

Contribution of quorum-sensing systems to virulence of *Pseudomonas aeruginosa* in an experimental pyelonephritis model

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Background and Purpose: *Pseudomonas aeruginosa* has been reported to monitor its cell density as well as expression of virulence determinants by quorum-sensing signal mechanisms operative through autoinducers. In the present investigation, we studied the contribution of quorum-sensing signals during the course of *P. aeruginosa*-induced pyelonephritis in mice.

Methods: The standard parent strain of *P. aeruginosa* (PAO1), possessing functional *las* and *rhl* quorum-sensing systems and its isogenic mutant strains, PAO-JP1 (single mutant), harboring a mutated *lasI* gene and PAO-JP3 (double mutant), harboring mutated *lasI* and *rhlR* genes were employed. One uroisolate of *P. aeruginosa* belonging to serotype O8 and deficient in production of quorum-sensing signals was also used.

Results: The parent strain of *P. aeruginosa* was significantly more virulent compared to its isogenic mutant strains and quorum-sensing negative clinical strain, as assessed by neutrophil influx, malondialdehyde production, renal bacterial load and pathology induced in experimental animals.

Conclusions: Quorum-sensing systems play an important role in the pathogenicity of *P. aeruginosa* in pyelonephritis. Both the *las* and *rhl* quorum-sensing systems are important for the virulence of *P. aeruginosa* in the development of pyelonephritis.

Key words: Animal disease model, *Pseudomonas aeruginosa*, quorum sensing, virulence

Introduction

Successful adaptation of microorganisms to different niches depends on their ability to regulate gene expression in response to environment. Pathogens have been reported to use chemical signaling pathways to translate external signals into adaptive responses. These pathways, known as quorum-sensing signal mechanisms, help bacteria to monitor their population density [1]. They also control expression of specific genes, some of which have been implicated in virulence of organisms [2]. A variety of Gram-positive and Gram-negative bacteria, including *Pseudomonas aeruginosa*, have been shown to possess these quorum-sensing systems [3-6]. This

pathogen possesses a wide variety of factors, such as exotoxins, alginate, hemolysins, protease, elastase, phospholipase C and siderophores which have been implicated in its ability to cause acute and chronic infections [7-9]. Recently, it has been reported that *P. aeruginosa* controls expression of these virulence traits through quorum-sensing systems operative through autoinducers which include acylhomoserine lactones (AHLs) [10,11]. Two AHL-mediated quorum-sensing systems, *las* and *rhl*, have been identified in *P. aeruginosa*. The *las* system has been shown to regulate the expression of virulence factors such as elastase, alkaline protease, Las B protease, exotoxin A, pyocyanin, pyoverdine and hemolysin [12,13]. The *rhl* system is known to be involved in modulating the expression of several of virulence factors controlled by the *las* system [14,15]. Profiles of the AHLs produced depend on different environmental conditions as well as on the cell form (planktonic or biofilm) of the organism [16,17].

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In vivo studies with *P. aeruginosa* are available in relation to experimental models of burn wound, contact lens-induced keratitis and respiratory tract infection, where it has been demonstrated that mutant strains of *P. aeruginosa* which do not produce AHLs are less virulent than AHL-producing strains [18-22]. However, there is a paucity of literature regarding the role played by these signals in relation to *P. aeruginosa*-induced urinary tract infections (UTIs). *P. aeruginosa* is the third most common pathogen associated with hospital-acquired UTIs [23]. The majority of isolates encountered are known to possess quorum-sensing systems, which modulate several virulence factors through AHL production. However, recently it has been reported that quorum-deficient strains of *P. aeruginosa* also occur naturally and are associated with UTIs [24]. It is in this context that the present investigation was planned to study the role of quorum-sensing signals in the virulence of *P. aeruginosa* in an experimental mouse model of pyelonephritis, in order to more precisely define its role in vivo.

Methods

Bacterial strains

Bacterial strains used in this study were standard *P. aeruginosa* strain PAO1 and its isogenic mutants. PAO1 is an invasive laboratory strain that possess 2 functional quorum-sensing systems (*las* and *rhl*) [22]. PAO-JP1, a *lasI*-deleted isogenic mutant strain of PAO1, is defective in the synthesis of 3-oxo-C12-HSL (delta [Δ] *lasI*). PAO-JP3 is a mutant strain lacking both the *lasR* and *rhlR* systems (Δ *lasR* and Δ *rhlR*). All these strains were kindly provided by Dr. Barbara H Iglewski, University of Rochester, New York, USA. In addition, 50 strains of *P. aeruginosa* isolated from urine samples of catheterized patients suffering from complicated UTIs were screened for production of quorum-sensing signals both qualitatively and quantitatively. Qualitative determination of quorum-sensing signals was carried out according to the method of Stickler et al [25]. Briefly, Luria agar plates covered with 40 μ L of X-Gal (5-bromo-4-chloro-3-indolyl-D-galactopyranoside) [20 mg/mL stock solution in dimethyl formamide] was used for cross feeding assays. AHL reporter strain (*Escherichia coli* MG 4) was first streaked on plate followed by streaking of the test strain. Quantitative determination of quorum-sensing signals was carried out according to the bioassay method of Zhu et al [26]. Briefly, each bioassay tube consisting of 2 mL of reporter strain and 0.5 mL of test

supernatant was incubated at 30°C in a water bath for 5 h with rotation at 100 rpm. Beta-galactosidase activity was then measured according to the method of Pesci et al [27]. One quorum-deficient wild type clinical strain of *P. aeruginosa* (PA 15) belonging to serotype O8 as serotyped by laboratory of health care-associated infection, London was selected on the basis of above assays.

Preparation of inoculum

Bacterial strains were grown overnight in trypticase soya broth at 37°C without shaking, washed and cell count adjusted to 10⁸ spectrophotometrically. Bacterial concentration was confirmed by viable counts.

Animals

Female Swiss Webster (LACA) mice, 6-8 weeks old, weighing 25 \pm 5 g obtained from Central Animal House, Panjab University, Chandigarh, India were used. Animals were kept in clean polypropylene cages and given food and water ad libitum.

Induction of ascending pyelonephritis

For induction of ascending pyelonephritis, the method of Mittal et al [28] was employed. A soft intramedic polyethylene catheter, non-radiopaque (outer diameter 0.61 mm, Clay Adams, USA) was inserted in the bladder through the urethral meatus and 0.05 mL of inoculum containing 10⁸ colony forming units (CFU)/mL was slowly injected into the bladder. The catheter was kept in place for 10 min to avoid leakage and allow proper administration of the required dose, and then withdrawn carefully. No obstruction or further manipulation of the urinary tract was done. All animal experiments were carried out in duplicate in 2 groups of 8 mice. The study protocol was approved by the institutional ethics committee for animal experimentation.

Assessment of renal bacterial load

Animals were sacrificed on 1st, 3rd, 5th, 7th, 10th, 12th and 15th postinfection day. Kidneys were removed aseptically, weighed and homogenized in 1 mL of sterile phosphate buffer solution. Quantitative bacterial counts per gram of kidney tissue were calculated as reported by Harjai et al [29].

Assessment of renal pathology

Kidneys were fixed in 10% buffered normal saline and dehydrated in ethanol gradient of 30-100%. Tissues were then embedded in wax, sectioned and stained with

hematoxylin and eosin [29]. Medulla, cortex, calyx and subcalyx of each kidney were evaluated on a semiquantitative scale of 0 to 4. These individual scores were then added to obtain overall severity scores, which ranged from 0 to 16 [30]. The histopathological examination was done by a pathologist experienced in urinary tract pathology.

Assessment of neutrophil response

Tissue neutrophils were quantitated using myeloperoxidase (MPO) assay [28]. Kidney tissue was homogenized in 2 mL of 50 mM potassium phosphate, pH 6.0 with 5% hexadecyl-trimethylammonium bromide and 5 mM ethylenediamine tetra-acetic acid. Homogenates were sonicated and centrifuged. Supernatants were mixed in the ratio of 1:15 with assay buffer and absorbance read at 490 nm. MPO units were calculated as change in absorbance over time. Experiments were carried out in triplicate in 2 sets.

Malondialdehyde estimation

Malondialdehyde (MDA) was estimated following the method of Wills [31]. Briefly, 0.5 mL of tissue supernatant was added to 0.5 mL of Tris-hydrochloric acid (0.1 M, pH 7.4) and incubated at 37°C for 2 h. One mL of trichloroacetic acid was then added and the mixture centrifuged at 700 g for 10 min. One mL of supernatant was mixed with equal volume of thiobarbituric acid (0.67% w/v) and kept in a boiling water bath for 10 min. After cooling volume was made to 3 mL with double distilled water, absorbance was read at 532 nm. The amount of MDA formed was expressed in nanomoles per milligram (nmol/mg)

protein, for which protein content of tissue homogenate was calculated according to the method of Lowry et al [32].

Statistical analysis

Results were analyzed statistically by use of Student's *t* test and Fisher's 2-tailed exact test for calculating *p* values. Chi-squared test and Spearman rank correlation was used to find correlations between renal bacterial load, MDA production, renal severity scores and neutrophil influx.

Results

The pyelonephritic potential of *P. aeruginosa* standard parent strain (quorum-sensing positive), its isogenic mutants and uroisolate (quorum-sensing negative) was assessed and compared in the mouse model of ascending UTI at different postinfection time periods (from day 1 to day 15). Renal bacterial load was significantly higher in mice infected with parent strain compared to mice infected with *lasI* mutant or *lasR rhIR* double mutant or quorum-sensing negative clinical strain at all postinfection time intervals (Fig. 1) [$p < 0.001$].

The mutants and quorum-sensing negative clinical strain of *P. aeruginosa* started clearing from the kidneys of experimental animals after the third postinfection day and were not recoverable from renal tissue on the seventh postinfection day. On the contrary, parent strain was able to persist in the renal tissue of mice until the 12th postinfection day and kidneys were sterile on the 15th postinfection day. However, no significant difference in renal bacterial load was observed in mice

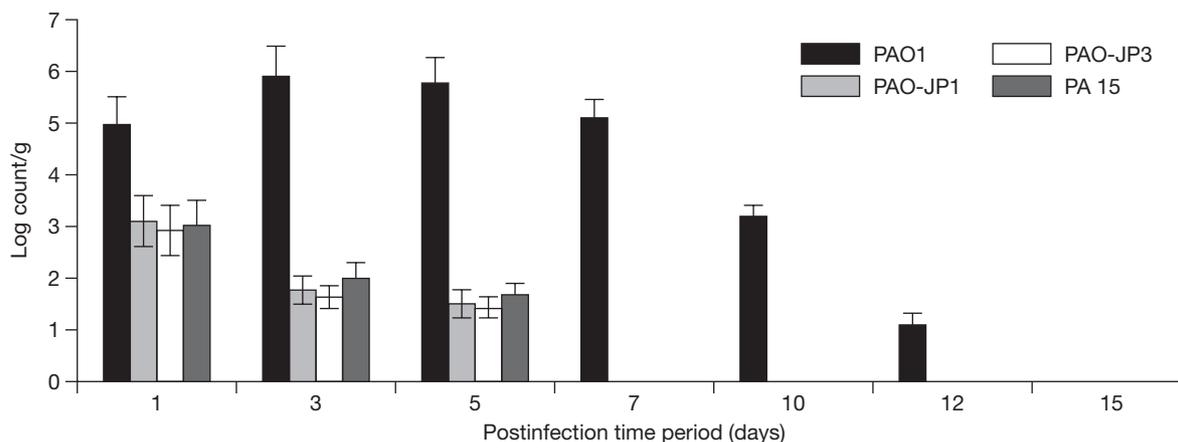


Fig. 1. Renal bacterial load following infection with *Pseudomonas aeruginosa* standard parent strain (PAO1), quorum-sensing deficient single (PAO-JP1) and double (PAO-JP3) mutants, and a quorum-deficient wild type strain (PA 15). Data represent mean of 64 kidneys studied \pm standard deviation.

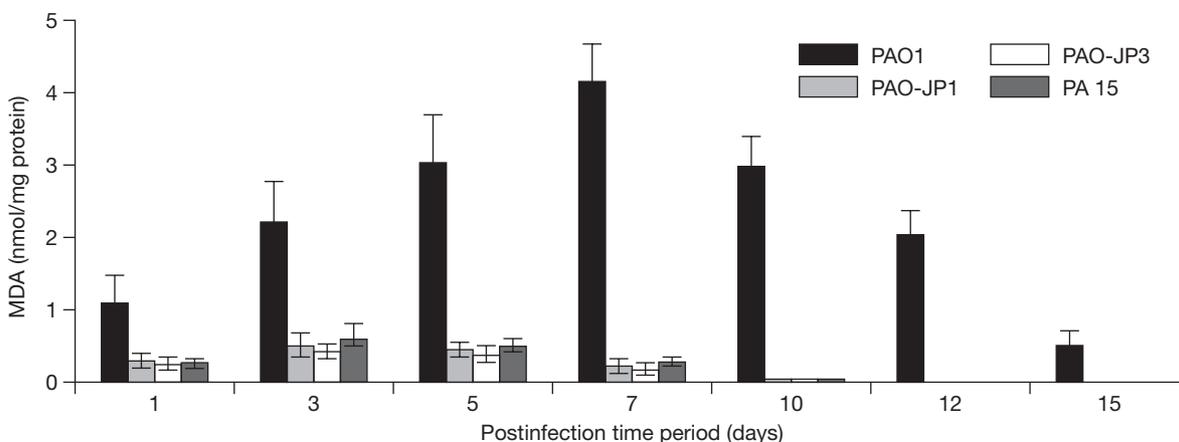


Fig. 2. Malondialdehyde (MDA) production in renal tissue of mice following infection with *Pseudomonas aeruginosa* standard parent strain (PAO1), quorum-sensing deficient single (PAO-JP1) and double (PAO-JP3) mutants, and a quorum-deficient wild type strain (PA 15). Data represent mean of 64 kidneys studied \pm standard deviation.

infected with single mutant, double mutant or quorum-sensing negative uroisolate of *P. aeruginosa* ($p>0.05$). Levels of MDA in renal tissue of mice increased significantly until the seventh postinfection day in mice infected with parent strain of *P. aeruginosa* (Fig. 2). Direct correlation between renal bacterial load and MDA production was observed in experimental animals as assessed by chi-squared and Spearman rank correlation test ($p<0.001$). However, in mutants and uroisolate, the levels of MDA remained significantly less than parent strain and ranged from 0.02 to 0.59 nmol/mg of protein.

Histopathological examination of renal tissue of experimental animals revealed severe inflammation along with destruction of tubules in mice infected with *P. aeruginosa* PAO1 strain compared to normal mice (Fig. 3 and Fig. 4). On the other hand, mild inflammation was observed in the case of mice infected with quorum-deficient mutant strains and uroisolate (Fig. 5). Severe glomerular infiltration was observed in kidneys of mice

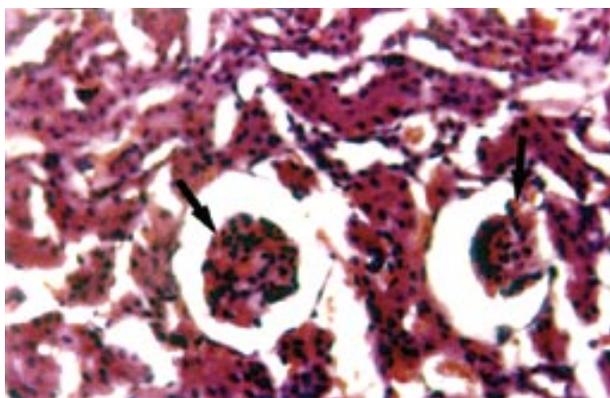


Fig. 3. Photomicrograph showing normal renal histology of uninfected mice (hematoxylin and eosin, $\times 250$).

inoculated with parent strain whereas very mild infiltration was observed in glomerulus with *P. aeruginosa* mutants and uroisolate (Fig. 6). Renal pathology scores varied from 1 to 2 in the case of mutants and uroisolate, compared to 2 to 5 with parent strain at different post-infection time periods (Fig. 7). MPO assay also revealed significantly increased neutrophil infiltration until the seventh postinfection day in renal tissue with parent strain compared to single or double mutant strains and uroisolate of *P. aeruginosa* (Fig. 8). These results correlated with renal severity scores when assessed by chi-squared and Spearman rank correlation test ($p<0.001$).

Discussion

In the present investigation, the role of quorum-sensing systems in an experimental model of pyelonephritis

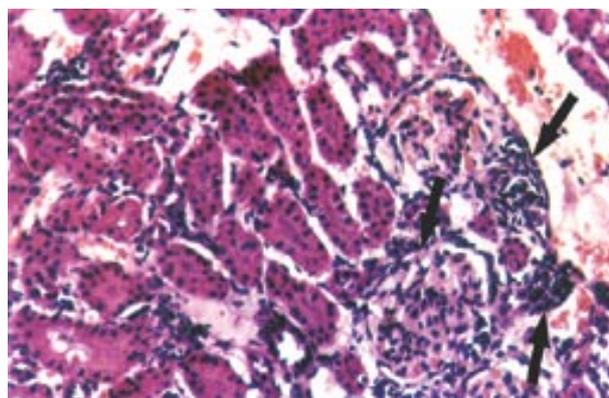


Fig. 4. Photomicrograph showing severe inflammation along with destruction of tubules in renal tissue of mice infected with standard parent strain of *Pseudomonas aeruginosa* (PAO1) [hematoxylin and eosin, $\times 250$].

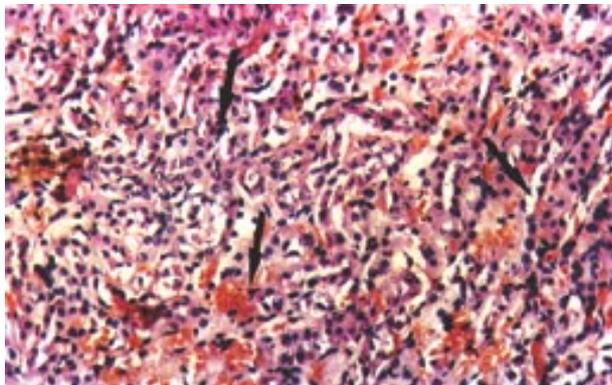


Fig. 5. Photomicrograph showing mild interstitial inflammation in renal tissue of mice infected with quorum-deficient strains of *Pseudomonas aeruginosa* (hematoxylin and eosin, $\times 250$).

was examined. Pyelonephritis was broadly defined by 2 criteria: presence of 10^4 CFU of *P. aeruginosa* per gram of kidney tissue and histopathological evidence of destruction of tubules and glomeruli along with phagocytic infiltration. For this, we employed parent strain of *P. aeruginosa*, PAO1, a reference laboratory strain, since it has been reported to be a virulent strain in a variety of animal models of infection [33–35]. We observed that out of 50 uroisolates screened for production of quorum-sensing signals, 5 strains of *P. aeruginosa* were not producing these signals.

In the present investigation, high renal bacterial counts were observed until the seventh postinfection day in mice infected with parent strain PAO1, possessing both *rhl* and *las* quorum-sensing systems, whereas a significant decrease in renal bacterial load in the kidneys of mice infected with mutant strains as well as quorum-deficient uroisolate of *P. aeruginosa* was observed after the third postinfection day. These findings suggest that both *las* and *rhl* quorum-sensing systems are important

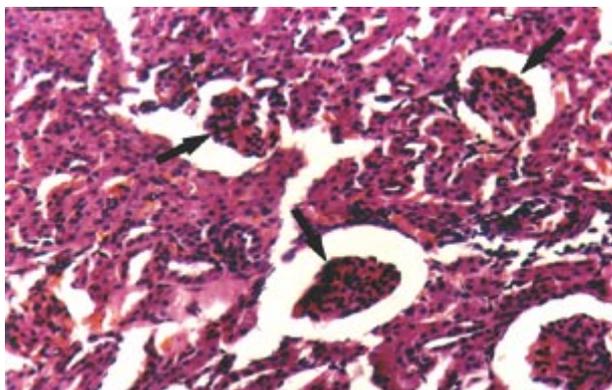


Fig. 6. Photomicrograph showing mild glomerular infiltration in renal tissue of mice infected with quorum-deficient strains of *Pseudomonas aeruginosa* (hematoxylin and eosin, $\times 250$).

for virulence of *P. aeruginosa* in the pyelonephritis model, particularly during the acute phase. Quorum-sensing signals have been shown to contribute to the virulence of *P. aeruginosa* in different infection models, such as pulmonary infection, mouse burn wound and keratitis. In *P. aeruginosa*-induced acute pneumonia [18] and chronic lung infection mouse model [21], a *lasI rhlI* double mutant was avirulent, whereas respective ‘single’ mutants showed reduced virulence when compared with the wild type strain. Similarly, in a mouse burn model of *P. aeruginosa* infection, quorum-sensing systems were shown to play an important role in the horizontal spread of *P. aeruginosa* within burned skin [19]. In relation to UTI, only 1 report is available, in which Stickler et al [25] demonstrated production of quorum-sensing signal molecules by biofilms on the surface of catheters both in a physical model of bladder (in vitro) as well as in vivo, indicating that quorum-sensing signals are produced in vivo in the urinary tract following infection with *P. aeruginosa* in catheterized patients. The present study is unique in highlighting the importance of these molecules in an experimental animal model of UTI.

In response to bacterial colonization in tissues, reactive oxygen species (ROS) are generated, leading to lipid peroxidation and tissue damage. In the present study, MDA levels were used to measure lipid peroxidation as an index of tissue damage, since MDA estimation offers speed, reliability, sensitivity and can be applied directly to complex tissues. Also, MDA is a stable product of oxidative attack of ROS on unsaturated fatty acids, an essential constituent of cell membranes. The significant increase in renal MDA level observed following infection with parent strain in comparison to mutants and uroisolate indicated that quorum-sensing signals are also essential for inducing renal tissue damage in pyelonephritis. Increased MDA levels have also been correlated with ischemia [36], chronic renal failure [37] and tubulointerstitial injury [38].

Assessment of renal severity scores is also a reliable and acceptable technique for checking the virulence potential of an organism in vivo. Results of renal pathology scoring provided additional evidence in support of a contribution of quorum-sensing systems to the virulence of *P. aeruginosa* in this model. Severe inflammation coupled with destruction of tubules was observed in kidneys of mice infected with quorum-sensing producer strain. In contrast, a significant decrease in renal severity scores was observed with mutants and uroisolate. Renal tissue of mice infected

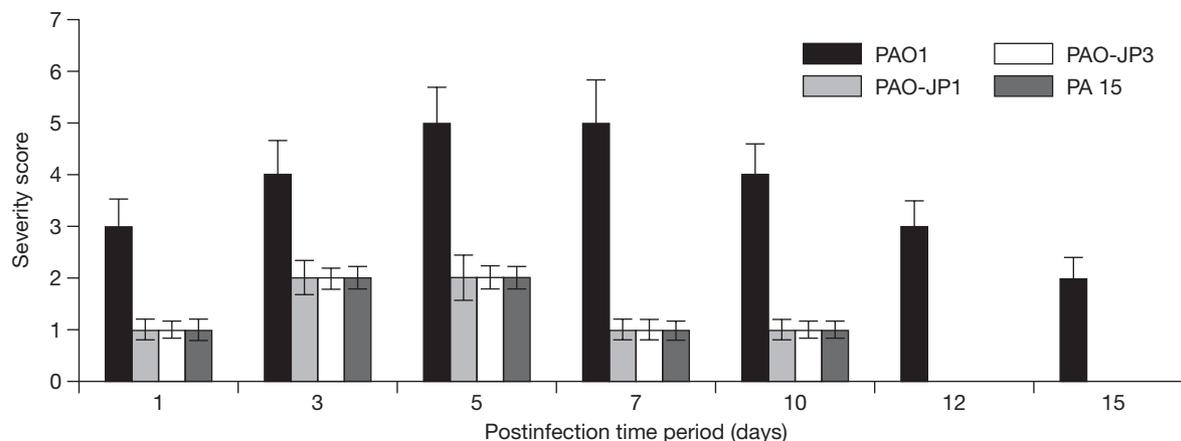


Fig. 7. Renal pathological scores following infection with *Pseudomonas aeruginosa* standard parent strain (PAO1), quorum-sensing deficient single (PAO-JP1) and double (PAO-JP3) mutants, and a quorum-deficient wild type strain (PA 15). Data represent mean of 64 kidneys studied \pm standard deviation.

with these strains showed mild inflammation with severity scores of 1-2. The importance of quorum-sensing signals in the pathogenesis of respiratory tract infections was highlighted in models of acute pneumonia in mice [18] and rats [21] where lung tissue pathology was studied. Animals infected with PAO1 strain showed more severe confluent pneumonia whereas mild focal pneumonia occurred in mice infected with quorum-deficient mutant strains.

In the present study, the level of MPO in renal tissue was taken as an index for tissue neutrophil recruitment. A significant decrease in MPO level was observed when mutant strains and quorum-deficient uroisolate were used to induce infection. This observation indicated that infection with quorum-deficient strains lacking a functional *las* system resulted in more mild infiltration

of neutrophils in kidney tissue compared with the parent strain. Other workers have also reported an immunomodulatory effect of the *las* system [39]. The *las* molecule is a potent inducer of the neutrophil chemokine interleukin-8 (IL-8) and the inflammatory mediator cyclooxygenase-2, both of which serve as neutrophil attractants and are associated with the pathophysiological process of inflammation and edema [40]. In an earlier study from our laboratory, production of macrophage inflammatory protein-2, a homologue of human IL-8 in mice, was directly related to neutrophil recruitment in the urinary tract following infection with *P. aeruginosa* [28].

The results of the present study indicate that quorum-sensing signals play a significant role in the evolution of *P. aeruginosa*-induced pyelonephritis. This

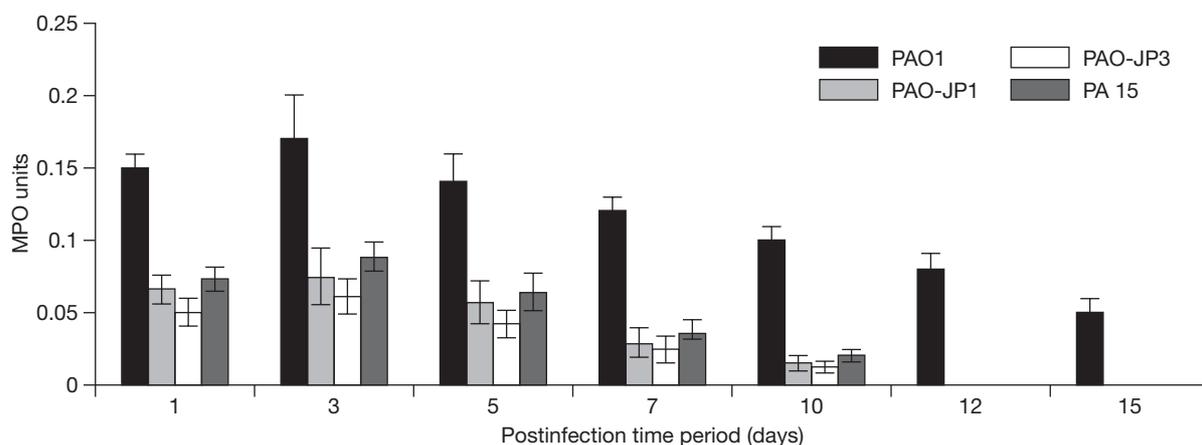


Fig. 8. Myeloperoxidase (MPO) activity in renal tissue of mice following infection with *Pseudomonas aeruginosa* standard parent strain (PAO1), quorum-sensing deficient single (PAO-JP1) and double (PAO-JP3) mutants, and a quorum-deficient wild type strain (PA 15). Data represent mean of 64 kidneys studied \pm standard deviation.

was demonstrated by reduced ability of the mutants and quorum-deficient clinical strain to colonize and cause mild pathological alterations, and also by decreased neutrophil influx and decreased MDA production in renal tissue of experimental animals compared to parent strain. Our study indicates that both the *las* and *rhl* quorum-sensing systems are important for the virulence of *P. aeruginosa* in the development of pyelonephritis. Since AHLs can serve as potential target molecules for developing an effective preventive strategy against UTIs caused by biofilms of *P. aeruginosa*, further studies employing purified molecules of AHLs are warranted in order to elucidate the role of quorum-sensing signals in the pathogenesis of *P. aeruginosa*-induced pyelonephritis.

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