International Journal of Advanced Research in Biological Sciences
ISSN: 2348-8069
www.ijarbs.com

Research Article

Molecular characterization of *Proteus mirabilis* and *Pseudomonas aeruginosa* isolates from catheter biofilm

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Abstract

Objective: Molecular characterization will help to identify the organism and also to find out the change in the sequence that promoted antibiotic resistance. **Methods:** The bacterial strains were isolated from urinary tract catheter biofilm (*P. mirabilis*), The DNA bands were observed on gel doc imaging system. The obtained genomic DNA was taken for sequencing studies, the molecular characterizations of the isolates were determined by 16S rRNA profile analysis method. The fragments were observed in Agarose gel electrophoresis and The BLAST database was analysed in National Centre for Biotechnology. **Results:** A GenBank (Eztaxon) BLAST search was performed for each of the sequences for the identification of the isolates. **Conclusion:** The gene sequencing study confirmed that identify of *P. mirabilis* and *P. aeruginosa* and validated the biochemical and morphological evaluation.

Keywords: Intrauterine devices (IUDs), Copper-T and cervical swab, Cystine lactose electrolyte deficient agar medium (CLED), microtitre plate (MTP), SEM, *Proteus mirabilis.*

Introduction

The rRNA is the most conserved gene in all cells. Portions of the rDNA sequence from distantly related organism are remarkable similar. This means that sequences from distantly related organisms can be precisely aligned, making the true differences easy to measure. For this reason, genes that encode the rRNA (rDNA) have been used extensively to determine taxonomy, phylogeny and to estimate rates of species divergence among bacteria. Thus the comparison of 16S rDNA sequence can show evolutionary relatedness among microorganisms.

In Bacteria, Archaea, Mitochondria, and Chloroplasts the small ribosomal subunit contains the 16S rRNA (where the S in 16S represents Svedberg units). The large ribosomal subunit contains the rRNA species (the 5S and 23S rRNA). Bacterial 16S, 23S, and 5S rRNA genes are typically organized as a co-transcribed operon, there may be one or more copies of the operon dispersed in the genome (for example, *P. mirabilis*). The Archaea contains either a single rDNA operon or multiple copies of the operon.

To infer relationships that span the diversity of known life, it is necessary to look at genes conserved through the billions of years of evolutionary divergence. An example of genes in this category is those that define the ribosomal RNAs (rRNA). Most prokaryotes have three rRNA, called the 5S, 16S and 23S rRNA. The 5S has been extensively studied, but it is usually too small for reliable Phylogenetic interference. The 16S and 23S rRNA is sufficiently large to be useful. The 16S rDNA sequence has hyper variable regions, where sequences have diverged over evolutionary time; these

are often flanked by strongly conserved regions. Primers are designed to bind to conserved regions and amplify variable regions. The DNA sequence of the 16S rDNA gene has been determined for an extremely large number of species. In fact, there is no other gene that has been as well characterized in as many species. Sequences from tens of thousands of clinical and environmental isolates are available over the internet through the National Centre for Biotechnology Information and the Ribosomal Database project. These sites also provide search algorithms to compare new sequences to their database.

The extraordinary conservation of rRNA genes can be seen in these fragments of the small sub unit (16S) rRNA gene sequences from organisms spanning the known diversity of life. All of the available molecular methods for evaluating Phylogenetic relationships (e.g., DNA and rRNA hybridization, 5S rRNA and protein sequencing, 16S rRNA oligonucleotide cataloguing, enzymological patterning, etc.) have advantages and limitations. In general, macromolecular sequences seem preferred because they permit quantitative inference of relationships (Lloyd *et al.,* 2007). Moreover, because they accumulate, sequences are most useful in the long term. Of the macromolecules used for Phylogenetic analysis, the ribosomal RNAs, particularly 16S rRNA, is found to be the most useful for establishing distant relationships because of their high information content, conservative nature, and universal distribution (Miller *et al.,* 2000). The principle of using rRNA sequences to characterize microorganisms has now gained wide acceptance (Murray *et al.,* 1984), and its general application can be anticipated if methods for determining rRNA sequences can be simplified. The approach described here rapidly provides partial sequences of 16S rRNA that are useful for Phylogenetic analysis. In the present study two major bacterial isolates were subjected to rRNA study. Molecular characterization will help to identify the organism and also to find out the change in the sequence that promoted antibiotic resistance.

Materials and Methods

Bacterial strain and cultivation

The bacterial strains were isolated from urinary tract catheter biofilm (*P. mirabilis*) collected from Cuddalore Government Hospital, Cuddalore, and Tamilnadu. The strain was maintained as freeze-dried (-20°C) stock culture. The bacterium was grown in 50% strength Tryptic Soya Broth (15g/k, TSB) by shaking (120-rev min^{-1}) at 20°C for 48 hours.

Isolation of DNA

Nutrient broth was prepared and sterilized. The bacterial isolates from catheter (*Proteus mirabilis*) biofilm were inoculated and incubated in a shaking incubator for overnight. A small amount of culture broth was centrifuged at 13,000 rpm for 5 minutes. The supernatant was discarded and 200µl of Tris 0.1mM, 200µl of lysis solution 1% sodium dodecyl sulphate (SDS) and sodium hydroxide (NaOH) were added to the pellet. The suspension was mixed in vortex and deproteinazed with 700ml phenol, chloroform, isoamyl alcohol (25:24:1v/v/v), homogenized and centrifuged 10 minutes at 13,000 rpm for 10 minutes. The DNA was precipitated by addition of 700µl of ice cold 95% ethanol and spinned. Then it was washed in 70% ethanol and centrifuged 10 minutes at 10,000 rpm. The supernatant was discarded. The pellet was dried in room temperature, suspended in TE buffer and stored in refrigerator.

Separation of genomic DNA by agarose gel electrophoresis

The pellet stored in TE buffer was prepared. One µl of ethidium bromide stain was incorporated into the gel. The gel casting tray was sealed on both sides with tape and agarose was poured into the tray. The comb was placed in the gel and allowed for solidification. After solidification, the comb and the tape were removed. The gel tray was placed in the electrophoresis tank and TE buffer was pored over to cover the gel. 3µl bromophenol blue (tracking dye) and 7µl of DNA sample were mixed well. Then the samples were loaded into the wells using micropipette. The power was switched on and the gel was run at 50V. The power was switched off when the tracking dye reached three fourth of the gel. The DNA bands were observed on gel doc imaging system. The obtained genomic DNA was taken for sequencing studies (Fig 1.3).

Molecular characterization

The molecular characterizations of the isolates were determined by 16S rRNA profile analysis method. The

fragments were observed in Agarose gel electrophoresis.

16S rRNA sequencing

Amplification of 16S rRNA gene of bacterial isolates using universal primer had been carried out. A large fragment of the 16S rRNA gene was amplified using primers FD1- AGAGTTTGATCCTGGCTCAG and RP2- ACGGCTACCTTGTTACGACTT. The PCR master mixture-genei (100µl) reaction contained 4µl 10ng bacterial DNA and 4 U of Taq DNA polymerase and 1µm of each specific primer. The PCR amplification program consisted of one cycle of 94°C for 5 min, then 30 cycles of 94°C for 20S, 57°C for 20S, and 72°C for 30S, and one cycle of 72°C for 5 min. Amplification products were separated on a 1.0 % agarose gel with ethidium bromide in 1X TBE buffer. Products were purified using DNA purification kit and sequenced.

The size range of the PCR products was around 1.4kp. A GenBank (Eztaxon) BLAST search was performed for each of the sequences for the identification of the isolates. The 16S rRNA gene sequences were deposited in GenBank.

Determination of phylogenetic relationship - BLAST analyses

The BLAST database was analysed in National Centre for Biotechnology. The results were compared with the resolved sequence of the strain with known 16S rRNA sequences. Determination of Phylogenetic relationships was analyzed by the program Phylogenetic Analysis Using Parsimony (PAUP) version 4.0b1 of Macintosh (Swofford, 1993). The robustness of the internal branches of the trees was estimated by bootstrap analyses using 1000 replications in a heuristic search with random stepwise addition (3 replications) (Miller *et al.,* 1990). Bootstrap majority-rule (> 50%) consensus trees were obtained.

Results and Discussion

Restriction fragment analysis of PCR amplified 16S rRNA gene was used to classify the bacterial strain. The traditional taxonomic methods based on morphological physiological, and biochemical characters are now accompanied by DNA based methods like DNA-DNA hybridization and sequencing of 16S rRNA genes (Grimont *et al.,* 1996; Hartung, 1998).

The analysis of the 16S rRNA gene sequences data for the strain isolated from catheter biofilms, *P. mirabilis*, and *P. aeruginosa* were to support a meaningful Pairwise analysis and construction of a Phylogenetic tree. The genetic relationships between the strain of biofilms and known members of other species of *Staphylococcus* genus were estimated by Pairwise analysis (Swofford, 1993; Altschul *et al.,* 1997) using historic search with TBR branch swapping (100 replicates). The bootstrap analyses were run with TBR MULPARS and 1000 replicates. Nine equally Pairwise similarity trees, which showed few differences in topology analysis is shown in (Fig 1.1&1.2). These findings support further taxonomic analysis of the isolates by sequencing of the full 16S rRNA gene by DNA/DNA hybridization and or by PCR analysis of other genes, preferably from noncoding DNA region.

From the repeat profile, 16 repeats were categorized at the nucleotide level. The size range of the PCR products was around 1.4kp. A GenBank (Eztaxon) BLAST search was performed for each of the sequences for the identification of the isolates. The 16S rRNA gene sequences were deposited in GenBank (JX857537 and JX857538). On BLAST search sequences from patterns sample I showed maximum similarity to *P. mirabilis* (Table 1.1) strain collected from MTCC culture. The sample II showed maximum variations and the isolates were *P. aeruginosa* (Table 1.2).

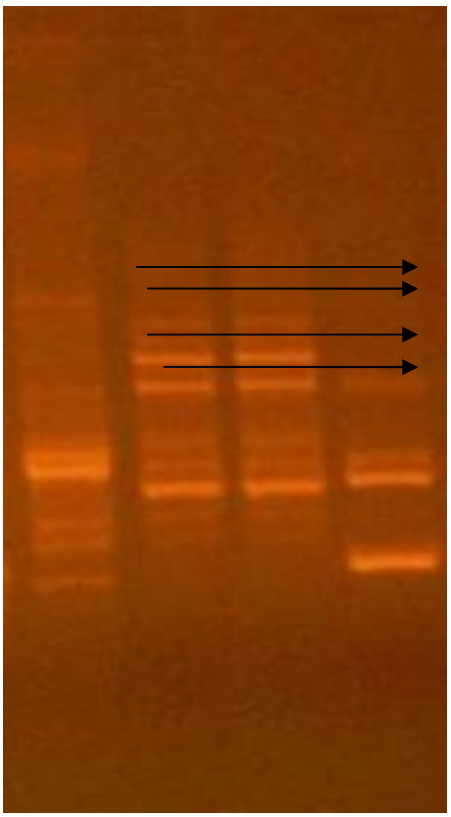
The gene sequencing study confirmed that identify of *P. mirabilis* and *P. aeruginosa* and validated the biochemical and morphological evaluation.

ATTAGCTAGTAGGTGGGGTAAAGGCTCACCTAGGCGACGATCTCTAGCTGGTCTGAGAGGATGATCAGCCACACT
GGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGC
AGCCATGCCGCGTGTATGAAGAAGGCCTTAGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGTGATAAGGTTAAT
ACCCTTGTCAATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGG
TGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCAATTAAGTCAGATGTGAAAGCCCCGAG
CTTAACTTGGGAATTGCATCTGAAACTGGTTGGCTAGATTCTTGTAGAGGGGGGTAGAATTCCATGTGTAGCGGTG
AAATGCGTAGAGATGTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAA
AGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCTGTAAACGATGTCGATTTAGAGGTTGTGGTCTT
GAACCGTGGCTTCTGGAGCTAACGCGTTAAATCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGA
ATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTAAATTCGATGCAACGCGAAGAACCTTACCTACTCTTG
ACATCCAGCGAATCCTTTAGAGATAGAGGAGTGCCTTCGGGAACGCTGAGACAGGTGCTGCATGGCTGT

Sequencing of *Pseudomonas aeruginosa*

GCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCG
TAGGTGGTTCAGCAAGTTGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCCAAAACTACTGAGCTAGAGT
ACGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGC
GACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCA
CGCCGTAAACGATGTCGACTAGCCGTTGGGATCCTTGAGATCTTAGTGGCGCAGCTAACGCGATAAGTCGACCGCC
TGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTA
ATTCGAAGCAACGCGAAGAACCTTACCTGGCCTTGACATGCTGAGAACTTTCCAGAGATGGATTGGTCCTTCGGGA
ACTCAGACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGGCCCGTA

Figure: 1.3 Agarose gel electrophoresis pattern of 16S rDNA from the biofilm forming bacterial isolates



C-Control, PM-*Proteus mirabilis*, PA- *Pseudomonas aeruginosa*

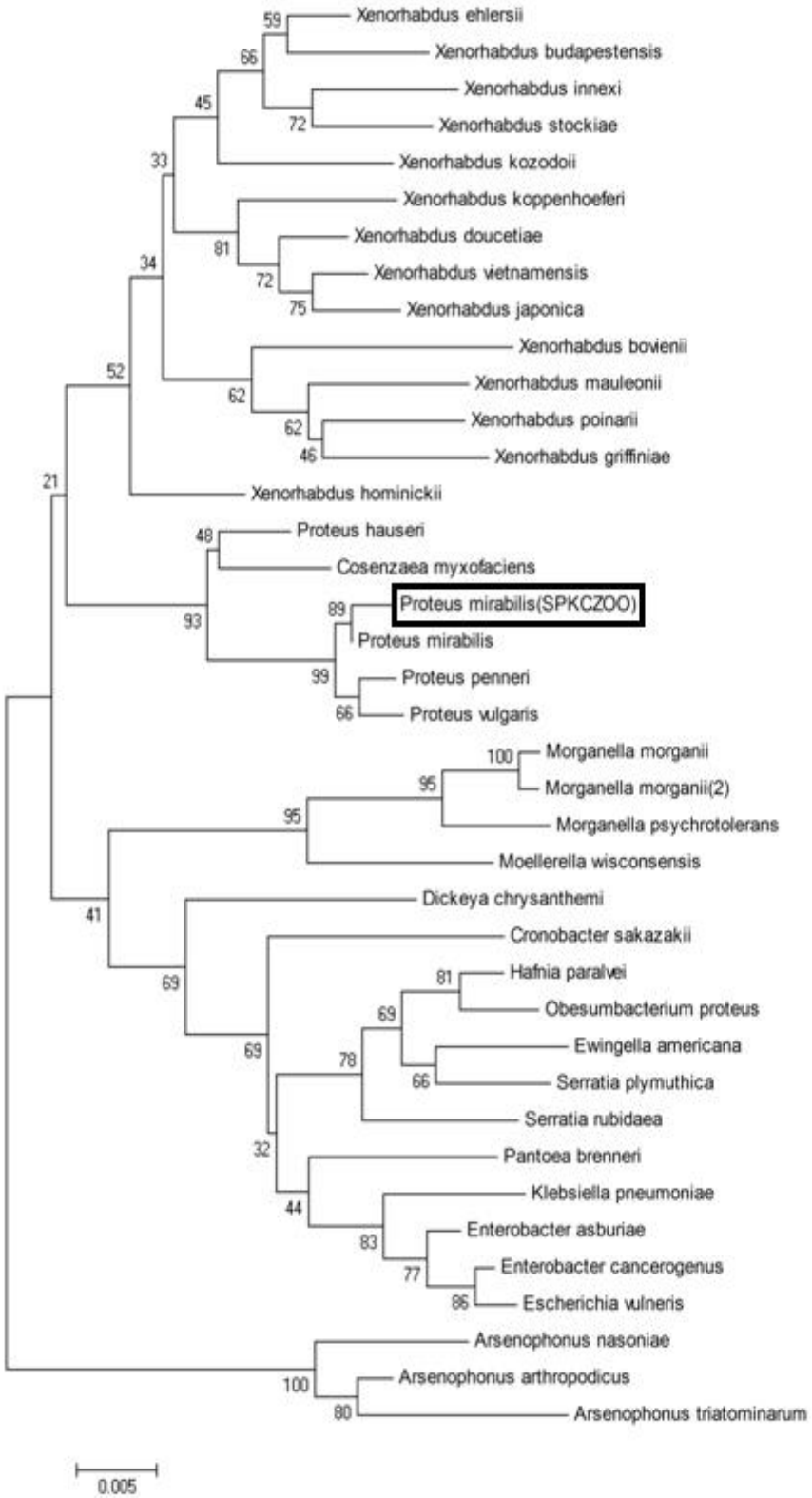


Figure: 1.1 The BLAST database of National Centre for Biotechnology Information (NCBI) was used to compare resolved sequence of the *Proteus mirabilis* (SPKC ZOO) strain with known 16S rRNA sequences. The Phylogenetic tree was reconstructed for the strain isolated from catheter biofilms.

Phylogeny tracing

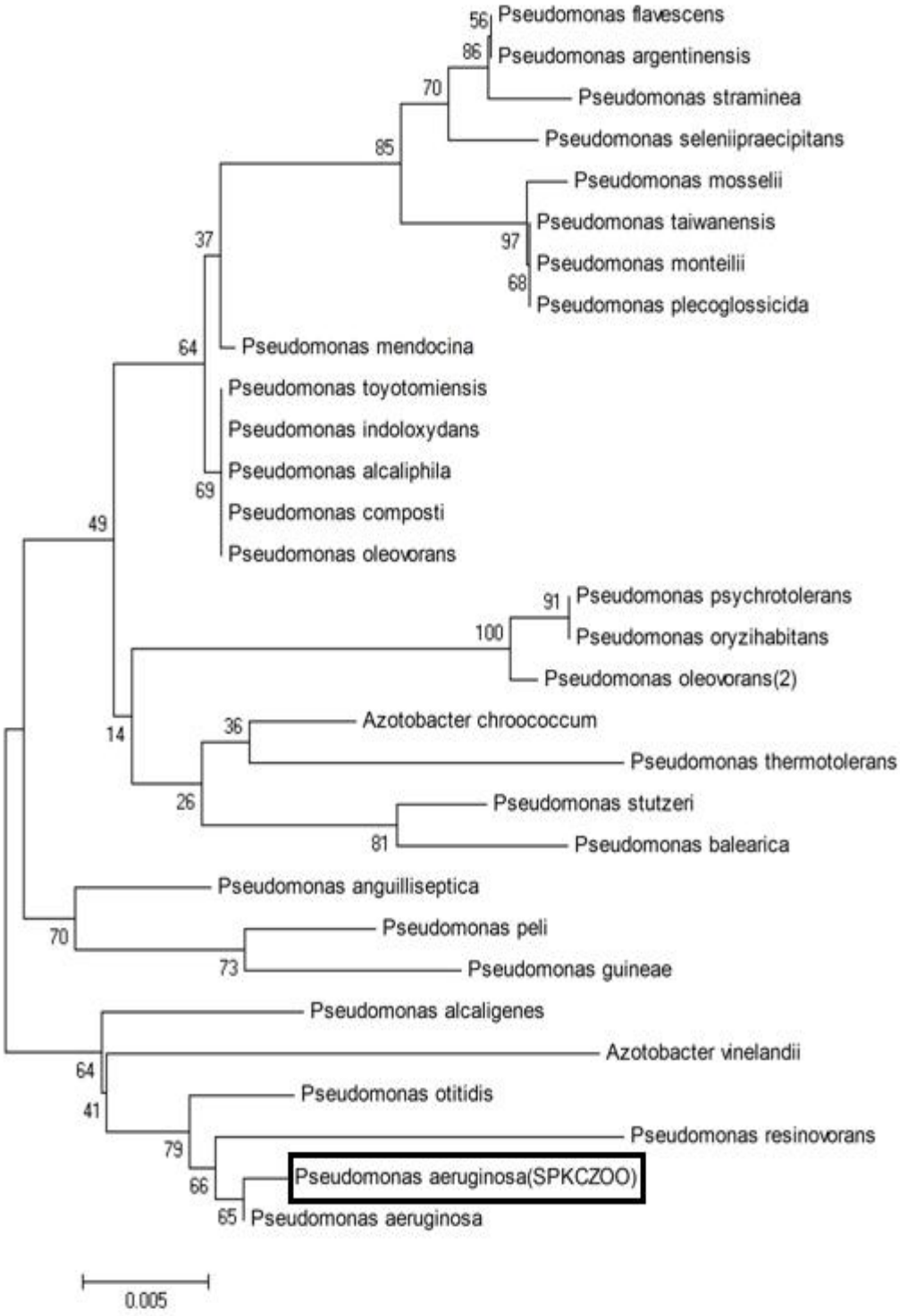


Figure: 1.2 The BLAST database of National Centre for Biotechnology Information (NCBI) was used to compare resolved sequence of the *Pseudomonas aeruginosa* (SPKC ZOO) *s*train with known 16S rRNA sequences. The Phylogenetic tree was reconstructed for the strain isolated from catheter biofilms.

Table: 1.1 BLAST database analyses for *Proteus mirabilis*

Rank	Name/Title	Authors	Strain	Accession	Pairwise Similarity	Diff/Total nt	mega BLAST score	BLASTN score
1	Proteus vulgarise	Hauser 1885	ATCC 29905(T)	DQ885257	99.756	2/820	1610	1610
2	Proteus penneri	Hickman et al. 1983	NCTC 12737(T)	DQ885258	99.268	6/820	1578	1578
3	**Proteus mirabilis**	**SPKC ZOO 2012**	**Biofilm isolates**	**JX857537**	**89.268**	**6/820**	**1578**	**1578**
4	Cosenzaea myxofaciens	(Cosenza and Podgwaite 1966) Giammanco et al. 2011	NCIMB 13273(T)	DQ885259	98.413	13/819	1516	1516
5	Proteus hauseri	O'Hara et al. 2000	DSM 14437(T)	FR733709	98.293	14/820	1514	1515
6	Xenorhabdus hominickii	Tailliez et al. 2006	KE01(T)	DQ211719	97.433	21/818	1441	1437
7	Xenorhabdus vietnamensis	Tailliez et al. 2010	VN01(T)	DQ205447	96.829	26/820	1419	1419
8	Xenorhabdus ehlersii	Lengyel et al. 2005	DSM 16337(T)	AJ810294	96.829	26/820	1419	1419
9	Xenorhabdus kozodoii	Tailliez et al. 2006	SaV(T)	DQ211716	96.581	28/819	1401	1388
10	Xenorhabdus koppenhoeferi	Tailliez et al. 2006	USNJ01(T)	DQ205450	96.463	29/820	1396	1396

Table: 1.2 BLAST database analyses for *Pseudomonas aeruginosa*

Rank	Name/Title	Authors	Strain	Accession	Pairwise Similarity	Diff/Total nt	mega BLAST score	BLASTN score
1	**Pseudomonas aeruginosa**	**(SPKC ZOO 2012)**	**Biofilm isolates**	**JX857538**	**99.832**	**1/596**	**1166**	**1160**
2	Pseudomonas otitidis	Clark et al. 2006	MCC10330(T)	AY953147	99.161	5/596	1134	1128
3	Pseudomonas alcaligenes	Monias 1928	LMG 1224(T)	Z76653	98.826	7/596	1118	1112
4	Pseudomonas anguilliseptica	Wakabayashi and Egusa 1972	NCIMB 1949(T)	X99540	97.819	13/596	1070	1065
5	Pseudomonas mendocina	Palleroni 1970	LMG 1223(T)	Z76664	97.808	13/593	1055	1053
6	Pseudomonas resinovorans	Delaporte et al. 1961	LMG 2274(T)	Z76668	97.797	13/590	1039	1037
7	Pseudomonas toyotomiensis	Hirota et al. (in press)	HT-3(T)	AB453701	97.651	14/596	1057	1043
8	Pseudomonas alcaliphila	Yumoto et al. 2001	AL15-21(T)	AB030583	97.651	14/596	1062	1057
9	Pseudomonas composti	Gibello et al. (in press)	C2(T)	FN429930	97.651	14/596	1062	1057
10	Pseudomonas indoloxydans	Manickam et al. 2008 (invalid)	IPL-1(T)	DQ916277	97.651	14/596	1062	1057

Acknowledgement

Authors are thankful to Dr. A.J.A.Ranjit singh, Principal, Sri Paramakalyani College, Alwarkurichi, Tirunelveli, Tamilnadu, lab facilities and encouragement. We thankful for supporting agency of Department of Biotechnology, Govt of India and New Delhi.

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