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Original Article

Pseudomonas aeruginosa associated pulmonary infections and *in vitro* amplification virulent rhamnolipid (*rhlR*) gene

Infecções pulmonares associadas a *Pseudomonas aeruginosa* e gene ramnolipídeo virulento (rhlR) da amplificação in vitro

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Abstract

Background: *Pseudomonas aeruginosa* is a common opportunistic pathogenic bacterium with the ability to develop a strong communication pathway by quorum sensing system and different virulent factors. Among the various important secretions of *P. aeruginosa* rhamnolipid is important biological detergent, believed to be involved in the development of the biofilm and intercellular communication. It readily dissolves the lung surfactants that are then easily catalyzed by the phospholipases and in this way is involved in the acute pulmonary infection. **Objective:** research work was designed to investigate virulence and gene associated with virulence in *P. aeruginosa* responsible for pulmonary infections. **Methods:** In current study polymerase chain reaction (PCR) was used for the detection of the *rhlR* (rhamnolipid encoding) gene of isolated strains. A number of assays were performed that ensured its virulent behavior. Disc diffusion method was used to check its antibiotic resistance. Isolated strains were resistant to a number of antibiotics applied. **Result:** It was found that males are more prone to respiratory infections as compared to females. Male members with age of 44-58 and 59-73 are at a higher risk, while females with age of 44-58 are also at a risk of pulmonary infections. Antibiotic resistance was observed by measuring zone of inhibition in strains GCU-SG-M_4, GCU-SG-M_3, GCU-SG-M_5, GCU-SG-M_2, GCU-SG-M_1 and GCU-SG-M_6. GCU-SG-M_2 was resistant to fluconazole (FLU), clarithromycin (CLR), cefixime (CFM) and Penicillin (P10). No zone of inhibition was observed. But it showed unusual diffused zone around the Ak and MEM antibiotic discs. *rhl R* gene and 16s *rRNA* gene were characterized and analyzed. **Conclusion:** Findings from current study would help in raising awareness about antibiotic resistance of *P. aeruginosa,* and also the sequence of *rhl R* gene can be used as the diagnostic marker sequence to identify the virulent *rhl R* gene sequence from the samples when isolated from sputum of Pneumonia patients.

Keywords: molecular characterization, risk factors, antibiotics resistance, virulence assays.

Resumo

Antecedentes: Pseudomonas aeruginosa é uma bactéria patogênica oportunista comum, com a capacidade de desenvolver uma forte via de comunicação pelo sistema de detecção de quorum e diferentes fatores virulentos. Entre as várias secreções importantes de P. aeruginosa rhamnolipid, há um importante detergente biológico, que se acredita estar envolvido no desenvolvimento do biofilme e na comunicação intercelular. Dissolve rapidamente os surfactantes pulmonares que são facilmente catalisados pelas fosfolipases e, dessa maneira, estão envolvidos na infecção pulmonar aguda. Objetivo: O trabalho de pesquisa foi desenhado para investigar a virulência e o gene associado à virulência em P. aeruginosa responsável por infecções pulmonares. Métodos: No presente estudo, a reação em cadeia da polimerase (PCR) foi utilizada para a detecção do gene rhlR (codificação ramnolipídeo) de cepas isoladas. Foram realizados vários ensaios que garantiram seu comportamento virulento. O método de difusão em disco foi utilizado para verificar sua resistência a antibióticos. As estirpes isoladas foram resistentes a vários antibióticos aplicados. Resultado: Verificou-se que os homens são mais propensos a infecções respiratórias em comparação às mulheres. Membros do sexo masculino com idade entre 44 e 58 e 59 e 73 anos correm maior risco, enquanto mulheres com idade entre 44 e 58 anos também correm risco de infecções pulmonares. A resistência aos antibióticos foi observada medindo a zona de inibição nas cepas GCU-SG-M4, GCU-SG-M3, GCU-SG-M5, GCU-SG-M2, GCU-SG-M1 e GCU-SG-M6. O GCU-SG-M2 foi resistente ao fluconazol (FLU), claritromicina (CLR), cefixima (CFM) e penicilina (P10). Nenhuma zona de inibição foi observada. Mas se notou uma zona difusa incomum ao redor dos discos antibióticos Ak e MEM. Os genes rhl R e 16s rRNA foram caracterizados e analisados. Conclusão: As conclusões do presente estudo ajudariam a aumentar a conscientização sobre a resistência a antibióticos de P.

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aeruginosa e, também, a sequência do gene rhl R pode ser usada como sequência de diagnóstico para identificar a sequência virulenta do gene rhl R das amostras quando isoladas do escarro de pacientes com pneumonia.

Palavras-chave: caracterização molecular, fatores de risco, resistência a antibióticos, ensaios de virulência.

1. Introduction

Pseudomonas aeruginosa bacterium is living in versatile environments. It is gram negative bacterium, metabolically able to regulate its systems and highly resistance to antibiotics causing it to spread in diverse habitats mainly in hospitals. *P. aeruginosa* is a human adaptable pathogen causing acute infections (bacteremia, pneumonia and urinary tract infections) in individuals with HIV infections, surgical wounds, cancer, carrying catheters or burns, or an organ transplantations (Gellatly and Hancock, 2013). The heterogeneous bacterial population of *P. aeruginosa* showed distinct microenvironments for biofilms (Ren et al., 2016). Metabolically active cell are at periphery and consume most of the oxygen, causing oxygen gradients in the biofilm (Su et al., 2016).

P. aeruginosa has vast ecological distribution with ability to fit in changed circumstances making it a successful opportunistic pathogen. According to American surveillance data, it is the third most common opportunistic pathogen. *Pseudomonas aeruginosa* releases hemolysins *i.e.* rhamnolipid as well as phospholipase-C that have very toxic consequences to the host cells and is responsible for the spread of pathogen throughout the target tissue. Both toxins act together for the catalysis to lipids and as well as lecithin. Structure of the rhamnolipid to some extent is similar to that of detergents. These rhamnolipids readily dissolve the phospholipids which are then easily cleaved by the action of phospholipase-C. Lung surfactants as a result are lost which initiate atelectasis that ultimately then leads towards chronic infections of lungs (Liu, 1966; Wardlaw et al., 2006). *P. aeruginosa's* exposure to the respiratory epithelial cells of human can result into the inhibition of mucociliary transportation and several ciliary actions (Read et al., 1992; Bhutta, 2007). Two Quorum sensing systems *i.e. las* QS and *rhlR* are present in this bacterium. *rhlR* QS releases many toxins including rhamnosyl transferase and rhamnolipid Both toxins ultimately cause lung infection. A number of scientific projects had worked on the determination of rhamnolipids functioning which is still not clear. Firstly, they were known as surfactants because they have ability to reduce the surface tension (Smyth and Openshaw, 2006). Their study became very important because the hydrocarbons *e.g.* alkanes were firstly solubilized by them and then their uptake is promoted (Beal and Betts, 2000). They also have the property to enhance the hydrophobic ends of a cell surface that promotes the decomposition of the molecules that previously were slightly soluble. However, the purpose of the rhamnolipid is not just to assimilate the substrates which are not soluble that is evident by their competent growth on the substrates that were soluble (Al-Tahhan et al., 2000). A number of virulent agents are produced by the *P. aeruginosa* and for the transformation of these factors it has a highly developed mechanism through which bacterial density is checked and these cells communicate with each other through signals. Once the bacterial biofilm has been formed it enables the pathogen to detect any change in its environment, and it can respond in a very appropriate manner after the processing of information received. Additional activities that have recently been observed these are thought to be (Gray, 1997). Aims and objectives of the current study included screening of *Pseudomonas aeruginosa* involved in acute pulmonary infections. Also to determine the virulence of *Pseudomonas aeruginosa* via detection the *rhlR* gene of *Pseudomonas aeruginosa* associated with acute pulmonary infections in humans.

2. Materials and Methods

2.1. Sample Collection

Blood samples, sputum samples and samples of lower respiratory tract pneumonic patients were provided from a running project in GCU Microbiology laboratory. Among these samples secretions of lower respiratory tract had been obtained by endotracheal suction catheter (nelton). Pediatric mucus extractor had been used for the samples from bronchoscopy whereas blood culture vials (TSB with SPS aerobic) were being used for blood samples (Figure 1).

2.2. Isolation and characterization of Pseudomonas aeruginosa

Bacterial isolation from sputum samples of pneumonic patients was done using citrimide agar medium (Figure 2). Pure culture was obtained by spread plate method and streaking method. Initial confirmation of *P. aeruginosa* was made by morphological and biochemical test (Figure 3) (Tables 1 and 2).

2.3. Molecular characterization of Pseudomonas aeruginosa and detection of rhlR gene

PCR based confirmation of isolated strains of *P. aeruginosa* was carried out using universal primers for 16srRNA. Marcogen genomics was used to manufacture universal primers and gene specific primers.

3. Results

Percentile of the pulmonary infections caused by *Pseudomonas aeruginosa* was determined. These infections include respiratory failure Type-I and Type-II, Septicemia, Asthma, pulmonary edema, pneumothorax and chest filial injury. According to the resulted percentile pneumonia can be declared as most common pulmonary infection in patients, and septicemia & Type-II respiratory failure as the 2nd most common pulmonary infection.

3.1. ETT v/s Blood Culture and Bronchoscopy

Percentage of gram negative bacterial content (*P. aeruginosa*) in three different cultures was compared

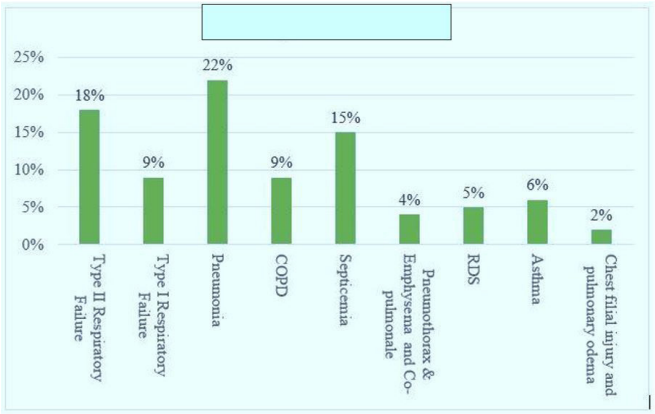

Figure 1. Showing Bar Graph of Pulmonary Infection Percentile of *P. aeruginosa*.

Figure 2. Showing *P. aeruginosa* Growth on Citrimide Agar Plates.

Figure 3. Showing β-Hemolysis by *P. aeruginosa* in Blood Agar Test.

with each other. Among all 57.7% (26/45) the highest observed value was in ETT-culture 24.4% (11/45) on the other hand *cocci* the gram positive bacterial content was observed higher in blood culture 76% (34/45) than in the ETT culture 42% (19/45). Similarly in bronchoscopy culture their higher percentage was observed 8/15 (53.3%).

3.2. Prevalence and Isolation of P. aeruginosa

Sample collection in the present study has shown that in males (60%) the rate of pulmonary infection is comparatively higher than females (40%). Male members within the age of 44-58 and 59-73 are at a higher risk. In addition to that some females within the age ranging from 44-58 are also at a risk of getting pulmonary infections. Citrimide agar medium is a selective bacterial medium that only allows the growth of *P. aeruginosa.* Using this media culture of strains i.e. GCU-SG-M_1, GCU-SG-M_2, GCU-SG-M_3, GCU-SG-M_4, GCU-SG-M_5, GCU-SG-M_6 were obtained.

3.3. Virulence Behavior assays

i. Blood Agar Test:

Strains showed beta hemolysis

ii. Protease Test:

The result of this activity was clearly observed by the presence of a hollow zone. Different strains provided different values and it is obvious that the sensitivity of each strain varies. These values were recorded as 9.5mm, 2.5mm, 9mm, 4mm, 7mm, and 8mm for GCU-SG-M_4, GCU-SG-M_3, GCU-SG-M_5, GCU-SG-M_2, GCU-SG-M_1 and GCU-SG-M_6 respectively.

iii. Lipase and Lecithinase Test:

Results are shown in Figure 4.

iv. Biofilm-Formation Assay:

All strains produced positive results for this assay indicating the how virulent behavior these pathogenic bacterial strains have.

v. Pigment Production

a. Qualitative Assay:

Different culture media were used to determine the quality of pigment produced by the pathogenic strains. Among all strains the two i.e. GCU-SG-M_6 and GCU-SG-M_4 were observed with a slight yellowish-green colored colonies that this is due to the production of pyoverdin pigment production (Figure 5). Remaining strains of *P. aeruginosa* probably produced another pigment *i.e.* Pyocyanin that's why these colonies appeared with blue-green colored colonies on different agar media e.g. king A, king B, citrimide, LB and MH agar media (Table 3).

b. Quantitative Assay:

Quantitative of the produced pigment was measured by quantitative assay using spectrophotometer. Optical density was measured at OD 520 nm. One by the pyocyanin producing strains i.e. GCU-SG-M_5 produced the highest value of optical density (23µg/ml). The lowest pigment yielding strain that showed about 1.7µg/ml.

Table 1. Showing results of different morphological tests.

STRAINS	COLOR	ODOUR	SHAPE	SIZE	SURFACE ELEVATIONS	SURFACE EDGES	APPEARANCE
GCU-SG-M$_1$	Dull Green	Fruity, grape like	spherical	Medium	Flat	Smooth	Semi transparent
GCU-SG-M$_2$	Dark green	Fruity, grape like	spherical	Large	Flat	Smooth	Translucent
GCU-SG-M$_3$	Yellow green	Fruity, grape like	spherical	Large	Flat	Rough	Translucent
GCU-SG-M$_4$	Yellow green	Fruity, grape like	spherical	Medium	Flat	Rough	Translucent
GCU-SG-M$_5$	Yellow green	Fruity, grape like	spherical	Small	Flat	Smooth	Translucent
GCU-SG-M$_6$	Yellow green	Fruity, grape like	spherical	Large	Flat	Smooth	Translucent

Table 2. Showing results of different tests performed for biochemical characterization.

	CT	OX	CIT	UR	GLU	LAC	EMB	M.C	G.S	E.S.S
GCU-SG-M$_1$	***	***	***	***	F	N.F	N.F Pinkish	N.F Colourless	-Ve rods	-Ve
GCU-SG-M$_2$	***	***	***	***	F	N.F	N.F Pinkish	N.F Colourless	-Ve rods	-Ve
GCU-SG-M$_3$	***	***	***	***	F	N.F	N.F Pinkish	N.F Colourless	-Ve rods	-Ve
GCU-SG-M$_4$	***	***	***	***	F	N.F	N.F Pinkish	N.F Colourless	-Ve rods	-Ve
GCU-SG-M$_5$	***	***	***	***	F	N.F	N.F Pinkish	N.F Colourless	-Ve rods	-Ve
GCU-SG-M$_6$	***	***	***	***	F	N.F	N.F Pinkish	N.F Colourless	-Ve rods	-Ve

Table 3. Showing results of qualitative assays.

Strains	Citrimide Agar	EMB Agar	LB Broth	CLED Agar
GCU-SG-M$_1$	B/G Pyocyanin	Pink colonies	Pyocyanin	Translucent colonies
GCU-SG-M$_2$	B/G Pyocyanin	Pink colonies	Pyoverdin	Translucent colonies
GCU-SG-M$_3$	B/G Pyocyanin	Pink colonies	Pyocyanin	Translucent colonies
GCU-SG-M$_4$	Y/G Pyoverdin	Pink colonies	Pyocyanin	Translucent colonies
GCU-SG-M$_5$	B/G Pyocyanin	Pink colonies	Y/G Pyocyanin	Translucent colonies
GCU-SG-M$_6$	Y/G Pyoverdin	Pink colonies	Y/G Pyoverdin	Translucent colonies

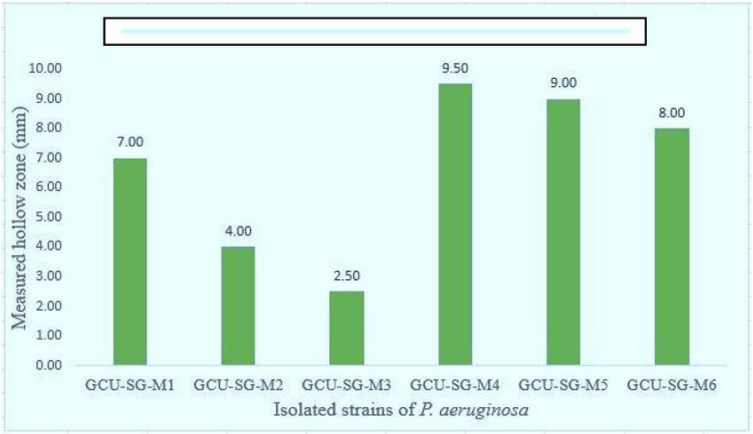

Figure 4. Bar graph showing proteolytic activity of isolated strains of *P. aeruginosa.*

3.4. Rhamnolipid Assay

Strains which showed the maximum Rhamnolipid production resulted 2.4µg/ml whereas, the minimum value obtained was 0.2µg/ml. Maximum Rhamnolipid yielding strains were those isolated from bronchoscopy samples as well (Table 4).

3.5. Antibiotic Susceptibility Test

GCU-SG-M_1 isolate was 100% resistant to the antibiotic disc of Amikacin AK, Fluconazole FLU, Meropenem MEM, Cefixime CFM, Clarithromycin CLR, and Penicillin PIG (Figure 6). GCU-SG-M_2 was 100% resistant to FLU, CLR, CFM and P10. No zone of inhibition was observed (Figure 7).

Table 4. Showing Results of Rhamnolipid Assay.

Sr. No.	Sample name	Rhamnolipid production (µg/ml)
I	GCU-SG-M_1	1.70
II	GCU-SG-M_2	2.01
III	GCU-SG-M_3	2.40
IV	GCU-SG-M_4	2.37
V	GCU-SG-M_5	0.25
VI	GCU-SG-M_6	1.96

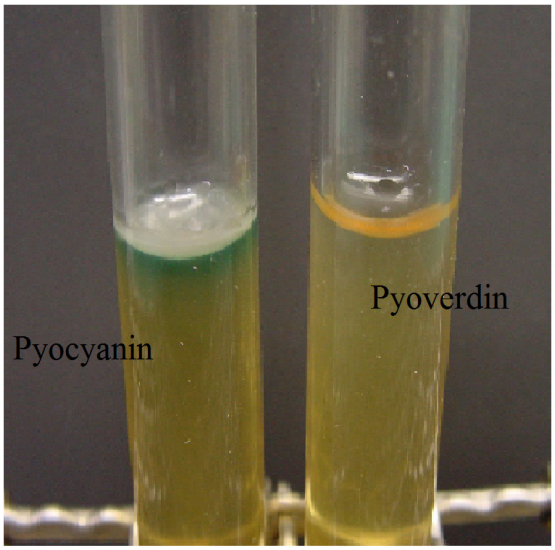

Figure 5. Shows production of Pyocyanin (Left) and Pyoverdin (Right) pigments in LB broth

But it showed unusual diffused zone around the Ak and MEM antibiotic discs (Tables 5 and 6).

3.6. Fluorescence of P. aeruginosa Isolated under UV rays

Fluorescence of the isolates was clearly observed which appeared as illuminating greenish light. This green light was considered as a confirmation for the present in fluorescent pigment present in the *P. aeruginosa* strains.

3.7. Molecular Characterization

Molecular identification with universal primers was done (Table 7) (Figures 8 and 9)

3.7.1. Aligned sequence of 16S rDNA of P. aeruginosa GCU-SG-M_3

CACGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTG
GTGGGGTAAAGGCCTACCAAGGCGACGATCCGTAACTGG
TCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGT
CCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGAC
AATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGA
AGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGG
AAGGGCAGTAAGTTAATACCTTGCTGTTTTGACGTTACCA
ACAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCG
GTAATACGAAGGGTGCAAGCGTTAATCGGAATTACTGGGC
GTAAAGCGCGCGTAAGTGGTTCAGCAAGTTGGATGTGATC
ACTACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGA
TCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACG
GGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGC
CTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGA
TTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAGTTA
ATACCTTGCTGTTTTGACGTTACCAACAGAATAAGCACCGG
CTAACTTCGTGCCAGCAGCC

3.8. Amplification of the Targeted rhlR gene

3.8.1. Aligned rhlR gene sequence of P. aeruginosa GCU-SG-M3 (Figures 10 and 11)

Aligned sequence of *P. aeruginosa* rhlR gene showed 99% homology with previously reported rhlR gene sequences.

AGGAATGACGGAGGCTTTTTGCTGTGGTGGGACGGTTT
GCGTAGCGAGATGCAGCCGATCCACGACAGCCAGGGCGT
GTTCGCCGTCCTGGAAAAGGAAGTGCGGCGCCTGGGCTT
CGATTACTACGCCTATGGCGTGCGCCACACGATTCCCTTCA
CCCGGCCGAAGACCGAGGTCATGGCACCTATCCCAAGGCC
TGGCTGGAGCGATACCAGATGCAGAACTACGGGGCCGTG
GATCCGGCGATCCTCAACGGCCTGCGCTCCTCGGAAATGG

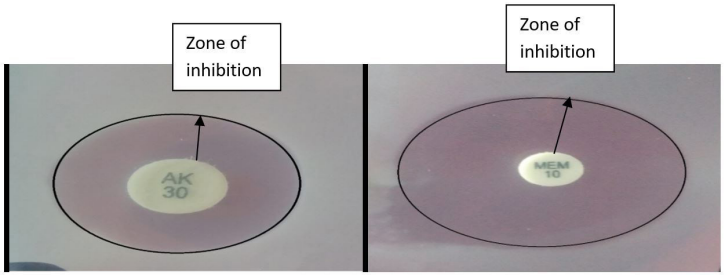

Figure 6. Inhibition zone around Amikacin (AK) and Meropenem MEM.

Table 5. Results of Different Virulence Behavior Assays.

Strains	Lip	Lec	Prot	Pyo	Rham	Bio	Hem
GCU-SG-M_1	+ve	+ve	+ve	Y/G	+ve	+ve	β
GCU-SG-M_2	+ve	-ve	-ve	Y/G	+ve	+ve	β
GCU-SG-M_3	+ve	+ve	+ve	B/G	+ve	+ve	β
GCU-SG-M_4	+ve	-ve	+ve	B/G	+ve	+ve	β
GCU-SG-M_5	+ve	+ve	+ve	Y/G	+ve	+ve	β
GCU-SG-M_6	+ve	+ve	-ve	Y/G	+ve	+ve	β

Note: Lip; Lipase **Lec;** Lecthinase **Prot;** Protease **Pyo;** Pyocyanin **Rham;** Rhamnolipid **Hem;** Hemolysis **Mot;** Motility **Bio;** Biofilm **CR;** Congo red assay **B/G;** Blue green **Y/G;** Yellow green.

Table 6. showing antibiotic discs used in disc diffusion method.

Sr. No.	Name of antibiotic	Symbols	Potency
I.	Fluconazole	FLU	25mcg
II.	Clarithromycin	CLR	15mcg
III.	Amikacin	AK	30mcg
IV.	Cefixime	CFM	5mcg
V.	Penicillin	PIG	10mcg
VI.	Meropenem	MEM	10mcg

Table 7. Universal primers for 16srRNA and Primers for rhamnolipid gene.

1492R Sequence	5'- GGCTACCTTGTTACGACTT -3'
27F Sequence	5'- AGAGTTTGATCCTGGCTCAG-3'
Primers for rhamnolipid gene	
Oligo F Sequence	5'-CTCCCCGGGCAACTCCAAAAG-3'
Oligo R Sequence	5'-AATTTGCTCAGCGTGCTTTC-3'

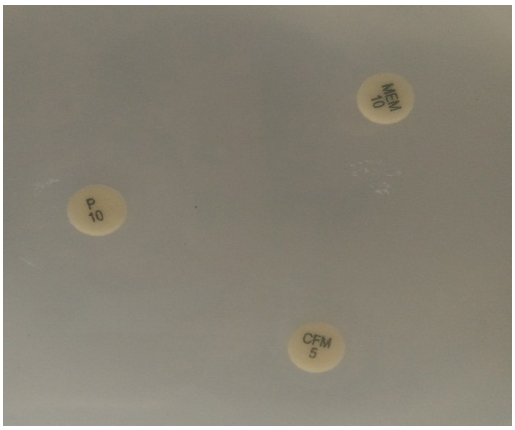

Figure 7. Resistance of *P. aeruginosa* having no inhibition zone around antibiotic disc

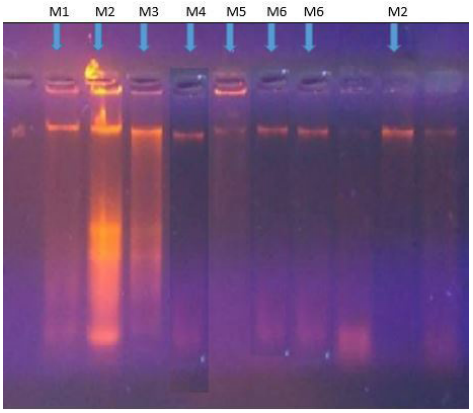

Figure 8. Showing bands of DNA under UV light in Gel Electrophoresis

TGGTCTGGAGCGACAGCCTGTTCGACCAGAGCGGATGCTC
TGGAACGAGGCTCGCGATTGGGGCCTCTGTGTCGGCGCG
ACCCTGCCGATCCGCGCGCCGAACAATTTGCTCAGCGTGC
TTTCCGTGGCGCGCGACCAGCAGAACATCTCCAGCTTCGA
GCGCGAGGAAATCCGCCTGCGGCTGCGTTGCATGATCGAG
TGCTGACCCAGAAGCTGACCGACCTGGAGCATCCGATGCT
GATGTCCAACCCGGTCTGCCTGAGCCATCGCGAACGCGAG
ATCCTGCAATGGACCGCCGACGGCAAGAGCTCCGGGGAA
ATCGCCATCATCCTGAGCATTTCCGAGAGCACGGTGAACT
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CAAGACGCTGGCTGCCACCTACGCCGCGGCGCTGGGCCTC
ATC

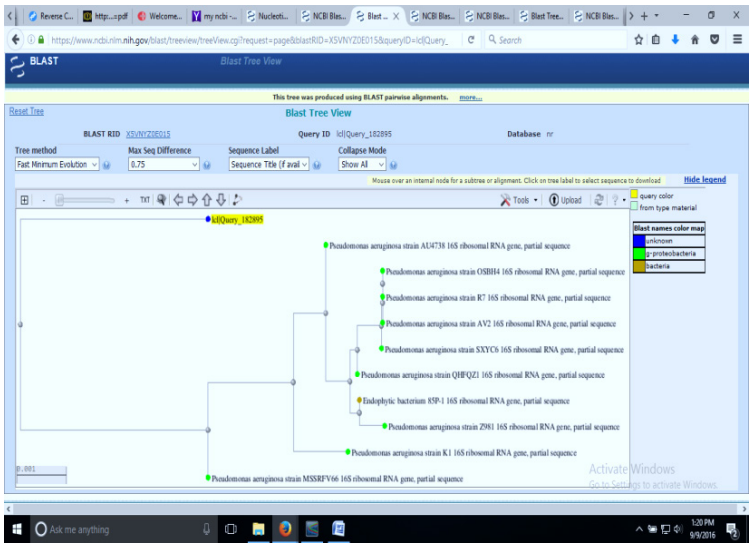

Figure 9. Dendrogram showing similarity of *P. aeruginosa*

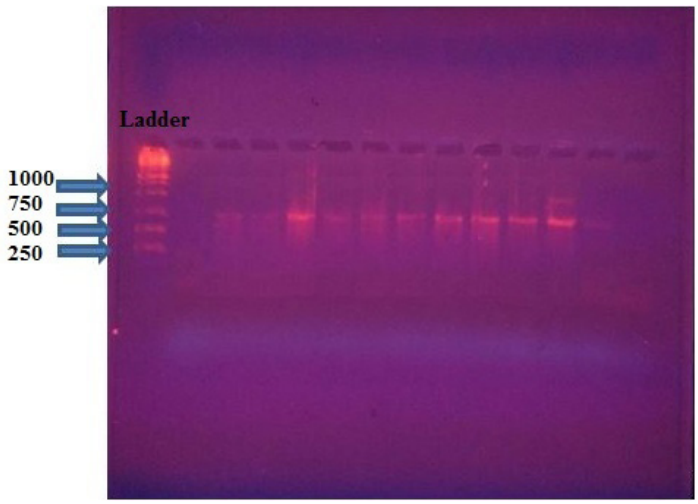

Figure 10. Bands glowing closer to the ladder band with 750 bp are representing PCR products.

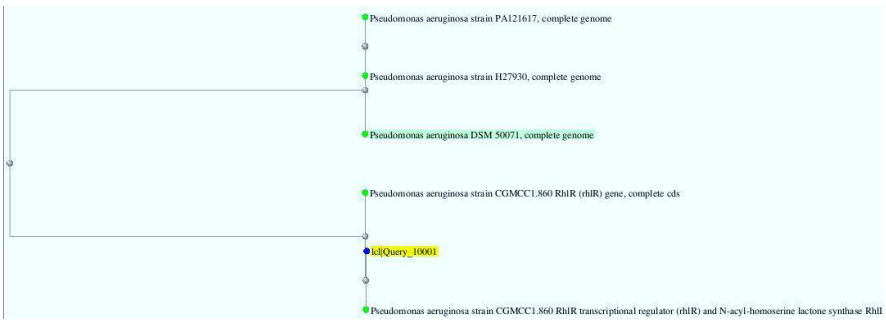

Figure 11. Showing dendrogram of *P. aeruginosa* GCU-SG-M_3.

4. Discussion

Among all pathogenic bacteria the most common is *P. aeruginosa* that is responsible for 57.7% of VAP infections and 53.3% of cystic fibrosis is also caused by this pathogen. Our results are supported by the research work of Kumar et al. (2005) according to which the most common pathogenic bacteria is *P. aeruginosa* (63.5%) which is followed by *Staphylococcus aureus* (33.3%), *Acinobacter lwoffi* (22.6%) in the spread of VAP infections. Similar results had been reported in different studies from other

regions (Arora et al., 2011). Some of the previous studies have shown that the infection of P. aeruginosa are more common in females than males. For example 48% of male individuals and 52% of the female individuals and effected according to Read et al. (1992). Similarly the research work of Khattak *et al*., 2013 have shown that females are at higher risk of infection (54%) than in males (45%). But in present study higher rate of infection is scene in the males (*i.e*. 60%) but in females it is 40%. These results are supported by the previous work of Rashid and Kornberg (2000) and Khattak et al. (2013) that show higher rate of susceptibility in male (56%, 60%) respectively than in females (44%, 40%). This variation can be explained by the difference in climatic condition of various geographic regions of the world. Virulence behavior assays and antibiotic resistance to a wide range of antibiotics have shown that *P. aeruginosa* involved in hospital acquired pneumonia, ventilator acquired pneumonia is highly efficient multi drug resistant pathogenic bacteria. There are a number of genes that play important role in formation of *P. aeruginosa* biofilm (that contributes in its resistant nature), in the production of rhamnolipid as well as in synthesis of pyocyanin. Which are the virulent expressions of these genes in other words (Ochsner et al., 1994). There are undoubtedly number of these type of genes either involved in the reaction of autoinducers or in the secretion of virulent products. The results of all virulent assays of present study supports the results of previous studies of Lee et al. (2006) as *P. aeruginosa* have showed positive results of all virulence assessing assay (Ali et al., 2015). Our findings would help in raising awareness about antibiotic resistance of *P. aeruginosa* associated with pulmonary infections in patients. Arasu et al. (2016) reported that there was a 70.7% susceptibility of *P. aeruginosa* isolates to Meropenem but also concluded Ciprofloxacin was the most active agent (85.4% susceptibility) against *P. aeruginosa* strains along with Amikacin (95.1% susceptibility).

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