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Original Research Article

Prevalence study of quorum sensing groups among clinical isolates of *Pseudomonas aeruginosa*

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A B S T R A C T

The opportunistic human pathogen *Pseudomonas aeruginosa* regulates production of numerous virulence factors via the action of two separate but coordinated quorum sensing systems, *las* and *rhl*. These systems control the transcription of genes in response to population density through the intercellular signals N-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL) and N-(butanoyl)-L-homoserine lactone (C4-HSL). Also plays a significant role in the transcription of multiple *P. aeruginosa* virulence genes. A total of 60 clinical isolates of gram negative bacteria primarily identified as *P. aeruginosa* were obtained from different teaching hospitals in Baghdad**,** the isolates were confirmed as *P. aeruginosa* by PCR which was performed by housekeeping gene *(rpsl* gene) and also screened of four quorum sensing genes (*rhlR, rhlI, lasR, lasI*) and linked with four virulence factor (*exoU, exoS, PilB, protease IV*). Sequence analyses of these isolates showed that the *lasR, lasI, rhlR* and *rhlI* genes had mutations. The combination of these mutations probably explains virulence factor deficiencies. Results of this study suggest that QS (quorum sensing) deficient clinical isolates occur and are still capable of causing clinical infections in humans.

Introduction

Pseudomonas aeruginosa is a bacterium of environmental origin considered an essentially opportunistic pathogen infecting hospitalized and immune-compromised patients (Pitt *et al.*, 2006). In Iraq, *P. aeruginosa* is an important cause of nosocomial infections and is considered the first cause of UTI (Salih *et al.*, 2011). While Al-Habib *et al.* (2011) showed the predominant microorganism with second and third degree burns was *P. aeruginosa*. Some virulence factors favor this pathogen's infection, such as the formation of pyocyanin, hemolysin, gelatinase and biofilm which act to increase tissue damage and protecting *P. aeruginosa* against the recognition of the immune system and the action of antibiotics (Cevahir *et al.*, 2008). Pathogenesis involves production of both extracellular and cell-associated virulence

factors (Wagner, 2008). Many virulence factors are expressed through a cell density-dependent mechanism known as quorum sensing. These additional virulence factors include elastase, lipase, protease, and several cytotoxins, encoded by *exo* genes. Elastase and alkaline protease are known to degrade a large variety of tissue components such as proteinaceous elements of connective tissue and cleave the cell surface receptors on neutrophils (Lomholt., 2001).

There are generally three major classes of bacterial quorum sensing systems based on the type of auto inducer signals and the receptors used for its detection. In gram negative bacteria typically use Lux I/R *quorum-sensing* (Yang, 2009). The *las* system consists of the *lasR*–transcriptional regulator and the *lasI*–synthase protein. *lasI* is essential for the production of the AHL signal molecule *N*-(3-oxododecanoyl)-L-homoserine lactone (3O-C_{12}-HSL). *lasR* requires 3O-C_{12}-HSL in order to become an active transcription factor. It was recently demonstrated that, in the presence of 3O-C_{12}-HSL, *lasR* forms multimers, and that only the multimeric form of this protein is able to bind DNA and regulate the transcription of multiple genes and it regulates expression of *lasA* (lasAprotease), *apr* (alkaline protease), *toxA* (exotoxinA), *lasI* (the PAI-1synthase), and *lasB* (elastase) at high cell density.

A second QS system in *P. aeruginosa* consists of the *rhlI* and *rhlR* proteins. The *rhlI* synthase produces the AHL *N*-butyryl-L-homoserine lactone (C_4-HSL), and *rhlR* is the transcriptional regulator. Only when *rhlR* is complexed with C_4-HSL does it regulate the expression of several genes. *rhlI*, *rhlA*, and *rhlB* an operon, coding for rhmnosyl transferase which is required for rhamnlipid production and *rpoS* a stationary –phase sigma factor.

Materials and Methods

Sampling: Between September and December 2013, 60 samples were taken from different teaching hospitals in Baghdad. The swab samples were taken from patients with burn wound and ear infections, sputum samples from respiratory tract infection (RTI), urine samples were taken from patients suffering from urinary tract infection (UTI) and blood samples from suspected bacteremia patients (Table 1).

Phenotypic tests: The specimens were inoculated directly onto 5%blood agar, *pseudomonas agar* and MacConkey's agar which were incubated at 37°C for 24 hours and with further 48 hours incubation if there is no growth. Identification of the isolates was relied upon their colonial morphology, gram reaction and standard biochemical tests. Further confirmative diagnostic tests for *P. aeruginosa* were attempted including growth at 42°C in brain heart infusion, oxidase test, catalase test, urease test and confirmatory by api*20E* kit.

DNA Extraction: Template DNA was prepared as described by Ruppé *et al.* (2009). Briefly, few isolated colonies of overnight growth bacteria were suspended thoroughly in 1 mL distilled water and boiled in a water bath, for 10 min. After centrifugation at 10000 rpm for 5 min, the suspension was taken as a template.

Application of PCR: In order to confirm the isolates as *P. aeruginosa*, PCR assay that based on housekeeping gene *(rpsL* gene) sequence with specific primers as described by Xavier *et al.* (2010), was carried out in 25 µL reaction volumes composed from 12.5 µl of GoTaq®Green Master Mix, template DNA–5µl, forward & reverse primers–1.5 µl for each, and 4.5 µl of

Deionized Nuclease–Free water was added to PCR mixture to get final volume of 25 µl. PCR mixture without template DNA was used as a negative control. PCR was run under the following conditions : primary denaturation step at 95°C for 5 min, 30 repeated cycles start with denaturation step at 94°C for 30 sec, annealing at 57°C for 30 sec, and 1 min at 72°C as extension step followed by final extension step at 72°C for 7 min.

PCR amplification procedure:

Detection of virulence genes was performed by amplifying the genes by multiplex PCR. The primers sequences were previously reported and obtained from Alpha DNA company (USA). Amplification was performed in a thermal cycler (Eppendorf, Germany), using the following primers for *pilB*F(**5′-** ATG AAC GAC AGC ATC CAA CT′**- 3′**); *pilB*R (**5′-**GGG TGT TGA CGC GAA AGT CGA T′**- 3′**); *ExoU* F(**5′-**GGG AAT ACT TTC CGG GAA GTT′**- 3′**), *ExoU*R(**5′-**CGA TCT CGC TGC TAA TGT GTT′**- 3′**); *ProteaseIV* F(**5′-**TAT TTC GCC CGA CTC CCT GTA′**- 3′**); *ProteaseIV*R(**5′-**AAT AGA CGC CGC TGA AAT C′**- 3′**) the reactions mixtures included an initial denaturation at 94°C for 5 min consisted of 35 cycles of 94ºC for 30 seconds, specific annealing temperature 60◦C for 30 seconds and 72ºC for 5 min 30 seconds and a final extension at 72ºC for 10 min for *ExoS*F(**5′-**CTT GAA GGG ACT CGA CAA GG′**- 3′**); *ExoS*R(**5′-**TTC AGG TCC GCG TAG TGA AT′**- 3′**) gene the reactions mixtures included an initial denaturationat 94°C for 5 min consisted of 35 cycles of 94ºC for 30 seconds, specific annealing temperature 65°C for 30 seconds and 72ºC for 5 min in and a final extension at 72ºC for 10 min in Thermal Cycler. While all quorum sensing gene reaction mixtures included an initial denaturation at 94°C for 5 min consisted of 35 cycles of 94ºC for 30 seconds, specific annealing temperature 50°C for 30 seconds and 72ºC for 30 min and a final extension at 72ºC for 10 min. The detection PCR products was performed on 0.8 to 1% agarose gels by electrophoresis and visualized under UV light.

DNA sequence analysis of *lasR, rhlR, lasI* and *rhlI* genes: *lasR, rhlR, lasI* and *rhlI* genes from all isolates were PCR amplified using the primer sets described below. After purification the PCR products were sequenced on an Applied Biosystem DNA sequencer ABI3730 XL. For PCR amplification and sequencing the following primers were used.

lasR start 5- ATGGCCTTGGT TGACGGTT-3

lasR stop 5-GCAAGATCAGAGA GTAATAAGACCCA-3

lasI start 5- ATGATCGTACAA ATTGGTCGGC-3

lasI stop 5- GTCATGAAACCGCC AGTCG-3

*rhlR*start 5- GCCATGATTTTGCCGTATC GG-3

rhlR stop 5- CGAGCATGCGGCAGGAG AAGC-3

rhlI start 5- GGAGTATCAGGGTAGGG ATGC-3

rhlI stop 5- CGAGCATGCGGCAGGAGA AGC-3

Detection of amplicon: Following amplification, aliquots (10 µl) were removed from each reaction mixture and λ phage ladder 100-bp are examined by electrophoresis (70V, 45 min) in gels composed of 1.5% (w/v) agarose (Promega, USA) in 1X TBE buffer (40 mMTris, 20mM boric acid, 1 mM EDTA, pH 8.3), stained with ethidiumbromide (5 µg/100 ml). Gels were visualized under UV illumination using a gel image analysis system.

Results and Discussion

In this study, sixty isolates of *P. aeruginosa* were isolated from different hospitals in Bagdad the source of these isolates were as follows: 22 isolates collected from burn patients, 18 isolates from wounds infections, 9 isolates from sputum taken from patients suffering from respiratory tract infection, 6 isolates from blood, 2 isolates from urinary tract infections (UTI), and the last 3 isolates from ear swab.

Microscopic examination of *P. aeruginosa* showed negative gram reaction, very small rods occur as single bacteria or in pairs. For other biochemical tests, *P. aeruginosa* showed a positive result for oxidase, and catalase, while negative result for urease test. Final identification for the isolate have been done at two levels: The first was by using conventional method (api 20E) that characterized as the typical easy and rapid one. The second step have been performed by housekeeping gene (*rpsL*) using polymerase chain reaction technique (PCR) all the 60 isolates gave positive result in both of the previous two steps. Salman *et al.* (2013) pointed to the beneficial use of housekeeping gene in species detection. Moreover Caltoir *et al.* (2000) suggested that PCR is the technique that offers a fast (<1.5h) tool with high sensitivity and specificity for the detection of *P. aeruginosa* as compared to conventional methods.

The polymerase chain reaction (PCR) is a powerful technique that has rapidly become one of the most widely used techniques in molecular biology because it is quick, inexpensive and simple. In this study, two techniques were used for the detection by PCR: multiplex and uniplex. This technique is very sensitive, easy to perform, specific for gene families and very efficient compared with the other methods (Bradford, 2001).

Sixty isolates from different hospitals in Baghdad used to study four virulence factor [*exoU, exoS, PilB, ProteaseIV (TC*)] which was screened by PCR and the results which showed high frequency of virulence factor genes in local isolates. Beginning with the genes codifying for the type III secretion system (T3SS), the *exoS* and *exoU* were differently distributed among the tested strains. 91.6% (55/60) harbored TTSS genes and the results showed that most of the isolates contain either *exoS* or *exoU*. 33 (55%) isolates showed *exo U +/ exo S-* while 16 (26.6%) isolates showed (exo U -/ exo S +) whereas 5 (8.3%) isolates showed (*exoU +/exoS*+) and the last 7 (11.6%) isolates show (*exo U -/exo S* -). Those which harbor *exoU* gene are referred to as cytotoxic and which those harbor *exoS* are referred to as invasive and those that do not harbor any of these genes are considered neither as cytotoxic nor invasive. Therefore, there are three phenotypes of *P. aeruginosa*, cytotoxic, invasive and neither cytotoxic nor invasive (Zhu *et al*., 2006; Choy *et al.,* 2008).

For pilB gene, the results showed that 21.6% (13/60) of *P. aeruginosa* harbor this gene, and the majority of these isolates were from male patients the results showed a widespread dissemination of this gene in *P. aeruginosa* isolated from burn infection 61.5% (8/13) followed by wound infections 30.7% (4/13) and one isolates 7.6% (1/13) from sputum this result partially agree with (Holban *et al.,* 2013) who showed that 35% isolates of *P. aeruginosa* harbor this gene, and widespread dissemination of this gene in *P. aeruginosa* 60% of isolates from wound infections, 40% of ear isolates, then 20% for each of urine and burn isolates. In the last *proteaseIV* (*TC*) gene the results showed that 46.6% (28/60) isolates *of P. aeruginosa* harbor this gene. Widespread of this gene in *P. aeruginosa*

isolated from surgical wound and burn patients 64.2% (18/28) is due to that contribute with tissue injuries followed by sputum 21.4% (6/28) this results agree with Holban *et al.* (2013) who showed most of surgical wound isolates harbor this gene and disagree with Smith *et al.* (2006) who showed that the *protease IV* gene highly conserved among CF lung isolates, which suggests that *protease IV* may have an important role in the pathogenesis of *P. aeruginosa* at this site and contribute to acute lung infection in young CF patients.

QS genes (*lasI, lasR, rhlI, rhlR*), were screened by multiplex PCR technique, the results showed that 81.6% (49 /60) isolates were positive for one or more QS genes while only 18.3% (11/60) were negative for all these genes. The results was 65% (39/60) isolates were positive for *rhlR*, 43.3 % (26/60) isolate were positive for *rhlI*, while 5% (3/60) positive for *LasR* and the last 78.3% (47/60) were positive for *lasI*. The role of QS in the pathogenesis of *P. aeruginosa* was examined and the results show high frequency of virulence factor genes in local isolates., suggesting that these isolates were QS proficient.

This observation confirms the crucial role of QS in *P. aeruginosa* virulence in the present study, among the 60 isolates we identified 2 isolates (P24 and P56) that were defective in production of all virulence factors tested PCR analysis of these isolates for the presence of QS genes revealed that P24 isolate contained *lasR, lasI, rhlR* and *rhlI* genes while P56 isolate was negative for *lasR* and *rhlI*. Bosgelmez *et al.* (2008) explained this results and confirm that point mutation in QS gene cause that result, they reported QS mutation isolates that were unable to produce the C4-HSL signaling molecule and C4-HSL dependent virulence factors as a result of mutations in the QS genes but still cause infections in humans, also the results show 1 isolate P12 out of 60 negative for all QS genes and virulence factor genes. This results agree with Schaber *et al.* (2004) who identified QS deficient clinical isolate which lost all virulence factors tested, yet still caused a wound infection suggested that besides known virulence factors, there may be additional factors yet uncharacterized involved in the pathogenesis of *P. aeruginosa.* Another possibility that may lead a QS deficient strain to cause infection is the presence of multiple *P. aeruginosa* strains in the infection site. A single patient may be infected by both QS proficient and deficient strains of *P. aeruginosa*. This study pointed to that there may be different types of isolates from the same patients.

Isolates (P5, P6, P7, P10, P12, P13, P14, P39, P40, P43, and P45) show negative for all QS gene but contain one or more virulence factor. This agree with Dénervaud *et al.* (2004) who confirm there may be other virulence factors which may not be stringently controlled by QS, the results of this study confirm that the QS systems play an important role in the pathogenesis of *P. aeruginosa* and indicate that *P. aeruginosa* is capable of causing clinical infections in humans despite of QS deficient contradict the theory that QS plays a major role in *P. aeruginosa* pathogenicity and not all virulence factor controlled by QS.

To determine if QS genes have mutations, sequenced *lasR, lasI, rhlR* and *rhlI*, results showed different types of mutation. Investigations of QS functionality and connected phenotypes, expressed by biofilm and non-biofilm producer isolates, showed that a significantly higher proportion of mutation in biofilm isolates compared to their non-biofilm.

Table.1 Prevalence *P. aeroginosa* of in clinical specimens

Type of specimen	No. of isolates	*P. aeroginosa*isolates, no. (%)[a]	*Gender*, no. (%)[a]		Dwelling-place, no. (%)[a]	
			Male	Female	Urban	Rural
Burn	22	36.66	15	21.6	31.6	5
Wound	18	30	26.6	3.3	18.3	11.3
Sputum	9	15	6.6	8.3	5	10
Blood	6	10	10	-	8.3	1.6
Ear swab	3	5	5	-	5	-
Urine	2	3.3	1.6	1.6	3.3	-
Total	60	100	64.8	34.8	71.5	27.9

[a]Percentage of the number of isolates with respect to the total number of isolates.

Figure.1 Agarose gel electrophoresis (1%agarose, 7 V/cm^{2}for 60min) of *rpsL* gene (201bp amplicon). Lane M 100bp DNA ladder, lanes 1-7 represent of bands

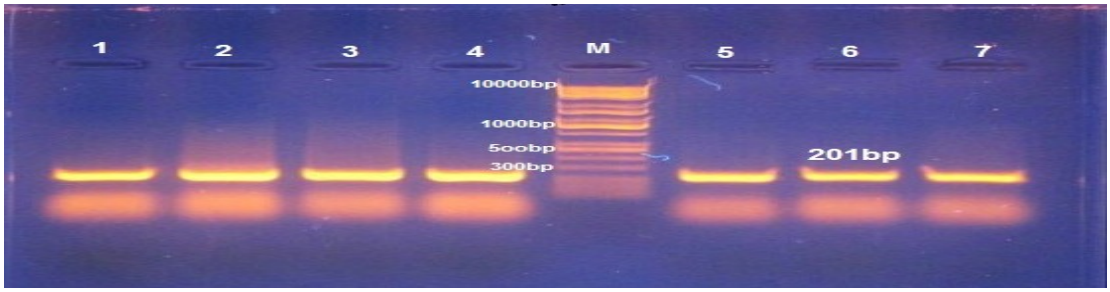


Fig.2 Agaros gel electrophoresis (1% agarose, 7 v/cm^2 for 60 min)of *exoS*gene (504bp amplicon) lane M100 bp DNA Ladder); lanes1-12 represent of bands

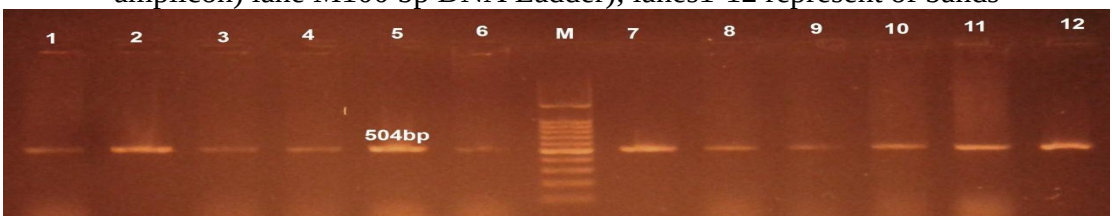


Fig.3 Agaros gel electrophoresis (1% agarose, 7 v/cm^2 for 60 min)of *PilB* gene (826 bpamplicon) lane M100 bp DNA Ladder); lanes1-6 represent of bands

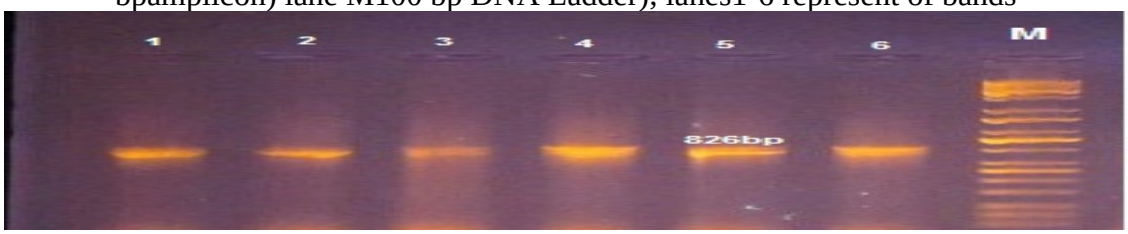


Fig.4 Multiplex PCR: Agarose gel electrophoresis (1% agarose , 7 v/cm^2 for 60 min) of (*exoU,proteaseIV*) genes (428bp,752bp amplicon respectively). Lane M 100bp DNA Ladder lanes 1–24 represent of bands

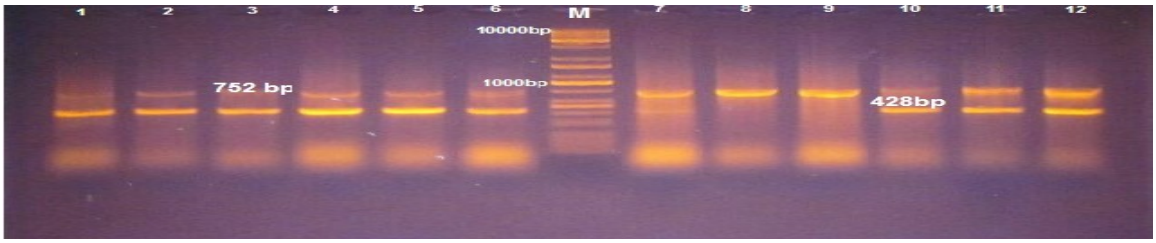
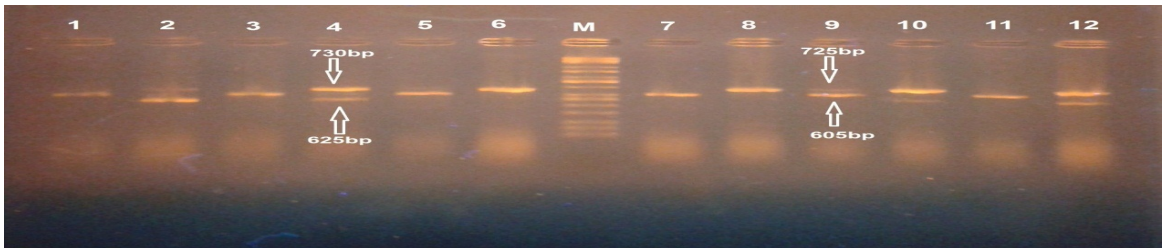


Fig.5 Multiplex PCR : Agarose gel electrophoresis (1% agarose, 7 v/cm^2 for 40 min).of (QS)genes (1,3, 5,7,9,11) multiplex for (*lasR,lasI*) size products (725 bp , 605 bp) respectively While (2 ,4, 6, 8,10) multiplex for (*rhlR , rhlI*) size product (730bp , 625) lane M 100bp DNA Ladder



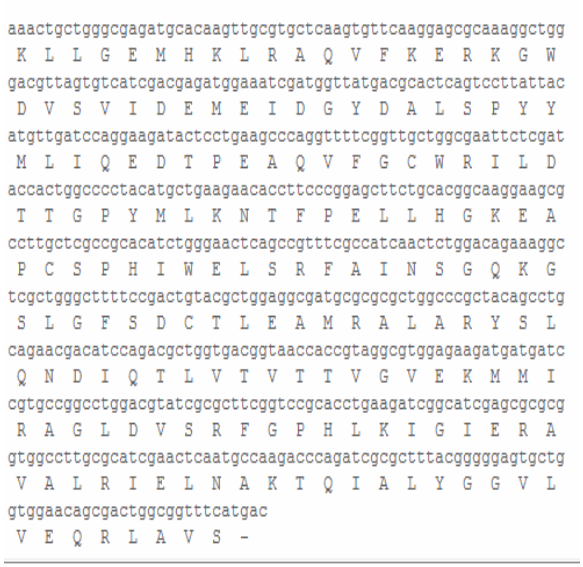


Figure (3-9). Mutations in the*LasI* gene of *P. aeruginosa* isolates fromwound. The nucleotide sequence alterations were identified by alignment with the strain PUPa3 sequence. A: The PUPa3sequence; B: The nucleotide in local isolates, insertions (Ins) are indicated. Under the amino acid sequence, frame shifts (fs) are indicated in yellow bold.Cited by: http://web.expasy.org/translate/.

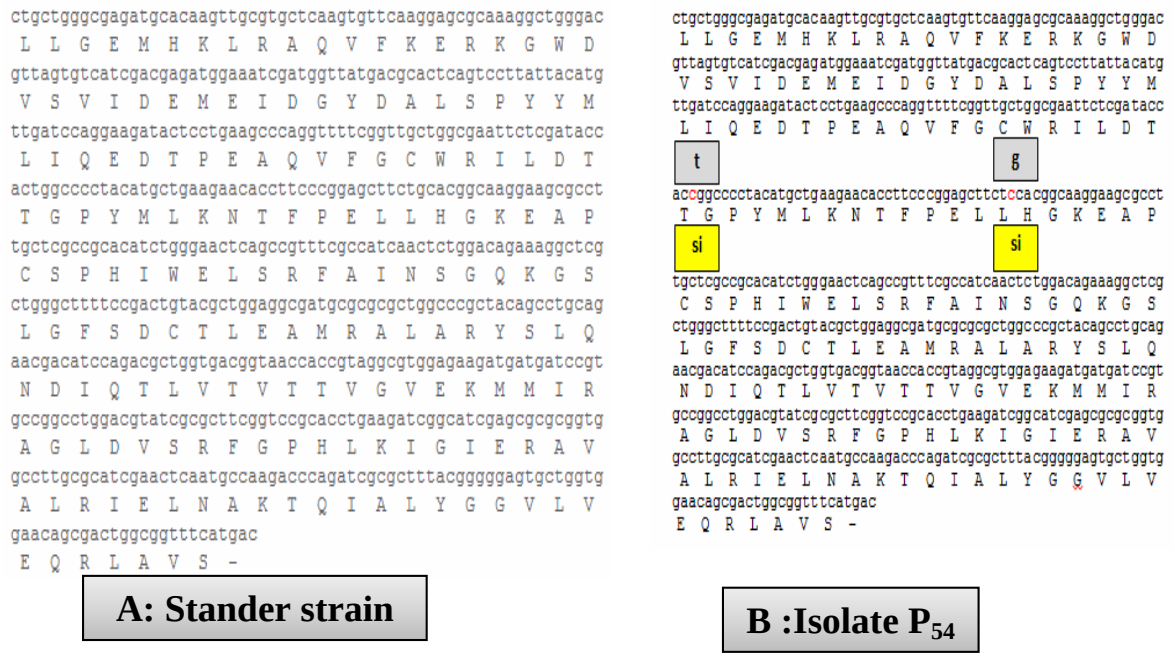
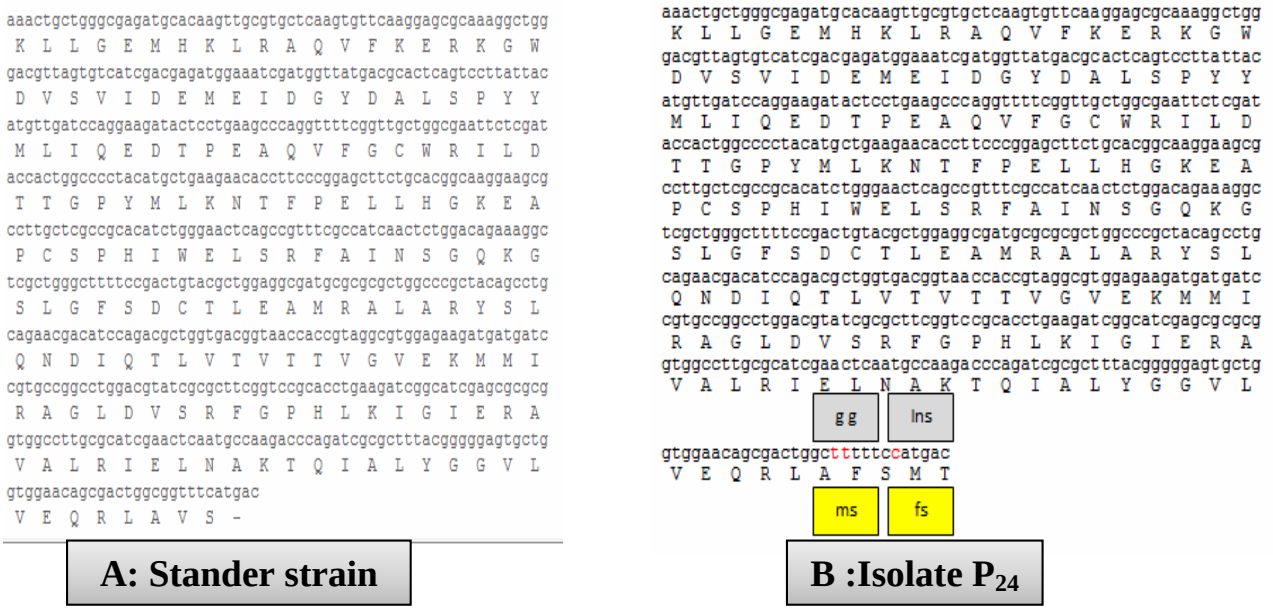
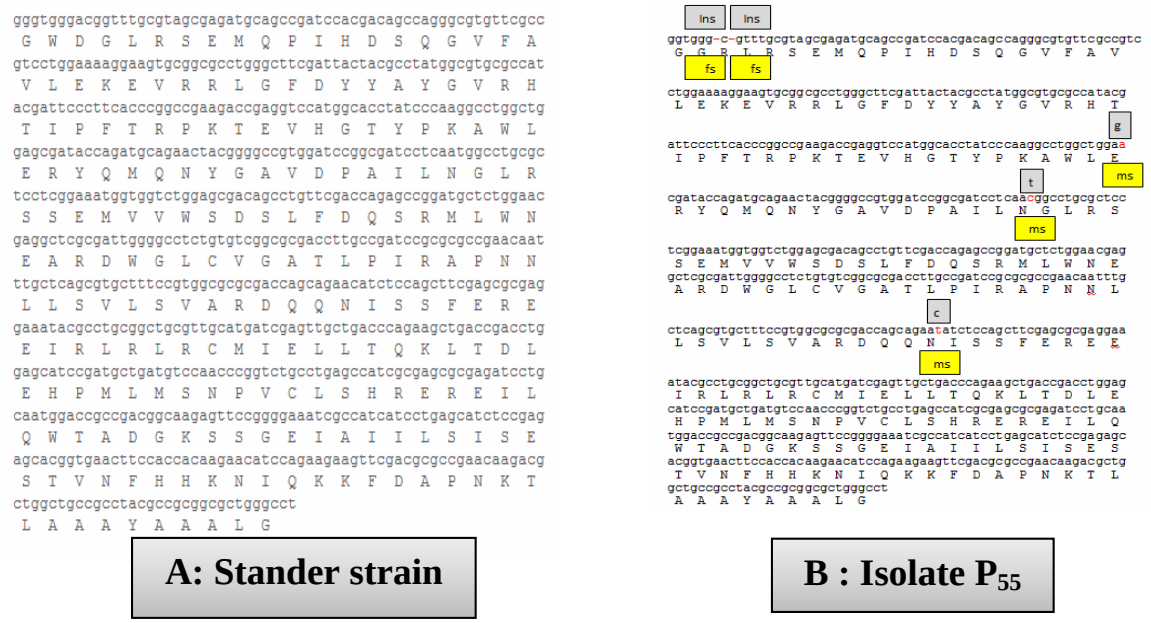


Figure (3-10) Mutations in the*lasI* gene of *P. aeruginosa* isolates from wound patients. The nucleotide sequence alterations were identified by alignment with the strain.M18 sequence. A: The M18 sequence; B: The nucleotide substitutions in local isolates, substitutions are indicated. Under the amino acid sequence, silent (si) are indicated in yellow bold. Cited by: http://web.expasy.org/translate/.

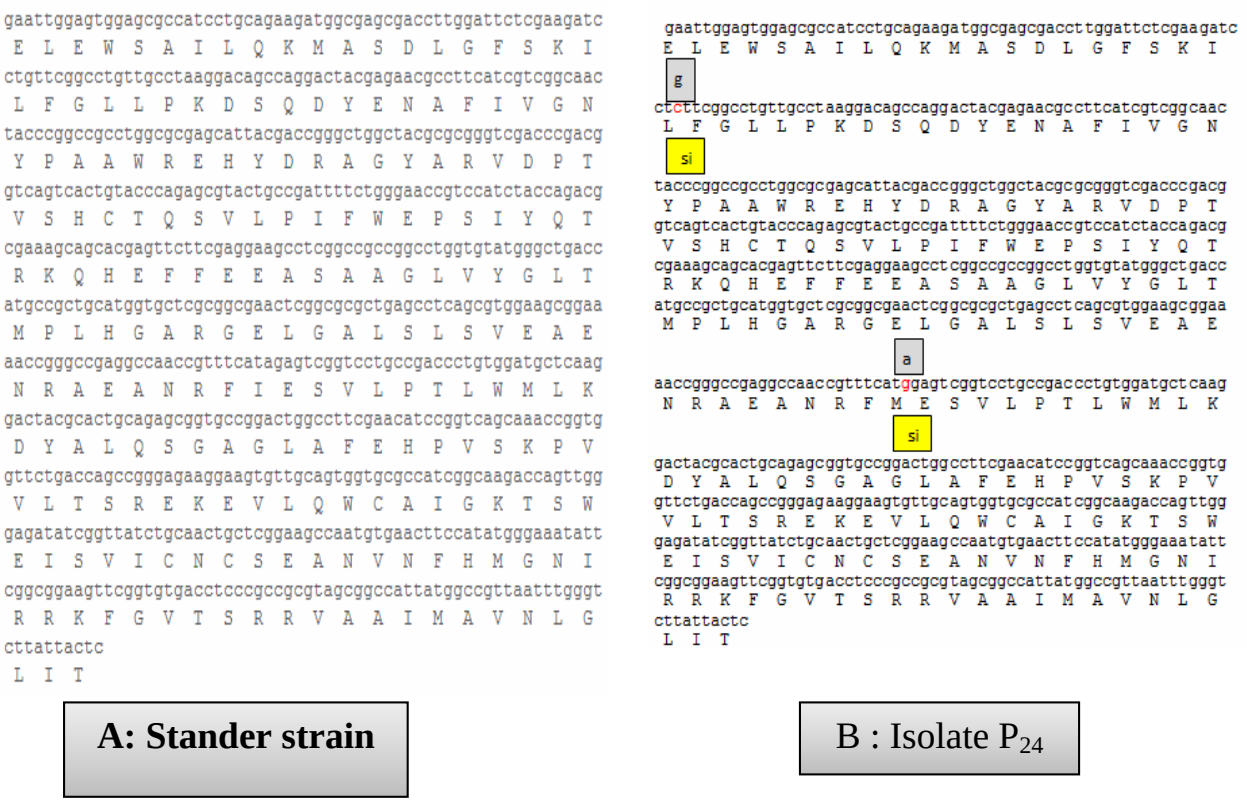


Figure(3-11). Mutations in the*lasI* gene of *P. aeruginosa* isolates from earswab. The nucleotide sequence alterations were identified by alignment with the strain PUPa3 sequence. A: The PUPa3 sequence; B: The nucleotide in local isolates, insertions (Ins), substitutions are indicated. Under the amino acid sequence, frame shifts (fs), missense (ms) are indicated in yellow bold. Cited by: http://web.expasy.org/translate/.

Figure(3-12) . Mutations in the*lasI* gene of *P. aeruginosa* isolates from burn patients. The nucleotide sequence alterations were identified by alignment with the strain PUPa3 sequence. A: The PUPa3 sequence; B: The nucleotide in local isolates, insertions (Ins), substitutions are indicated. Under the amino acid sequence, frame shifts (fs), missense (ms)and silent (si) are indicated in yellow bold. Cited by: http://web.expasy.org/translate/.



Figure(3-13). Mutations in the*rhlR* gene of *P. aeruginosa* isolates from burn patients. The nucleotide sequence alterations were identified by alignment with the strain PUPa3 sequence. A: The PUPa3 sequence; B: The nucleotide in local isolates, insertions (Ins), substitutions are indicated. Under the amino acid sequence, frame shifts (fs) and missense (ms) are indicated in yellow bold. Cited by: http://web.expasy.org/translate/.



Figure(3-14). Mutations in the*lasR* gene of *P. aeruginosa* isolates from ear swab. The nucleotide sequence alterations were identified by alignment with the strain PUPa3 sequence. A: The PUPa3 sequence; B: The nucleotide in local isolates, substitutions are indicated. Under the amino acid sequence, sense (si) are indicated in yellow bold. Cited by: http://web.expasy.org/translate/.

This difference in the functionality of the QS system between mucoid and non-mucoid isolates strongly support that different adaptation strategies are employed by the two phenotypes (Bjarnsholt *et al.,* 2009). Furthermore, mutation was found to correlate with urban female in patient. This result indicated that the high incidence rate of mutation in hospital environments compared to other represent best media to recombination between related species.

Analysis showed that the wild-type sequences of QS genes (*lasR* and *rhlR* as well as of the genes *lasI* and *rhlI* encoding the signal molecule) were conserved among the *P. aeruginosa* isolates. From 10 isolates, we only found a 2 occurrence of a loss of function mutation in *lasI* gene and intact *lasR*. This isolate is P60 and have mutations in the *lasI* gene of *P. aeruginosa* isolated from wound, insertions (Ins) tat position+571. This isolate positive for *rhlI, lasI*, *lasR* while harbor *exoU, TC, PilB* from virulence factors genes insertion mutation leading to frame shifts and point mutation (both transitions and transversions) resulting in either stop codons or substitutions in conserved semi-conserved or non conserved amino acid.

Moreover, mutations in *lasI* gene of P27 isolate from burn patients. This isolate positive for *rhlR, lasR, lasI* while negative to *rhlI* gene also harbored *exoU, exoS* and

TC from virulence factor gene insertion cat position+564 may lead to frame shift & missenceat position+557, +558 and single nucleotide polymorphism at position 111+ (t/c) that silent mutation. Also mutations in *lasI* gene of P24 isolate from ear swab. Insertion c at position +564 may lead to frame shift and missenceat position+560, +561. This isolate positive for *rhlI, lasI, rhlR* and *lasR* while negative for all virulence factor. As well as mutations in the *lasI* gene of P54 isolates from wound patients. Single nucleotide polymorphism at position 572+(t/c) & 601(g/c) that silent mutation, no change translate amino acid, This isolate positive for *rhlI, lasI* while negative for *rhlR, lasR* gene also harboured only *exoU* from virulence factor gene. However, *lasR* for the P24 isolate showed two silent mutation at position +74(g/c) & +398(a/g). Bjarnsholt *et al.* (2009) indicates that these silent mutations have no effect on the functionality of the gene and its encoded product. Mutations preferentially occurred in the genes encoding the regulatory proteins, in accordance with previous observations (Heurlier *et al.*, 2006).While many mutation observed in *rhlR* , like isolate P55 from wound have insertion mutation at position+14&+16 and three missence are indicated at position+186,+235,+403.this isolates have only *rhlR* and harbour only *exoU* from virulenc factors genes

Bjarnsholt *et al.* (2009) approved that increase in mutation frequencies leading to a weak mutator phenotype of the isolates was found to correlate with the loss of functionality of either *lasR* or *rhlR*.

This also suggest that a treatment with drugs interfering with QS is useful, but in some cases when virulence factors encoding in different strategies beside QS these drug remain non useful.

References

Al-Habib, M.H., Al-Gerir, A., Hamdoon, M.A. 2011. Profile of *Pseudomonas aeruginosa* in burn infection and their antibiogram study. *Ann. Coll. Med. Mosul.*, 37(1): 57–65.

Bjarnsholt, T., Jensen, P.O., Fiandaca, M.J., Pedersen, J., Hansen, C.R. 2009. *Pseudomonas aeruginosa* biofilms in the respiratory tract of cystic fibrosis patients. *Pediatr. Pulmonol.,* 6: 547–558.

Bosgelmez, G.T., Ulusoy, S. 2008. Characterization of N-butanoyl-l-homoserine lactone (C4-HSL) deficient clinical isolates of *Pseudomonas aeruginosa. Microbial. Pathog.,* 44: 13–19.

Bradford, P.A. 2001. Extended – spectrum β-lactamases in the 21st century: characterization, epidemiology and detection of this important resistance threat. *Clin. Microbiol. Rev.,* 14: 933–951.

Caltoir, V., Gilibert, A., Glaunec, J.M., Lannay, N., Bait, L., Legr, D. 2010. Rapid detection of *Pseudomonas aeruginosa* from positive blood cultures by quantitative PCR. *Annals. Clin. Microbial. Antimicrob.,* 9(21): 1186–1476.

Cevahir, N., Demir, M., Kaleli, I., Gurbuz, M., Tikvesli, S. 2008. Evalution of biofilm production, gelatinase activity, and mannose-resistent hemagglutination *Acinetobacter baumannii* strains. *J. Microbiol. Immunol. Infect.,* 41: 513–518.

Choy, M., Stapleton, F., Willcox, M., Zhu. 2008. Compaction of virulence factors in *Pseudomonas aeruginosa* strains isolated from contact lens-and non contact lens –related keratitis. *J. Med. Microbial.*, 57: 1539–1546.

Dénervaud, V., TuQuoc, P., Blanc, D.,

Favre, B.S., Krishnapillai, V., Reimmann, C., Haas, D., van Delden, C. 2004. Characterization of cell-to-cell signaling deficient *Pseudomonas aeruginosa* strains colonizing intubated patients. *J. Clin. Microbiol.*, 42: 554–562.

Heurlier, K., Denervaud, V., Haas, D. 2006. Impact of quorum sensing on fitness of Pseudomonas aeruginosa. *Int. J. Med. Microbiol.*, 2(3): 93–102.

Holban, A.M., Chifiriuc, M.C., Bleotu, C., Grumezescu, A. 2013. Virulence markers in *Pseudomonas aeruginosa* isolated from hospital-aquired infection occurred in patients with underlying cardiovascular disease. *Romanian Biotechnol. Lett.*, 18(6): 8843–885.

Lomholt, J.A., Poulsen, K., Kilian, M. 2001. Epidemic population structure of *Pseudomonas aeruginosa*: evidence for a clone that is pathogenic to the eye and that has a distinct combination of virulence factors. *Infect. Immun.*, 69(10): 6284–6295.

Pitt, T.L., Simpson, A.J., Gillespie, S.H., Hawkey, P.M. 2006. Principles and practice of clinical bacteriology. 2nd edn., John Wiley & Sons, London. Pp. 427–443.

Ruppé, E., Hem, S., Lath, S., Gautier, V., Ariey, F., Sarthou, J.L., Monchy, D., Arlet, G. 2009. CTX-M β-Lactamases in *Escherichia coli* from community-acquired urinary tract infections, Cambodia. *Emerg. Infect. Dis.*, 15(5): 741–748.

Salih, H. A., Abdulbary, M., Abdulride, A. S. 2011. Susceptibility of *Pseudomonas aeruginosa* isolated from urine to some antibiotics. *AL-Qadisiya J. Vet. Med. Sci.*, 10(2): 201–208.

Salman,M., Ali, A., Haque, A. 2013. Novel multiplex PCR for detection of *Pseudomonas aeruginosa.* A major cause of wound infection *Pak J Med.*, 29(4): 957–967.

Schaber, J.A., Triffo, W.J., Suh, S.J., Oliver, M., Hastert, M.C., Griswold, J.A., Auer, M., Hamood, A. N., Rumbaugh, K.P. 2007. *Pseudomonas aeruginosa* forms biofilms in acute infection independent of cell-to-cell signalling. *Infect. Immun.*, 75(8): 3715–3721.

Smith, L., Rose, B., Tingpej, P., Manos, J., Bye, B. 2006. Protease IV production in *Pseudomonas aeruginosa* from the lungs of adults with cystic fibrosis. J. Med. Microbiol., 55(10): 1641–1644.

Wagner, V.E., Filiatrault, M.J., Picardo, K.F., Iglewski. 2008. *Pseudomonas aeruginosa*: virulence and pathogenesis issues, In: Cornelis P, (Ed). *Pseudomonas* genomics and molecular biology. Caister Academic Press, Norfolk. Pp. 129–158.

Xavier`, D.E., Renata, C.P., Raquel, G., Lorena, C.C.F., Ana, C.G. 2010. Efflux pumps expression and its association with porin down – regulation and β- lactamase production among *Pseudomonas aeruginosa* causing bloodstream infections in Brazil. *BMC Microbiol.*, 10: 217.

Yang, L. 2009. *Pseudomonas aeruginosa* quarm sensing. A factor in biofilm development and an antipathogenic drug target. Ph.D. thesis, Department of System Biology. Technical University of Denmark.

Zhu,H., Conibear, T.C.R., Bandara, R., Aliwarga, R., Stapleton, F. (2006). Type III secretion system - associated toxins, proteases, serotypes, and antibiotic resistance of *Pseudomonas aeruginosa* isolate associated with keratitis. *Curr. Eye Res.*, 31: 297–306.