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Molecular Characterization of Virulent Genes in Neonatal Respiratory Infections Associated Pathogenic Strains of *Pseudomonas aeruginosa* Using PCR Technique

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ABSTRACT

The current research study anticipated the identification of toxic genes in *Pseudomonas aeruginosa* involved in quorum sensing (QS), as many virulence factors are regulated by QS. The objective of the current research was to isolate and screen the pathogenic strains of *P. aeruginosa* from neonate's sputum samples and molecular characterization of virulent genes in these strains. The *P. aeruginosa* was screened out in the Microbiology Laboratory, Department of Zoology, Government College University Lahore. Different identification tests and Molecular characterization was conducted. Molecular characterization of virulent genes was performed by using specific primers. Products of PCR were sequenced in order to get accession numbers from the NCBI site. The identification of genes *LasR*, *LasI*, *rhlR*, and *rhlI* was made in the *P. aeruginosa* strains (SS5, SS6, and SS11). Recent studies have disclosed that these virulent genes play a significant role in *P. aeruginosa* strains' resistance against various antibacterial agents. *P. aeruginosa* possesses diverse metabolic capabilities to manage survival in various conditions and can exhibit extreme resistance to antibiotics, facilitating its spread among different environments especially in hospitals. These genes involved in overall pathogenicity of *P. aeruginosa*.

Keywords: Opportunistic pathogen,Virulent genes, Respiratory distress syndrome,, QS system, Bacterial infections

INTRODUCTION

Bacteria are one of the attractive microbes that change the planetary history and play an important role for their existence. Bacteria are known for spreading infectious diseases and play an important role in the health maintenance of many other ecosystems (Wess, 2023; Magnabosco *et al.,* 2024). They are involved in nitrification and many other activities. Pink coloration is retained by Gram negative and purple by Gran positive bacteria. *P. aeruginosa, Citrobacter, Klebsiella pneumonia* and *Escherichia coli* are common Gram negative bacteria that retained pink coloration after Gram staining, a technique utilized for differentiate between Gram negative and Gram positive bacteria by their ability to retain pink or purple color (Kohlerschmidt *et al.,* 2021; Simukoko, 2021; Parasuraman *et al.,* 2024).

Mucosal and hair in Upper respiratory tract play a crucial role in the respiratory immune system. Upper respiratory tract (Nose, Throat) disrupted by different pathogens and these pathogens are captured by these hair like cells and mucous (Legendre *et al.,* 2021). Mucous produced by lower airway passage captured and trapped foreign particles and expelled them outside by coughing and by upward movement of hair. Different defensive cells identify a number of microbes in respiratory tract which causes mild to severe infections and eliminate them (Deshmukh *et al.,* 2020; Honish, 2021; Abbott, 2024).

Acute and chronic respiratory infections caused by *P. aeruginosa* (Berglund *et al.,* 2020; Conceição-Silva *et al.,* 2021). The key factors contributing to illness and deaths are type three leakage factors (TTSF), Exo S, Exo T, and Exo U, especially confirmed in models with acute minor respiratory infection. Respiratory infections in humans affected by the presence or absence of TTS components. A blue pyocyanin pigment with antimicrobial properties produced by *P. aeruginosa*. Intranasal infection in adult mice shows that pyocyanin production also causes lung damage. *P. aeruginosa* quorum collection systems, including, *LasI*, *LasR*, *rhlR* and *rhlI* also play a role in severe infectious diseases (Ali *et al.,* 2020; Morin *et al.,* 2021; Govers *et al.,* 2022; Akhand & Ahsan, 2023).

Infection has shown that many severe strains lead to the acquisition of quorum, particularly the reduction in *LasR* mutants. Results of different studies

indicate that the antibiotic resistance increases by the loss of ability of *LasR*, like it increase in beta-lactam activity. Diminishing the function of *LasR* helping to enhance the strains that cause chronic infections (Thi *et al.,* 2020; Pelegrin *et al.,* 2021; Shouman *et al.,* 2023; Chaudhry *et al.,* 2024; Wu *et al.,* 2024). Initially *P. aeruginosa* survive in planktonic form among host's tissues but later the cells were seductively converted in biofilms. Biofilms have a wide range of populations with phenotypic and genotype diversity, and are formed by several species. (Guillaume *et al.,* 2022; Jo *et al.,* 2022).

Metabolic active cells are primarily present in the peripheral area and use excessive oxygen to produce oxygen levels in the biofilm. Organisms having low metabolic activity are present on the top layer of biofilm and are anoxic. Therefore, active-growing peripheral bacterial cells within the biofilm are more sensitive to antibiotics that struggle to pass through the biofilm layer to reach deep cells (Jurado-Martín *et al.,* 2021). They help maintain the channels and gaps of the biofilm, and some nutrients can flow according to the matrix, which can also contribute to the diffusion of the cells of the biofilm. Thus, properties of biofilms can change by a number of factors (Al Dawodeyah *et al.,* 2018; Karmegham *et al.,* 2020; Michael *et al.,* 2023; Awari *et al.,* 2024).

MATERIALS AND METHODS

Samples Collection: Samples of newborns suffering from pneumonia linked to RDS were obtained from qualified and experienced pulmonologists at Fatima Memorial Hospital Lahore.

Clinical trial number: Not applicable.

Isolation of Bacteria: The samples were placed on McConkey agar plates and incubated at 37 °C overnight. The plates were examined after the incubation period to confirm whether the isolated bacterial strains were gram negative or gram positive. (Michael *et al.,* 2023) (Figure 1).

Figure 1. Growth of pinkish colonies of *P.aeruginosa* on Mac-Conkey agar

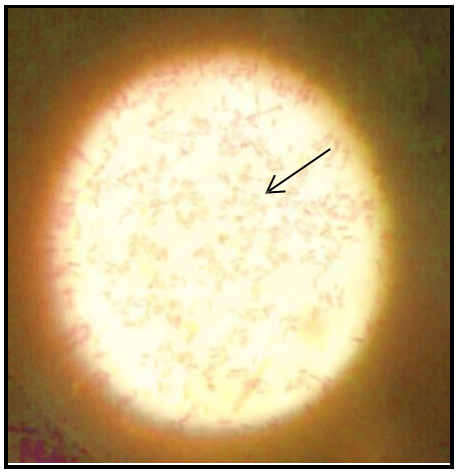


Figure 2. Gram's staining slide showing Gram negative *P.aeruginosa*

Gram's staining: Gram's staining procedure was utilized to isolate the Gram negative bacteria from samples and screened out under microscope (Figure 2).

Figure 3. Pyocyanin Test

Qualitative Assay: To confirm the existence of *P. aeruginosa*, bacterial colonies were streaked onto the King B media, and incubated at 37ºC for 24 hours. After the incubation period, the analysis of colonies under a transilluminator was done to estimate the productivity of fluorescein and pyocyanin (Awari *et al.,* 2024) (Figure 3).

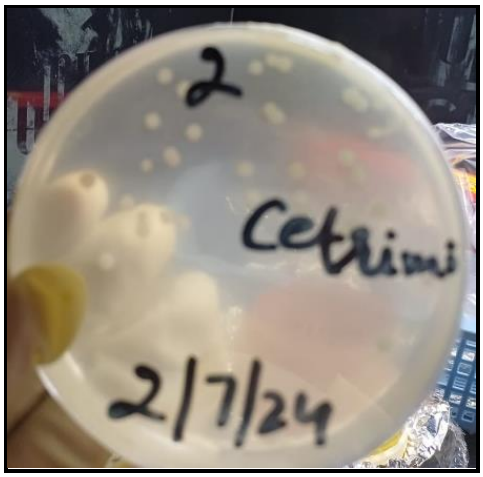


Figure 4. Cetrimide agar

Isolation of Pure culture: A medium was prepared with Cetrimide to isolate pure colonies of *P.aeruginosa*, and this medium is specific for the growth of *P. aeruginosa*. (Gautam, 2022). After the incubation, the media was poured into plates and allowed to cool. Once the media solidified, bacterial colonies were streaked and incubated for 48 hours at 37ºC. After the incubation period, the analysis of plates was done to see if desired bacterial strains grow on the Cetrimide media or not. (Sawa *et al.,* 2020) (Figure 4).

Pathogenicity Test: For pathogenic strains of *P. aeruginosa*, pathogenicity test also known as blood agar test. Non-coagulated blood was poured into sterilized nutrient agar to prepare blood agar media. Plates with blood agar were prepared and pure culture of *P.aeruginosa* was streaked onto blood agar plates and incubated for overnight (Figure 5).

Figure 5. Pathogenicity Test showing Beta hemolytic strains

Glycerol stock preparation: After sterilization nutrient broth was poured into test tubes and inoculation of pure pathogenic bacterial strain was done into broth and incubated for overnight at 37ºC. To prepare glycerol stocks, Eppendorf's were sterilized with, 200µl glycerol and then 800 µL of bacterial strains was added into Eppendorf's and stock was stored at 20ºC (Karmegham *et al.,* 2020).

Morphological characterization: The colonies of *P. aeruginosa* isolates were observed on different media. The colonies of *P. aeruginosa* on cetrimide agar were also observed under UV lamp at 250 nm. The morphology characteristics of the isolated bacteria were Gram stained and observed under 100 X objective of microscope.

Molecular characterization of Pthogenic strains of *P. aeruginosa:* Genomic DNA isolation was done by using Phenol: chloroform extraction method

Buffered phenol: It was prepared by mixing equal amount of 0.5 M Tris HCI and melted phenol at 60°C to 65 °C; the mixture was stirred slowly for one hour on the magnetic stirrer. After an hour stopped and the magnetic stirrer and remove the top layer. Add equal volume of 0.5 M Tris HCI and placed the mixture on the magnetic stirrer overnight, next day discard the upper layer and again add equal amount of 0.5 M Tris HCI, stir it on the magnetic stirrer. Continue the stirring process until the mixture attains the pH up to 7.4 to 7.8. For the storage of buffered phenol 0.IM Tris HCI was added. Now buffered phenol is ready to use.

Procedure for genomic DNA isolation: First of all, prepare the 50 ml nutrient agar medium was prepared and for 15 minutes autoclaved at 121°C, 15lb pressure. Inoculated the bacterial culture with the help of inoculating loop in the laminar air flow cabinet under aseptic conditions and incubatde it at 37°C for overnight incubation. Next day broth culture was centrifuged at 90 rpm for 10 min. then discard the supernatant and washed the pallet 1ml (1000ul) TEN buffer and mixed the TEN buffer with pallet by vortex. Mixture was centrifuged at 90rpm for 10 min to form pallet again, supernatant was discarded and washed the pallet with 500 ul SET buffer. Mix it by vortex and add 120ul lysozyme, then incubate the mixture at 37°C for 15min. After 15min incubation 100ul TEN and 20ul 25% SDS were added. Inverted the test tube gently until lysis occurred and then again incubated at 60°C for 15 minutes. After incubation allowed it to cool down at room temperature and added 20ul of 5M NaCI. Then again centrifuged the tube at 5500rpm for 10 min. transferred the upper layer into separate eppdorf and added equal volume of buffered phenol and chloroform mixture (1:1) and mix it with the upper layer separated in eppdorf. Centrifuge it at 5500rpm for 10 min and again separated the top layer in new eppdorf and mixed it with double volume of chilled 100% ethanol. Then the tube was placed in the refrigerator overnight for precipitation of DNA. 2nd day tube was centrifuged at 90rpm for 10 min and top layer was discarded then wash the pallet with 70% ethyl alcohol two times. The tube was allowed to dry and finally DNA was stored in 50ul TE buffer at -20°C.

Gel electrophoresis: Gel Electrophoresis was performed to check the isolated DNA bands. 0.6 g agarose gel was dissolved into 60 ml of 1X TAE buffer. Mixed the agarose in TAE buffer by heating in the microwave oven, add 0.2ul ethidium bromide (it binds with the DNA and produce fluorescent by absorbing UV rays and transmit visible red light) in the mixture, mixed them and poured the mixture in the gel plate. Gel plate was prepared by fixing the transparent tape at the open ends. After pouring fixed the comb in the gel plate, allowed the gel plate to solidify at room temperature. After solidification, Comb was removed from the gel carefully. 5ul sample was taken in the eppdorf mixed 2ul of loading dye load the sample in the wells present in the gel. And loaded the ladder one side of the gel. Put the gel in the gel caster after removing the tape from uncovered sides. Allow the gel to run in TAE buffer for 30-40 minutes.

16s RNA gene amplification: The gene16S rRNA is accountable for the encoding the rRNA which is the part of small ribosomal subunit *i.e.* 30S. This gene is amplified by PCR then itis sequenced. The 16S rRNA gene usually has 9 hyper variable regions due o which there is a vast variety in bacterial species. These hyper variable regions are denoted as V1 - 19. In these hyper variables regions of 16S rRNA gene, a sequence which is specified for particular strain is present that sequence is used to identify the particular bacterial strain. In medical field, this method is commonly used in the diagnosis purposes. The 16S rRNA gene is approximately, 1,550 base pairs long. This method is extensively used mainly because of three reasons *i.e.* 16S rRNA gene is present in the DNA of all bacteria, its function is covered over a long time and this gene is usually large enough to provide the required information. If any mutation occurs in this gene then it can be tolerated. These hyper variable regions of 16S rRNA are usually flanked which are used in PCR to amplify the 16S rRNA gene of a variety of bacterial strains by conserved sequence which enables us to develop universal primers.

PCR protocol: Protocol for 50 µL reaction was followed by using Universal primer for 16s ribosomal DNA 16S-27F 5`AGAGTTTGATCMTGGCTCAG 3` and 16S-1492R 5`TACGGYTACCTTGTTACGACTT 3` for identification of *strains.* Reagents were mixed into 0.5 ml PCR tubes were kept on ice in a safety cabinet. The suitable program on thermo cycler was precisely set and all tubes are kept for PCR. First of all, 50ul of PCR master mix was prepared by adding 16ul MgCl2, 4ul 10Xpolymerase, 2ul dNTPS, 4ul of reverse and

forward primers each, with the help of micro pipette4ul genomic DNA sample 0.8ul Taq. DNA polymerase, and 15.2ul deionized water in an eppdorf, that eppdorf was placed in the ice box. This master mix was taken by the micro pipette and transferred to the PCR tube. The PCR tubes were placed in the thermocycler and the polymerase chain reaction was started for 35 cycles. The temperature was raised to 94°C for 5 minutes for initial denaturation, and then the temperature was decreased up to 60°C for the annealing of primers to the specific region of the DNA. Then, the temperature was increased to the 72°C for the polymerase to add deoxyribonucleotides at the 3' end. This process was repeated many times and the final extension was done at 72°C for 10 minutes.

Gel electrophoresis: Gelelectrophoresis was done by using 1.5% agrose gel for the confirmation of amplified product. The gel was run for 30-35 minutes at 120V. The band of PCR product was visualized when the gel was seen in the UV trans-illuminator.

Sequencing of PCR Products: PCR products of isolated strains for sequences were sent to Malaysia and sequence blast was done on National Center for Biotechnology Information (NCBI) for identification at species level. Accession numbers were obtained from NCBI.

RESULTS

Samples were taken from the patients suffering from acute and chronic lung infections. 250 samples were collected from blood, endotracheal tube and sputum (Table 1).

Table 1. Sources of sampling

Sr. no.	Hospital	Source of samples		
		Blood	**Sputum**	**Endotracheal tube**
1	Jinnah Hospital	68	37	13
2	Mayo Hospital	37	28	15
3	District headquarter hospital Kasur	37	15	-
Total samples =250		**142**	**80**	**28**

Initial Isolation of *P. aeruginosa*: *P. aeruginosa* were isolated using cetrimide agar plates. The positive isolates which showed growth on the selective media cetrimide agar were 72 from blood, 43 from sputum and 19 from endotracheal samples (Table 4.2). Table 3 represents the data of clinical isolates as per their age and gender. Sampling and data were collected on the availability of the patient admitted in the hospitals (Table 2-3).

Table 2. Initial isolation of *P. aeruginosa* from different samples

Sr. No.		Positive Isolates
1.	Jinnah Hospital	Blood=47
		Sputum=15
		Endotracheal tube=10
2.	Mayo Hospital	Blood=26
		Sputum=11
		Endotracheal tube=06
3.	District Headquarter Hospital Kasur	Blood=11
		Sputum=08
		Endotracheal tube=0

Table 3. No. of isolates (*P. aeruginosa*) from male and female patients

No. of isolates (*P. aeruginosa*)	Female	Male	Age in years	Source
34	03	31	25-35	Blood, Sputum and Endotracheal aspirates
47	11	36	36-45	
53	13	40	46-55	
Total=134	**27**	**107**		

Cetrimide agar: For *P. aeruginosa* Cetrimide agar was used as selective medium. The florescence of colonies was observed in Trans-illuminator at 230nm. Colonies of all isolates were observed florescent under UV radiation.

MacConkey agar: MacConkey agar was used as a selective medium for gram negative bacteria (appendix I) pink colonies on MacConkey agar showed positive test. All colonies appeared in yellow which confirmed that these isolates were lactose non fermenters.

Blood Agar Test: Blood agar was used to observe the hemolytic activity of *P. aeruginosa*. Isolates showed β-hemolysis.

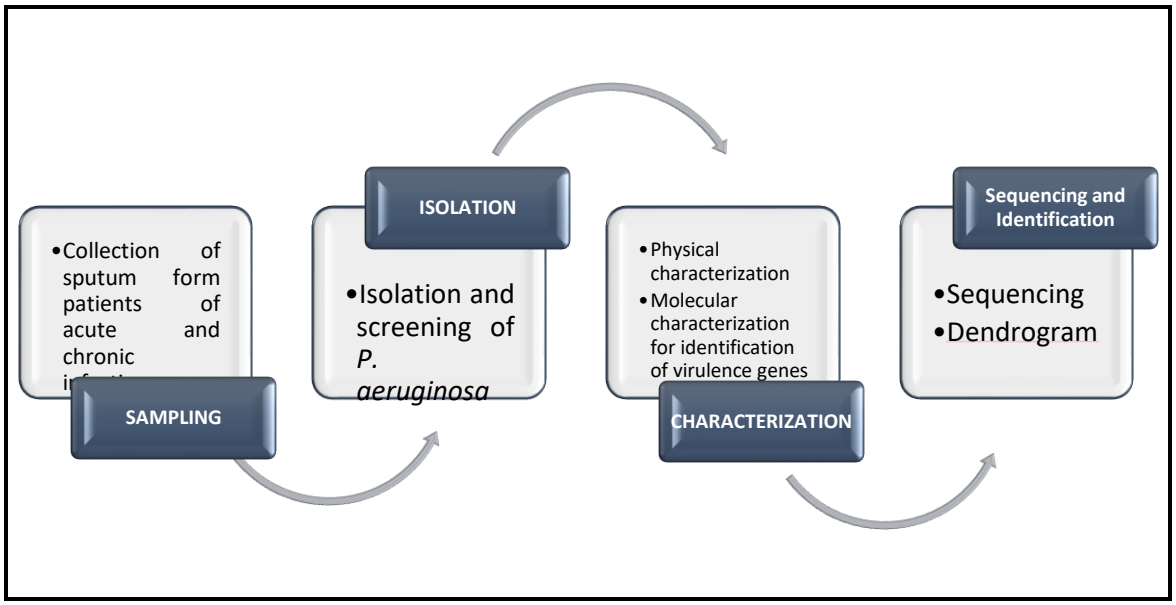
Characterization of bacterial isolates

Morphological characterization: The colonies of *P. aeruginosa* appeared as light blue yellowish green on Cetrimide media. All colonies were observed to be round, smooth, elevated and flat edge mucoid. The

colonies of *P. aeruginosa* were observed under UV lamp at 230 nanometer (nm) showed florescence. *P. aeruginosa* colonies on MacConkey agar appeared as Pale yellowish colonies which showed these isolate are non-lactose fermenters.

Table 4. Morphological characterizations of *P. aeruginosa*

Isolates of *P. aeruginosa*	Morphological Characteristics			
	Colony Color	**Morphology**	**Appearance on cetrimide agar plate under UV light**	**Appearance of colony on MacConkey agar plate**
134 isolates from human blood, endotracheal tube and sputum samples were processed on selective media Cetrimide	Light Blue yellowish green	Small smooth rounded elevated colonies	Colonies showed florescence	Pale yellow colonies showed non lactose fermenters



Schematic representation of Methodology and results of current research work

Molecular characterization: The bacterial were identified by sequencing of 16S rDNA gene and virulence genes. Genomic DNA was extracted. Extracted DNA samples were run on agarose gel and bands were observed in trans-illuminator under ultraviolet radiations and images were captured by using gel imager for records (Figure 6A). Using universal primer and specific gene primers for *P. aeruginosa*, genes were amplified (Figure 6B).

Sequencing: DNA samples were amplified by using universal and specific gene primers and after sequencing of PCR product from ABI Sequencer Malaysia following results were obtained. Phylogenetic distance tree were obtained by using Clustal W (Figures 7ABCandD). NCBI alignment Sequences were compared with NCBI repository (Tables 5-9).

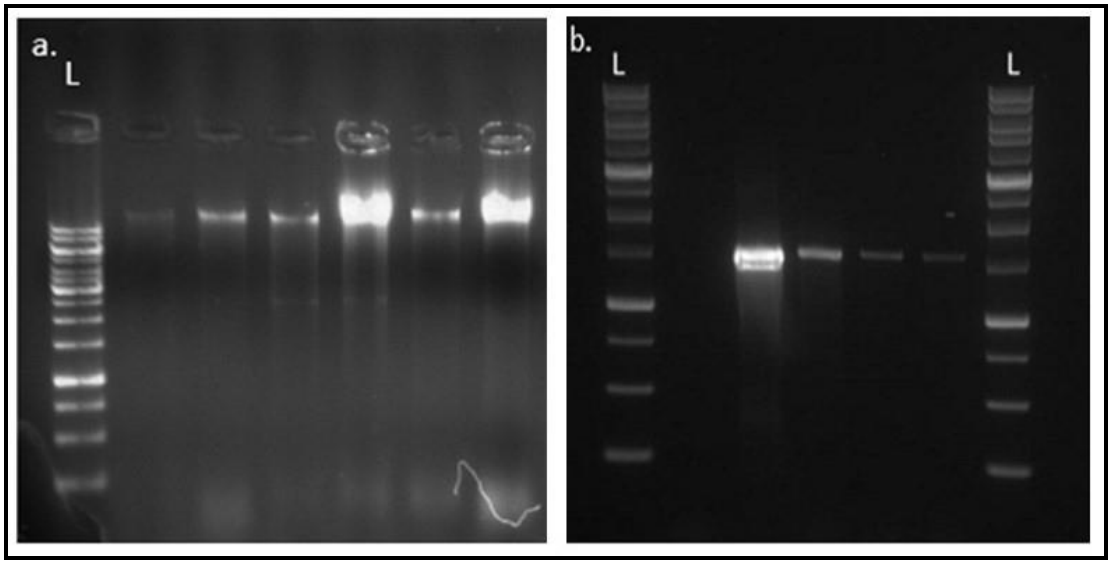


Figure 6. (A) Bacterial Genomic DNA bands. (B) PCR product of 1.5kb

Table5. Universal and specific genes primers

Sr. no.	**Genes**	**Primers**	**Sequence of nucleotide**
1	Universal	27F	AGAGTTTGATCMTGGCTCAG
		1492R	TACGGYTACCTTGTTACGACTT
2	*rhlR*	F	TGCATTTTATCGATCAGGGC
		R	CACTTCCTTTTCCAGGACG
3	*lasI*	F	CGTGCTCAAGTGTTCAAGG
		R	TACAGTCGGAAAAGCCCAG
4	*rhlI*	F	TTCATCCTCCTTTAGTCTTCCC
		R	TTCCAGCGATTCAGAGAGC
5	*lasR*	F	AAGTGGAAAATTGGAGTGGAG
		R	GTAGTTGCCGACGACGATGAAG

Table 6. Virulent genes in *P. aeruginosa (ss5)*

Sr. no.	Genes	Accession no.
1	*rhlR*	MH373641
2	*lasI*	MH373643
3	*rhlI*	MH373645
4	*lasR*	MH373649

Table 7. Virulent genes of *P. aeruginosa (ss6)*

Sr. no.	**Genes**	**Accession no.**
1	*rhlR*	MH373642
2	*lasI*	MH373644
3	*rhlI*	MH373646
4	*lasR*	MH373650

Table 8. Virulent genes of *P. aeruginosa (ss11)*

Sr. no.	Genes	Accession no.
1	*rlhR*	MH388293
2	*lasI*	MH373651
3	*rlhI*	MH388292
4	*lasR*	MH373652

Table 9. Molecular characterization of pathogenic strains of *P. aeruginosa*

Sr. No	Strains	Species name	Base pair	Accession No.
1	PA 1 *ss5*	*P. aeruginosa*	1159 bp	PQ567246
2	PA 29 *ss6*	*P. aeruginosa*	1333 bp	PQ569151
3	PA 25 *ss11*	*P. aeruginosa*	1050 bp	PQ568379

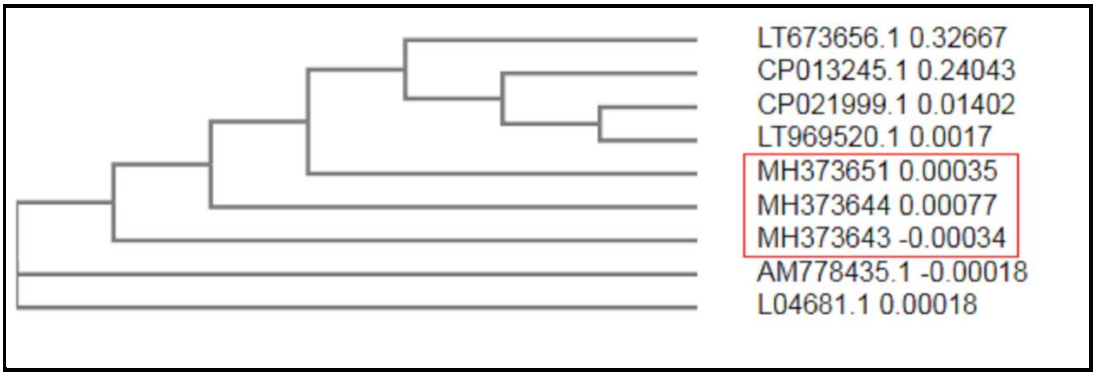


Figure 7A. Dendrogram of ss5, ss6, ss11 (*P. aeruginosa)*

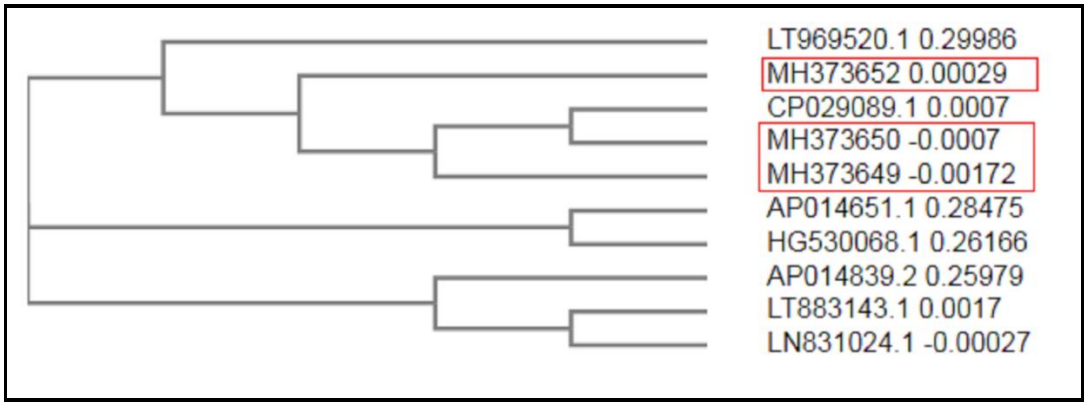


Figure 7B. Dendrogram of ss5 (*P. aeruginosa)*

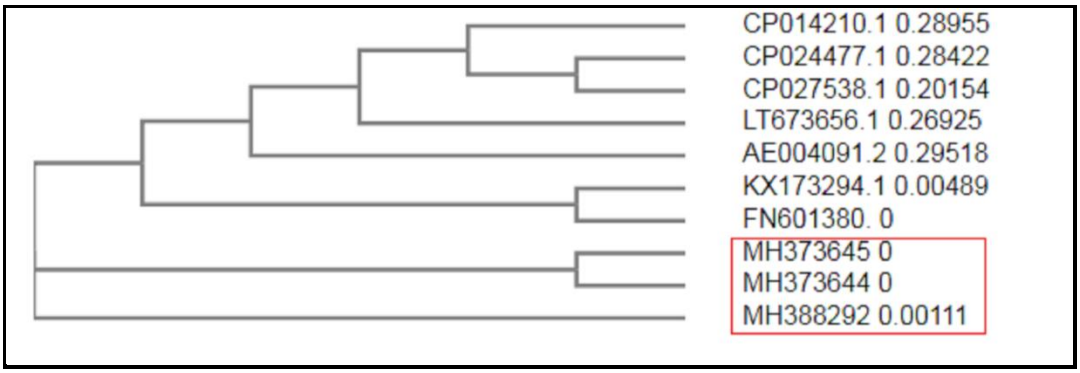


Figure 7C. Dendrogram of ss5 (*P. aeruginosa*)

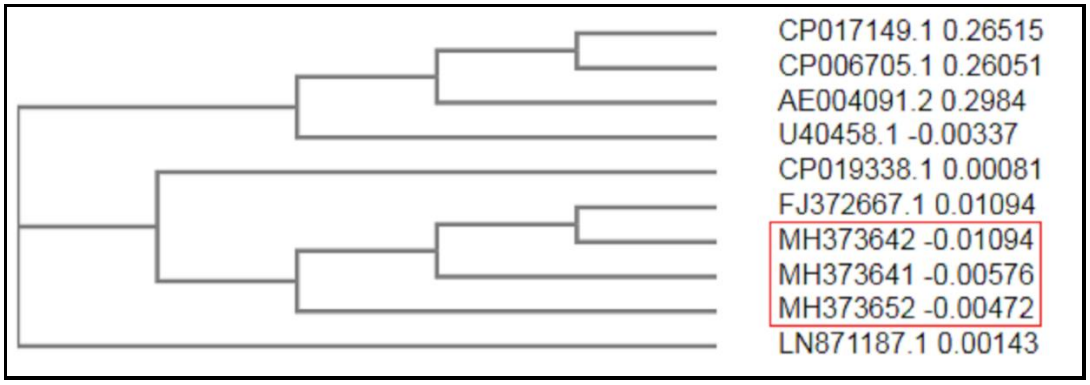


Figure 7D. Dendrogram of ss5, ss6, and ss11 (*P. aeruginosa*)

DISCUSSION

The types of *Pseudomonas* are ubiquitous gram-negative motile rod-shaped bacteria. It grows on various cultural mediums, forming smooth, round colonies resembling grapes. The synthesis of pyocyanin and pyoverdin results in blue and green colors production respectively. In the present study, 134 isolates of *P. aeruginosa* were isolated and

virulence factors involved in the pathogenicity were studied. Likewise, Different virulent genes were amplified and detected in strains of *P. aeruginosa*. These virulence factors are cause of spread of this pathogen in the hospital environment. These genes are involved in virulence in this particular bacterium and the mechanisms that facilitate bacterial clearance without causing excessive immune pathology needs further investigations in management dealing with sepsis and Pseudomonas pneumonia. *P. aeruginosa* is a very adjustable according to environment and it adapted for newer lifestyle and pathogenicity (Parasuraman *et al.,* 2024).

The identification of genes linked with the virulence of *P. aeruginosa* was also carried out. Genes studied in current research work were *LasR*, *LasI*, *rhlR*, and *rhlI*. The identification and characterization of genes *LasR*, *LasI*, *rhlR*, and *rhlI* was done in the *P. aeruginosa* strains named SS5, SS6, and SS11.The increasing resistance against various available antibiotics is also due to these factors. Some are already reported such as *LsrR* and *LsrK*, which are regulating the *lsr* regulon. Quorum sensing system comprises of these cup/cluster genes. The Quorum Sensing signal AI-2 is regulated by *LsrR* which is a transcriptional regulator. The role of rhamnolipids in the biofilm formation of *P. aeruginosa* has been investigated. It was reported that rhamnolipids production is under control of *RhlR*. Rhamnolipids are required for the pillar structures to maintain water channel structures in biofilms. Rhamnolipids not only provide the nutritional balance of the biofilm but also prevent invasion of bacteria to open spaces from colonization. In one study, Strain PA01 at the same chromosomal locus in all variants had a *LasB* gene compatible with the *LasB* gene sequence and with a sequence identity of 95-99%. These genes were all characterized at molecular level in all clinical types of *P. aeruginosa*. The sequence showed 98-100% identity. *LasB* sequences and quorum sensing reports can't be described without *LasB* activity (Nag *et al.,* 2021).

Infections are caused when external proteases like antimicrobial agents are produced by *P. aeruginosa*, and their production is affected by quorum system of. *P. aeruginosa*. In another study, *LasR* mutants of this bacteriumshowed resistance to commonly used antibiotics. *LasB*, another gene, is also involved in production of extracellular protease that damages tissue in *P. aeruginosa* infections. The factors of the virulence are managed by the *LasB* and APRA through the *Las* quorum system. Loss of *LasB* is seen when *LasR* and *RhlR* disable *LasB*. The sequencing and comparing of genome of strains by using bioinformatics highlighted areas of genomes that are adaptable between pathogenic and non-pathogenic bacterial strains. The individual gene variations between pathogenic and less virulent strains can be identified by using the bioinformatics tools. To address issues of antibiotic resistance and potential countermeasures, their occurrence, geographic manifestations and potential for spread needs to be continuously monitored. Having knowledge of these virulent genes can lead to better understanding of mechanism of virulence of pathogenic bacteria and role of these associated virulence genes ((Wang *et al.,* 2022; Sharma *et al.,* 2023).

CONCLUSION

P. aeruginosa is an opportunistic pathogen and associated with different types of respiratory infections. This virulence is because of the presence of virulent genes constituting the quorum sensing system in this pathogen. There is need to identify and characterize these virulent genes which can develop better understanding of mechanism of infection or virulence caused by these factors.

DATA AVAILABILITY

The sequences are deposited in NCBI, and Accession numbers are provided. The data is available on NCBI website. Accession numbers of bacterial strains of *P.aeruginosa* and virulent genes are given in Table 2-5.

AUTHORS' CONTRIBUTIONS

Conceptualization: Nazish Mazhar Ali, Investigation: Nazish Mazhar Ali, Maham Chaudhry, Safia Rehman. Methodology: Maham Chaudhry, Safia Rehman, Asif Rasheed, Areej Hassan. Statistical analysis: Maham Chaudhry, Saima shokat. Review and editing: Nazish Mazhar Ali, Asma Riaz, Samreen Riaz. Supervision: Nazish Mazhar Ali, Validation, Nazish Mazhar Ali, Maham Chaudhry

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