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Screening of Clinical Isolates to produce Quorum-sensing molecules

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Abstract

Background: Quorum sensing enables bacterial communication and virulence regulation, contributing to antimicrobial resistance in clinical settings. Screening clinical isolates for QS molecules is essential for developing novel anti-virulence therapies. **Objectives:** This study aimed to collect and process clinical samples (blood and pus) from patients with suspected bacterial infections, isolate and identify Gram-negative bacterial strains using morphological and biochemical tests, and screen them for N-acyl homoserine lactone (AHL) quorum-sensing molecules via biosensor-based agar plate bioassays to evaluate their potential role in pathogenesis. **Methods:** Clinical bacterial isolates were obtained from mixed cultures using selective media and streak plating to achieve pure cultures on multiple plates. Five distinct strains were purified and identified via colonial morphology, Gram staining, and biochemical tests. All isolates were screened for N-acyl homoserine lactone (AHL) quorum-sensing (QS) molecules using agar plate bioassays with Chromobacterium violaceum CV026 and Agrobacterium tumefaciens A136 biosensors, monitoring violacein pigmentation in double-layer overlays. **Results:** The isolates were identified as three Pseudomonas spp. (MIC1, MIC2, MIC3), one Klebsiella sp., and one Escherichia coli strain. Only Pseudomonas sp. MIC1 exhibited a strong positive response with CV026, producing brown violacein coloration indicative of short-chain AHLs, with no reaction observed with A136. The remaining four isolates showed no QS activity in either assay. **Conclusion:** This study highlights the selective production of AHL-mediated QS molecules in clinical Pseudomonas isolates, underscoring their potential role in pathogenesis. These findings support the development of quorum-quenching strategies to mitigate virulence and antimicrobial resistance in nosocomial infections, warranting further genomic and multi-pathway investigations.

Keywords:
N-acyl homoserine lactones, Quorum sensing, Clinical bacteria, Bioassay screening, Pseudomonas isolates

1. Introduction

Bacterial cell-to-cell communication via chemical signals is facilitated by quorum sensing (QS). This phenomenon occurs when the cell density within a bacterial population reaches a critical threshold. The Gram-negative bacterium *Vibrio fisheri* was the first organism in which this mechanism was discovered and described nearly fifty years ago [1,2].

It is now well established that both Gram-negative and Gram-positive bacteria share this chemical communication pathway. The signaling molecules responsible for this process are known as autoinducers (AIs), which are produced and secreted into the extracellular environment. Depending on their molecular composition, AI molecules may be actively or passively transported from the cytoplasm to the cell exterior. When the concentration of these signaling molecules exceeds a specific threshold, changes in gene expression and metabolic activity occur throughout the bacterial community.

QS molecules enable communication not only among bacteria of the same species but also between different species and even with higher organisms. They regulate various cellular processes such as energy metabolism,

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enzyme synthesis, bioluminescence, plasmid conjugative transfer, DNA replication, and the production of polysaccharides and antibiotics [3–5].

Several studies have shown that signaling molecules can profoundly influence the expression of genes essential for bacterial survival and adaptation. These genes govern processes such as biofilm formation, toxin and virulence factor production, horizontal gene transfer, and interspecies communication [4,6–8]. The type and characteristics of the bacterial habitat determine the amount of signaling molecules produced and, consequently, the regulation of genes involved in environmental adaptation. Autoinducers are therefore a critical evolutionary tool for modulating gene expression and coordinating population-wide behavior [9].

The classification of bacterial autoinducers is based on their distinct molecular structures, which are used for communication. Differences exist not only among bacterial species but also between Gram-positive and Gram-negative groups [10]. Traditionally, it was believed that Gram-negative bacteria use *N*-acyl-*L*-homoserine lactones (AHLs) as autoinducers, whereas Gram-positive bacteria utilize oligopeptides. However, recent studies have demonstrated that both bacterial types can produce AHL molecules [11–14].

The present study aimed to collect and process clinical samples (blood and pus) from patients with suspected bacterial infections, isolate and identify Gram-negative bacterial strains using morphological and biochemical methods, and screen them for *N*-acyl homoserine lactone (AHL) quorum-sensing molecules using biosensor-based agar plate bioassays to evaluate their potential role in pathogenesis.

2. Materials and Methods

This study was approved by the Advanced Studies and Research Board (ASRB), University of Sindh, Jamshoro. This study was carried out from 5 August 2024 to 20 December 2024.

2.1. Collection of Clinical Samples

A total of fifteen clinical samples (ten blood and five pus) were collected from patients suspected of having bacterial infections at Liaquat University Hospital and other healthcare facilities in Hyderabad, Pakistan. All samples were collected following informed patient consent and strict aseptic procedures to minimize contamination, in accordance with NIH Good Clinical Laboratory Practice (GCLP) guidelines.

Trained clinicians and phlebotomists followed biosafety measures, including the use of gloves, sharps disposal bins, and unbreakable plastic containers to prevent needlestick injuries. Each sample was labeled with patient details (name, age, gender, ID, date/time of collection, collector's initials, clinical presentation, and requested tests) to ensure traceability and reproducibility.

2.2. Collection of Blood Samples

Blood samples (approximately 3 mL) were collected intravenously from the antecubital vein after disinfecting the skin with 70% isopropyl alcohol and applying a tourniquet. A sterile 21-gauge syringe and needle were used. Samples were promptly transferred into aerobic blood culture bottles (e.g., BD BACTEC or equivalent). According to CLSI M47 guidelines, bottles were incubated at 37°C for 24–48 hours or until turbidity was observed.

2.3. Collection of Pus Samples

Pus specimens were collected from wound sites using sterile cotton swabs, targeting both the surface and edges of the exudate. All procedures were performed under biosafety level 2 (BSL-2) conditions following NIH GCLP and WHO biosafety protocols. Samples were placed in Amies transport medium, maintained at 4°C, and transported within 2 hours to the Microbiology Laboratory, University of Sindh. Transport complied with NIH Category B infectious substance protocols (UN 3373).

2.4. Isolation of Bacterial Isolates from Clinical Specimens

Processing was carried out in a laminar flow cabinet under BSL-2 conditions to minimize aerosol risks. Blood cultures showing turbidity were subcultured by streaking 10 µL onto nutrient agar, blood agar, and MacConkey agar plates using sterile loops. Pus swabs were directly streaked onto the same media. Plates were incubated aerobically at 37°C for 18–24 hours. Distinct colonies were subcultured to obtain pure isolates and stored at −80°C in 20% glycerol for later analyses. All procedures adhered to CLSI M100 standards.

2.5. Preparation of Media

All media were prepared aseptically according to manufacturer instructions (e.g., HiMedia or Oxoid) and NIH GCLP quality control (QC) standards. Each batch was inspected for defects, pH verified, and tested for sterility.

- **Nutrient Agar:** 28 g nutrient agar powder was dissolved in 1 L distilled water, heated to dissolve com-

pletely, and autoclaved at 121°C for 15 minutes. After cooling to 45°C, the medium was poured into sterile Petri dishes under laminar flow. Plates were labeled and QC-tested using *E. coli* ATCC 25922.

- **Luria–Bertani (LB) Agar:** Prepared using 10 g/L peptone, 5 g/L yeast extract, 10 g/L NaCl, and 15 g/L agar; pH adjusted to 7.0. Autoclaved and tested for sterility and growth performance. Used primarily for biosensor assays.

Blood agar and MacConkey agar were either prepared in-house or purchased pre-made, with QC documentation including results from positive and negative control strains.

2.6. Identification of Bacterial Isolates

Pure isolates were identified to the genus level using morphological and biochemical tests, focusing on Gram-negative rods with potential quorum-sensing (QS) activity (e.g., *Pseudomonas* spp.), following CLSI M35 and NIH GCLP procedures. All tests were performed in triplicate.

- **Gram Staining:** Conducted according to Cheesbrough (2006) and CLSI methods. Smears were fixed, stained sequentially with crystal violet, iodine, ethanol, and safranin, and examined under oil immersion (100×). Gram-negative rods appeared pink-red.
- **Oxidase Test:** A colony was rubbed on filter paper impregnated with N,N,N ,N -tetramethyl-*p*-phenylenediamine reagent. Development of a deep blue color within 10 seconds indicated a positive result (e.g., *P. aeruginosa*).
- **Catalase Test:** A colony was mixed with 3% hydrogen peroxide; immediate bubbling indicated a positive result.
- **Citrate Utilization Test:** Inoculated onto Simmons' citrate agar and incubated at 37°C for 24–48 hours. A color change from green to blue indicated a positive reaction.
- **Indole Test:** Cultures grown in tryptophan broth were treated with Kovac's reagent; a red ring indicated a positive result (negative for *P. aeruginosa*).
- **Coagulase Test:** Performed for Gram-positive isolates using rabbit plasma. Clot formation within 4 hours indicated positivity.
- **Urease Test:** Inoculated onto urea agar and incubated at 37°C for 24 hours. A pink color indicated a positive result.

ATCC reference strains were used as controls, and all data were recorded following GCLP documentation standards.

2.7. Screening of Clinical Isolates for Quorum-Sensing Molecules

Detection of acyl homoserine lactones (AHLs) was performed using biosensor strains *Chromobacterium violaceum* CV026 (for short-chain AHLs) and *Agrobacterium tumefaciens* A136 (for long-chain AHLs), following established bioassay protocols.

Agar Plate Bioassay: Pure isolates were streaked on LB agar and incubated at 37°C for 24 hours. Biosensor strains were grown overnight in LB broth, mixed with soft LB agar (0.7%) containing X-gal (50 µg/mL for A136) and appropriate antibiotics, and overlaid on the plates. Plates were incubated at 30°C for 24 hours. AHL production was indicated by purple pigmentation (*C. violaceum* CV026) or blue coloration (*A. tumefaciens* A136). Zone diameters were measured and compared with synthetic AHL standards as positive controls. All assays were conducted in triplicate per GCLP QC protocols.

3. Results

3.1. Collection of Samples (Pus and Blood)

A total of fifteen clinical samples, including ten pus and five blood samples, were collected from indoor and outdoor patients in various clinical laboratories and hospitals in Hyderabad. Pus, a whitish-yellow or brown-yellow protein-rich exudate (*liquor puris*), accumulates at the site of infection, while blood serves as a transport medium delivering nutrients and removing waste products. All samples were cultured to isolate both Gram-negative and Gram-positive bacteria.

3.2. Isolation and Identification of Clinical Isolates

Several bacterial strains were isolated using selective and differential media. Colonies were characterized based on morphology, color, elevation, and texture. Mixed cultures were further purified through repeated streaking to obtain distinct colonies. A total of five pure isolates were obtained and characterized through Gram staining and biochemical tests. Based on these results, three isolates were identified as *Pseudomonas* sp. (MIC1, MIC2, MIC3), one as *Klebsiella* sp., and one as *Escherichia coli*. All isolates were Gram-negative rods with variable biochemical properties consistent with their respective genera.

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3.3. Screening of Clinical Isolates for Signaling Molecules

All five isolates were screened for acyl homoserine lactone (AHL) production using biosensor strains *Chromobacterium violaceum* CV026 (short-chain AHLs) and *Agrobacterium tumefaciens* A136 (long-chain AHLs). Among these, only *Pseudomonas* sp. MIC1 showed a positive reaction in the CV026 bioassay, indicated by the formation of purple (violacein) pigmentation. No color development was observed in the A136 bioassay, suggesting the absence of long-chain AHLs. The remaining four isolates were negative for AHL production in both systems.

3.4. Agar Plate Bioassay Observations

In the agar plate bioassay, violacein pigmentation was observed around the inoculated *Pseudomonas* sp. MIC1 colony, confirming the secretion of short-chain AHL molecules detectable by the CV026 biosensor. No pigment production occurred around other isolates, indicating the absence of detectable quorum-sensing activity. These findings demonstrate that among the clinical isolates analyzed, only *Pseudomonas* sp. MIC1 possessed active quorum-sensing capabilities through AHL signaling, highlighting potential strain-specific variability in QS-mediated communication.

4. Discussion

The isolation and identification of bacterial strains from clinical samples represent a critical step in understanding microbial diversity and potential pathogenic mechanisms. In this study, five distinct bacterial isolates were purified from mixed cultures using selective media and streak plating, followed by characterization via Gram staining, colonial morphology, and biochemical tests. These isolates were identified as three *Pseudomonas* spp. (MIC1, MIC2, MIC3), one *Klebsiella* sp., and one *E. coli* strain. This approach aligns with standard microbiological protocols for clinical isolate identification, ensuring purity and reliability for downstream analyses (15). The predominance of *Pseudomonas* spp. in our isolates is consistent with their frequent recovery from clinical settings, particularly in immunocompromised patients, where they contribute to nosocomial infections (16).

Screening for quorum-sensing (QS) signaling molecules, specifically N-acyl homoserine lactones (AHLs), revealed that only *Pseudomonas* sp. MIC1 produced detectable AHLs, as evidenced by a positive reaction with the biosensor *Chromobacterium violaceum* CV026, manifesting as violacein pigmentation in the agar plate bioassay. No response was observed with the *Agrobacterium tumefaciens* A136 biosensor, indicating the production of short-chain AHLs (e.g., C_4–C_8) rather than medium- or long-chain variants (17). The agar overlay method further confirmed this interaction, with color formation observed at the interface between the clinical isolate and biosensor layers. This selective positivity underscores the role of AHL-mediated QS in *Pseudomonas* spp., which is well-documented in regulating virulence factors such as biofilm formation, exopolysaccharide production, and antibiotic resistance in clinical isolates (18–19). Similar AHL production has been reported in multidrug-resistant *Pseudomonas aeruginosa* strains from hospital environments, where QS contributes to persistent infections (20).

In contrast, the remaining isolates—*Pseudomonas* spp. MIC2 and MIC3, *Klebsiella* sp., and *E. coli*—exhibited no detectable AHL production with either biosensor. This lack of response in the other *Pseudomonas* strains may reflect strain-specific variations in QS activation, potentially influenced by mutations in *lasI*/*rhlI* synthase genes or environmental cues that were not replicated in our *in vitro* conditions (21). Such heterogeneity is not uncommon; studies on clinical *Pseudomonas* isolates have shown that QS proficiency can vary, with some strains exhibiting reduced AHL output due to evolutionary adaptations in chronic infections (22). For *Klebsiella* sp. and *E. coli*, the negative results are attributable to their reliance on alternative QS systems, primarily the AI-2/LuxS pathway, which utilizes furanosyl borate diester rather than AHLs for intercellular communication (23–24). *Klebsiella pneumoniae*, in particular, employs Type 2 QS involving AI-2 to modulate biofilm formation and virulence, but AHL-based systems are rarely dominant in this genus (25). Similarly, *E. coli* clinical isolates predominantly use AI-2 signaling through the LsrR regulator to control gene expression related to adhesion and motility, explaining the absence of AHL detection in our assays (26).

These findings highlight the specificity of AHL-based QS to certain Gram-negative pathogens like *Pseudomonas*, with implications for targeted therapeutic interventions. Disrupting QS in AHL-producing strains could attenuate virulence without promoting resistance, as demonstrated by quorum-quenching strategies in *Pseudomonas* infections (27). However, the limited sample size and focus on AHLs alone represent study limitations; future work should incorporate broader QS molecule screening (e.g., AI-2 assays) and genomic sequencing to elucidate regulatory networks. Overall, this screening provides foundational insights into QS diversity among clinical isolates, paving the way for anti-QS agents in combating antimicrobial resistance.

5. Conclusions

This study successfully isolated and identified five clinical bacterial strains—three *Pseudomonas* spp. (MIC1, MIC2, MIC3), one *Klebsiella* sp., and one *E. coli*—from mixed cultures using selective media and biochemical assays. Screening via biosensor-based agar plate bioassays revealed that only *Pseudomonas* sp. MIC1 produced AHL-type quorum-sensing molecules, eliciting a violacein response with *Chromobacterium violaceum* CV026, indicative of short-chain AHLs, while other isolates were negative. These findings underscore the strain-specific prevalence of AHL-mediated quorum sensing in *Pseudomonas* clinical isolates, highlighting its potential role in virulence and offering a foundation for developing targeted quorum-quenching therapies to combat antimicrobial resistance in nosocomial infections. Future research should expand to genomic analyses and broader quorum-sensing pathway evaluations for comprehensive therapeutic insights.

6. List of abbreviations

QS	Quorum sensing
AI	Autoinducer
AHL	*N*-acyl homoserine lactone
ASRB	Advanced Studies and Research Board
BSL-2	Biosafety Level 2
CV	*Chromobacterium violaceum*
MIC	Microbial isolate code

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The author gratefully acknowledges the support and assistance received in completing this research.

8. Authorship

The author has granted their consent for this version to be published, meets the authorship requirements set by the International Committee of Medical Journal Editors (ICMJE), and is responsible for the integrity of the work.

9. Authors' Contributions:

H. Shaikh: Conceptualization, methodology, investigation, data curation, formal analysis, validation, resources, project administration, writing – original draft, and writing – review & editing. She approved the final manuscript.

10. Conflicts of interest

None

11. Funding

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12. Consent for publication

Not Applicable

13. AI Use Disclosure

None

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