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Screening & profiling of quorum sensing signal molecules in *Pseudomonas aeruginosa* isolates from catheterized urinary tract infection patients

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***Background & objectives*: Catheter associated urinary tract infections are the second most common nosocomial infections and *Pseudomonas aeruginosa* is the third most common organism responsible for these infections. In this study *P. aeruginosa* isolates from catheterized urinary tract infection patients were screened and profiled for the presence of different type of quorum sensing (QS) signal molecules.**

***Methods*: Screening and quantitation of AHLs was done by using cross feeding assay and by determining β-galactosidase activity respectively using *Escherichia coli* MG4 as reporter strain. Further, AHL profiles were determined by separating AHLs on TLC coupled with their detection using *Chromobacterium violaceum* CV026 and *Agrobacterium tumifaciens* A136 biosensor strains.**

***Results*: All uroisolates from catheterized patients having urinary tract infections were found to be producers of QS signal molecules. There were differences in amounts and type of AHL produced amongst uroisolates of *P. aeruginosa*. Several AHLs belonging to C4-HSL, C6-HSL, oxo-C6-HSL, C8-HSL, C10-HSL and C12-HSL were determined in these strains.**

***Interpretation & conclusions*: Simultaneous use of more than one reporter strain and assay method proved useful in determining the AHLs profile in uroisolates of *P. aeruginosa*. Observed differences in the amounts and types of AHLs may reflect differences in virulence potential of *P. aeruginosa* to cause UTIs which can be further confirmed by employing animal model system. The present study speculates that production of QS signal molecules may act as a new virulence marker of *P. aeruginosa* responsible for causing catheter associated UTIs and can be considered as futuristic potential drug targets towards treatment of UTIs.**

Key words *Agrobacterium* A136 - AHL - biosensors - *Chromobacterium* CV026 - *Pseudomonas aeruginosa* - TLC - UTI

Pseudomonas aeruginosa regulates the expression of cell associated and extracellular virulence factors in response to their population density by producing quorum sensing (QS) signal molecules[1-11]. QS signal molecules belongs to acyl homoserine lactones (AHLs) family in *P. aeruginosa*. These AHL molecules incorporate a homoserine lactone ring which differs with respect to the length and C3 substitution of the N-linked

acyl side chain. Accumulation of the AHL molecule above a threshold level results in its interaction with a member of the *LuxR* family of activators allowing transcription of target genes[7].

Importance of QS system has been demonstrated in various infections like cystic fibrosis, burn wound, respiratory tract infections, microbial keratitis and urinary tract infection caused by *P. aeruginosa*[12-16]. Catheter associated urinary tract infections (UTIs) are the second most common nosocomial infections and *P. aeruginosa* is the 3rd most common pathogen associated with 35 per cent of nosocomial catheter associated UTIs[17,18]. These infections are a major cause of concern because of recurrence and chronicity among several susceptible patient populations. Due to formation of biofilms on the catheter surface which are resistant to antibiotics and host defence mechanism, *P. aeruginosa* is difficult to eradicate.

Analysis of QS signal molecules can be useful in various infections including UTI caused by *P. aeruginosa.* Detection and identification of QS signals can give an idea about the type of community, density of population and expression of virulence components of the infecting pathogen. Moreover, these QS regulatory mechanisms are also being proposed as a novel target for developing innovative strategies to control infections[19]. Since AHLs produced by bacteria differ only in the length of the acyl-chain moiety and substitution at position C3, which can be either unmodified or carry an oxo- or hydroxyl group, screening for production of these compounds may require use of many reporter organisms as well as techniques.

In the present study, analysis of QS signals in the uroisolates of *P. aeruginosa* has been rendered possible mainly by the use of four bacterial biosensors which were able to detect the presence of exogenous AHLs. These biosensors cannot produce their own AHL but carry functional *LuxR* family protein cloned together with a cognate target promoter positively regulating the transcription of a reporter gene (*e.g.* biolumiscence, β- galactosidase, GFP and violacein pigment). Most of the biosensors respond to limited number of acyl-HSLs due to the specificity of their R protein[20,21]. In addition to choice of different biosensor strains, choice of detection method also becomes important and critical. There is a need to use of more than one biosensor strains and different methods for screening and profiling of QS signal molecules in *P. aeruginosa* isolated from catheterized urinary tract infection patients for evaluation of complete range of AHLs produced by uroisolates of *P. aeruginosa,* and screen the urine samples of patients directly for the detection of QS signal molecules.

Material & Methods

Bacterial strains: A total of 50 uroisolates of *P. aeruginosa* consecutively isolated from catheterized patients having UTI, attending Government Medical College and Hospital, Chandigarh, India, over a period of two years (November 2006 to December 2008) were used. Study protocol was approved by the Panjab University Ethical Committee. *P. aeruginosa* standard strain PA01 was also used along with clinical isolates.

For the detection and measurement of AHLs, reporter strain *Escherichia coli* MG4 (pKDT17) was used (ampicillin-100 µg/ml). AHL biosensors *Chromobacterium violaceum* CV026 (LB, kanamycin-20 µg/ml) and *Agrobacterium tumifaciens* A136 (Minimal A media, tetracycline-50 µg/ml and streptomycin-25 µg/ml) were used for TLC. Reporter strain PAO-JP2 (pECP61.5) (carbenicillin-20 µg/ml) was also used as a specific reporter strain for C4-HSL.

Cross feeding assay for AHL detection: Luria agar plates covered with 40 µl of X-Gal (20 mg/ml) were streaked 1 cm apart with reporter strains *E. coli* MG4 or PAO-JP2 and culture to be tested. AHLs produced and diffused through the agar results in appearance of blue colour in the reporter strain.

Extraction of AHLs: Overnight grown culture supernatants (10 ml) were extracted twice with equal volume of acidified ethyl acetate. Pooled extracts were dried over anhydrous magnesium sulphate and were evaporated to dryness. Residues were re-suspended in 50-100 µl of HPLC grade ethyl acetate.

Analytical thin layer chromatography (TLC): To evaluate the profiles of AHLs, TLC was carried out according to the method of Shaw *et al*[21]. Briefly, 4 µl of sample was applied to the silica gel $C18_{RP}$ TLC plates (Merck, Germany). The chromatograms were developed with methanol: water (60:40 v/v). Once the solvent front migrated to within 2 cm of the top, plates were air dried. Plates were then overlaid with a thin film of agar seeded with biosensor strain *C. violaceum* CV026 or *A. tumifaciens* A136. In case of A136, agar was supplemented with X-Gal (65 µg/ml). All experiments were done in triplicates. AHLs were identified by comparing the retention factor of synthetic standard AHLs and test AHL spots.

AHLs quantification: Culture supernatant was extracted from overnight grown culture for β-galactosidase

activity. Reporter culture was diluted 1:1 in Z buffer and assayed for β-galactosidase activity by using *o*-nitrophenyl-D-galactopyranoside (ONPG) as a substrate as described by Miller[22].

Results & Discussion

Identification of AHLs is becoming important in clinical settings since QS cascades are now becoming futuristic possible drug target factors to combat *P. aeruginosa* infections[6]. In the present study, four biosensor strains were employed for screening, identification and quantitation of AHLs in different *P. aeruginosa* uroisolates. *E. coli* MG4, used in cross-feeding assay can detect wide range of exogenous AHLs (C8 to C14-HSLs) but cannot detect shorter chain and 3-hydoxy AHLs[23]. In the presence of exogenous AHLs, *lacI:Z* gene gets activated and transcripts the reporter gene for the production of blue colouration by the activation of β-galactosidase. Initial screening showed all the uroisolates to be producers of QS signal molecules based on the development of blue colouration in reporter strain (Fig. 1). This method has an advantage of being rapid, and easy to perform. It can directly screen strains isolated from patients for AHL production. All uroisolates of *P. aeruginosa* have also been found to be producing AHLs earlier using *A. tumifaciens* reporter strain by cross feeding assay[24]. These workers made first demonstration of AHL production by biofilms developed on the catheters both *in vitro* and *in vivo* in patient's bladder in a clinical setting. However, type of AHL produced by uroisolates of *P. aeruginosa* has not been reported so far. Biosensor strain PAO-JP2 employed for detection of C4-HSL, contains plasmid pECP61.5 with *lacI:Z* gene insertion. By employing PAO-JP2 biosensor strain, it was observed that all the uroisolates of *P. aeruginosa* were C4-HSL producer in the extracts of their supernatants by cross-feeding assay.

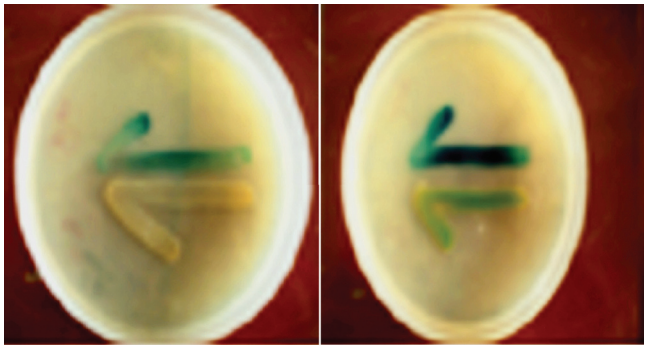

Fig. 1. Agar plate cross-feeding assay for screening of acyl homoserine lactone (AHL) production by uroisolates of *P. aeruginosa*. Evidence for the production of AHL is indicated by blue colouration of the reporter strain *E. coli* MG4.

Separation of AHLs by TLC coupled with their detection by AHL biosensor strains gives an identifying index of the AHLs produced by the test bacteria. Keeping this in view, we tried to identify the types of AHLs produced by uroisolates using TLC and two biosensor strains, *C. violaceum* CV026 and *A. tumifaciens* A136. *C. violaceum* CV026 is a violacein and AHL negative double mini Tn5 mutant strain. Transposons are inserted into the *CviI* AHL synthase gene and violacein repressor gene. This strain can produce pigment violacein after the use of exogenous AHL. *C. violaceum* CV026 can detect C4 to C8-HSLs but most strongly C6-HSL[25]. Identification of AHLs was done by separating bacterial extracts by TLC ($C18_{RP}$ Silica gel plates, Merck, Germany) and subsequently development with biosensor *C. violaceum* CV026. Synthetic AHL standards C4-HSL, C6-HSL, oxo-C6-HSL, C8-HSL, C10-HSL and C12-HSL were also run simultaneously. Purple colour spots parallel to the position of synthetic standards C4 and C6-HSL on plates were observed (Fig. 2). Relative retention factor (Rf) was calculated and compared with that of standards. A total of 74 per cent isolates showed the presence of C6-HSL. Although, *C. violaceum* CV026 biosensor is reported to detect C4-HSL, C6-HSL and C8-HSL[26,27], but other than C6-HSL no other AHL was detected in the present study. Non-detection may be due to low production of C4-HSL and C8-HSL which are reported to be detected by *C. violaceum* CV026 when produced at higher concentrations only[25]. Negative

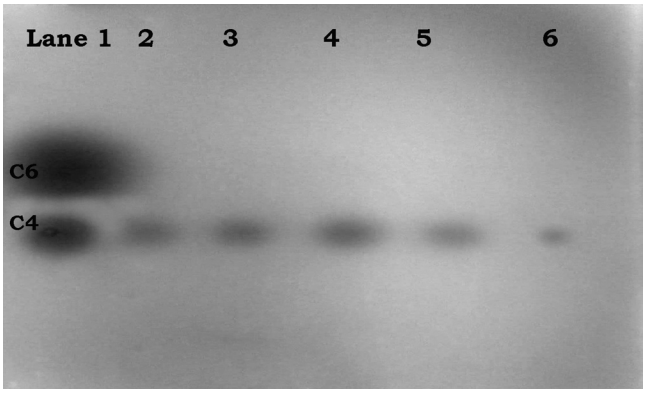

Fig. 2. Separation and detection of acyl- HSL standards [mixture of C4 (BHL), C6 (HHL), C8 (OOHL)] (lane 1) and acyl- HSLs produced by uroisolates of *P. aeruginosa* (lane 2-6) by employing thin layer chromatography. Spots were visualized with biosensor *Chromobacterium violaceum* CV026. Tentative identification of spots, based on migration of standards, is indicated.

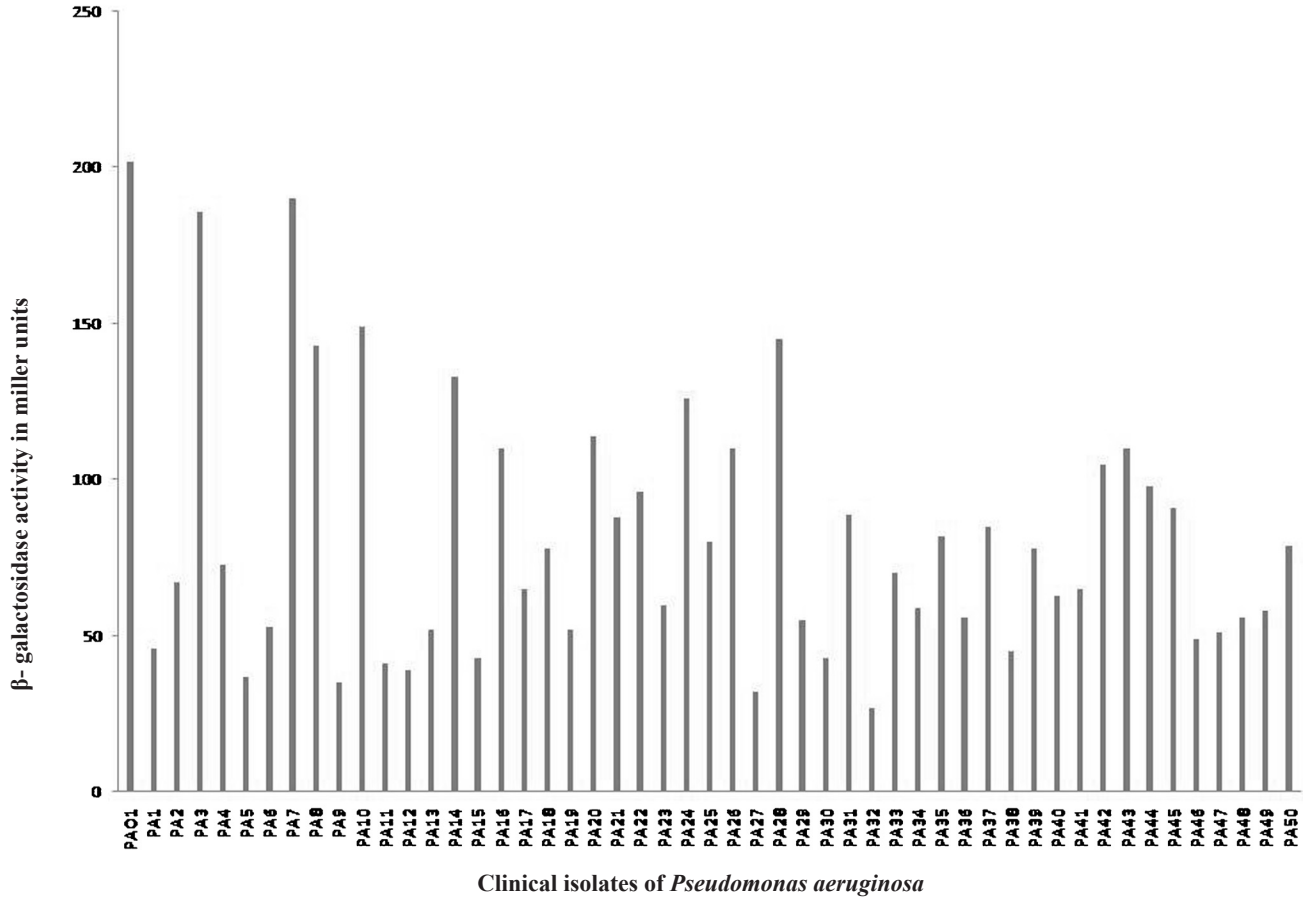

Fig. 4. Quantitative β- galactosidase activity expressed in miller units (MU) for determining the levels of quorum sensing signal molecules in the extracts of culture supernatants of Standard strain PA01 (bar 1) and uroisolates of *P. aeruginosa* (bar 2-50).

results from *P. aeruginosa* isolates from ICU patients for AHLs with *C. violaceum* CV026 has been reported earlier and it was suggested that the isolates either failed to produce short chain AHLs or the level of signals was very low[28]. The oxo-C6-HSL and C8-HSL were detected with a 6-fold and C4-HSL with a 30-fold less activity[7]. However, detection of C4-HSL and C8-HSL was made possible only by using violacein inhibition assay by *C. violaceum* CV026[27,29,30].

Since *C. violaceum* CV026 cannot detect AHLs with longer acyl side chains, broad range biosensor, *A. tumifaciens* A136 was employed thereafter for TLC. *A. tumifaciens* A136 contains a plasmid with *traR* promoter and *traG*::*lacZ* transcriptional fusion. *traG*::*lacZ* gets activated in the presence of exogenous AHL and results in appearance of blue colour. This reporter strain can detect C8 to C12-HSLs including oxo-C6-HSL and is quite sensitive to even low level of longer acyl side chain AHLs[31]. All the isolates

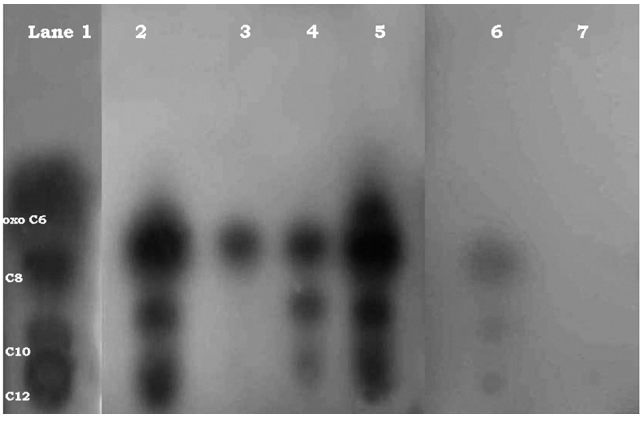

Fig. 3. Separation and detection of acyl- HSL standards [mixture of oxo C6 (OHHL), C8 (OOHL), C10 (ODHL) and C12 (OdDHL)] (lane 1), acyl- HSLs produced by uroisolates of *P. aeruginosa* (lane 2-5) and wild type PAO1 and mutant JP2 (lane 6-7) by employing thin layer chromatography. Spots were visualized with biosensor *A. tumifaciens* A136. Tentative identification of spots, based on migration of standards, is indicated.

showed production of different types of AHLs on the basis of relative retention factor (Fig. 3). Only 26 per cent isolates were positive for the production of oxo-C6-HSL, 90 per cent isolates for C8-HSL, 58 per cent isolates for C10-HSL and 54 per cent isolates for C12-HSL.

Two to six different AHLs were detected in most of the test isolates by employing two biosensor strains in TLC. Presence of at least four AHLs like C12-HSL, C6-HSL, oxo-C6-HSL and C4-HSL was also detected earlier by employing TLC using *C. violaceum* CV026 and *A. tumifaciens* A136 as reporter strains in microbial keratitis isolates[27]. Erickson *et al*[14] showed the production of different AHLs like C8-HSL, C10-HSL and C12-HSL in sputum samples of cystic fibrosis patients indicating their production during lung infections. Recently, we have also shown the production of QS signal molecules qualitatively in renal homogenates of mouse model of UTI indicating their role in pathogenesis of UTIs[16].

Results of quantitation of β-galactosidase activity showed production of variable levels of AHLs by *P. aeruginosa* uroisolates (Fig. 4) indicating that *P. aeruginosa* isolated from the same source also produces different amounts of AHLs. These findings point towards strain level differences in AHL production. Involvement of different phenotypes in infections among numerous strains of *P. aeruginosa* has also been indicated previously[32]. Various phenotypes of isolates from same source have been reported to produce different levels of AHLs in microbial keratitis. Invasive isolates from keratitis patients produced high levels of AHLs whereas cytotoxic isolates produced low levels of AHLs[27].

In conclusion, results of the present study indicated that use of more than one biosensor strain and assay methods was useful in determining the profiles of AHLs. All the methods proved to be useful for the detection of AHLs directly from pure cultures. Further, production of AHL was one of the important properties possessed by uroisolates of *P. aeruginosa* and hence indicating a definitive association of QS with urinary tract infection. This approach can further be exploited for detection of QS signal molecules directly in the urine samples of patients indicating presence of pathogen.

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