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RESEARCH ARTICLE

PREVALENCE OF AI-1 TYPE OF QUORUM SENSING MOLECULES IN *KLEBSIELLA PNEUMONIAE* CLINICAL ISOLATES.

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Abstract

Klebsiella pneumoniae is one of the most frequently isolated bacterial pathogen from cases of nosocomial and community acquired infections. In the present study, 50 clinical strains (Burn wound and urological isolates) along with two standard strains of *K. pneumoniae* were screened for the presence of recognised quorum sensing system. Although all the isolates were found to be positive for AHL production but the burn wound isolates showed higher AHL production. The clinical isolates showed variable type of signal production therefore bioassay guided expression of quorum sensing signal molecules (AI-1) was employed for qualitative estimation of C12-HSL, C8-HSL, C6-HSL and C4-HSL production by employing *A. tumefaciens* A-136 (pDSK519) *E. coli* MG4 (pKDT17), *E. coli* MG4 (λ1.4), *P. aeruginosa* PAO-JP2 (pECP61.5), *C. violaceum* CV026 as their reporters respectively. 100% of the strains of *K. pneumoniae* were found to produce AHLs, the quorum sensing signal molecule which was subsequently confirmed by HPLC. These results confirm that *K. pneumoniae* is an avid producer of AI-1 quorum sensing signal molecule.

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Introduction:-

Quorum sensing (QS) is a process that allows bacteria to communicate and regulate their population density by using the secreted chemical signaling molecules called autoinducers. It is an important global gene regulatory mechanism that enables individual bacteria to communicate and coordinate their behavior in population density. It arms them with a mechanism for minimizing host defenses by delaying the production of virulence factors until sufficient bacteria have amassed to overwhelm the host defense mechanism (Bjarnsholt *et al.,* 2005).

N-acylhomoserine lactones (AHLs) are arguably the most studied QS signaling molecules. AHLs are usually produced by a protein homologous to LuxI and they in turn bind to LuxR protein. Once the threshold level of an AHL is detected, transduction leads to the induction of genes that control a variety of survival functions, including the production of antimicrobial substances and protection against host's innate defence mechanisms (Yin *et al.,* 2012).

AHLs are composed of a homoserine lactone ring (HSL) with an acyl side chain which varies in length from C4 to C18 (Fuqua *et al.,* 1999 and Marketon *et al.,* 2002). In some AHL molecules, this acyl chain can be modified by a 3-oxo, a 3-hydroxy, or a terminal methyl branch with various degrees of unsaturation (Theil *et al.,* 2009). The acyl

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side chain length and the substitutions on the side chain provide signal specificity (Khajanchi *et al.,* 2011). The specificity of AHL molecule is attributed to the length of the acyl side chain and substitution on it.

K. pneumoniae communicates by using two types of QS signal molecules autoinducer-1(AI-1) and autoinducer-2(AI-2), which link the surrounding cell density by coordinating the appropriate the cell function. AI-2 (4, 5-dihydroxy-2, 3-pentanedione) gets cyclizes by the addition of borate and loss of water and get converted in to final AI-2 signaling molecule (Zhu *et al*., 2012). AI-2 system is luxS dependent QS system that regulates biofilm formation (Balestrino *et al.,* 2005) and lipopolysaccharide synthesis (Araujo *et al.,* 2010). Only two reports are available in literature which confirms that *K. pneumoniae* isolates produce *N*-octanoylhomoserine lactone (C8-HSL) and *N*-3-dodecanoyl-L-homoserine lactone (C12-HSL). One isolate has been shown to produce *N*-hexanoyl-homoserine lactone (C6-HSL) (Yain *et al*., 2012, 2013). The producer gene related to the production of AI-1 is not known in *K. pneumoniae* till now. *K. pneumoniae* also produces AI-1 type of quorum sensing molecule, which are *N*-acylhomoserine lactones (AHLs) produced by a protein homologous to *luxI* (Yin *et al.,* 2012). Hitherto no study known to evaluate the effect of AI-1 autoinducer molecule on virulence factors or physical attributes of *K. pneumoniae.* So there is lack of information probably because of few studies reported. Hence, the present study was conducted to analyses the prevalence of AHL production in clinical isolates of *K. pneumoniae*.

Materials and methods:-

Bacterial Strains:-

Standard strain of *K. pneumoniae* B5055 was procured from Dr. M. Trautman, Germany, *K. pneumoniae* 43816 from Pennsylvania State University USA and clinical isolates were collected from Department of Medical Microbiology, PGIMER and Government Medical College and Hospital, Sector 32, Chandigarh, India. Study protocol was approved by the Panjab University Ethical Committee. Their ability to produce quorum sensing molecules was screened by using biosensor reporter strains *E. coli* MG4 carrying plasmid pKDT17 (Pearson *et al.,* 1995), *E. coli* MG4 carrying plasmid λ1.4 (Passador *et al.,* 1993) and *A. tumefaciens* A136 carrying plasmid pDSK519 (Fuqua *et al.,* 1996). Biosensor reporter strain *E.coli* MG4 (pKDT17), *E.coli* MG4 (λ1.4) and PAO-JP2 (pECP 61.5) were obtained from Prof. Barbara H. lglewski, USA. *C. violaceum* CV026 and *A. tumefaciens* A136 (pDSK519) from Dr. K. H McClean (Texas Health University, USA).

Cross feeding and well plate assay for AHL Detection:-

T streak assay and well plate assay were employed for the qualitative detection of AHLs by using biosensor reporter strains *E.coli* MG4 (pKDT17), *E.coli* MG4 (λ1.4), PAO-JP2 (pECP 61.5), *C. violaceum* CV026 and *A. tumefaciens* A136 (pDSK519) respectively. Clinical isolates and standard strain of *K. pneumoniae* B5055, *K. pneumoniae* 43186 were screened using cross feeding assay as described by Stickler *et al.,* (1998). The Luria agar plates containing 40 µl of X-Gal (20 mg/ml) were seeded with the reporter strain and 50 µl of ethyl acetate extract was added in the wells bored with a punch. AHLs diffused through the agar and formed blue colour around the wells.

Extraction of AHLs from spent bacterial culture:-

The AHLs were extracted from each bacterial strains by incubating an overnight culture in 100 ml broth media. *K. pneumoniae* strains ware grown under optional environmental growth conditions. Cells were grown to OD_{600} of 2.0 and spent culture supernatant was extracted twice with an equal volume of ethyl acetate. Extracts were pooled, concentrated and dreid in rota evaporator to dryness. The dry content was resuspended in 500µl of HPLC grade ethyl acetate and stored at -20°C.

AHLs Quantification:-

Quorum sensing signal molecules were quantifed in the culture supernatant of all the *K. pneumoniae* strains on the basis of it's 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 Zhu *et al.,* (2002).

Separation and detection of AHLs by analytical thin layer Chromatography (TLC):-

TLC was performed according to the protocol of Shaw *et al.* (1997). A TLC tank was filled with 20ml methanol: water (60:40 ratio) and left for 1hour to enable saturation. The extracted 50µl sample and 5 µl of C8-HSL (10mm) standard (dissolved in Acetonitrile) were chromatographed on TLC plate (RP C18 aluminium silica gel plate, Merck, Germany). C8-HSL purchased from Sigma-Aldrich. After Chromatography, the TLC plate was air dried and overlaid with film of X-gal containing *A. tumifaciens* A136 culture with 0.8% agar. The TLC plate was then

incubated for 48 hours at 28°C. AHL were detected on the basis of blue pigmentation on the TLC plate and the results were digitally recorded.

Analytical high performance liquid chromatography (HPLC) analysis of AHLs:-

Sample preparation for HPLC:-

Detection of natural C8-HSL was performed by taking standard C8-HSL (Sigma) as a reference. 1mg of synthetic C8-HSL was dissolved in 1ml HPLC grade acetonitrile and then filtered through 0.22 µm syringe filter (Pall). Similarly, extracted AHL sample (Polled preparative TLC) was prepared. These samples were finally subjected to HPLC.

Sample analysis by Analytical HPLC:-

For the detection of synthetic as well as natural C8-HSL, samples were applied on analytical HPLC and chromatographed. For this purpose, Waters HPLC, analytical Sunfire C-18 reverse phase column (250X4.6mm, 5µm particle size) with oven was used. Waters Binary HPLC Pump 1525 was used. System was coupled to Waters Autosampler 2707 with Waters Photodiode Array Detector 2998. HPLC system was equilibrated with mobile phase HPLC grade acetonitrile and water prior to sample application. 20 µl of synthetic C8-HSL was applied to analytical HPLC for analysis. HPLC was performed under isocratic solvent system with acetonitrile:water (70:30) at a flow rate of 1ml/min. Sample of extracted natural AHLs dissolved in acetonitrile was also processed under similar set of conditions.

Retention time of synthetic C8-HSL and extracted AHLs in HPLC chromatogram was compared and analysed. Natural C8-HSL was also confirmed by comparing its λmax with synthetic C8-HSL via HPLC Waters Photodiode Array Detector with scanning wavelength range of 190-800 nm.

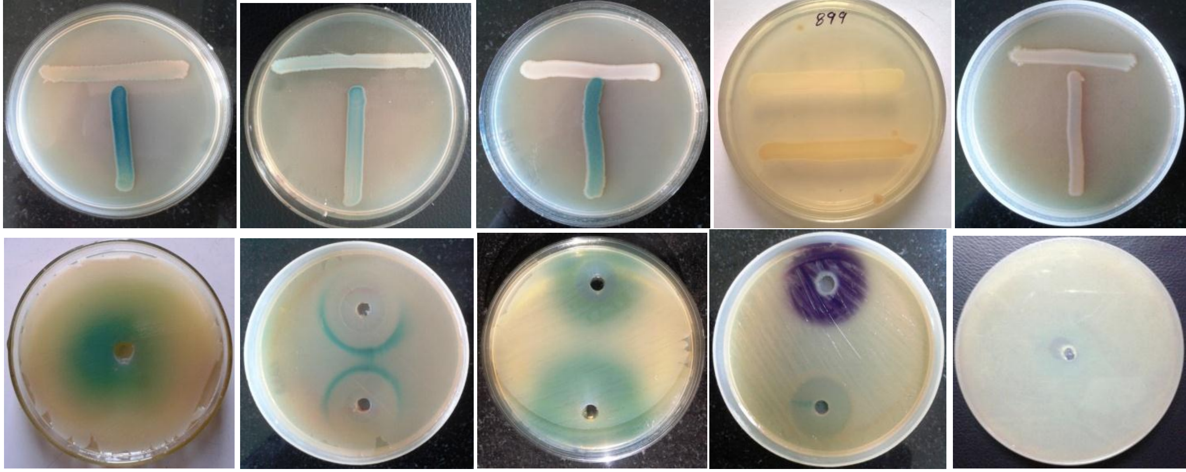


Figure 1:- Plates showing qualitative screening of QSSM by different *K. pneumoniae* clinical strains using *A. tumefaciens* A136 , *E. coli* MG4, *E. coli* λ1.4, *C. violaceum* CV026 and *Pseudomonas* JP2 reporter strains.

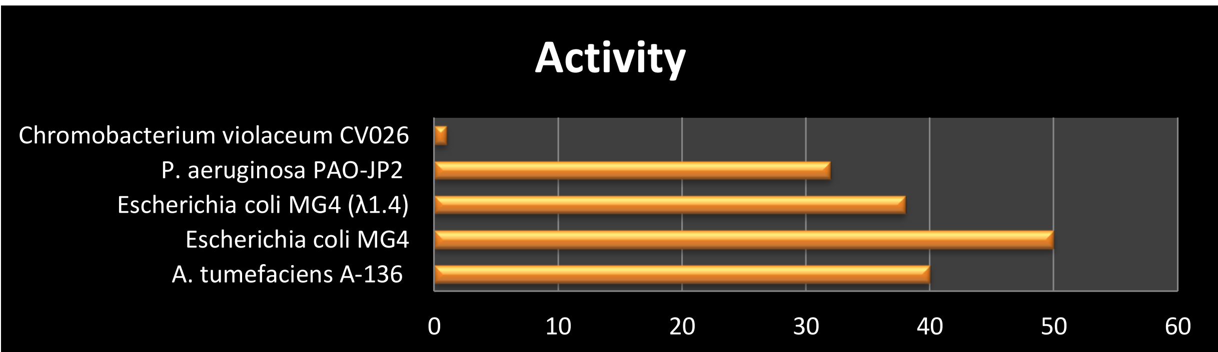


Figure 2:- Showing positivity of *K. pneumoniae* clinical isolates screened for quorum sensing signal molecule production using different bioreporter strains.

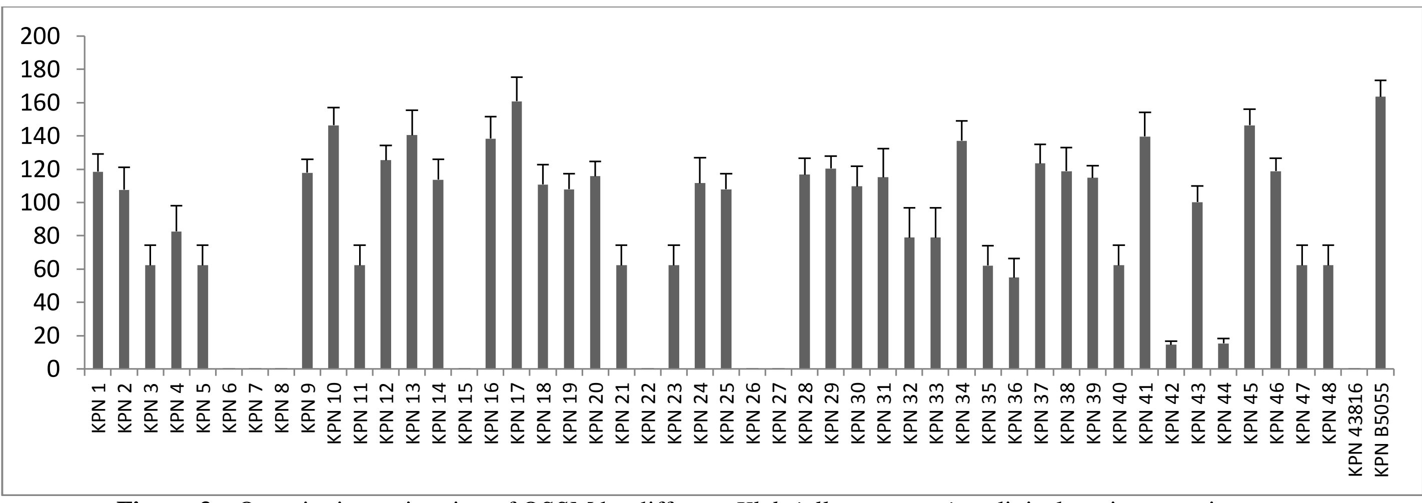


Figure 3:- Quantitative estimation of QSSM by different *Klebsiella pneumoniae* clinical strain was using *A.tumefaciens* A136 reporter strain.

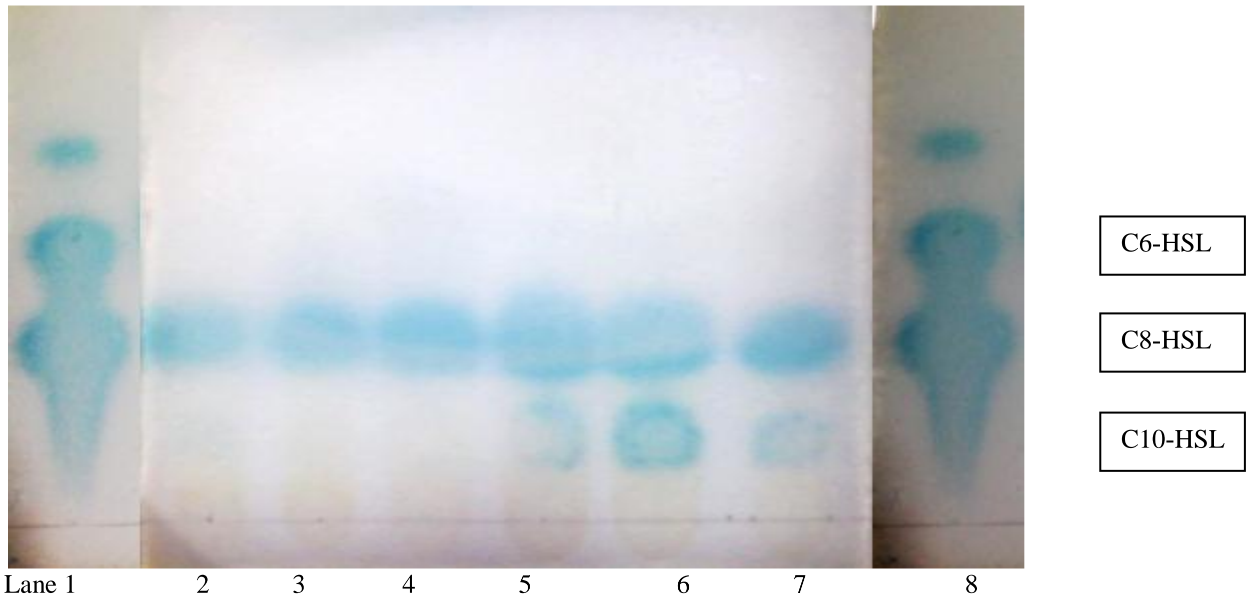
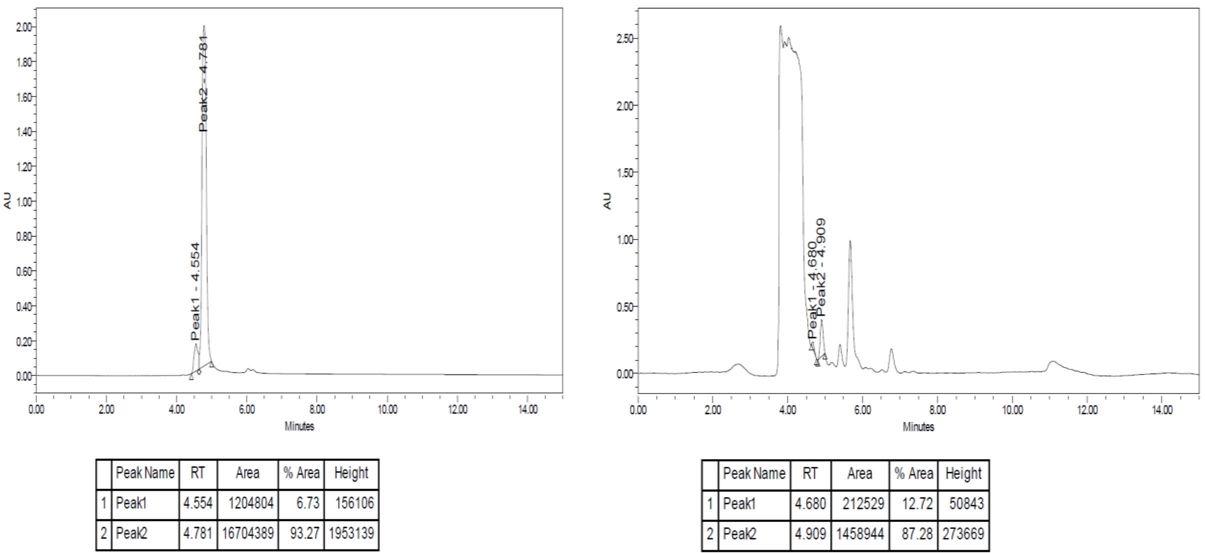


Figure 4:- TLC profile of QSSM by different *K. pneumoniae* clinical strain was using *A. tumefaciens* A136 reporter strain.



	Peak Name	RT	Area	% Area	Height
1	Peak1	4.554	1204804	6.73	156106
2	Peak2	4.781	16704389	93.27	1953139

	Peak Name	RT	Area	% Area	Height
1	Peak1	4.680	212529	12.72	50843
2	Peak2	4.909	1458944	87.28	273669

Figure 5:- HPLC chromatogram of synthetic C8-HSL and Extracted AHL of *K. pneumoniae.*

Results and Discussion:-

Identification of AHLs is becoming important in clinical settings since QS cascades are now being recognized as futuristic drug targets to combat *K. pneumoniae* infections. In the present study, six biosensor strains were employed for the screening, identification and quantitation of AHLs in different *K. pneumoniae* isolates. The epidemiological study revealed that the burn wound isolates show better AHL production. The clinical isolates show variable type of signal production therefore bioassay guided expression of quorum sensing signal molecules (AI-1) was determined qualitatively for C12-HSL, C10-HSL, C8-HSL, and C6-HSL production by employing *A. tumefaciens A-136* (pDSK519) *E. coli* MG4 (pKDT17), *E. coli* MG4 (λ1.4), *P. aeruginosa* PAO-JP2 (pECP61.5), *C. violaceum* CV026 as their reporters respectively. *E. coli* MG4, used in cross-feeding assay can detect wide range of exogenous AHLs (C8 to C14-HSLs) but cannot detect short chain and 3-hydoxy AHLs (Cha *et al.,* 1998). 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 isolates to be producers of QS signal molecules based on the development of blue colouration in the reporter strain (Fig. 1). All isolates of *K. pneumoniae* were found to be positive for AHLs using *A. tumifaciens*A-136*, E. coli MG4, and E. coli* λ1.4 reporter strain by cross feeding assay.

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 C4-HSL was not produced by any isolate of *K. pneumoniae* in this study. When *C. violaceum* CV026 was employed for detection of C4-HSL, C6-HSL and C8-HSL it was observed that only one isolate of *K. pneumoniae* was positive. Yai *et al.,* (2013) also showed that the *K. pneumoniae* isolate produce the C6-HSL molecule.

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 isolates 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 *Cvi*I 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 (McClean *et al.,* 1997). Identification of AHLs was done by separating bacterial extracts by TLC (C18RP Silica gel plates, Merck, Germany) and subsequently development with biosensor *C. violaceum* CV026. Synthetic AHL standards C4-HSL, C6-HSL, and C8-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 2% isolates show positive resonance when screened with *C. violaceum* CV026 biosensor. Although, *C. violaceum* CV026 biosensor is reported to detect C4-HSL, C6-HSL and C8-HSL (McClean *et al.,* 1997), but other than C6-HSL no other AHL was detected in the present study. Non-detection may

be due to low sensitivity for C8-HSL which are reported to be detected by *C. violaceum* CV026 when produced at higher concentrations only (McClean *et al.,* 1997).

Since *C. violaceum* CV026 cannot detect AHLs with longer acyl side chains, broad range biosensor, *A. tumifaciens* A-136 was employed thereafter for TLC. *A. tumifaciens* A-136 contains a plasmid with traR promoter and *tra*G::*lac*Z transcriptional fusion. *tra*G::*lac*Z 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 AHL (Oger *et al.,* 2002). All the isolates showed production of different types of AHLs on the basis of relative retention factor (Fig. 3). 100 per cent isolates showed production of C8-HSL, 90 per cent isolates showed C10-HSL and 80 per cent isolates showed C12-HSL production. To check the profile of AHL molecules, high AHL producing and low AHL producing strains extract were selected out. These ethyl acetate extracts of *K. pneumoniae* were applied on the reverse phase TLC plates. Samples were spotted on TLC plates and run with methanol/water (60:40, vol/vol). AHL molecules were detected by overlaying the plate with biosensor strain *A. tumifaciens A-136*. It detected both standard AHL molecules (C8-HSL) (Fig. 4 lane 8) as well as extracted AHLs (Fig.4 lane 2-7) following run on the TLC plate. The *Rf* value of C8-HSL was 0.42 and natural AHL was 0.42.

Three different AHLs were detected in most of the test isolates by employing two biosensor strains in TLC. Presence of at least three AHLs like C12-HSL, C10-HSL and C8-HSL was also detected earlier by employing TLC using *C. violaceum* CV026 and *A. tumifaciens* A-136 as reporter strains in microbial isolate Yai *et al.,* (2012) showed production of different AHLs like C8-HSL, C12-HSL isolation of *K. pneumoniae* from the posterior dorsal surface of the human tongue and human intestines.

In the present study, the presence of C8-HSL molecules was also confirmed by performing HPLC. Extract AHL sample was analyzed along with the synthetic C8-HSL molecules. In analytical HPLC chromatogram, a single, sharp peak of synthetic C8-HSL was detected with a retention time (*Rt*) of 4.781 min and peak area 93.27%. The λmax for synthetic C8-HSL was recorded to be 196 nm in λ scan from 190-800 nm via Waters PDA detector 2998 (Figure 5).

Results of quantification of β-galactosidase activity showed production of variable levels of AHLs by *K. pneumoniae* isolates (Fig. 4) indicating that *K. pneumoniae* isolated from the same source also produces different amounts of AHLs. These findings point towards strain level differences in AHL production.

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 in *K. pneumoniae*. 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 isolates of *K. pneumoniae* and hence indicating a definitive association of QS with different infections caused by *K. pneumoniae*. Show the relevance of future therapies against *K. pneumoniae* by targeting C8-HSL molecules.

50 clinical strains along with standered strains of *K. pneumoniae* were used to screen for the production of AI-1 type of quorum sensing molecules, the production of short as well long side chain type AI-1 molecules. All the isolates were found to be positive for AHL production. All the positive strains of *K. pneumoniae* were found to be the producer of C8-HSL, the quorum sensing signal molecule which was subsequently confirmed by HPLC. These results confirm that AI-1 quorum sensing signal molecules are produced by *K. pneumoniae*.

Reference:-

1. Arrand S. K., Qin Y., Oger P., (2002) Quorum-sensing system of *Agrobacterium* plasmids: analysis and utility. *Methods Enzymol* ; *358* : 452-84.
2. Balestrino D., Haagensen J. A., Rich C., Forestier C. (2005) Characterization of type 2 quorum sensing in *Klebsiella pneumoniae* and relationship with biofilm formation. *J. Bacteriol*. 187 : 2870-80.
3. Bjarnsholt T., Jensen P. O., Burmolle M., Hentzer M., Haagensen J., Hougen H., Calum H., Madsen K., Moser C., Molin S., Hoiby N. and Givskov M., (2005). *Pseudomonas aeruginos* tolerance to tobramycin, hydrogen peroxide and polymorphonuclear leukocytes is quorum sensing dependent. *Microbiology* 151 : 373–83.

4. Cha C., Gao P., Chen Y. C., Shaw P. D., Farrand S. K., (1998) Production of acyl-homoserine lactone quorum-sensing signals by gram-negative plant associated bacteria. *Mol Plant Microbe Interact 11* : 1119-29
5. De Araujo C., Balestrino D., Roth, L., Charbonnel N., Forestier C., (2010) Quorum sensing affects biofilm formation through lipopolysaccharde synthesis in *Klebsiella pneumoniae*. *Res. Microbiol.* 161 : 595–603.
6. Fuqua, C., and Eberhard, A., (1999). In: Cell-Cell Communication in Bacteria. *American Microbiology Association Press*; 1999.
7. Khajanchi B. K., Kirtley M. L., Brackman, S. M. and Chopra, A. K. (2011) Immunomodulatory and Protective Roles of Quorum-Sensing Signaling Molecules N-Acyl Homoserine Lactones during Infection of Mice with *Aeromonas hydrophila. Infection and Immunity* 79 : 2646–2657.
8. Marketon M. M., Gronquist M. R., Eberhard A. and Gonzalez J. E., (2002) Characterization of the *Sinorhizobium meliloti sinR/sinI* Locus and the Production of Novel *N*-Acyl Homoserine Lactones. *Journal of Bacteriology* 184 : 5686-95.
9. McClean K. H., Winson M. K., Fish L., Taylor A., Chhabra S. R., Kamara M (1997) Quorum sensing and *Chromobacterium violaceum*: exploitation of violacein production and inhibition for the detection of N-acylhomoserine lactones. *Microbiology 143* : 3703-11.
10. Passador L., Cook, J. M., Gambello M. J., Rust, L. and Iglewski B. H. (1993). Expression of *Pseudomonas aeruginosa* virulence genes requires cell-to-cell communication. *Science*, *260* : 1127-30.
11. Pearson J. P., Passador L., Iglewski B. H., and Greenberg, E. P. (1995) A second N-acylhomoserine lactone signal produced by *Pseudomonas aeruginosa. Proc Natl Acad Sci U S A.* 28: 92 (5) :1490-4.
12. Stickler D. J., Morris, N. S., Mclean R, J., Fuqua C. (1998) Biofilms on indwelling urethral catheters produce ruorum-sensing signal molecules in situ and *in vitro. Appli Environ Microbiol* 64 : 3486-71.
13. Thiel V., Kunze B., Verma P., Wagner-Dobler I. and Schulz, S. (2009). New structural variants of homoserine lactones in bacteria. *A European Journal of Chemical Biology* 10 : 1861-68.
14. Yin F.N., Cheng H. J., Chan J. W., Yin W.F., Chan K.G. (2013) Short Chain N-Acylhomoserine Lactone Production by Clinical Multidrug Resistant *Klebsiella pneumoniae* Strain CSG20. *Sensors* 13 : 15242–251.
15. Yin W.F., Purmal K., Chan S., Chan X.Y., Chan, K.G. (2012) Long chain N-acyl homoserine lactone production by enterobacter sp. isolated from human tongue surfaces. *Sensors* 12 : 14307–314.
16. Zhu H. et al., (2002) Determination of quorum-sensing signal molecules and virulence factors of *Pseudomonas aeruginosa* isolates from contact lens-induced microbial keratitis. *J Med Microbiol* 51 : 1063-70.
17. Zhu H., Liu H. J., Ning S. J. and Gao Y. L. (2012) The Response of Type 2 Quorum Sensing in *Klebsiella pneumoniae* to a Fluctuating Culture Environment. *DNA and Cell Biology* 31 : 455-59.