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Detection, identification and characterization of pathogenic bacteria from wound infection

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Abstract
This study was aimed to isolate & identify the pathogenic bacteria from collected wound samples by selective culture medium, standard biochemical tests and 16s rRNA sequencing. Purulent materials from different wound infection were collected aseptically with the aid of sterile swab sticks from 100 out of 25 patients of Namakkal district. Seven types of bacterial species were isolated and identified by selective culture medium and standard biochemical tests from the collected wound samples. 16S rRNA gene PCR and subsequent were applied to 4 bacterial wound isolates after enrichment by automated culture. Each wound samples showed one or more bacterial isolate, totally 78.9% of samples exhibited 43 isolates. The most predominant bacterial isolate was *Staphylococcus aureus* (36%) followed by *Pseudomonas aeruginosa* (18%), *Klebsiella pneumoniae* (14%) and *Escherichia coli* (11%) from 100 different wound samples were identified and characterized 16S ribosomal RNA gene sequencing. *Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae* and *Pseudomoas aeruginosa* isolates were characterized by sequencing process. The results were confirmed the identity of the isolates. All the clinical isolates were 100% similarity with respective species. 16S rRNA gene sequencing allowed bacterial identification that was reproducible and more accurate then that obtained by phenotypic testing. 16S rRNA gene sequencing analysis would be helpful in timely and correct identification of pathogens.

Keywords: Wound pathogens, 16S rRNA gene sequencing, PCR, Phylogenetic Tree

Introduction
A wound infection is defined by the US Centre for Disease Control and Prevention (CDC) as surgical site infection (SSI). Surgical site infections do not include stitch abscesses, episiotomy infections, new-born circumcision scars, or infected burn wounds. Wounds have been defined as a disruption of normal anatomical structure and more importantly function. Therefore, healing is the complex and dynamic process that results in the restoration of anatomical continuity and function. The most common causative organisms associated with wound infections include *Staphylococcus aureus*, *Streptococcus pyogenes*, *Enterococci*, *E.coli* and *Pseudomonas aeruginosa*. Louis Pasteur proved that bacteria did not spontaneously generate but were introduced into wounds from a foreign source (Cohen, 1998) [6].

The human skin and mucous membranes represent a diverse environment of bacteria, the normal microfloras (Sewell *et al.*, 1994) [17]. Probably the most important bacteria of this microflora are members of the genus *Staphylococcus*. The genus *Staphylococcus* is currently divided in 38 species and 17 subspecies, half of which are indigenous to humans (Kloos & Bannerman, 1998) [9]. *Staphylococcus* generally has a benign or symbiotic relationship with their host. However, they may develop into a pathogen if they gain entry into the host tissue through trauma of the cutaneous. Barrier, inoculation by needles, or implantation of medical devices.

Staphylococcus aureus is one of the major causes of wide spread gram positive bacterial nosocomial infections, especially the post-surgical wound infections (Mallick & Basak, 2010) [13]. Its dis-ease manifestations range from minor skin infections to life-threatening diseases such as pneumonia, sepsis etc. The ability of *Staphylococcus aureus* has developed resistance to antibiotics and remains non-responsive to treatment (Chambers & DeLeo, 2009) [3].

The pathogenic potential of different strains of *S. aureus* varies greatly owing to colonization to different tissues and host species or adaptation to varying microenvironment. The pathogenic strains are known to be capable of producing more than 30 extracellular enzymes and toxins (Thakker *et al.,* 1998) [19]. Strain discrimination of bacteria has been found useful in epidemiological studies mainly in determining clonal relationship of the bacterial isolates and tracing the origin and course of spread of the organism in a population (Sleander *et al.,* 1986) [18]. This study aimed at isolating and identifying multidrug resistant *Staphylococcus aureus* from various types of wound sources using the 16S rDNA sequencing technique.

Materials and Methods

Study area and collection samples. Study Area and Collection of Samples Wound samples for microbiological analysis were collected randomly from hospitals in Namakkal and properly labeled indicating the source and age of patients. Different types of wound samples were collected namely accident wound (N=20), post-operation sepsis (N=4), skin infection (N=32), abscesses (N=12) and burn wound (N=32). The samples were transported soon to the laboratory after being obtained

Isolation and identification of *Staphylococcus aureus* Culture plates of Blood agar, MacConkey agar, Nutrient agar, Baired parker agar and Mannitol salt agar (Hi Media, India) were used. The swab sticks used for the collection of the samples were streaked directly on the labeled agar plates and incubated at 37 °C for 24 h. After incubation, cultures were examined for significant growth. Subcultures were then made into plates of nutrient agar and incubated for another 24 h. The primary identification of the bacterial isolates was made based on colonial appearance and pigmentation. Biochemical tests were performed to identify the isolates (Cowan & Steel, 1985) [7].

DNA Sequencing

PCR Amplification of 16S rDNA, Pure culture of isolates was grown in Luria Bertani (LB) broth and genomic DNA was extracted following the protocol (Sambrook *et al.,* 1989) [16]. PCR amplification of the 16S rRNA gene was performed using the universal primers 27F 5´-AGAGTTTGATCCTGGCTCAG-3´ (Lane, 1991) [10] and 1492R 5´-GGTTACCTTGTTACGACTT-3´ (Turner *et al.,* 1999) [20], (Bioseve, Hyderabad). The reaction mixture (50 µL) contained 30mM Tris (pH 8.4), 50mM KCl, 1.5mM MgCl2, 50 mM concentrations of each deoxynucleoside triphosphate, 10pmol of primer, and 1 U of Taq polymerase (Genei, Bangalore). PCR reaction conditions in MJ Research DNA Engine Tetrad were, 1 cycle of 95 °C for 5min, 30 cycles of [94 °C for 1min, 55 °C for 1min, 72 °C for 1min], 1 cycle of 72°C for 1min. Purification of PCR Product and 16s rRNA gene sequencing. 15µL of amplified DNA products were dissolved in 50µL of PCR cleanup solution mixed well and incubated at 55°C for 15min. The mixture was centrifuged at 12000 rpm for 15min to remove the supernatant. DNA pellet was precipitated by the addition of 600µL of 80% ethanol and centrifuged at 12,000rpm for 15min. Finally, the DNA pellets were dried and dissolved in 15µL of Milli Q water (Millipore, USA). Purified 16S rDNA product was extended using the primers 27F 5´AGAGTTTGATCCTGGCT-CAG3´ and 1492R 5´GGTTACCTTGTTA-CGACTT3´.

The extension products were purified by isopropanol precipitation. The purified extension products were separated using Big Dye chemistry in the ABI 3730xl DNA Analyzer (Applied Biosystems Inc.) by capillary electrophoresis. Sequence data analysis was done using Sequencing Analysis software.

Data Analysis

16S rRNA gene sequences of isolates were compared to the non-redundant sequences database (GenBank, EMBL and DDBJ) using the BLASTn program in the National Centre for Biotechnology Information (NCBI) website (http://blast.ncbi.nlm.nih/gov/Blast.cgi.). Multiple sequence alignment was performed for homologous sequences and a phylogenetic tree was constructed using the neighbour joining method. The 16S rRNA gene sequences were also compared with database sequences at the Ribosomal Database Project (RDP), (Wang *et al.,* 2007) [22] using the RDP classifier programme (http://rdp.cme.msu.edu) for identification of the strains.

Results and Discussion

In this study, highest occurrence were observed in skin wound samples (44.2%) and second most is the burn wound samples (27.9%) followed by accident wound samples (16%). *Staphylococcus aureus* was the predominant bacterial strain in all samples followed by *P. aeruginosa*. Similarly, various bacterial isolates from various wound infections were reported (Lawrence & Lilly, 1972; Riaz & Babar, 1996; Church *et al.,* 2006; Muhammad *et al.,* 2012; Valarmathi *et al.,* 2013) [11, 15, 4, 14, 21].

Percentage of bacterial incidence in different pus samples was varied from 2% to 36%. A total of 43 bacterial isolates were isolated from the 25 pus samples. The most predominant bacterial isolate was *Staphylococcus aureus* (36%) followed by *Pseudomonas aeruginosa* (18%), *Streptococcus pyogenes* (14%), *Klebsiella pneumoniae* (14%), *Escherichia coli* (11%), *Enterococcus faecalis* (5%) and *Proteus mirabilis* (2%). These organisms are involved in wound infection in human beings. About 14 samples showed mixed infection and four samples had polymicrobial infection. This kind of infection may be treated with multi antibiotic ointments. Pus samples directly inoculated on selective recovery of different wound infection causing organisms. Eleven different media were used for the recovery of wound isolates. EMB agar, XLD agar, MacConkey agar are used for the recovery and differentiation of gram negative bacteria like *Escherichia coli*, *Proteus* sp., and *Klebsiella* sp. *Pseudomonas* sp is recovered and differentiated by making use of Mac Conkey agar and cetrimide agar. Blood agar and its different forms were used for the differentiation of *Staphylococcus* sp., and *Streptococcus* sp., Chromogenic agar medium is used for the differentiation of *Proteus* sp., KF Streptococcus agar is used for the selective isolation of *Enterococcus faecalis*. It produces pin pointed reddish brown colonies on KF Streptococcus agar Microscopic features and biochemical features of the wound isolates were indicated in table 5.8. Based on the biochemical features E4 isolate was identified as *Escherichia coli.* Similarly S7 as *Staphylococcus aureus*, Kp4 as *Klebsiella pneumoniae*, P1as *Proteus mirabilis*, Pa2 as *Pseudomonas aeruginosa*, St4 as *Streptococcus pyogenes* and En2 as *Enterococcus faecalis*. Totally Twelve isolates were selected from the group (Table 1).

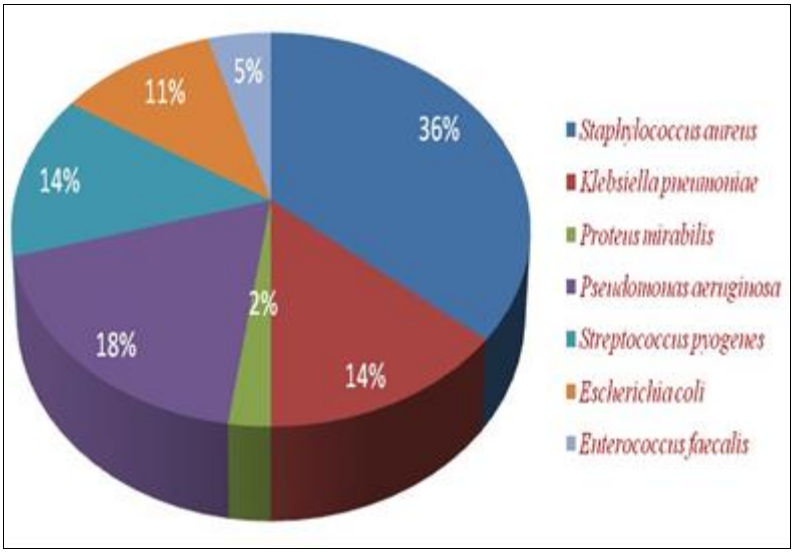


Fig 1: Bacterial incidence in pus samples collected from different wound infections

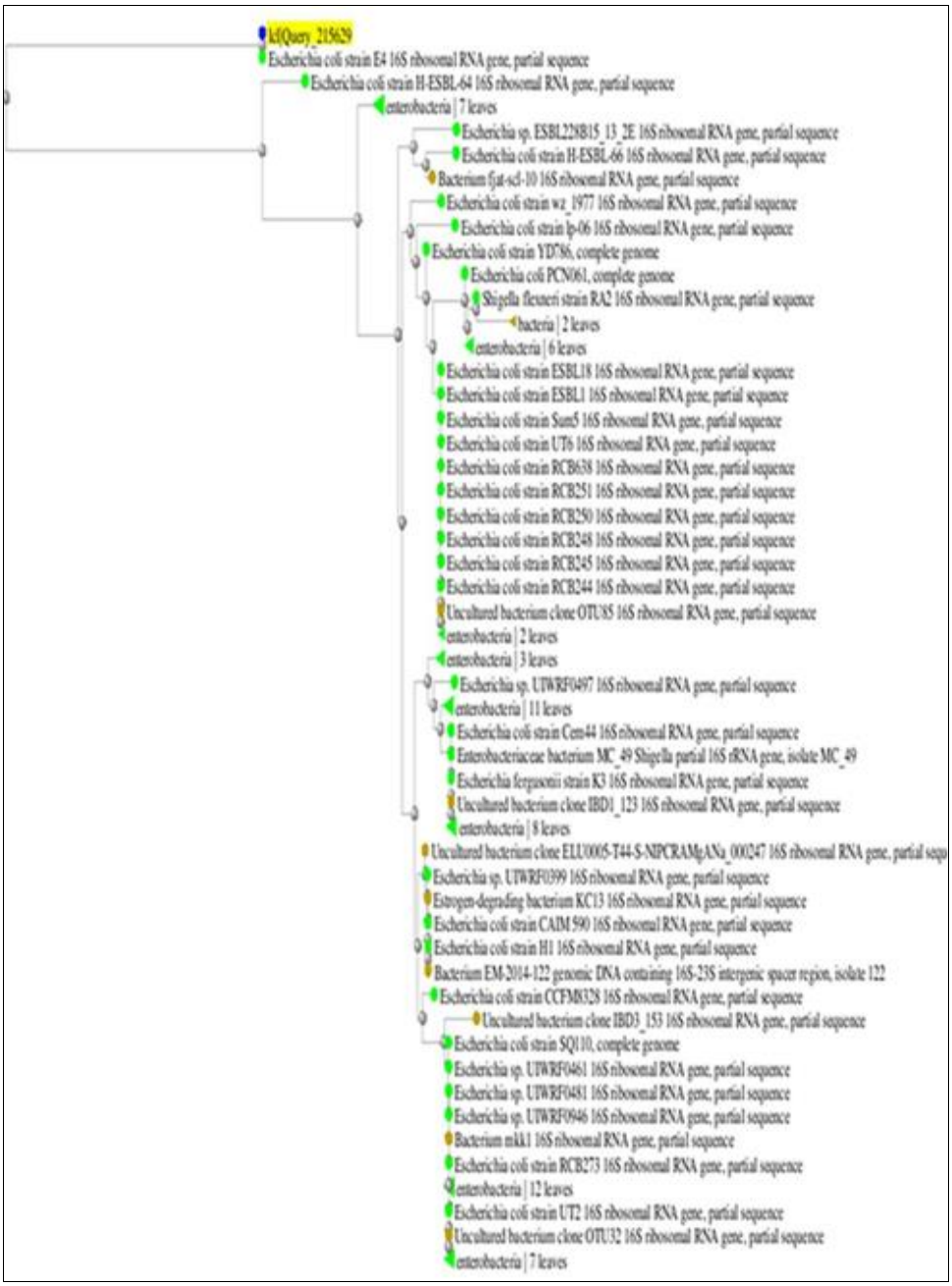


Fig 2: Phylogenetic tree of *E. coli* with distance created by blast

Table 1: Identification characters of different wound isolates

S. No	Character	E4	S7	Kp4	P1	Pa2	St4	En2
1	Simple staining	Rod	Cocci	Rod	Rod	Rod	Cocci	Cocci
2	Gram Staining	-	+	-	-	-	+	+
3	Motility	+	-	-	+	+	-	-
4	Indole	+	-	-	+	-	+	-
5	Methyl red	+	+	-	-	-	-	-
6	Voges proskauer	-	-	+	-	-	-	+

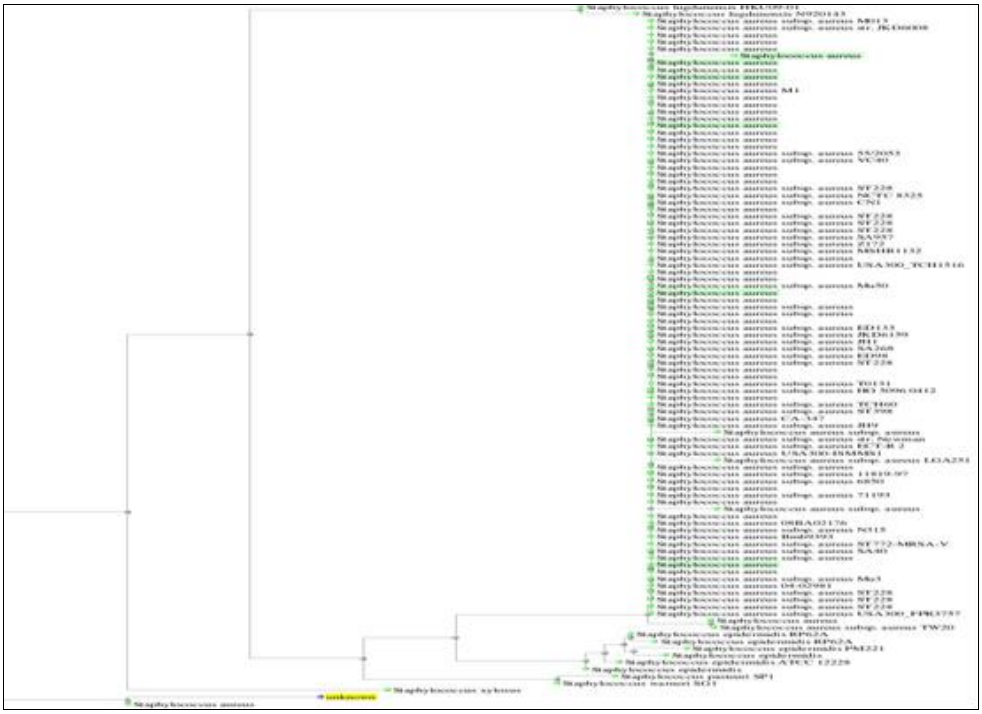


Fig 3: Phylogenetic tree of *Staphylococcus aureus* with distance created by blast

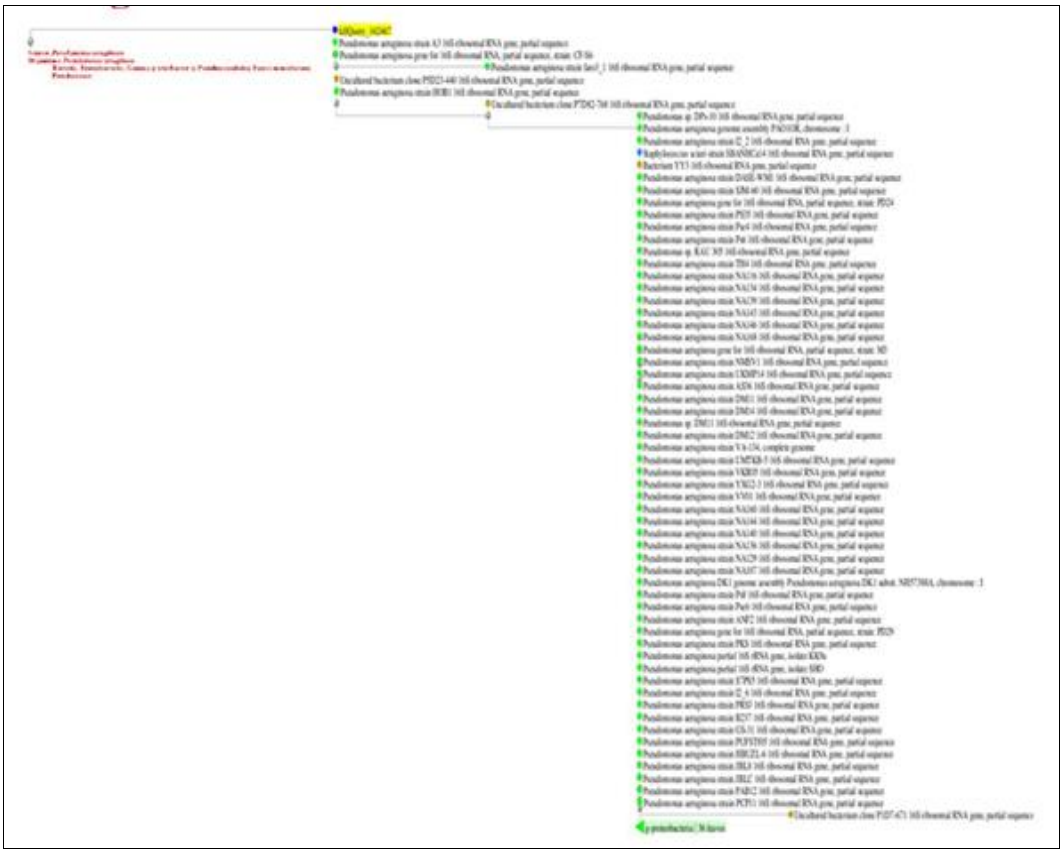
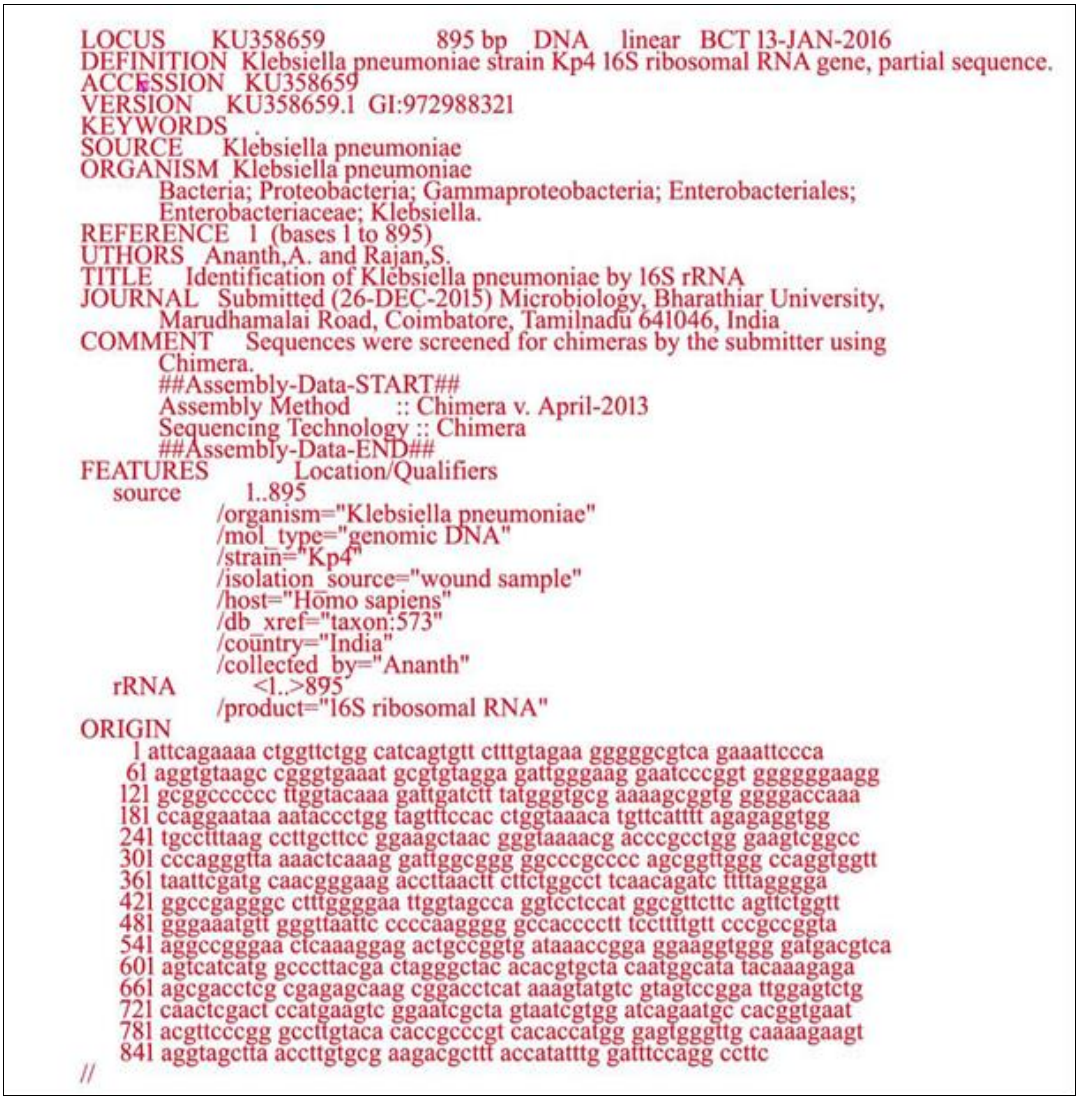


Fig 4: Phylogenetic tree of *Pseudomonas aeruginosa* with distance created by blast



```
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DEFINITION  Klebsiella pneumoniae strain Kp4 16S ribosomal RNA gene, partial sequence.
ACCESSION   KU358659
VERSION     KU358659.1  GI:972988321
KEYWORDS    .
SOURCE      Klebsiella pneumoniae
ORGANISM  Klebsiella pneumoniae
          Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales;
          Enterobacteriaceae; Klebsiella.
REFERENCE   1  (bases 1 to 895)
UTHORS   Ananth,A. and Rajan,S.
TITLE     Identification of Klebsiella pneumoniae by 16S rRNA
JOURNAL   Submitted (26-DEC-2015) Microbiology, Bharathiar University,
          Marudhamalai Road, Coimbatore, Tamilnadu 641046, India
COMMENT     Sequences were screened for chimeras by the submitter using
          Chimera.
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          Assembly Method       :: Chimera v. April-2013
          Sequencing Technology :: Chimera
          ##Assembly-Data-END##
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                     /mol_type="genomic DNA"
                     /strain="Kp4"
                     /isolation_source="wound sample"
                     /host="Homo sapiens"
                     /db_xref="taxon:573"
                     /country="India"
                     /collected_by="Ananth"
     rRNA            <1..>895
                     /product="16S ribosomal RNA"
ORIGIN
        1 attcagaaaa ctggttctgg catcagtgtt ctttgtagaa gggggcgtca gaaattccca
       61 aggtgtaagc cgggtgaaat gcgtgtagga gattgggaag gaatcccggt ggggggaagg
      121 gcggccccccc ttggtacaaa gattgatctt tatgggtgcg aaaagcggtg ggggaccaaa
      181 ccaggaataa aatacccctgg tagtttccac ctggtaaaca tgttcatttt agagaggtgg
      241 tgcctttaag ccttgcttcc ggaagctaac gggtaaaacg acccgcctgg gaagtcggcc
      301 cccagggtta aaactcaaag gattggcggg ggcccgcccc agcggttggg ccaggtggtt
      361 taattcgatg caacgggaag accttaactt cttctggcct tcaacagatc tttagggga
      421 ggccgagggc ctttggggaa ttggtagcca ggtcctccat ggcgttcttc agttctggtt
      481 gggaaatgtt gggttaattc ccccaagggg gccacccctt tccttttgtt cccgccggta
      541 aggccgggaa ctcaaaggag actgccggtg ataaaccgga ggaaggtggg gatgacgtca
      601 agtcatcatg gcccttacga ctagggctac acacgtgcta caatggcata tacaaagaga
      661 agcgacctcg cgagagcaag cggacctcat aaagtatgtc gtagtccgga ttggagtctg
      721 caactcgact ccatgaagtc ggaatcgcta gtaatcgtgg atcagaatgc cacggtgaat
      781 acgttcccgg gccttgtaca caccgcccgt cacaccatgg gagtgggttg caaaagaagt
      841 aggtagctta accttgtgcg aagacgcttt accatatttg gatttccagg ccttc
//
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Fig 5: Genbank Flat File of *Klebsiells pneumoniae*

Ezekiel *et al.*, (2014) [8] stated that *Staphylococcus aureus* was the most frequent organism isolated accounting for 23 (18.3%) of a total of 126 isolates. Other organisms were *Pseudomonas aeruginosa* and *Bacillus* sp., 11.1% each; *Escherichia coli* 10.3%; *Candida* sp., 8.7%; Coagulase negative *Staphylococci* 8.7%; *Pseudomonas* sp., 6.3%; *Serratia odorifera* 4.7%; Bacteroides 4.0%; *Enterococcus* spp 3.2%; the remaining isolates were other enterobacteria. Sensitivity of the bacterial isolates to antibiotics varied. In general, resistance to the β-lactam antibiotics was above 98%, whilst more than 70% of isolates were resistant to erythromycin, fusidic acid and tobramycin. This study doesnot showed any *Bacillus*, *Candida*, *Serratia* and coagulase negative *Staphylococci. Escherichia coli*, *Staphylococcus aureus, Klebsiella pneumoniae* and *Pseudomoas aeruginosa* isolates were characterized by sequencing process. The results were confirmed the identity of the isolates. All the clinical isolates were 100% similarity with respective species. Gene of *Pseudomonas aeruginosa* showed 99% similarity with *Pseudomonas aeruginosa* strain Pa2 16S ribosomal RNA gene partial sequence (Figure 4). Similarly *Klebsiella* showed 100% sequence similarity with *Klebsiella pneumoniae* (Figure 5) strain Kp4 16S ribosomal RNA gene partial sequence (GenBank: KU358659.1). *E. coli* (Figure 2) and *Staphylococcus aureus* (Figure 3) sequences were submitted to genbank and obtained accession number KT971133.1 (*Escherichia coli*) and KT971132.1 (*Staphylococcus aureus*).

Previous studies have also reported the use of 16S rRNA gene sequencing for identification of bacterial strains from water sources. The identification of *E. coli* from using 16S rRNA gene and 16S-23S rRNA internal transcribed spacer region sequences (Magray *et al.*, 2011) [12]. The observed similarity in 16S rRNA sequences recovered from fresh water bacteria from lakes in North America and Europe (Zwart *et al.*, 1998) [23].

RDP rank classifier programme for 16S rRNA sequence classified the isolates ECPH2 and ECPul2 as strains belonging to the genus *Staphylococcus aureus* with a confidence level of 95% (Ananth A and Rajan S, 2014) [1].

Conclusion

This study revealed the presence of wound infection causing bacteria, those are capable of causing various human illness. The bacterial isolate screened in various wound infections. The bacterial isolates of predominately wound infections were *S. aureus* (37.2%), compared with others. In this study we used the 16S rDNA sequencing technique to identify four bacterial strains S7, Kp4, Pa2 and E4 isolated from Wound sources of Hospitals around namakkal district, tamilnadu. Quick and accurate identification of microorganisms is essential for clinical as well as

epidemiological studies during disease outbreaks. 16S rRNA gene sequencing can offer a more consistently reliable and accurate method for identification. The frequency of misidentification by phenotypic metods compared to 16S rRNA gene sequencing and its impact on patient care are unknown and warrant further study.

Conflicts of interest
The authors declare that there are no conflicts of Interest.

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Conflict of Interest
Not available

Financial Support
Not available

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