and Dendrogram for the sequenced data using Tamura –Nei genetic destine model and UPGMA tree build method.

Materials and Methods

Collection and diagnosis of bacterial isolates: Twenty six clinical isolates of *P. aeruginosa* were isolated from patients submitted to Baghdad /Iraq during October 2013- to January 2014. Twenty two isolates were from different nosocomial infections and 4 isolates from CF patients. The nosocomial isolates were obtained from midstream urine from patient suffering urinary tract infections (n: 4), bronchial wash (n:2), sputum samples (n:4), surgical wounds or abscesses (n:3), blood samples (n:6) and ear infections (n: 3). Each isolate was identified on the basis of colony morphology, conventional biochemical testes according to atlas *et al*. (1995) followed by complementary API 20E test.

Genotyping detection for isolates: *RpsI* gene (a house keeping gene accession no. CP006985.1) was used for genotypic diagnosis. Specific primers listed in Table (1) were employed and the amplified size was 201bp. Template DNA was prepare by simple boiling method (Xavier *et al.,* 2010). Briefly, few isolated colonies of overnight growth bacteria were suspended thoroughly in 5ml distilled water and boiled in a water bath for 10min. After centrifugation, supernatant was separated and used as template DNA. PCR mixture was composed from 12.5 of GoTaq® Green Master Mix (2x), 5µl template DNA, 1.5 µl forward and reverse primers (for each) final concentration 0.6pmol/µl, and nuclease free water up to 25µl (4.5µl). 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 57°C for 30sec and 1min at 72°C as extension step followed by final extension step at 72°C for 7min (Xavier *et al.,* 2010).

Amplification of Virulence genes: The prevalence of virulence genes, exoenzyme S (*exoS*), exotoxin A (*toxA*) and putative Silidase or neuraminidase genes (*nan1*) were determined by PCR. The genes were amplified with primers selected on the basis of the published PAO1 sequence (Table 1) (Stover *et al.,* 2000). PCR mixture was composed from 5µl template DNA prepared by boiling method as previously described, 12.5 µl of GoTaq ® Green Master Mix (2x), 1.5µl from forward and reverse primers final concentration 1pmol\µl for each gene, then the volume was complete to 25µl using nuclease free water 4.5µl). PCR condition (Table 2) were optimized according to this study by repeated changing annealing temperatures for each primer (from 60 to 68ºC) and number of cycles (35) till being fixed at the condition listed in Table 2. Usually the process started with initial denaturation step (95ºC for 5 min) followed by repeated cycles which consists from denaturation step (95ºC), annealing step (depends on the primers) then the extension step (mostly at 72°C) followed by final extension step (usually at 72°C). PCR products were separated in 1% agarose gel for 1h at 75V, stained with ethidium bromide and detected by UV transilluminator (Sambrook and Russell, 2001).

DNA sequencing and analyzing: Amplified genes were identified and sequenced by sending 25µl of PCR products to NICEM Company, USA. The results were analyzed by using Geneious software version NO. 7.1.2R (2013). Phylogenic analysis and Dendrogram for the sequenced data were also performed with same software using Tamura –Nei genetic destine model and UPGMA tree build method.

Results and Discussion

P. aeruginosa is a metabolically versatile bacterium that can cause a wide range of severe opportunistic infection in patients with serious underlying medical conditions (Gellatly and Hancock 2013).The critical traits contributing to the pathogenic potential of *P. aeruginosa* are the production of a myriad of virulence factors, formation of biofilms and resistance (Deepak *et al.,* 2012). Chronic *P.aeruginosa* lung infection is the cause of much of morbidity and most of the mortality in CF patients (Høiby, 2011). In this study, 26 isolates of *P.aeruginosa* were collected from different source including CF patients. 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 *rpsI* gene for detection the same bacteria and reported positive result for all isolates. Figure (1) shows positive agarose gel electrophoresis results for *rpsI* gene products (amplified).

Table (1): Primers used for PCR amplification

Gene	Primer sequence (5`→3`)	Product size(bp)	Origin	Reference
rpsl-f rpsl-r	GCAAGCGCATGGTCGACAAGA CGCTGTGCTCTTGCAGGTTGTGA	201	Alpha DNA Co. (Canada).	Xavier *et al*., (2010)
exoS-f *exoS-r*	CTTGAAGGGACTCGACAAGG TTCAGGTCCGCGTAGTGAAT	504	Alpha DNA Co.	Stover *et al*., (2000)
toxA-f *toxA-r*	GGTAACCAGCTCAGCCACAT TGATGTCCAGGTCATGCTTC	352	Alpha DNA Co.	Stover *et al*., (2000)
nan1-f *nan1-r*	AGGATGAATACTTATTTTGAT TCACTAAATCCATCTCTGACCCGATA	1316	Alpha DNA Co.	Stover *et al*., (2000)

Table (2): PCR react condition according to this study

Genes	Initial denaturation	No: of cycles	Denaturation	Primer Annealing	Elongation	Final extension
Rpsl	95ºC /5 min	35	95ºC/30 sec	57ºC/30sec	72ºC/1min	7min/72 °C
exoS	95ºC /5 min	35	95ºC/30 sec	64ºC/30sec	72ºC/1min	7min/72 °C
toxA	95ºC /5 min	35	95ºC/30 sec	68ºC/30sec	72ºC/1min	7min/72 °C
nan1	95ºC /5 min	35	96ºC/30 sec	63.4ºC/30sec	72ºC/1min	7min/72 °C

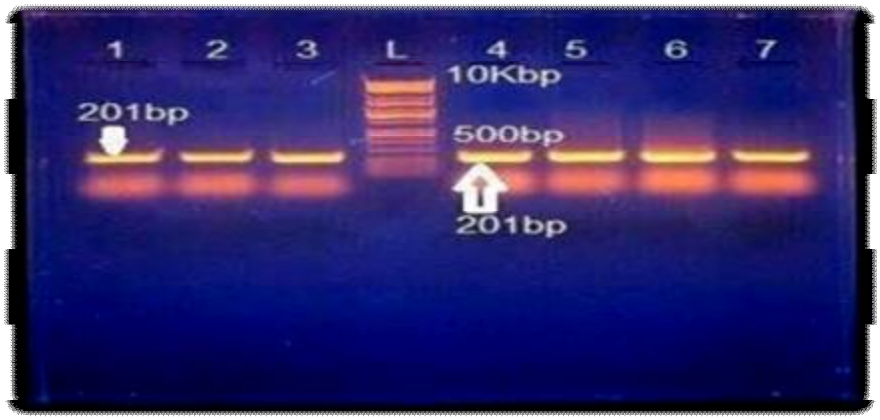

Figure (1): Agarose gel electrophoresis (1% agarose, 7 V/cm for 60min) of *rpsl* gene PCR product (201bp amplicon). Line L, DNA ladder, lines 1-7positive results

The virulence of *P. aeruginosa* is multi factorial 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 (Döring, 1987). In this study the presence of *exoS* gene (approximately 500bp) was detected in 21/26 (80.7%), distributed between 19 different clinical source and 4 in CF samples (Figure 2 and Table3). Lanotte *et al.* (2004) reported that 75.3% of their isolate gave positive results for *exoS* which is the line of the current study, while the results of Mitov *et al*. (2010) and Nikbin *et al*. (2012) disagreed with our study, when they reported that only 58.6%, 65.0% of their isolates respectively harbored this gene. According to source (Table 3), the presence of *exoS* gene in isolates from cystic fibrosis patients, wound, urine, and blood was significantly higher than sputum and brancheal washer.

Results of DNA sequencing (fig 4) and phylogenic analysis (Figure 5) using Tamura –Nei genetic destine model and UPGMA tree build method revealed that some changing took place in nucleotide sequencing as compared with *exoS* gene control (accession no.L27629).

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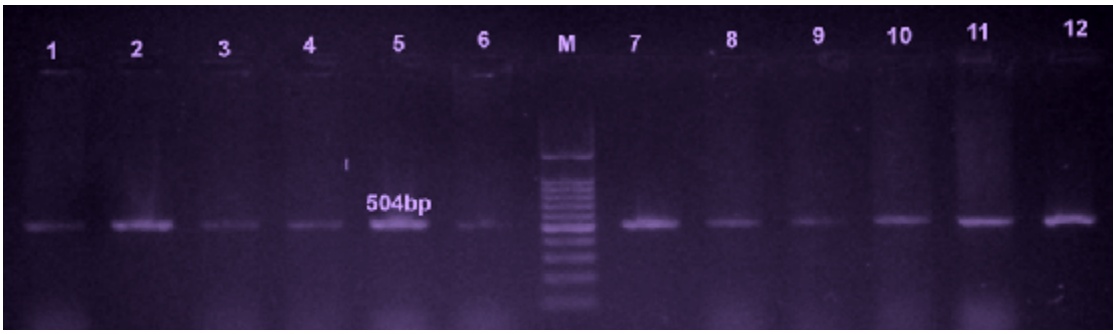

Figure (2): Agaros gel electrophoresis (1% agarose,7v/cm2 for 60 min)of *exoS* gene (504bp amplicon) lane M DNA Ladder); lanes1-12 represent positive results.

Most isolates display different point mutation as compared with the control. Point mutation type transversion occur in residue no.17 base 52 in *P. aeruginosa exoS* BW1 isolated from bronchial washer causes alteration T to A thus alteration the amino acid leucine residue to glutamine. Also a point mutation type transition occur in *P. aeruginosa exoS* B1, *P. aeruginosa exoS* U1 and *P. aeruginosa exoS* CyF1 in residue no.18 base 55 when the G convert to A causes conversion of serine residue to asparagine (Figure 4). *P. aeruginosa exoS* B3 and *P. aeruginosa exoS* CyF2 isolated from blood and cystic fibrosis respectively showed high similarity made them segregate within the same group or clone (Figure 5). *P. aeruginosa exoS* U 2 and U3 also within the same clone but not with *P. aeruginosa* exoSU 1 nor U4. Such difference may explain the highly virulence in some isolates.

Results of *toxA* gene prevalence revealed that total 19/26(73%) of the isolates were harbored this gene (fig 6). Nikbin *et al*. (2012) illustrated that this gene was reported in 90.6% of their isolates which is higher than our percentage, while Rawya *et al*. (2008) percentage was 89.4%. All CF isolates were positive to this gene as with *exoS*.

Results of DNA sequencing revealed that a point mutation was detected in *P. aeruginosa toxA* CyF2 (Figure 7). Nitrogen base G no.36 residue 12 was changed to a causes the conversion of arginine to histidin. Also mutation occur in *P. aeruginosa toxA* CyF 1, 2, 3, 3, *P. aeruginosa toxA* U1,2,3,4 and *P.aeruginosa toxA* BI, BW1 when nitrogen base no.134 residue 44 A was converted to G causes the conversion of threonine to alanine. Another point mutation occurs with the same above isolates when nitrogen base A no.233 side 78 changed to G causes conversion of asparagine to serine.

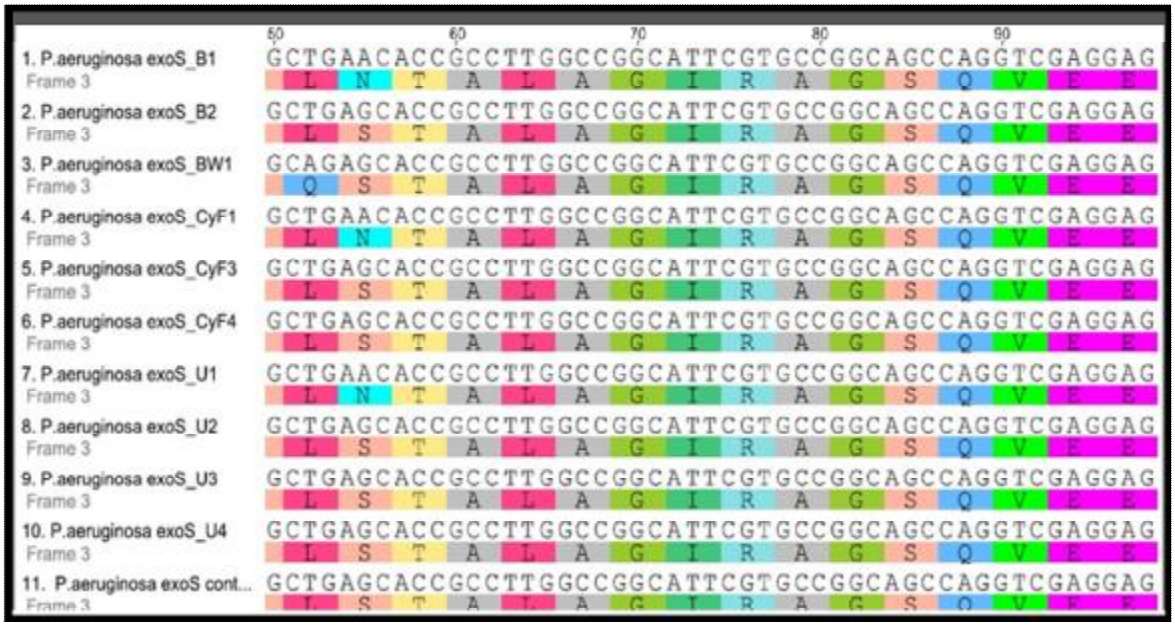

Figure (4): Part of DNA sequencing for *exoS* gene illustrating point mutation in some *P.aeruginosa* isolates .L :Leucine changed to G:Glutamine in bp no.52 residue 17 and S: Serine to N: Asparagine bp no. 55 residue 18.

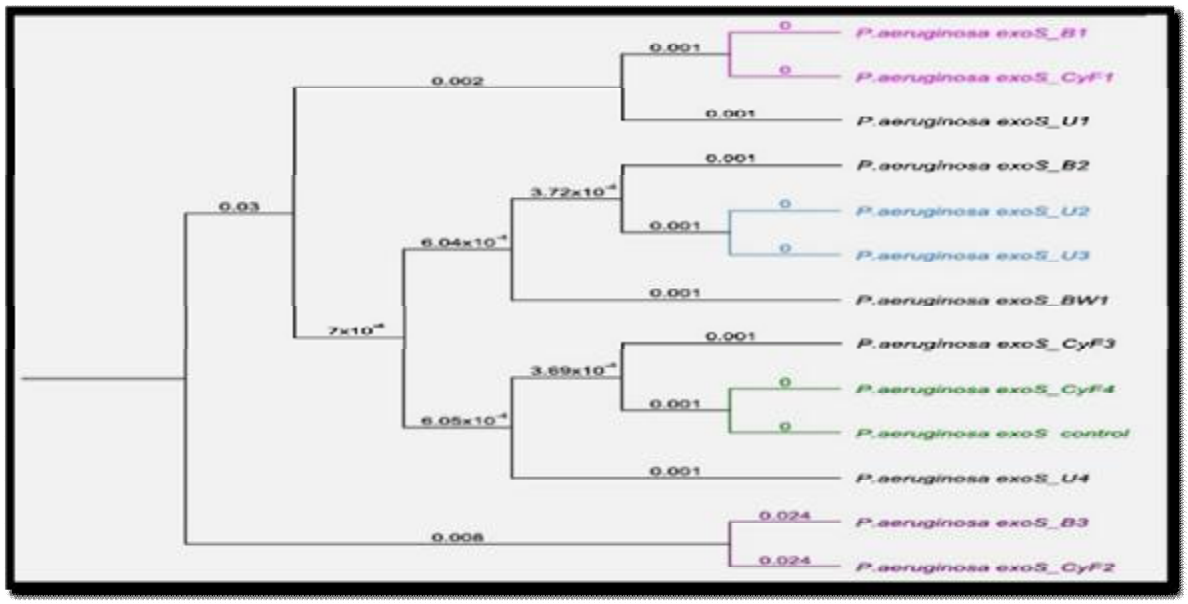

Figure (5): Dendrogram illustrating phylogenic analysis of *exoS* gene sequencing. The data separated to 2 groups. . Similarity in sequence was found between *P. aeruginosa exoS* B1 and CyF1, *P. aeruginosa exoS* U2 and U3 and between *P. aeruginosa exoS* B3 and CyF2.

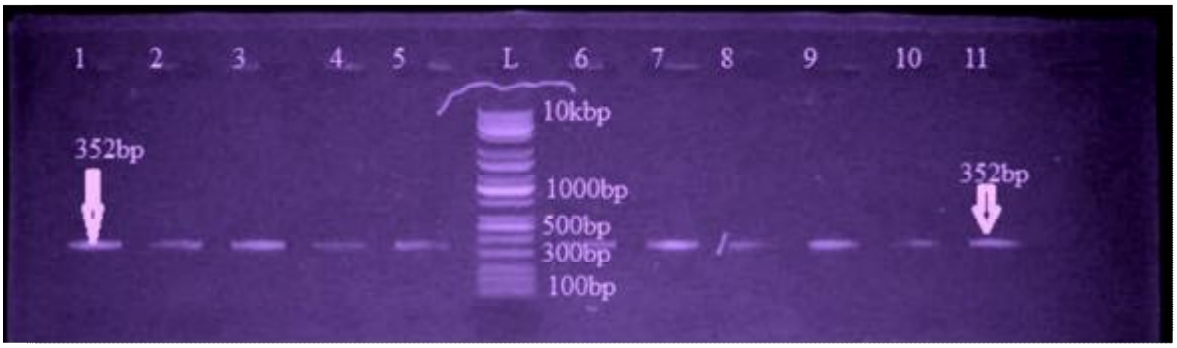

Figure (6): Agaros gel electrophoresis (1% agarose,7v/cm2 for 60 min) of *toxA* gene (352bp amplicon). Lane L DNA Ladder: lane1-11represent positive results.

Figure (7): Part of DNA sequencing for *toxA* gene illustrating point mutation in some *P. aeruginosa* isolates. Nitrogen base no.134 residue 44; A converted to G causes the conversion of threonine to alanine.

For the dendrogam (Figure 8), the isolates were divided to two groups, the first one contain *P. aeruginosa toxA* B1and B2 while the second group contain the rest isolates. Three clones were detected in group two and they were clone 1 contain *P. aeruginosa toxA* ,B2 ,Ear and the control, clone 2 contain *P. aeruginosa toxA* BW1, CyF and U3. The last clones contain *P. aeruginosa toxA* CyF 3&4. Such identity may indicate that they are oriented from the same source.

There was a borderline significant difference in the prevalence of *nan1*gene when it was found only in 1\26 (3.8%) of total isolate and 25% of CF isolates (Figure 4 and Table 3). Mitov *et al*. (2010) reported the percentage of *nan1*prevalence was 16.9% which disagreed with our result but similar result reported by Nikbin *et al*. (2012) when the prevalence of this gene was 26.8% (Mitov *et al*., 2010; Nikbin *et al*., 2012). In another study carried by Lanotte *et al*. (2004) 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%) (Lanotte *et al.,* 2004). Prevalence of *toxA* among parochial washer and urine isolates in the current study was significantly higher than wound isolates.

Mitov *et al.* (2010) reported that the frequency of *nan1*gene was low in non-CF isolates as compared with CF isolates which is a good agreement with the results of the current study. Still the frequency of *nan1* gene emergence was very low when only a single positive result for in pulmonary tract of CF patients and disappearance for this gene in the other sources (Table 3). Our result may support Mitov *et al*.(2010) hypothesis that the molecular-genetic detection of *nan1* gene may be used as an indirect measure of CF pulmonary disease evolution and considered as an important virulence factor in CF rather than non – CF infections. A study conducted by Antonov *et al*. (2010) on 36 isolates of *P. aeruginosa* found that *toxA, exoS* and *nan1* were prevalent and these virulence factors could play important roles in pathogenesis of this bacteria (Mitov *et al.,* 2010; Antonov *et al*., 2010).

Conclusions

The results of the current study focused on the prevalence of 3 important virulence factors. Most of CF and non-CF isolates harbored *toxA, exoS* gene while *nan1* was detected in only one CF isolates .Results of sequencing revealed that different point mutation occurred type transversion or transition causes alteration type of amino acid.

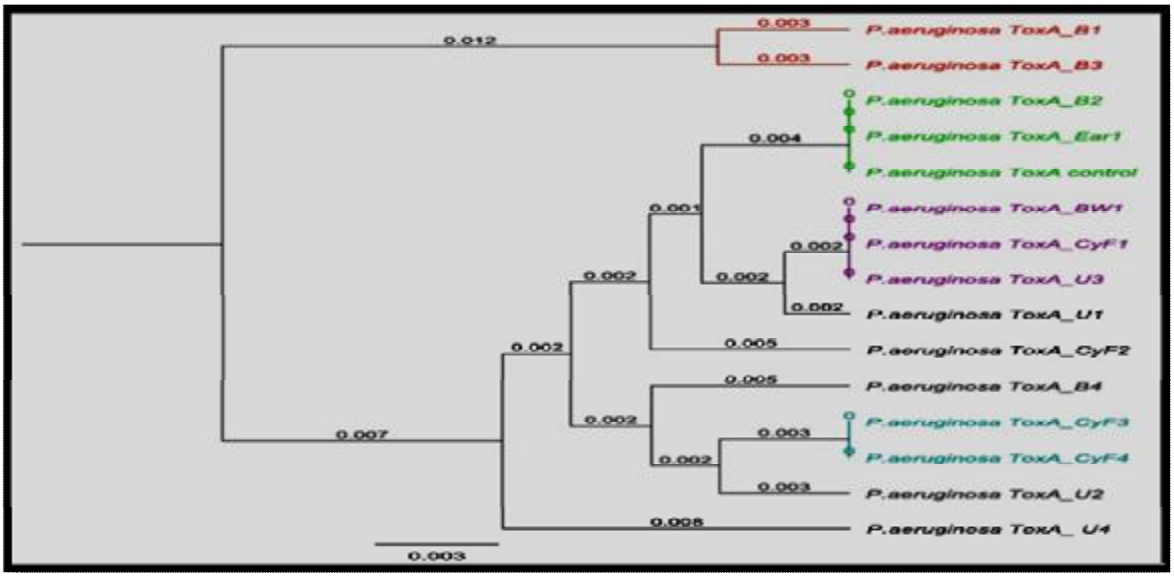

Figure (8): Dendrogram illustrating phylogenic analysis of *toxA* gene sequencing. The data separated to 2 groups A and B. Similarity in sequence was found between *P. aeruginosa toxA* B1 & B3, and between B2, Ear1and control finally between *P. aeruginosa toxA* CyF2 &4.

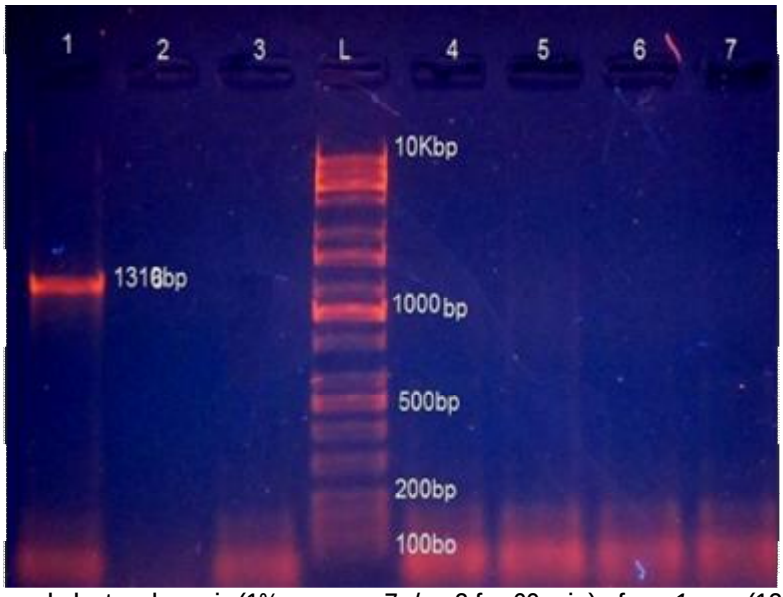

Figure (4): Agaros gel electrophoresis (1% agarose, 7v/cm2 for 60 min) of *nan1*gene (1316bp amplicon) lane L DNA Ladder); (lane1) represent positive result for CF isolate.

Table (3): Prevalence and percentage of some virulence genes among *P. aeruginosa* obtained from various sources.

Virulence gene	Cystic fibrosis 4(%)	Wound 3(%)	Urine4(%)	Blood 6(%)	Sputum 4(%)	Bronchial washer 2(%)	Ear infection 3(%)	Total no.26(%)
exoS	4(100)	3(100)	4(100)	6(100)	2(50)	1(50)	1(33.3)	21(80.7)
toxA	4(100)	1(33.3)	2(50)	6(100)	4(100)	1(50)	1(33.3)	19 (73)
nan1	1(25)	–	–	–	–	–	–	1(3.8)

(-)*negative result

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