Quorum Sensing Mediated Pathogenicity, Virulence Genes and Class 1 Integron in Carbapenem-Resistant Clinical Pseudomonas Aeruginosa Isolates

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Research Article

Keywords: carbapenem-resistant Pseudomonas aeruginosa, QS system, virulence genes, class 1 integron

Posted Date: December 9th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-1148175/v1

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Abstract

Carbapenems are the most effective agents for treating carbapenem-resistant clinical P. aeruginosa (CRPsA) infections. During an infection, a quorum-sensing (QS) system and its regulating virulence genes have a great role. The aim of the study was to detect the presence of a las and rhl QS system and related virulence genes and a class 1 (Cls1) integron. A total of 52 CRPsA isolates obtained from Kastamonu, Turkey was analyzed. A conventional culture method, an oprL gene-based molecular assay for P. aeruginosa isolates, and an automated VITEK-2 compact system were applied for the isolation and identification of CRPsA isolates. The oprL gene was detected in all of the isolates tested. At least one of the las or rhl system genes was detected in 98.07% of the isolates, and the percentage of las system genes (98.07%) were higher than that of rhl system genes (90.38%). algD, lasB, toxA and aprA genes were detected in between 46.15% and 88.46% of the isolates, and co-existence of four genes were detected in 40.38% of the isolates. Cls1 integron and slime production using Congo Red Agar (CRA) were present in 51.92% and 67.30%, respectively, of the isolates. There was a significant positive correlation (p < .10) between the las system and the rhl system and a strongly positive correlation (p < .01 or p < .05) between the rhl system-four virulence genes and slime production-and among some virulence genes. In conclusion, the CRPsA isolates tested in the study are highly virulent and QS systems have a significant role in pathogenesis.

Introduction

Carbapenems (imipenem, ertapenem, meropenem and doripenem) are β-lactam, antibiotic class members and one of the most effective antipseudomonal drugs for curing infections commonly caused by P. aeruginosa. However, recent study results have shown that P. aeruginosa is becoming increasingly resistant against carbapenem (Tacconelli et al., 2018; Hu et al., 2019). According to the World Health Organization reports, carbapenem-resistant clinical P. aeruginosa (CRPsA) ranks second based on most criteria for bacteria among 20 antidrug-resistant bacterial species (Tacconelli et al., 2018). There are several major causes of carbapenem resistance, such as extensive clinical use of this antibiotic, overexpression of efflux systems, alterations or losses in outer membrane porin D (OprD) and carbapenemase production. The most common and globally reported carbapenemase genes, known as metallo-β-lactamases (MBLs) are transported from mobile genetic elements, specifically via integrons (Rojo-Bezares et al., 2016). In addition to these antibiotic resistance mechanisms of P. aeruginosa, its ability to produce biofilms and its success in escaping from the host immune system are related to the infection formation that is difficult to treat. Because bacterial biofilms have multiple tolerance mechanisms for antibiotic therapy, they cause biofilm infections to persist despite antibiotic exposure (Radovanovic et al., 2020). This result is owing to extracellular polymeric substances (EPS), which are detected around biofilm-forming bacteria and form a barrier against antimicrobial agents and host immune system elements (Brindhadevia et al., 2020; Chakraborthy et al., 2020).

Bacteria work together to carry out cell-to-cell communication by secreting small signaling molecules (autoinducers, AIs). When the population number reaches the threshold level, they start to form a biofilm due to EPS. This communication procedure is referred to as QS. The QS system of P. aeruginosa contains two major AI signaling molecules (AHL: 3-oxo-C₁₂-HSL and C₄-HSL). Each system (Las and Rhl) is encoded by two components: AI synthesis (lasI and rhlI genes) and their cognate transcriptional activating protein (lasR and rhlR genes). There is also a third system in P. aeruginosa known as the Pseudomonas quinolone signal, which is induced by 2-heptyl-3-hydroxy-4-quinolone and controlled by the las and rhl systems (Brindhadevia et al., 2020; Sirken et al., 2020; Elnegery et al., 2021). These systems in the QS mechanism cause the bacteria to have a successful role in
pathogenicity by adjusting the population densities and activating the relevant virulence factors. *P. aeruginosa* has many virulence factors. Some virulence factor-encoding genes, such as elastase (*lasB*), toxin A (*toxA*), and alkaline protease (*aprA*), are governed by the las and rhl synthesis genes (*lasI/lasR* and *rhlI/rhlR*) (Attiah et al., 2021). These virulence genes have been shown to host many proteins hydrolyses, causing host tissue damage, disrupting immune response, and supporting inflammation (Iiyama et al., 2017; Haghi et al., 2019; Everett and Davies, 2021). For instance, alginate is responsible for biofilm formation and has a great role in the structural preservation and stability of biofilms (Gholami et al., 2017).

The *oprL* gene encodes the structural membrane lipoprotein of *P. aeruginosa*. The presence of this specific outer membrane protein also has a key role in the hereditary resistance of *P. aeruginosa* to many antibiotics (efflux transport system or membrane selectivity). The gene is also employed for the determination of *P. aeruginosa* in clinical and other samples by polymerase chain reaction (PCR) at the species level or by reverse transcription (RT)-PCR assay (De Vos et al., 1997).

Due to the above-mentioned findings, understanding the QS system involved in pathogenicity and the regulatory mechanisms that regulate the virulence genes expression is critical for the development of alternative curing approaches for the monitoring and prevention of pseudomonal infections. Therefore, this study aimed to analyze (i) the presence of las and rhl QS genes and their relation to the role of regulation of virulence factors, (ii) the presence of *lasB*, *algD*, *toxA* and *aprA* virulence genes, (iii) the presence of Cls 1 integron, and (iv) biofilm formation.

### Material And Methods

#### Isolation, identification, carbapenem-resistance profile of *P. aeruginosa*

A total of 52 carbapenem-resistant *P. aeruginosa* isolates obtained from various clinical samples [tracheal aspirate (*n* = 37), blood (*n* = 8), urine (*n* = 5) and wound (*n* = 2)] at the Clinical Microbiology Laboratory of Kastamonu Training and Research Hospital in Turkey, were employed as a material. The isolation and identification of the isolates were performed using a conventional method (determination of Gram and oxidase reaction, beta-hemolytic activity on sheep blood agar, colony morphology, pigment production, growth at 42°C) and the VITEK 2 compact system, and the carbapenem-resistance profile of the isolates was detected by the VITEK 2 compact system (BioMéreux, France) (Nakasone et al., 2007). Molecular confirmation of the isolates was applied using PCR assay based on *P. aeruginosa* species-specific *oprL* gene region detection according to Ahmadi and Roodsari (Ahmadi and Roodsari, 2016).

#### Slime production

Slime production was determined by cultivation of the isolates on CRA (including 37 g/L BHI broth, 10 g/L agar base, 50 g/L sucrose, 1 L water and 0.8 g/L Congo Red indicator) plates, as described by Freeman et al (Freeman et al., 1989).

The isolates were kept in nutrient broth with 15% glycerol at -86 °C for further analysis. *P. aeruginosa* ATCC 15692 was employed as a positive reference strain for detection of the *oprL* gene region, two QS systems and four virulence genes, and slime production tested.

#### DNA extraction
The isolates were sub-cultured on Trypticase Soya Agar (TSA) and a maximum of five colonies grown on TSA were collected. DNA extraction of the isolates was carried out using boiling method according to Katvoravutthichai et al. (Katvoravutthichai et al., 2016). The sequence, product size of the primers and amplification program (TurboCycler Lite 9020, Blue-Ray, Biotech) for the PCR assay utilized in this study are depicted in Table 1.

Detection of QS system genes

For this purpose, four gene regions (lasI/R, rhlI/R) associated with the las and rhl QS system were analyzed in the CRPsA isolates using PCR assay, previously described by Schaber et al. (Schaber et al., 2004).

Detection of QS-related virulence factors and class 1 integron

For this purpose, lasB, algD, toxA, and aprA virulence gene regions were determined using single target PCR assay with minor modifications to the protocol proposed by Martins (Martins et al., 2014). The total PCR mixture was 25 µL, including 1XPCR Buffer, 2.5 mM of MgCl₂, 0.2 mM of dNTPs, 0.2 µM of each primer, 1U of Taq polymerase, and 1 µL of template DNA.

Detection of Cls1 integron: For the presence of Cls1 integron, the integrase gene (intI1) was determined using the PCR assay previously described by Bass (Bass et al., 1999).

Statistical analysis

Fisher’s exact test was performed to analyze the relationship among the las and rhl QS systems, virulence gene distribution and biofilm formation. A statistical analysis was carried out using SPSS/20.0 software.

Results and discussion

CRPsA infections are one of the most serious healthcare-related infections because they are most commonly utilized for the last-choice antibiotic to cure P. aeruginosa infections (Morita et al., 2014). Many researchers stated that CRPsA’s infections cause problems such as increased mortality, longer hospital stay duration, and increased medical costs. According to available data from Turkey and other countries, the maximum human clinical CRPsA ratio is 60% (Baumgart et al., 2010; Bocharva et al., 2020; ECDC, 2020; Walters et al., 2019; Akgün et al., 2020; Çeken et al., 2021).

There have been limited data on the QS system and the presence of virulence genes in CRPsA isolates in contrast to its genetic diversity (Bogiel et al., 2021; Kumar et al., 2009; Ellappan et al., 2018; El-Mahdy, El-Kannishy, 2019). It is well known that the P. aeruginosa’s virulence genes expression is a highly complex procedure that is generally governed by las and rhl QS system genes. Kumar et al. stated that QS system-deficient strains that fail to create successful infection were associated with a decrease in virulence factors expression (Kumar et al., 2009). In the present study, two QS system genes were detected in almost all isolates (98.7%) and compared with the presence of the two QS system genes, the percentage of las system genes (98.07%) were higher than that of rhl system genes (90.38%). In addition, there was a positive correlation between two QS system genes (p < .10). Ellappan et al. reported that lasR genes and rhlR genes were identified in 81% and 84%, respectively, of the clinical CRPsA isolates (Ellappan et al., 2019). The current study results are higher [lasR (94.23%) and rhlR (82.69%)] than Ellappan et. Al.’s study results but lower than El-Mahdy and El-Kannishy’s study findings, in which QS lasR and rhlR genes were detected in all of the isolates (El-Mahdy, El-Kannishy, 2019).
Bacteria with QS systems that govern virulence factors and biofilm formation are more resistant to most treatment agents, such as carbapenems and next-generation antibiotics (Tanveer et al., 2020). The present study results supported this viewpoint to some extent. Hence, four virulence genes were identified at a ratio between 46.15% and 88.46% for 52 CRPsA isolates. When considering two QS system genes and slime production (67.30%) with four virulence genes, it can be concluded that the CRPsA isolates are highly virulent.

Pathogenesis of *P. aeruginosa* involves many stressful conditions (interferon, IFN), etc.) created by the host immune system, and to ensure that bacteria overcome many stressful factors and survive, a wide range of virulence genes are expressed, particularly by the las and rhl QS systems (Gonçalves et al., 2017). For instance, IFN-α produced by T-cells coordinates many different immunological responses (Schroder et al., 2004). IFN-α binds to *P. aeruginosa* outer membrane protein E (OprF). When the binding step occurs, the rhl QS system activates for the production of some virulence factors, such as lecA (encodes lecA, which are cytotoxic and adhesive factors) and pyocyanin. Afterward, the rhl QS system induces cytotoxic exoproducts such as exotoxin A to enter the host cell and then cause biofilm formation (Laughlin et al., 2000). Therefore, *toxA* gene is an important virulence factor in encoding exotoxin A (exoA). Our result of 86.53% is consistent with the results of Gonçalves et al. (Gonçalves et al., 2017). (87.3%) and Bogiel et al. (93.9%). These findings indicate that the *toxA* gene is highly common among CRPsA strains (Bogiel et al., 2021). In addition, according to some researchers there is a positive correlation among rhl system, exoA and biofilm formation, and the results of the present study also show agreement with the results (Laughlin et al., 2000; Diggle et al., 2006).

Another virulence factor is AprA, which also has a role in *P. aeruginosa* pathogenesis due to the degradation of wide proteins and destroys the host defense system (Hoge et al., 2010). In our study, the *aprA* gene was detected in 88.48% of the isolates, whereas Rojo-Bezares et al. and Bogiel et al. detected the gene in 100% of the isolates. There was also a positive significant correlation between the rhl system and *aprA* (*p* < 0.01) (Rojo-Bezares et al., 2016; Bogiel et al., 2021).

As an important virulence factor, the *algD* gene has a crucial role, especially in chronic lung infections and alginate biosynthesis. During infection and antibiotic therapy, the bacteria are transformed from the nonmucoid phenotype into mucoid-producing bacteria and start to produce alginate (Dogget, 1969). In the late stage of infection, mucoid-producing bacteria are dominant and cause deterioration, leading to a high mortality rate (Davis et al., 1980). In the current study, the *algD* gene was detected in 46.15% of the isolates. The results indicate that nearly half of the isolates have the mucoid-producing property and that there were significant correlations between *algD* and the rhl system (*p* < 0.05), between *algD* and slime production (*p* < 0.01), between *algD* and *lasB* (*p* < 0.05) and between *algD* and *toxA* (*p* < 0.05). Bogiel et al. and Ellappan et al. detected the *algD* gene in 92.5% and 93%, respectively, of the CRPsA isolates. The results of both studies are higher than our study results (Ellappan et al., 2018; Bogiel et al., 2021).

In our study, the CRPsA isolates were capable of biofilm formation due to *algD* gene (46.15%) and slime production (67.30%). These co-existing properties were present in 22 (42.30%) of the CRPsA isolates. From 37 tracheal origin isolates, 72.97% and 51.33% of the CRPsA isolates were capable of slime production and carried the *algD* gene, respectively. Thus, the majority of the tracheal origin CRPsA isolates have the potential of biofilm formation. According to Bogiel et al.’s study results, there was a positive correlation between *toxA* genes and *algD* genes (*p* < 0.05). Our study supports their results in terms of a positive correlation between *toxA* genes and *algD* genes (Bogiel et al., 2021).
There is limited research on biofilm formation of CRPsA isolates (Kumar et al., 2009). In El-Mahdy and El-Kannishy’s study, biofilm formation was detected in 65.2% and 94.1% of carbapenem-sensitive strains and carbapenem-resistant strains, respectively, and lasR and rhlR genes were identified in all CRPsA isolates (El-Mahdy and El-Kannishy, 2019). The authors concluded that biofilm formation was significantly related to carbapenem-resistant isolates. Kumar et al.’s study results support this conclusion. Similarly, in our study, slime production was determined in 67.30% of the isolates, and there was a positive correlation between the rhl QS system and slime production. A significant correlation between slime production and algD, as well as lasB genes (Table 3), was also detected (Kumar et al., 2009).

As a protease enzyme, elastase B (lasB) (pseudolysin) is encoded by the lasB gene. LasB is associated with cystic fibrosis due to elastinolytic activation and with vascular inflammation due to elastin fiber’s disorganization in vascular tissue due to protease degradation by lasB (Schultz and Miller, 1974). Similar to aprA, lasB also degrades some proteins, such as INF-γ, tumor necrosis factor-α and interleukin-6 (Horvat et al., 2010). lasB has an important role in the differentiation of pseudomonal biofilms (Yu et al., 2014). Tielen et al. showed that overexpression of lasB gene was not applicable to hard biofilm, but it contributes to the altering of the extracellular polymeric substances of the biofilm structure, such as reducing the alginate content but increasing the rhamnolipids concentration (Tielen et al., 2010). In this respect, in our study, lasB was detected in 69.23% of the CRPsA isolates, and there was a positive correlation between lasB and slime production and between lasB and algD genes. In the present study, lasB was identified in 69.23% of the CRPsA isolates. The ratio was quite lower than the ratios indicated by Ellappan et al. and Rojo-Bezares et al.’s study results. They detected the gene in CRPsA isolates at a ratio of 94% and in all of the imipenem resistance P. aeruginosa isolates, respectively (Rojo-Bezares et al., 2016; Ellappan et al., 2018).

CRPsA occurs primarily due to chromosomal mutation in P. aeruginosa isolates (Bocharva et al., 2020). The carbapenemase genes of the bacterium generally carries on mobile genetic elements such as integrons, and the gene spreads the resistance within and between species by integrons (Castanheira et al., 2009; Bocharva et al., 2020). Similar to this study, certain studies investigate the presence of integrons among CRPsA isolates, and a corresponding ratio between 67% and 13.6% has been obtained (Sung et al., 2009; Estepa et al., 2015; Liapis et al., 2019; Bocharva et al., 2020). Liapis et al. reported that most blaIMP genes in P. aeruginosa isolates are carried by Cls 1 integrons (Liapis et al., 2019). In the current study, Cls 1 integron was detected in 51.92% (27/52) of the CRPsA isolates, which is higher than the results of Sung et al. (13.6 %) and Bocharova et al. (44.1 %) but lower than the results of Estepa et al. (67%) (Sung et al., 2009; Estepa et al., 2015; Bocharova et al., 2020). Similar to our study, all study results indicate that carbapenemase gene can be transferred among bacteria due to the presence of integrons.

In conclusion, based on the findings of the current study, there is a significant positive correlation between las-rhl system, and between the QS system and four virulence genes and slime production. Cls 1 integron is common in the tested CRPsA isolates. Therefore, the CRPsA isolates are highly virulent and QS systems have a significant role in pathogenesis. Carbapenemase gene can be transferred among bacteria. All of the results indicate that CRPsA isolates are great concerns in terms of clinical aspects and to control of spread of the carbapenemase gene.

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**Declarations**

**Author contributions**

C.B., BS and İ.E designed the study; E.F.T and Ç.K. collected carbapenem-resistant \textit{P. aeruginosa} isolates and carried out phenotypic identification; C.B. and B.S. performed the molecular assay; C.B., B.S., E.F.T., Ç.K., Ö.E., and İ.E. prepared and revised the manuscript. All authors gave the final approval of the version to be published.

**Disclosure Statement**

All the authors report that they have no competing interests in this work.

**Financial Disclosure**

There is no financial support.

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Tables
| Primer     | Primer Sequence 5' → 3'                          | Product size (bp) | Amplification program                                                                 | References                      |
|------------|-------------------------------------------------|-------------------|---------------------------------------------------------------------------------------|---------------------------------|
| Species specific |                                                 |                   | groom denaturation; cycles program; extension                                          |                                 |
| OprL-F     | ATGGAAATGCTGAAATTCCGGC                          | 504               | 94°C for 5 min 30 cycles: 94°C for 1 min, 52°C for 1 min, 72°C for 1 min              | Ahmadi and Roodsari et al., 2016|
| OprL-R     | CTTCTTCAGCTCGACCGACGG                           |                   | 72°C for 10 min                                                                       |                                 |
| Virulence genes |                                                 |                   |                                                      |                                 |
| lasB-F     | GGAATGAACGAAAGCGTTCTC                           | 300               |                                                        |                                 |
| lasB-R     | GGTCCAGTAGTGACGGTTGG                           |                   |                                                        |                                 |
| algDF      | ATGCGAATCAGCATCTTTTGT                          | 1310              | 94°C for 3 min, 30 cycles of denaturation at 94°C                                      |                                 |
| algDR      | CTACCAGCAGATGCGCTTGG                           |                   | 30 cycles: 94°C for 30 s, 55°C for 1 min, 72°C for 1 min                              | Martins et al., 2014           |
| toxA-F     | GGTAACCAGCTCCAGCGCACAT                         | 352               | 72°C for 5 min                                                                       |                                 |
| toxA-R     | TGATGTCAGGTCAATGCTTC                          |                   |                                                        |                                 |
| aprA-F     | ACCCTGTCTATTTCCGGTC                           | 140               |                                                        |                                 |
| aprA-R     | GATTGCAGCGACAACCTTGG                           |                   |                                                        |                                 |
| QS system genes |                                                |                   |                                                      |                                 |
| lasF       | TCGACGAGATGGAAATCGATG                         | 363               |                                                        |                                 |
| lasR       | GCTCGATGCGATCTTTCAG                           |                   |                                                        |                                 |
| lasR-F     | TGCCGATTTCGGTGAACC                          | 362               |                                                        |                                 |
| lasR-R     | CCGCCGAATTTTCATCATG                          |                   | 95°C for 5 min                                                                       | Schaber et al., 2004           |
| rhlF       | CGAATTGCTCTCTGAATCGCT                         | 143               | 34 cycles: 94°C for 30 s, 59°C for 30 s, and 72°C for 2 min                           |                                 |
| rhlR       | GGCTCATGCGACGATGTA                            |                   | 72°C for 10 min                                                                       |                                 |
| rhlR-F     | TCGATTACTAGCCTATTGGCG                          | 207               |                                                        |                                 |
| rhlR-R     | TTCCAGAGCATCCGGCTCT                          |                   |                                                        |                                 |

Integrate gene for Class 1 integron
| Primer | Primer Sequence 5' → 3' | Product size (bp) | Amplification program | References |
|--------|-------------------------|------------------|-----------------------|------------|
| intl 1-F | CCT CCC GCA CGA TGA TC | 280              | 94°C for 5 minutes, 30 cycles followed by 1 min at 94°C followed by 1.5 30 30 cycles: 94°C for 1 min; 55°C for 1.5 min, 72°C for 1 min | Bass et al., 1999 |
| intl 1-R | TCC ACG CAC TGT CAG GC | 72°C for 7 min    |                       |            |
Table 2
PRESENCE OF TWO QS SYSTEM GENES, FOUR VIRULENCE GENES, BIOFILM PRODUCTION AND CLASS 1 INTEGRON IN CLINICAL CARBAPENEM-RESISTANT PSEUDOMONAS AERUGINOSA ISOLATES (n=52)

| Sample | oprL | Two QS system genes (n=51, 98.07%) | Virulence genes | Biofilm | Class1 |
|--------|------|-----------------------------------|----------------|---------|--------|
|        |      | lasI lasR rhlI rhlR               | lasB algD toxA aprA | CRA     | Intl1   |
| 1^U    | +    | + + + + + + + + + + + - - +     |               |         |        |
| 2^U    | +    | + + + + + + + + + + - - -       |               |         |        |
| 3^U    | +    | + + + + + - - - - + - -       |               |         |        |
| 4^U    | +    | - - - - - - - + + - - -       |               |         |        |
| 5^U    | +    | + + + + + + - - + + + + +     |               |         |        |
| 6^B    | +    | + + + + + + + - + + + + + + + |               |         |        |
| 7^B    | +    | + + + + + - - - + + + - -     |               |         |        |
| 8^B    | +    | + + + + + + + + + + + + + + + |               |         |        |
| 9^B    | +    | + + + + + + - + - - - -       |               |         |        |
| 10^B   | +    | + + + + + + - - - - + + -     |               |         |        |
| 11^B   | +    | + + + - - - - - - - - -       |               |         |        |
| 12^B   | +    | + + + + + + + + + + + + + + + |               |         |        |
| 13^B   | +    | + + + + + + - + + + + + + -   |               |         |        |
| 14^W   | +    | + + + + + + + - + + + + + -   |               |         |        |
| 15^W   | +    | + + - - - + - - - - - +       |               |         |        |
| 16^T   | +    | + + + + + + + + + + + + + + + |               |         |        |
| 17^T   | +    | + + + + + + - + + + + + + +   |               |         |        |
| 18^T   | +    | + + + + + + + + + + + + + + + |               |         |        |
| 19^T   | +    | + + + + + + - + + + + + + +   |               |         |        |
| 20^T   | +    | + + + + + + + + + + + + + + + |               |         |        |
| 21^T   | +    | + + + + + + + + + + + + + + - |               |         |        |
| 22^T   | +    | + + + + + + + + + + + + + + + |               |         |        |

U: Urine origin; B: Blood origin; T: Tracheal origin
| Sample | oprL | Two QS system genes (n=51, 98.07%) | Virulence genes | Biofilm | Class1 |
|--------|------|-----------------------------------|-----------------|----------|--------|
|        |      | lasI lasR rhlI rhlR (n=47, 90.38%) | lasB algD toxA aprA | CRA | IntI1 |
| 23T    | +    | + + + + + + + + + + + - |                             |        |        |
| 24T    | +    | + + + + + - - + + + - - |                             |        |        |
| 25T    | +    | + + + + + + + + + + + + |                             |        |        |
| 26T    | +    | + + + + + + + + + + + + |                             |        |        |
| 27T    | +    | + + + + + + + + + + + + |                             |        |        |
| 28T    | +    | + + + + + + + + + + + + |                             |        |        |
| 29T    | +    | + + + + + + - + + + + + + |                             |        |        |
| 30T    | +    | + + + + + + + - + + + + + |                             |        |        |
| 31T    | +    | + + + + + + + - + + + - - |                             |        |        |
| 32T    | +    | + + + + + + + + + + + - |                             |        |        |
| 33T    | +    | + + + + + + + + + + + + |                             |        |        |
| 34T    | +    | + + + + + + + + + + + + |                             |        |        |
| 35T    | +    | + + + + + + + + + + + + |                             |        |        |
| 36T    | +    | + + + + + + + - + + + - - |                             |        |        |
| 37T    | +    | + + + + + + + - + + + + + |                             |        |        |
| 38T    | +    | + + + + + + + - + + + - - |                             |        |        |
| 39T    | +    | + + + + + + + - + + + + + |                             |        |        |
| 40T    | +    | + + + + + + - - + + + + + |                             |        |        |
| 41T    | +    | + + + + + + + + + + + + |                             |        |        |
| 42T    | +    | + + + + + - - + + + - - |                             |        |        |
| 43T    | +    | + + + + + + + + + + + + |                             |        |        |
| 44T    | +    | + + + + + + + + + + + + |                             |        |        |
| 45T    | +    | + + + + + + - - + + + + + |                             |        |        |

U: Urine origin; B: Blood origin; T: Tracheal origin
| Sample | oprL | Two QS system genes (n=51, 98.07%) | Virulence genes lasI lasD rhlI rhlR (n=51, 98.07%) | Biofilm CRA | Class1 IntI1 |
|--------|------|-----------------------------------|-----------------------------------------------|------------|-------------|
| 46T    | +    | + + + + + + - + + - - - - - - - |
| 47T    | +    | + + + + + + + + + + + + - - - - |
| 48T    | +    | - + + + + + + - + + + - - - - - |
| 49T    | +    | + + - - - - - - + - - - - - - -|
| 50T    | +    | + + + - - - - - - - - - - - + -|
| 51T    | +    | + + + - - - - - - - + - - - - -|
| 52T    | +    | + + + + + + - + + + + + + + + +|
| Total number | 52  | 50   | 49   | 46   | 43   | 36   | 24   | 45   | 46   | 35   | 27   |
| %      | 100.00 | 96.15 | 94.23 | 88.46 | 82.69 | 69.23 | 46.15 | 86.53 | 88.46 | 67.30 | 51.92 |

U: Urine origin; B: Blood origin; T: Tracheal origin
### Table 3
STATISTICAL ANALYSES RESULTS USING FISHER'S EXACT TEST

| Correlation                  | p-values |
|------------------------------|----------|
| $\text{lai}$ and $\text{rhl}$ system | 0.096*   |
| $\text{lai}$ system and $\text{laiB}$ | 0.3077   |
| $\text{lai}$ system and $\text{toxA}$ | 1        |
| $\text{lai}$ system and $\text{aprA}$ | 0.1154   |
| $\text{lai}$ system and CRA | 0.3269   |
| $\text{rhl}$ system and $\text{laiB}$ | 0.001*** |
| $\text{rhl}$ system and $\text{algD}$ | 0.054**  |
| $\text{rhl}$ system and $\text{toxA}$ | 0.013**  |
| $\text{rhl}$ system and $\text{aprA}$ | 0.0002*** |
| $\text{rhl}$ system and CRA | 0.002*** |
| $\text{algD}$ and CRA | 0.0008*** |
| $\text{laiB}$ and CRA | 0.024**  |
| $\text{laiB}$ and $\text{algD}$ | 0.014**  |
| $\text{toxA}$ and $\text{algD}$ | 0.011**  |

*Significant of 10% level ($p \leq 0.10$); ** significant of 5% level ($p \leq 0.05$); *** significant of 1% level ($p \leq 0.01$)