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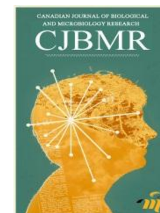

MULTIPLE RESISTANT ACTIVITIES AND MOLECULAR CHARACTERISATION OF *Pseudomonas aeruginosa* ISOLATED FROM TIRUPUR TEXTILE EFFLUENTS

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ABSTRACT

A potential bacterial strains of *Pseudomonas aeruginosa* were isolated and selected from textile effluent discharged parts of Noyyal river, Tirupur, Tamilnadu, India. Virulence factors of *P.aeruginosa* were detected through protease, phospholipase and slime test. Strong slime production was found in two strains; higher protease (13 mm) was found in P2 and P3 strains; positive phospholipase activity were identified in 4 isolates of *P.aeruginosa*. Nucleotide sequences of multiple resistances *P.aeruginosa* were confirmed by 16S r DNA sequencing with universal bacterial primers (16S-UP-F, UP-R) which was deposited in Gene Bank (Accession Number KT175510), and phylogenetic tree was constructed. The sequences showed 100% similarity with the already established the species *P.aeruginosa*. From this study, the isolate was confirmed as multiple resistant *P.aeruginosa* which causes dangerous diseases to human beings and aquatic organisms.

INTRODUCTION

Rapid industrialization and urbanization have resulted in large amount of wastes into the environment causing major pollution problem. Among many pollutants, textile industry effluents are the major source of environmental pollution. Synthetic dyes are widely used in the textile, pharmaceutical, cosmetic, and food industries. However, at least 10–15% of the dyes used in textile processing are released into wastewater [1]. *This* wastewater contains enormous amount of multiple resistant microorganism due to their favorable environment which causes dangerous diseases to humans, fishes and aquatic organisms. In the past few decades, a worldwide is increased in the incidence of multiple resistant bacterial infections especially *Pseudomonas sp* which is mainly due to the frequent use of synthetic chemicals and their frequent use has led to the emergence of resistant strains. Most famous and effective synthetic drugs became ineffective due to the virulence of multiple resistant. The Gram-negative bacterium *Pseudomonas aeruginosa* is an opportunistic, nosocomial pathogen of immune compromised individuals, *P. aeruginosa* typically infects the airway, urinary tract, burns, wounds, and also causes other blood infections in human and also aquatic organisms. Generally, bacterial flora of the fish including *Pseudomonas* reflects the microbial population of the aquatic habitat and is influenced by the bacterial load in the water and salinity.

As *Pseudomonas* species are so wide spread and numerous they may become involved in the disease processes and act as secondary invaders of fish compromised by the pathogens or other factors. *Pseudomonas aeruginosa* being the most abundant form of life on earth, it has been isolated from the environment as diverse as water, jet plane fuel and disinfectant solutions due to its ability to utilise many different organic compounds and survive in the apparent absence of nutrients.

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Most pathogens, including species of *Pseudomonas*, have developed an effective putative virulence factors and specific strategies to assist in their ability to colonize host tissues, cause disease and overcome host defenses. The factor that contributes to the process of virulence is hydrolytic enzyme production, which is known to play a central role in the pathogenicity of bacteria, protozoa, and pathogenic yeasts [2]. Although many microorganisms possess a variety of hydrolytic enzymes, proteases are by far the most commonly associated with virulence. *P.aeruginosa* produces hydrolytic enzymes such as phospholipases, secreted aspartyl proteinases (SAPs) and slime which make the host predisposed to pathogenic attack [3].

The slime production of *P.aeruginosa* is one of the pathogenicity factor which play an important roles in the adherence, colonization on host cells and protects itself from host defences [4].The phospholipase productions in *P.aeruginosa* play an important role in the pathogenicity. Extracellular phospholipases facilitate the ability of organisms to injure, invade, egress from various host cells and remove processed antigens from the surface of antigen present in cells. The level of phospholipase activity secreted by each particular isolate of *P.aeruginosa* is directly correlated with the pathogenicity of the isolate [5]. Among many hydrolytic enzymes produced by *P.aeruginosa*, Secreted aspartyl proteinases are considered to be the most important virulence factors and all of them are involved in pathogenicity [6]. For these reasons, the present study was carried out to evaluate the "Multiple resistant activities and Molecular characterisation of *Pseudomonas aeruginosa* isolated from tirupur textile effluents".

MATERIALS AND METHODS

Bacterial isolates and culture condition

Textile effluent samples were collected from Noyyal river (11°10'N to 11°22'N latitude and 77°21'E to 77°50'E longitude) Tirupur, Tamilnadu, India. A total 10 isolates of *P.aeruginosa*, were isolated from Tirupur textile effluent, in Pseudomonas isolation agar at 37^{0}C. Nutrient broth cultures were maintained at -20 ^{0}C in glycerol till further use.

SCREENING OF VIRULENCE FACTORS IN *P.aeruginosa*

Phospholipase production by *P.aeruginosa*

Price [7] described plate method for the detection of phospholipase activity in *P.aeruginosa* When isolates of *P.aeruginosa* grown on this medium indicating as phospholipase-positive and it forms distinct, well-defined, dense white zone of precipitation around the colony. This white zone is probably due to the formation of calcium complex with the fatty acids released by the action of phospholipase on the phospholipids present in the egg yolk. In this assay, phospholipase activity is defined as the ratio of colony diameter to the diameter of the dense white zone of precipitation around phospholipase positive colonies.

Protease production by *P.aeruginosa*

Protease production of *P.aeruginosa* species was checked on the skim milk agar medium as per method described by Gokce [8]. Skim milk agar medium possesses peptone from casein 5.0; yeast extract 2.5; skim milk powder (no inhibitors) 1.0; glucose 1.0; agar-agar 10.5.and distilled water 1000 ml maintained at pH 5.0. Ten µl samples (suspension) were introduced on a sterile paper disk placed on the surface of skim milk agar medium. The inoculated plates were incubated at 37°C for two days and diameters of zones of inhibition around the disks were measured for determination of protease activity.

Slime production by *P.aeruginosa*

A method of screening slime production was described by Freeman[9]. The composition of brain heart infusion medium (BHIM) contains 37 g/l, glucose 80 g/l, agar 10 g/l, and Congo red 0.8 g/l. The Congo red stain was prepared in aqueous solution which was autoclaved separately at 121 ºC for 15 min and it was added when the agar had cooled to 55 ºC. Plates were inoculated and incubated aerobically at 35 ºC for 48 h. Slime production was evaluated according to the "Congo red phenomenon". Isolates that produced dark red colonies showed as strong slime positive, whereas those shows pink or white colonies are indicating as moderate and light brown colour representing as weak positive slime producer.

GENOTYPIC ANALYSIS OF MULTIPLE RESISTANT ACTIVITY IN *P.aeruginosa* USING PCR ANALYSIS

DNA isolation

Five ml of overnight culture were washed and suspended in TE buffer, PH 8.0. Genomic DNA was isolated as per the method of Schmalenberger [10].

PCR conditions

All the reaction mixtures contained 1X PCR buffer (10 mM Tris HCl, pH-9.0, 50 mM KCl and 0.01% gelatin); 100 mM concentration of each dNTPs and 0.75 unit of Taq polymerase. The final concentration of $MgCl_2$ was adjusted to 1.5 mM in PCR-Ribotypng. In PCR-ribotyping $MgCl_2$ concentration was adjusted to 3 mM. Each primer concentration was 20 pmol for PCR-Ribotypng. PCR-ribotyping was carried out according to the method of Franciosa .The primers for the amplification of DNA spacer regions between the 16S-5S genes were F (50 -TTG TAC ACA CCG CCC GTC A-30) and R (50 -GCT TAA CTT CCG TGT TCG GTA TGG G-30). The amplification was carried out by after initial denaturation at 94 1C for 2 min, followed by 35 cycles at 94 1C for 1 min, 35 1C for 1 min and 72 1C for 2.5 min, with a ram time of 2 min between 35 and 72 1C; a final extension was performed at 72 1C for 5 min.

Agarose gel electrophoresis

The PCR products (10 ml) were separated by electrophoresis on a 1.5% agarose gel containing ethidium bromide (0.5 mg/ml) at 100 V for 1 h. in 1X TBE (Tris-Boric acid-EDTA) buffer. The gel images were digitized through UV gel image acquisition camera (Kodak, Japan).

16S r DNA sequencing analysis

The PCR amplified DNA was sequenced in ABI 3730 DNA Analyzer (Applied Biosystems). The sequence quality was checked using Sequence Scanner Software v1 (Applied Biosystems). Sequence alignment required editing of the obtained sequences were carried out using Geneious Pro v5.

Phylogenetic analysis

The 16s rDNA sequences of P2 isolates aligned with the sequences of similar species which were retrieved from the Gene bank database. The sequences were converted in to FASTA format. The retrieved sequences were fed with ClustalW for multiple sequence alignment to observe sequence homology. Evolutionary tree was inferred by using the neighbour-joining method [11]. Dendrogram was constructed by neighbour-joining method using PHYLIP software package.

RESULTS

Screening of virulence factors of *P.aeruginosa*

Phospholipase production by *P.aeruginosa*

All 10 isolates of *P.aeruginosa* were responsible for invasive diseases and were able to grow on egg yolk agar plates (Table.1and Fig.1). Among these isolates, six isolates of *P.aeruginosa* were negative and remaining four isolates were positive producing Phospholipase production. Among the positive isolates, P2 and P10 were significantly scored more than P7 and P8.

Protease production by *P.aeruginosa*

Protease production by *P.aeruginosa* on skim milk agar medium was observed (Fig.1). The higher protease (13 mm) production was found in P2 and P3 isolates and moderate protease (10 mm) was detected in P1, P6 and P9 isolates. However, the lowest protease production was observed in P7 and P10 (Table 1).

Slime production by *P.aeruginosa*

Isolates of *P.aeruginosa* showed the different ranges of slime production on brain heart infusion agar medium supplemented with Congo red (Fig.1). Slime production was demonstrated in all of *P.aeruginosa* isolates, (Table.1). Strong slime production was found in two isolates such as P2 and P4 which appeared as thick black colour. White layer with black bubble formation exhibited in two isolates (P6 and P9) indicating as moderate and one isolates (P5) showed light brown colour representing as weak positive slime producer.

16S r DNA sequencing analysis

After the determination of colony colour, the morphological, physiological and biochemical characteristics, the isolate selected by the PCR analysis was tentatively identified as *P.aeruginosa* and it was confirmed by the 16S r DNA sequencing with help of universal bacterial primers, 16s-UP-F and 16s-UP-R. Sequence of the *P.aeruginosa* isolate showed partial 16S r DNA sequences, consisting of 1258 nucleotides which was submitted to the Gene bank (National Center for Biotechnology Information, USA) and an Accession Number (KT175510) was obtained. Phylogenetic tree was deduced from species of *P.aeruginosa* using Neighbour-joining method. The sequences showed 100% similarity with the already established the species *P.aeruginosa.* From this study, the isolate was confirmed as the isolate of the species *P.aeruginosa.*

Table 1. Virulence factors of *P.aeruginosa* identified by Slime, Phospholipase and Protease tests

S.No.	Isolates No	Virulence Factors		
		Slime test	Phospholipase test (mm)	Protease test (mm)
1.	P 1	-	-	10
2.	P 2	S+	7	13
3.	P 3	-	-	13
4.	P4	S+	-	12
5.	P 5	W+	-	11
6.	P 6	M+	-	10
7.	P 7	-	6	8
8.	P 8	-	6	9
9.	P 9	M+	-	10
10.	P 10	-	9	8

P **1-** P **10 - Isolates of *P.aeruginosa;***

S+- strong, M+- Moderate, W+-weak;

Figure 1. Virulence factors of *P.aeruginosa*

Phospholipase activity of *P.aeruginosa*

Protease production by *P.aeruginosa*

Slime production by *P.aeruginosa*

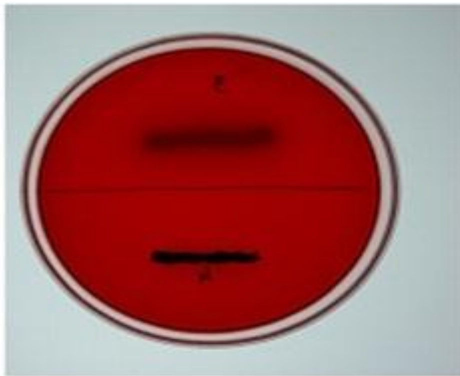
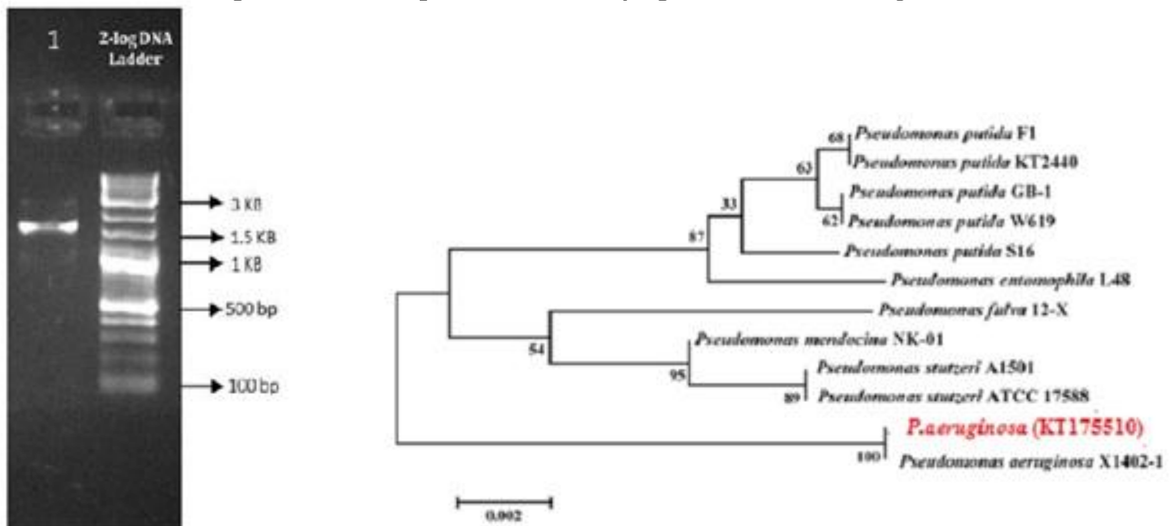


Figure 2. PCR Amplification and Phylogenetic tree of *P.aeruginosa*



DISCUSSION

The pathogenicity of *P.aeruginosa* has been attributed to various structures, most of which are situated on its surface. The high morbidity and mortality caused by this microorganism are still poorly understood, and the list of virulence factors is probably far from complete. One group of factors, such as the capsule and a recently identified protein[12] provides resistance to phagocytosis and thus promotes the escape of *P.aeruginosa* from the host immune defense. Other factors, including cell wall components and the intracellular toxin autolysin. This autolysin produced enzyme like protease, hydrolyase, amylase, and phospholipases which involved mainly in the inflammation caused by infection. The inflammation process fully develops only after lysis of bacteria by autolysin. Since inflammation is thought to induce most of the symptoms of Pseudomonas disease [13], this group of virulence factors directly responsible for the morbidity and mortality caused by *P.aeruginosa*. Pseudomonas enzymes, such as autolysin, neuraminidase, hyaluronidase and IgA1 protease, have also been suggested to play a role in the pathogenesis of Pseudomonas disease. [14].

Extracellular Phospholipases facilitate the ability of organisms to injure, invade the tissue of various host cells [15]. In the present study, all 10 isolates of *P.aeruginosa* were responsible for invasive diseases and were able to grow on egg yolk agar plates (Table 1 and Fig 1). Among these isolates, six isolates of *P.aeruginosa* were negative and remaining four isolates were positive producing Phospholipase production. Among the positive isolates, P2 and P10 were significantly scored more than P7 and P8.

Besides, in the present study it was found that the enhanced protease production by isolates of *P.aeruginosa* using skim milk agar medium is corresponding to the development of new multiple drug resistance activity. Protease production by *P.aeruginosa* on skim milk agar medium was observed. The higher protease (13 mm) production was found in P2 and P3 isolates and moderate protease (10 mm) was detected in P1, P6 and P9 isolates. However, the lowest protease productions was observed in P7 and P10. Biofilms are structured populations of microorganisms adhered to a surface (or interface) and embedded in an extracellular matrix consisting mainly of exopolysaccharides (sometimes bound together by proteins and DNA) [23]. In nature, these communities can be mono or more frequently, multispecific [16] and show a modified phenotype in terms of their growth rate and different gene expression patterns. Biofilms have been likened in their level of organization to a eukaryotic organism, undermining the frontier between the biology of eukaryotes and prokaryotes [17]. The polysaccharide surrounding the biofilm is frequently composed of one or more anionic uronic acids. Over 60% of bacterial infections (and up to 80% of chronic infections) are currently considered to involve microbial growth in biofilms. This peculiar form of life poses an array of problems in human clinical practice, from infections associated with the implant of prosthetic devices and dental plaque formation to diseases such as cystic fibrosis, otitis media, and endocarditis [18] Biofilms are also produced by *P.aeruginosa* and this bacterium often colonizes the upper airways in humans as a normal commensal, yet it may spread to other areas of the body, causing otitis media, pneumonia, or invasive diseases such as bacteremia and meningitis. The capacity of *P.aeruginosa* to form biofilms had not been explored until recently. Several newly developed invitro systems have allowed to test the capacity of *P.aeruginosa* to form biofilms, and to analyze the influence of several factors, including DNA and proteins which play a role in the virulence of this “supergerm” in the formation and development of biofilms [19].

In the present study, isolates of *P.aeruginosa* showed the different ranges of slime production on brain heart infusion agar medium supplemented with Congo red (Fig 1). Slime production was demonstrated in all of *P.aeruginosa* isolates, (Table 1). Strong slime production was found in two isolates such as P2 and P4 which appeared as thick black colour. White layer with black bubble formation exhibited in two isolates (P6 and P9) indicating as moderate and one isolates (P5) showed light brown colour representing as weak positive slime producer.

Multiple resistant *P.aeruginosa* infection is a life-threatening disease; however, it is proven that early diagnosis improves clinical outcomes. Recently developed molecular methods could allow an early and make it possible to achieve the accurate etiological diagnosis of bacterial infection [20]. PCR tests are available as diagnostic procedures for specific organisms (such as *P. aeruginosa, N. meningitidis*, *H. influenzae*) [21]. The use of broad-range bacterial PCR could help to improve our knowledge of the etiological spectrum of bacterial meningitis, allowing the detection of bacteria infrequently cultivated, or not yet recognized as causative agents of meningitis [22]. Amplification using 16S rDNA primers has seen various applications,. Recently it has been proposed as a strategy for the diagnosis of culture-negative bacterial meningitis and applied in daily microbiological practice [23]. Commercial tests are available for the identification of bacterial species based on the nucleic acid sequences of 16S rRNA [22]. The Micro Seq 500 16S ribosomal DNA (rDNA) based bacterial identification system (Applied Biosystems Division) has been marketed for rapid and accurate identification of bacterial pathogens: the first 500-bp of the 16S rRNA gene of the bacterial strain are amplified, sequenced and analysed using the database provided by the system or, alternatively, public databases such as Gene Bank, NCBI and further the organism was confirmed by phylogenetic tree [24]. Similarly, in the present study, 16S r DNA sequencing of *P.aeruginosa* done by universal bacterial primers, 16s-UP-F, R4 these universal bacterial primers were amplified with template DNA yielded bands at 1.6bp which were specific

for the strain of *P.aeruginosa.* In this study, DNA was isolated from the P2 isolates and its quality was checked by loading in 1% agarose gel with the Lambda DNA Hind III digest DNA marker which showed the intact DNA. 16S r DNA region of the P2 isolate was amplified through PCR which showed the molecular weight of 1.6 kb corresponding to that of the 1.5kb DNA ladder in 1% agarose gel (Fig.2) and this positive amplification of 1500 kb specific for the 16S r DNA regions of *P.aeruginosa* genes .The amplified product was purified to remove the excess primer for sequencing and it was sequenced using the automated DNA sequencer. Sequence of the P2 isolate showed partial 16S r DNA sequences, consisting of 1258 nucleotides (Figs. 2) which was submitted to the Gene bank (National Center for Biotechnology Information, USA) and an Accession Number (KT175510) was obtained.

Further, Phylogenetic tree was deduced from species of *P.aeruginosa* using Neighbour-joining method. The dendrogram placed the P2 isolate in a separate line of descent within the genus *P.aeruginosa* representing a distinct phylogenetic lineage. The P2 isolate of multiple resistant *P.aeruginosa* showed 100% similarity with the already established the species *P.aeruginosa.* From this study, P2 isolate was confirmed as the isolate of the species *P.aeruginosa*.

CONCLUSION

Multiple Resistance *P.aeruginosa* possess serious challenges and issues to combating infectious diseases in human and aquatic organisms. Environmental pollution is the major source for Multiple Resistant *P.aeruginosa*. Resources against multidrug-resistant pathogenic infections are now limited and identification of that organisms is very complicated. Further investigation is required indeed. Therefore the present study explained that *Pseudomonas aeruginosa* is naturally resistant to many antibacterial drugs and it was arise from textile industry effluents and Synthetic dyes. Multiple Resistance *Pseudomonas aeruginosa* was identified through Phospholipase Protease and slime test. These strains are morphologically similar but genetically different and it was confirmed through 16S rDNA gene sequence. Further the phylogenetic relationship was constructed using 16S rDNA gene sequences which were amplified by new set of primer pairs. Designing of new primers will help to identify Multiple Resistance *P.aeruginosa* at molecular level in future.

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CONFLICT OF INTEREST:
The authors declare that they have no conflict of interest.

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