Molecular identification of Quorum sensing and Quorum quenching bacteria isolated from soil and hospital setup

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Abstract

N-Acyl-homoserine lactones, the Quorum sensing signaling molecules predominantly found in gram-negative bacteria, which regulate several bacterial genes including virulence and antibiotic resistant genes. The study was aimed to identify and characterize QS and QQ bacteria from different samples. 5 samples with different ecological background were collected from soil and 10 samples from hospital setup. 31 different bacteria were isolated with either QS or QQ activities all together. CV026 and A136 biosensor strains were used for the detection of QS and QQ positive strains. QS activity was observed by cross streaking test of bacteria against CV026, it was affirmed that 13 isolates from the soil and 5 from hospital equipment's showed positive QS activity. QQ activity of each isolate was tested by well diffusion assay, C6-HSL and C12-HSL were our candidate AHL molecules. The AHL molecule degradation was detected in 4 isolates of soil and none from the samples obtained from hospital setup. The total of 6 strongly positive QS and QQ isolates were identified and selected for 16S rRNA gene sequencing. The phylogenetic analysis revealed that these isolates were closely related to *Pseudomonas*, *Bacillus* and *Exiguobacterium* genera. In contrast, 1 Gram positive bacterial isolate was also purified with QS potential.

Key words: Quorum sensing; Quorum quenching; AHL molecules; 16S rRNA.

INTRODUCTION

Quorum sensing (QS) signaling molecules control many social and physiological activities in bacterial cells such as development of biofilm, proteinaceous or peptidic toxins produced by bacteria which are called bacteriocins, expression of virulence genes in pathogenic bacteria, spore formation and even regulating gene expression [1; 2]. In bacterial cells communication different

types of signaling molecules have been studied, most of which regulate bacterial pathogenicity such as *N*-acyl-homoserine lactone (AHL) and methyl dodecenoic acid (DSF), hydroxyl palmitic acid methyl ester (PAME), Autoinducing peptide (AIP) and a furanosyl borate diester or tetrahydroxy furan are examples of Autoinducer-2 (AI-2). Depending on specie these are some signaling molecules used in QS [3]. These signaling molecules, known as autoinducers, are produced in response to environmental changes or at a particular phase of growth, which induces an intensive response when these molecules reach to a critical concentration [4]. It is believed that these signaling molecules when reach to a sufficient level can induce gene expression either directly or indirectly especially, when the number of bacterial cells re-aches to a threshold concentration [5; 6].

Perhaps, most investigated QS signals are N-acylhomoserine lactones (AHL) [7]. The structure of AHL signal consists of two parts, homoserine lactone ring (HSL) which is conserved part and an acyl chain. The length of acyl side chain is different in different AHL molecule varies from four to eighteen carbon chain and different in structure by the degree of saturation, and at the C3 position the occurrence of a hydroxy-, oxo- or some time no substituent [8]. AHL acyl homoserine lactone signals are considered as QS signals in gram-negative bacteria [9; 10].

QS mechanism and QS signals are different in gram-positive and gram-negative bacteria [9; 10]. In bacteria three classes of QS systems are present and for each class different signaling molecules have been recognized and responded by a precise controlling system and detecting apparatus [11-13]. QS system is used or adopted by many bacteria to achieve maximal competition advantage in a community of bacterial population, that is why, some other bacteria use QQ mechanism to counter measure the benefits of QS mechanism [14]. This QQ mechanism is widely distributed both in prokaryotes and eukaryotes which plays a vital role in host-pathogen and microbe-microbe interactions [15]. The mechanism is known for just to disturb or interfere with the QS process or to disturb the expression of certain functions, but it does not kill or inhibit the growth of bacteria [16].

In current viewpoint of increasing antibiotic resistance in bacterial species as compared to increased antibiotic discovery, QQ is significant approach which develops a selective pressure on pathogenic bacteria and a supportable bio-control procedure. QQ procedures targets AHL based QS itself to control AHL based QS function in pathogenic bacterial strains [17].

In soil samples the phenomenon of QS and QQ in bacterial population has been investigated and documented extensively, but from hospital environment/setup research has not been conducted on QS and QQ bacteria. The study was aimed to target bacterial isolates with QS and QQ potential purified from soil and samples obtained from hospital setup. The wealth of information obtained in the research will further help to control various animals and plant diseases based on AHL degrading bacteria.

Material and methods

Sampling and isolation of bacteria

For isolation of bacterial strains, a total 5 soil samples were collected at a depth of 10cm below the soil surface from different areas of district Mansehra including Dhodial, Baffa, and Shinkiari. Different ecological backgrounds were targeted for sampling such as farming soil, infertile land, mud and forestry soil. Samples were collected in falcon tubes from the targets and were immediately transported to the laboratory. A soil suspension of each sample was prepared by mixing the soil sample (1g) with PBS buffer by vigorous vortex. The soil suspension was serially diluted and spread onto Luria Bertani and nutrient agar. Different bacterial isolates with different color and morphology were isolated after incubation for 24h at 28°C. Pure colonies were obtained with three time streaking on LB agar and nutrient agar [18].

Eight samples were collected from the setup of Ayub medical complex Abbottabad using sterile cotton swabs. The cotton swabs were rubbed on surfaces like table, benches, surgical equipment, washroom's basin and hospital floor and were transferred in nutrient broth to laboratory for further process. Cotton swabs were streaked on nutrient and LB agar media. Different bacterial colonies were appeared after 24 hours of incubation at 37°C. Specific colonies with characteristic morphology and color were selected and streaked on appropriate culture medium. For obtaining bacterial pure colonies, the cultivation by streaking was performed repeatedly i.e. three times and preserved at -20°C for short time and at -80°C for prolong preservation.

Biological material

Chromobacterium violaceum (CV026) and A136 were used as bioreporter strains for the detection of QS and QQ bacteria identified in this study. *Bacillus cereus* was used as a known AHL-degrading strain [19]. *Chromobacterium violaceum* is commonly used to detect short chain AHLs molecules, whereas, *Agrobacterium tumefaciens* A136 can be used to detect long chain AHL

molecule. For cultivation of reporter strain LB agar media was used (Luria Bertani) (0.5% yeast extract, 0.5% NaCl and 1% tryptone) with addition of suitable concentration of antibiotics (spectinomycin 50ug/ml and tetracycline 4.5ug/ml; Kanamycin 20µg/ml). AT medium was used for growth of A136 at an optimum temperature of 28°C for 24 hours of incubation period. Bioreporter strain *A. tumefaciens* A136 have (pCF218) (pCF372) plasmid and in presence of this plasmid, A136 produce indigo color in the presence of X-Gal (5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside) in response to *N*-acyl chain length from six to 12 carbons of QS signal molecule AHLs.

Characterization and identification of bacteria

The isolated bacterial strains were identified through their morphology, colony color, texture, surface elevations, size etc. by following gram-staining [20], motility test, Catalase and oxidase tests [21]. Ability of the bacteria to produce acids from fermentation of glucose is detected by methyl red test [21]. Voges-Proskauer (VP) test was also performed [21]. Hydrolysis of starch was determined on peptone-beef extract agar. Urease and Nitrate reduction test was evaluated as described by [22].

Detection of QS and QQ bacteria for AHL production

In case of QS, AHL production from hospital and soil isolates were observed on LB agar media by using CV026 biosensor strains as described by the previous study [23]. While in case of QQ, the synthetic AHLs used in this assay include C6-HSL (N-hexanoyl-L-homoserine lactone) and C12-HSL (N-decanoyl-L-homoserine lactone) which were substrates for AHL degrading enzymes [24]. AHL biosensors, CV026 (*Chromobacterium violaceum*) and A136 were used for the screening of AHL-degrading bacteria. Cultures were grown on LB medium and were incubated for 15h at 30°C. AHL was dissolved with dimethyl sulfoxide (DMSO) at a specific concentration. The potential QQ activity of the isolates was tested by well diffusion agar-plate assays [25]. Homoserine lactone rings in AHL molecule, hydrolyzed by AHL-lactonase can be re-circularized in acidic solutions at lower than pH 2 [26]. The degraded C6-HSL was treated with 0.1 M HCl and re-circularized C6-HSL was detected by the CV026 reporter strain.

16S rRNA gene sequencing and phylogenetic analysis

The 16S rRNA gene amplification of each isolate was performed by colony PCR using set of bacterial universal primers 27F (5́AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'ACCTTGTTACGACTT-3') [27]. After confirmation of exact band size for different isolates,

the amplified fragments were sent for nucleotide sequencing. The retrieved nucleotide sequences were confirmed by running on National center for biotechnological information (NCBI) and EzTaxon-e server databases, it also helps in the phylogenetic analysis and relevant data retrieval from these databases and from other published articles. The phylogenetic neighbor or top hits similarity bacterial strains nucleotide sequences were copied from different data bases like NCBI and EzTaxon-e server (http:// eztaxon-e.ezbiocloud.net/) [28] and aligned by method as directed by the previous study [29]. 16S rRNA gene was also analyzed in Green-gene, Arb-Silva and RDP-II databases. Phylogenetic tree was constructed using Neighbor-Joining Methods with Kimura two-state parameter model analyses [30] by MEGA software version 5 [31]. In each case, bootstrap values were calculated based on 1000 replicates [32]. *Kurthia sensgalensis* JC8E, (**Fig. 2**) *Bacillus halmapalus* DSM 8723 (**Fig. 3**) and *Domibacillus mangrove* SAOS44 (Fig 4) were used as out group in phylogenetic study. 16S rRNA gene sequences were submitted to NCBI GenBank database and accession numbers are mentioned in **(Table 1)**.

Results

Bacterial strain isolation and identification

A total of 31 bacterial isolates were purified from soil and hospital environment on LB and nutrient agar media and then preserved. Out of 31, six bacterial isolates were further identified and confirmed by 16S rRNA gene amplification, sequencing, blasting in NCBI, Eziocloud server and phylogenetic analysis using MEGA software. Sam 1 and Sam 2 were isolated from hospital environment and shared closeness to members of genus *Pseudomonas*. Sam 3 was isolated from soil and it was identified as a member of genus *Bacillus*. The three other bacterial isolates collected from soil including Sam 4 and Sam 5 were classified as members of genus *Pseudomonas* and Sam 19 was confirmed to be related to genus *Exigoubacterium* due to their close positions on the constructed phylogenetic tree and similarity level found through different databases against the each of the candidate as described in (**Tab. 1, Fig. 3 & 5**).

Table 1: Sample isolates, closest species and their accession number

Sample No/Isolates	Source	Closest species	Similarity (%)	Accession No.
Sam 1	Hospital	*Pseudomonas brenneri*	99.25	MK942504.1
Sam 2	Hospital	*Pseudomonas geniculata*	99.77	MK942505.1
Sam 3	Soil	*Bacillus pseudomucoindes*	98.22	MK942506.1

Sam 4	Soil	*Pseudomonas geniculata*	99.24	MK942507.1
Sam 5	Soil	*Pseudomonas brenneri*	98.59	MK942508.1
Sam19	Soil	*Exiguobacterium epidermidis*	98.03	MN795763.1

Characterization and identification of bacteria

The identification and characterization of different soil and hospital equipment's bacterial isolates were confirmed by 16S rRNA gene sequencing, different morphological and biochemical tests. During the study (**Tab.1**) it was confirmed that total of 31 bacterial isolates were detected from soil and hospital environments' in which 6 candidate bacterial isolates having QQ and QS activity were identified by 16S rRNA gene sequences. Sam1 and Sam 2 were gram-negative, non-motile, catalase and oxidase positive Methyl red (MR), VP and indole negative. Sam 3 was gram stain positive, motile, catalase positive and negative for oxidase MR, VP and indole test. Sam 5, 4 and 19 were isolated from soil samples. Sam 5 and 4 were gram stain negative and 19 was gram stain positive. All these four isolates were motile and catalase positive. All the four isolates were positive for indole test. All these were oxidase positive except Sam19 which was oxidase negative. Sam 5 and 4 were negative for MR and VP test and 19 was positive for MR and VP tests. Sam 1, Sam 2, Sam 3 and Sam 4 were positive for Starch hydrolysis. Sam 5 and Sam 19 were negative for starch hydrolysis (**Tab. 2**).

Table 2: Morphological and biochemical characterization of bacteria

Characteristics	**Sam 1**	**Sam 2**	**Sam 3**	**Sam 4**	**Sam 5**	**Sam 19**
Sample type	H	H	S	S	S	S
Shape	Rod	Rod	Rod	Rod	Rod	Rod
Colony colour	white	Pale yellow	yellow	Whit cream	Light yellow	orange
Motility	-	-	+	+	+	+
Gram staining	-	-	+	-	-	+
Catalase	+	+	+	+	+	+
AHL production	+	+	-	-	+	+
C6-HSL hydrolysis	-	-	+	+	-	-
C12-HSL	-	-	+	+	-	-
Oxidase	+	+	+	+	+	-
MR (Methyl red)	-	-	+	-	-	+

VP (Voges Proskauer)	-	-	+	-	-	+
Indole	-	-	-	-	-	-
Lipase	-	+	+	-	+	-
Gelatinase	+	+	-	-	+	-
Starch hydrolysis	+	+	+	+	-	-
Agar	-	-	+	-	-	+

Screening for AHL production

Sam1 and Sam 2 were obtained from hospital environments and had the ability to produce QS signals AHL molecules. Sam 5, 3 and 19 were isolated from soil samples and were able to produce AHL signals. After 48hour incubation at 30°C, the purple pigmentation appeared in some isolates indicated the presence of short chain AHLs **(Fig. 1).**

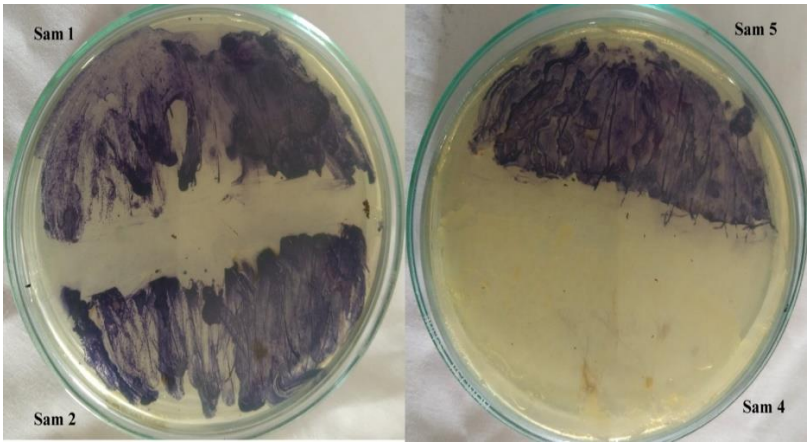

Figure 1: Screening of different soil and hospitals bacterial isolates by cross streaking method against *C. violaceum* CV026. Sam 1, Sam 2 and Sam 5 QS positive isolates triggered CV026 violacein production suggesting production of short chain AHL after 24 hours incubation. No color indicates negative QS isolates as Sam 4.

Screening for AHL degradation

Sam 4 was isolated from soil and had the ability to degrade AHL molecules. The C6- and C12-HSL degradative activities of the whole culture and supernatant was determined.

A136 was used as a reporter strain to detect the remaining residual AHL (**Fig. 2A**) **and CV**026 was used as reporter strain in the LB plate to notice the remaining AHL (**Fig. 2B**). In well number 3, AHL along with *Bacillus cereus* was used as a positive control, *Bacillus cereus* has the ability to degrade AHL molecule, so no purple and blue color indicated AHL absence. Well C was negative control, containing AHL molecule with LB media, so production of purple and blue color indicates the presence of AHL on culture plate. The previously boiled intracellular and

extracellular proteins obtained by sonication and through LB media respectively, were added in well 1 & 2 with AHL molecules which developed purple/blue color indicated absence of QQ enzyme as no degradation of AHL molecules was noticed. On the other hand, well 4 and 5 with extracellular/intracellular proteins and AHL molecule where AHL was degraded by enzymes produced by Sam 4 bacterial isolates resulted to no color production (Purple/blue) around the wells. The degraded C6-HSL was re-circularized by HCL so lactonase test was positive.

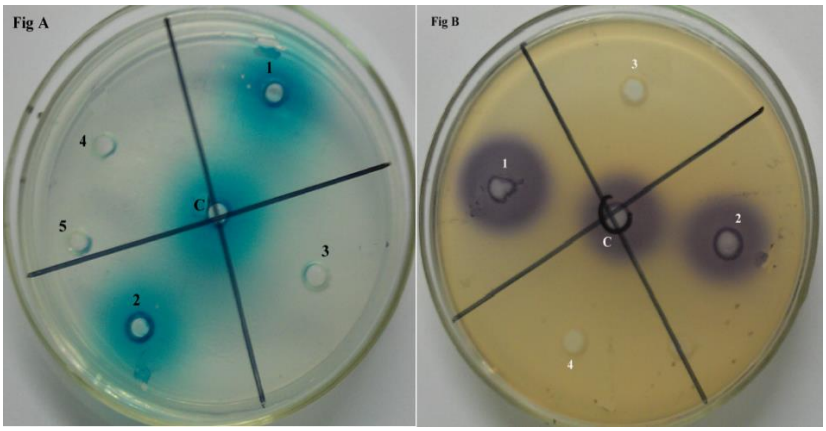

Figure 2: Detection of activity in intracellular and extracellular enzymes by Sam 4.

16S rRNA gene sequencing and construction of phylogenetic trees

16S rRNA gene sequences retrieved after sequencing indicated that Sam1 which was isolated from hospital environment showed 99.5% resemblance to *Pseudomonas brenneri* CFML 97-391^T. The 16S rRNA gene sequences of Sam 2, 4 and 5 were isolated from soil samples have shown 99.77% and 99.24% sequences similarity to *Pseudomonas geniculata* ATCC 19473^T and Sam 5 showed 98.59% sequences similarity to *Pseudomonas brenneri* CFML 97-391^T. Whereas, the retrieved nucleotide sequences of Sam 3 isolated from soil showed 98.22% sequence similarity to *Bacillus pseudomucoindes* DSM 12442^T and Sam 19 have shown 98.03% 16S rRNA sequence similarity to *Exiguobacterium enclenses* NIO-1109^T.

The blast result of Sam 1 showed 99.25% sequence similarity to *Pseudomonas brenneri* CFML 97-391^T with second top-hit sequence similarity to *Pseudomonas proteolytica* CMS 64^T i.e. 98.93%. In Neighbor-joining phylogenetic tree isolate Sam 1 resides in cluster of different species of genus *Pseudomonas* and makes a distinct branch between *Pseudomonas brenneri* CFML 97-391^T and *Pseudomonas proteolytica* CMS 64^T (**Fig. 3**).

By blasting 16S rRNA gene sequence of Sam 5 in NCBI and Ezbiocloud server it showed 99.77% sequence similarity to *Pseudomonas brenneri* CFML 97-391^{T} *and* Neighbor-joining phylogenetic tree Sam 5 made a sister branch with *Pseudomonas brenneri* with high bootstrap value.

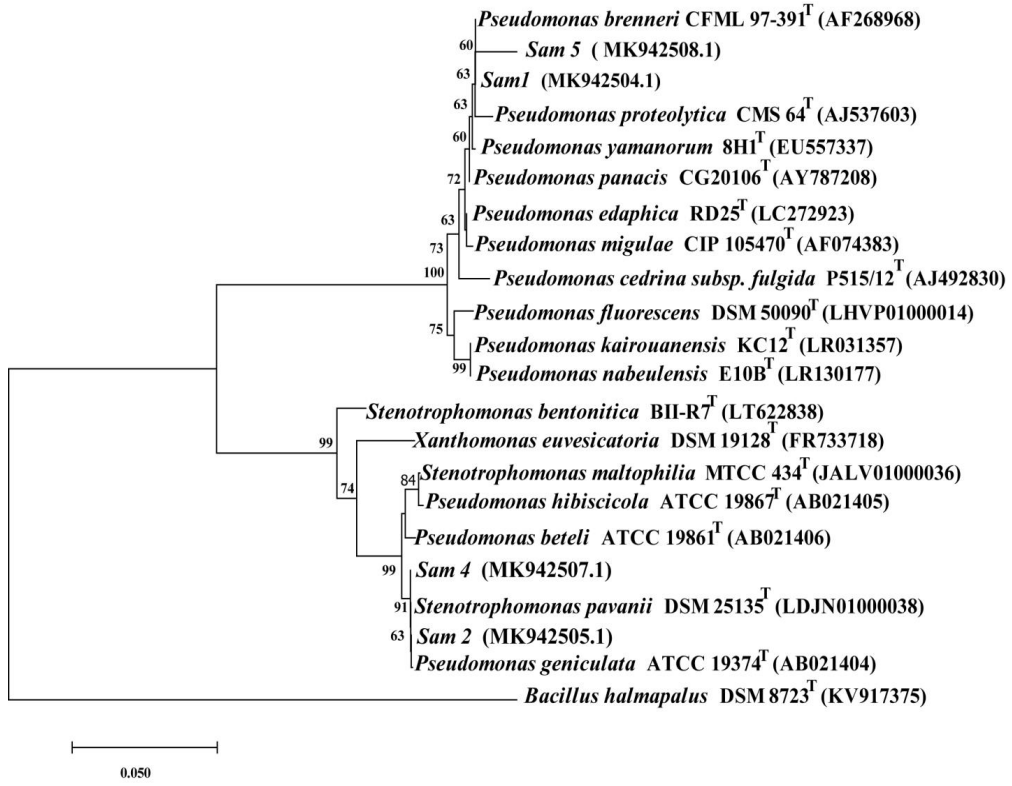

Figure 3: Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences showing the phylogenetic positions of isolate Sam 1, Sam 4, Sam 5 and Sam 2 and representative specie of genus *Pseudomonas* of the family *Pseudomonadaceae*. Bootstrap values (percentages) above 50 (1000 replicates) are shown at branch points. *Bacillus Halmapalus* DSM 8723 (KV917375) was used as an out-group. Bar, 0.05 substitutions per nucleotide position.

Smilarly most similar bacterial strain according to 16S rRNA gene sequence to Sam 4 is *Pseudomonas geniculata* ATCC 19473^{T}, which showed 99.24% of sequence similarity and after neighbour joining phylogenetic tree construction, the isolate Sam 4 contructed a branch with the *Pseudomonas* specie (**Fig. 3**). The phylogenetic analysis and similarity index obtained by blasting also reveals that, Sam 2 isolate reside between *Pseudomonas geniculate* and *Stenotrophomonas pavanii* and therefore, indicated its close relationship with both specie of *Pseudomonas* (**Fig. 3**).

The Sam 3 displayed close relationship with *B. pseudomycoides* (98.34%) and further validated when similar outcomes were found through phylogenetic tree, where Sam 3 pair with the similar

candidate of Bacillus as compare to the rest of representatives used in the tree. The phylogenetic tree is rooted by an outgroup *Kurthia senegalensis* JC8E^T (CAEW01000048) (**Fig. 4**).

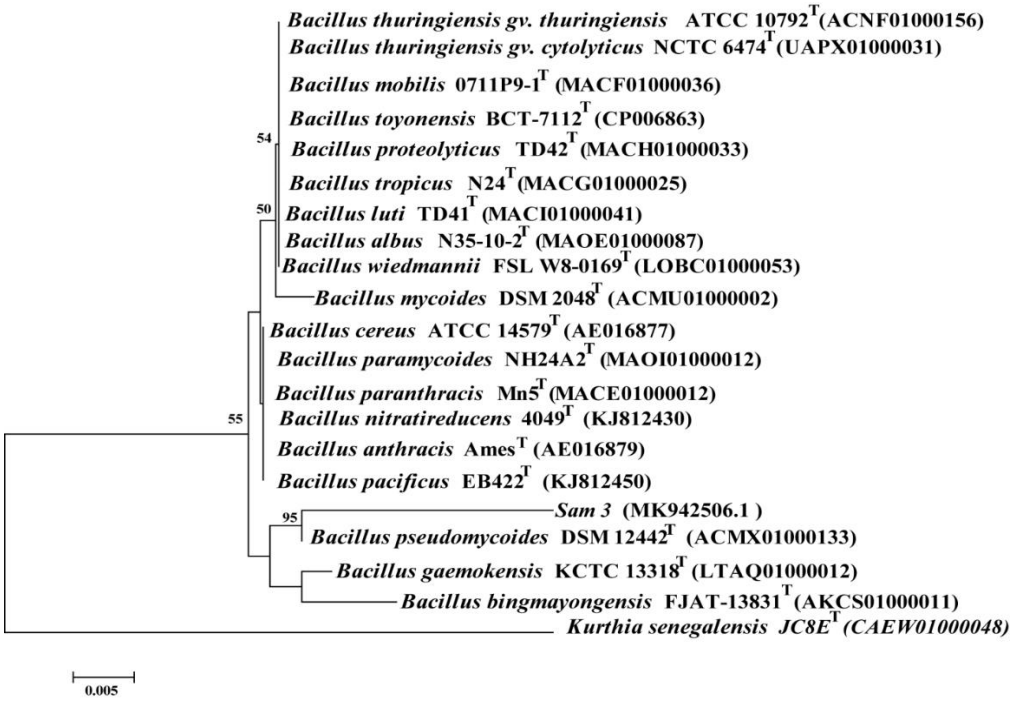

Figure 4: Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences showing the phylogenetic positions of isolate Sam 3 and other representative species of *Bacillus* of the family *Bacillaceae*. Bootstrap values (percentages) above 50 (1000 replicates) are shown at branch points. *Kurthia senegalensis* JC8E^T (CAEW01000048) was used as an out-group. Bar, 0.005 substitutions per nucleotide position.

Sam 19 (MN795763) clustered with the specie of genus *Exiguobacterium* and according to blasting results, it has shown close resemblance to strain *Exiguobacterium enclense, Exiguobacterium acetylicum and Exiguobacterium indicum* with sequence similarity level of 98.03%, 97.84% and 97.62% respectively. In Neighbor joining phylogenetic tree which was constructed with outgroup *Domibacillus mangrove* SAOS 44 the isolate Sam 19 made a cluster with most similar specie of *Exiguobacterium acetylicum* (**Fig. 5**).

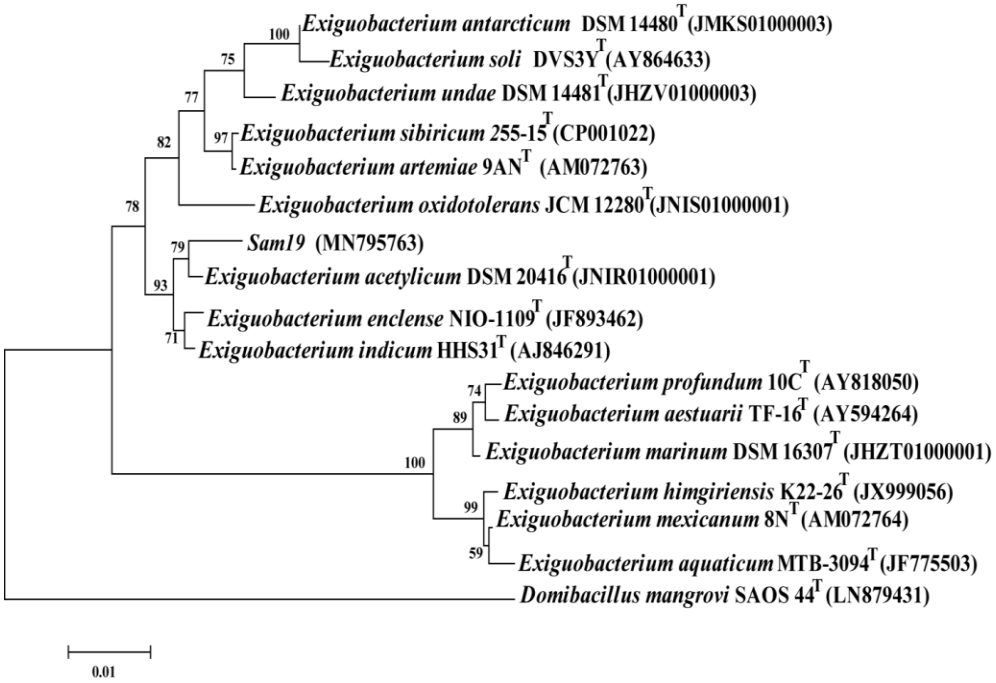

Figure 5: Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences showing the phylogenetic positions of isolate Sam 19 and representative species of genus *Exiguobacterium* of the family *Exiguobacteriaceae*. Bootstrap values (percentages) above 50 (1000 replicates) are shown at branch points. *Domibacillus mangrovi* SAOS44^{T} (LN879431) of genus *Domibacillus* family *Bacillaceae* was used as an out-group. Bar, 0.01 substitutions per nucleotide position.

Discussion

The frequent increase in the development of drug resistance in various microorganisms compels the scientists to find alternative ways of treatment for bacterial infections and to control biofilm formation [33]. QS signaling molecules play a vital role to regulate cell density and various other genes including virulence and antibiotic resistant genes. Some microorganisms especially bacteria have the ability to develop certain enzymes and molecules which can either degrade these QS molecules or inhibit their activity via inhibitor molecules by mechanism called as QQ. Thus, the discovery of both of these mechanisms in numerous bacterial specie represents a new breakthrough in biological research. A broad range of organism has been studied for enzymatic degradation of AHLs including bacteria, archaea, fungi, plants and mammalian [34; 35]. Different genes responsible for AHL degradation have been identified from bacterial cells from marine or terrestrial origin [36]. According to structure of AHL, enzymes which can degrade AHL can be

categorized into three categories, AHL lactonase which can degrade lactone ring in AHL molecule, AHL amidase which can break amide bond and AHL oxidase will usually modified AHLs [37].

The purpose to conduct this research was to explore the soil and hospital environment for QS and QQ bacterial specie. During the study, three species of genus *Pseudomonas* and one species of *Exiguobacterium* were found in QS phenomena. Among the QS positive *Pseudomonas* species two were isolated from hospital and one was isolated from soil. One species of *Pseudomonas* and *Bacillus* isolated from soil were involved in QQ phenomena. It is also presumed that, Sam 3 and Sam 4 bacterial isolates could produce either of the QQ enzyme i.e. AHL lactonase and AHL acylase and could only be validated by sequencing, targeted gene expression or by analyzing depleted substrate by any of these enzymes through advanced chromatographic techniques such as electrospray ionization mass spectrometry (ESI-MS) analyses. It was also detected that *Pseudomonas* specie designated as Sam 4 can release QQ enzyme both intracellular and extracellular.

Pseudomonas is a gram negative, opportunistic pathogen [38]. It is a common cause of hospital-acquired infections, mainly it causes infection in immune-compromised hosts [39]. Moreover, some strains exhibit high rates of resistance to antibiotics and are frequently multidrug resistant [40]. In recent years, it has been discovered that QS (QS) is involved in production and regulation of many virulence factors and the formation of biofilm by *P. aeruginosa* [41]. Similarly, *Pseudomonas aeruginosa* PAO1 gene pvdQ encodes an acyl-homoserine lactone (AHL) acylase which can degrade AHL [42].

In current study, Sam 19 which is a specie of *Exiguobacterium* isolated from soil is gram positive strain which has shown 98% sequence similarity to *Exiguobacterium epidermidis* and exhibit AHL production. First time Acylated homoserine lactone (AHL)-based QS in gram positive bacterium was reported in genus *Exiguobacterium* designated as MPO isolated from marine water in south India (GenBank: JF915892) [24].

Sam 3 which is a *Bacillus* specie isolated from soil has a QQ activity, it can degrade C6-HSL and C12-HSL. The first identified AHL lactonase, belonging to metallo-β-lactamase family was isolated from *Bacillus* sp. 240B1. This is an AHL-inactivating enzyme. It has been investigated in this research that strains from soil have the ability to degrade AHL QS signals which have shown that, soil is common inhabitant of QQ bacteria. These bacteria can disrupt the QS system of

pathogenic soil bacteria of plants and in this way, they can reduce a competitive environment for them in soil environment. Similar results are identified in another research, in which they screened total 500 isolates from soil and 37% bacteria have found QQ activity [43].

The molecular study of these six QS and QQ isolates indicates that AHL degrading bacteria were belonging to *Pseudomonas* and *Bacillus* specie, similar result were reported in another study, in which *Pseudomonas aeruginosa* PAO1 which have the ability of degrading AHL molecule to decrease biofilm formation [44]. It was also revealed in this study that, majorly QS bacteria were found associated with *Pseudomonas* and *Exiguobacterium* species. The similar results were also obtained in a study, where same kind of bacteria and their mechanism of QS ability was described by QS [45]. The present study revealed that *Pseudomonas* is the most important bacteria which have QS and also QQ ability.

Conclusion

It was elucidated that, diverse variety of QS and QQ bacteria are natural inhabitants of various forms of soil. Keeping in view that, the pathogenic bacteria also majorly communicate through QS system especially those responsible in nosocomial infections. The outcome from proposed data is that the soil is the natural habitat of QS and QQ bacteria. While QS is communication strategy of pathogenic bacteria to spread infection in hospital environment. The aim of this study was to detect the communication system in bacteria and to isolate novel type of bacterial species from soil and hospital equipment's with potential increased degradation of AHL molecules. Advance studies need to be performed for further investigation of both QS and QQ mechanism in the indigenous bacterial population including whole genome sequencing and gene expression for maximum results of QQ enzymes, which could be utilized to control pathogenic microorganism, biofilm formation etc.

Acknowledgements

Financial support from the higher education commission of Pakistan is gratefully acknowledged.

Conflict of interest

All the authors report no conflict of interest

5. REFERENCES

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