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Molecular Characterization of Multi-Drug Resistant Clinical Isolates from Healthcare Facilities

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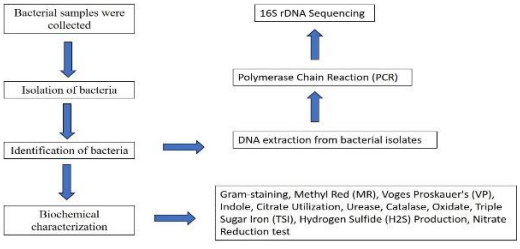
Abstract

This study aimed to analyze multi-drug resistant clinical isolates collected from healthcare facilities at the molecular level. Bacterial samples from a hospital were cultured on selective media to isolate Acinetobacter, E. coli, Klebsiella sp., and Pseudomonas sp., and characterized by Gram staining and biochemical tests. DNA was extracted from overnight cultures and used for PCR amplification of the V1-V3 and V3-V9 regions of the 16S rDNA. The PCR products were confirmed on agarose gels, purified, and sequenced. Sequences were analyzed using BLAST for species identification, and accession numbers were obtained from the NCBI GenBank database. Morphological and biochemical data for pathogenic bacteria, such as Acinetobacter (K1), E. coli (K2), Klebsiella (K3), and Pseudomonas sp. (K4) were analyzed. The 16s rDNA PCR products were sequenced using a commercial automated sequencer and were submitted to GenBank and NCBI. Bacterial sequencing analysis and comparison revealed that isolates K1, K2, K3, and K4 were Acinetobacter, E. coli, Klebsiella, and Pseudomonas sp. since they had a 100% homology with the library. The species-level identification of bacteria K1, K2, K3, and K4 is based on the highest identity (90%) and their GenBank accession codes are APK1, APK2, APK3, and APK4. This study suggests that samples from tertiary hospitals contain high loads of antibiotic-resistant bacteria. A change in the antibiotic sensitivity of the identified bacteria needs to be monitored, as there is limited availability of newer medicines, and the emergence of resistant bacteria far exceeds the rate of new antibiotic development.



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Introduction

Antibiotics have various effects that benefit resistant bacteria by reducing the growth or multiplication of vulnerable bacteria. Excessive antibiotic use by humans can cause bacteria to adapt to environmental conditions and can serve as vectors for the spread of antibiotic resistance. Skin, visitors, clothes, air, hands, postoperative surgery, and hospital wastewater are improperly managed healthcare wastes caused by the invasion and multiplication of resistant pathogenic microorganisms such as *Staphylococcus aureus, Escherichia coli, Streptococcus pyogenes, Klebsiella* sp., *Proteus* sp., *Enterobacter* sp., *Acinetobacter* sp., and *Pseudomonas aeruginosa* are the common etiological pathogens implicated in nosocomial (hospital-acquired) infections, and pyogenic infections are characterized by local inflammation, abscess, skin race, and pus formation. These events occur in the hospital environment and result in significant infection, long-lasting hospitalization, and economic burdens [1-3].

In the current clinical setting, antibiotic resistance and its effects on infection are the greatest health care and strength concerns in humans. The latest body of evidence implicates the environment as a critical component in the transmission, spread, and evolution of antibiotic-resistant AMR bacteria. However, clear evidence that directly links environmental factors to the emergence of bacterial resistance genes is currently lacking. Therefore, such research gaps identify more articulate explanations for the development of resistance genes and their transfer, mobilization, and dissemination in the surroundings [4-6]. Pathogens that were sensitive to antibiotics became resistant earlier because of mutations in the DNA-containing resistance base genes. However, developing countries are affected more by the widespread misuse of antibiotics, animal antibiotic use, improper making, poor quality of tablets, low cost, inadequate surveillance, factors associated with statistics, and national poverty [7-9]. National surveillance of antimicrobial resistance, significant gaps in surveillance, and lack of standards for methodology, procedures, processes, data sharing, and coordination [10-12]. This threatens the effective treatment of patients and results in the need to emphasize new treatment alternatives, continuous surveillance, infection control, monitoring, and appropriate antimicrobial prescriptions. In India, especially in South India, there is limited data concerning the resistance profiles of microorganisms isolated from hospitals or community waste. Therefore, this study was conducted to generate original local information and examine the magnitude of drug-resistant pathogens in hospital environments in the Karimnagar District, Telangana State.

Materials and Methods

Sample Collection

Bacterial samples were obtained in June at the tertiary hospital environment. Bacterial samples were kept in an ice box and delivered the same day to the Chalmeda Anand Rao Institute of Medical Sciences, Department of Microbiology, Bommakal Village, Karimnagar District, Telangana Teaching College for further analysis.

Isolation and Identification of Bacteria

Aliquots (0.1 mL) of 10^{-4} and 10^{-5} dilutions were uniformly disseminated over Chrom Agar (Hi-Media, India) for *Acinetobacter*, eosin methylene blue agar (Hi-Media, India) for *E. coli*, Chromo Agar (Hi-Media, India) for *Klebsiella* sp., and Cetrimide Agar (Hi-Media, India) for *Pseudomonas* sp. The plates were then incubated at 37°C for 24-48 h under aerobic conditions. All bacteriological studies were performed in triplicate, and representative colonies were selected and purified through consecutive subcultures [13, 14].

Biochemical Characterization

The isolates were primarily characterized by Gram-staining, Methyl Red, Voges Proskauer's, Indole, Citrate Utilization, Urease, Catalase, Oxidate, Triple Sugar Iron, Hydrogen Sulfide Production, and Nitrate Reduction test. The isolated pathogens were identified at the species level based on Bergey's Manual of Systematic Bacteriology [15].

Microscope

Gram staining was performed on overnight cultures of each strain, which were fixed on a slide and stained with crystal violet and iodine solution for 1 min each before being rinsed with water. The decolourized slides were stained with safranin for 30 seconds and then washed to remove excess safranin. Gram-negative isolates were detected by not retaining the purple stain and counterstaining pink with safranin. After drying the slides, 30% glycerol was added, and the slides were covered with a cover slip and examined under a photomicroscope.

16SrDNA Amplification and Sequencing

DNA Extraction from Bacterial Isolates

10 ml of overnight culture were harvested by centrifugation for 10 min at 5000 rpm, and the pellet was resuspended in 875 µl of TE buffer. 100 µl of 10 % SDS and 5 µl of Proteinase K (10 mg/ml stock) were again added and the mixture was incubated at 37˚C for 1 h. An equal volume of phenol-chloroform mixture was added, mixed by inversion, and incubated at room temperature for 5 min. The mixture was then centrifuged at 10,000 rpm for 10 min at 4°C and the supernatant was collected in a fresh tube. Next, 100 µl of 5 M sodium acetate was added and gently mixed. Then, 1 ml of isopropanol was added to the mixture and incubated at room temperature for 30 min. The mixture was centrifuged at 12,000 rpm for 10 min, and the pellet was washed with 70% ethanol 3 times. The pellet was dried at room temperature and resuspended in 50 µl of TE buffer.

Polymerase Chain Reaction (PCR)

Following an extensive literature survey, primers targeting the commonly used V-regions of 16S ribosomal DNA (rDNA) genes were meticulously chosen for the study. All samples were analyzed using PCR with the following primers.

Table 1. Forward and Reverse Primers for 16S rDNA Sequencing

V-Region	Primer Region	Forward Primer Sequence	Reverse Primer Sequence
V1-V4	27F - 805R	AGAGTTTGATCMTGGCTCAG	GACTACCAGGGTATCTAATCC
V3-V9	515F - 1492R	GTGCCAGCMGCCGCGGTAA	GGTTACCTTGTTACGACTT

Amplification of the V1 to V3 regions of the bacterial 16S rDNA gene was conducted using the universal primers 27F (forward primer) and 805R (reverse primer) (Table 1). The PCR was performed with EmeraldAmp® GT PCR Master Mix (Takara Bio Inc., Japan) under the following conditions: initial denaturation at 95 °C for 5 min, followed by 30 cycles of 95 °C for a 45 s denaturation, 55°C for a 30 s annealing, and 72°C for a 1 min elongation. The process concluded with a final extension for 7 min at 72°C. second primer set, 515F and 1492R, amplified V3 to V9 regions with PCR conditions: 95 °C for 5 min, 30 cycles at 95 °C for 30 s, 54 °C for 50 s, 72 °C for 45 s, and a final extension at 72 °C for 10 min. PCR band

sizes were confirmed on a 1.5% agarose gel, followed by purification using a NucleoSpin® Gel and PCR Clean-up Kit (MACHEREY-NAGEL GmbH & Co. KG, Germany), and quantified using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, USA).

16s rDNA Sequencing

16S rDNA gene fragments were sequenced at the Genomics Lab (Eurofins Genomics India Pvt. Ltd., India) using the automated ABI 3100 Sequencer and BigDye Terminator Cycle sequencing chemistry. Forward and reverse primers were used to analyze both DNA strands and electropherograms were evaluated using Chromas 2.6.6 software (Technelysium Pty Ltd, Australia). The obtained sequences were subjected to BLAST searches against the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify type strains that were closely related to the isolate sequences. Based on the maximum identity results, bacterial isolates of distinct colours were identified and subsequently submitted to NCBI GenBank through the bank program for the convenience of other researchers, and accession numbers were obtained.

Results

In the present study, morphological and biochemical data of pathogenic bacteria such as *Acinetobacter* (K1), *E. coli* (K2), *Klebsiella* (K3), and *Pseudomonas* sp. (K4) were assessed during the study period. Pathogenic bacteria were isolated from the selective nutrient agar. A total of four isolates were identified as *Acinetobacter*, *E. coli*, *Klebsiella*, and *Pseudomonas* sp. the highest experimental cultivation time. Visitors rising every day contribute to the bacterial species observed in clinical settings in Telangana. Moreover, some bacteria tend to survive for longer periods and thrive in clinical settings, such as *Acinetobacter*, *E. coli*, *Klebsiella*, and *Pseudomonas* sp., which can remain in the environment for prolonged periods. Based on these results, it is possible that the types of pathogenic bacteria could be identified as antibiotic-resistant (Table 2, Fig.2).

Biochemical Characterization

Microscopic strains of *Acinetobacter*, *E. coli*, *Klebsiella*, and *Pseudomonas* sp. are shown in Fig 1. The strain *Acinetobacter* (K1) is found to be gram reaction (-), Shape (Coccus), Methyl red (-), Catalase (+), Indole (-), Motility (non-motile), Voges Proskauer test (-), Nitrate reduction (-), Lactose (-), Oxidase (-), Citrate (+), and Urease (-). The strain *E. coli* (K2) was found to exhibit gram reaction (-), shape (Rod), methyl red (+), catalase (+), indole (+), motility (non-motile), Voges Proskauer test (-), nitrate reduction (+), lactose (+), oxidase (-), citrate (-), and urease (-). *Klebsiella* (K3) exhibited gram reaction (-), shape (Rod), methyl red (-), catalase (+), indole (-), motility (non-motile), Voges Proskauer test (+), nitrate reduction (+), lactose (+), oxidase (-), citrate (+), and urease (+). *Pseudomonas* sp. (K4) exhibited gram (-), shape (Rod), methyl red (-), catalase (+), indole (-), motility (non-motile), Voges Proskauer test (-), nitrate reduction (+), lactose (-), oxidase (+), citrate (+) and urease (-).

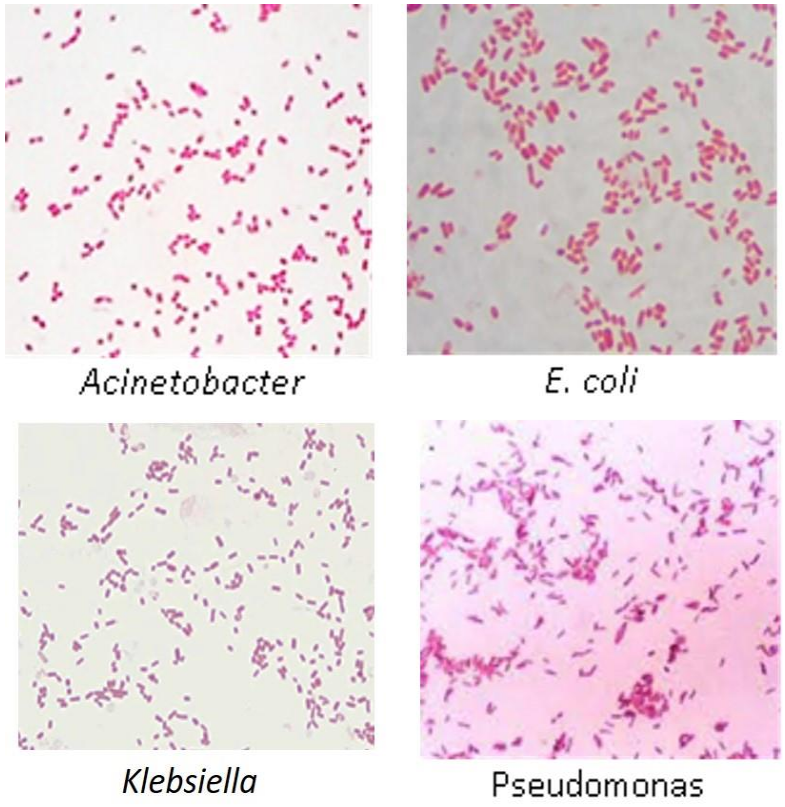

Fig.1. Microscopical View of Gram Staining Procedure

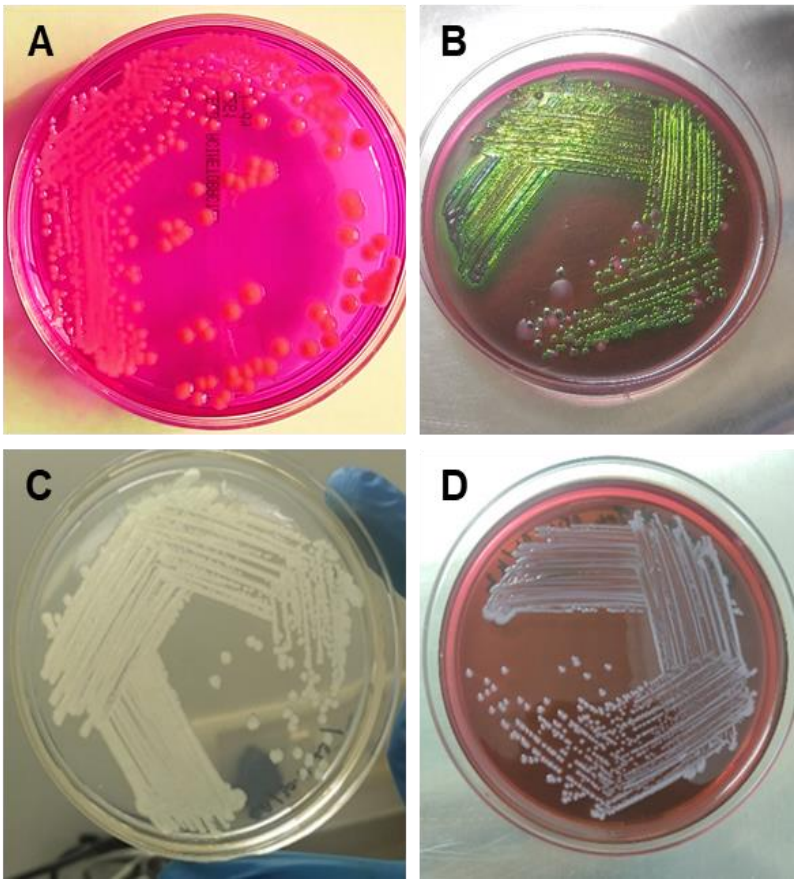

Fig. 2. Pure culture of isolated colonies (A- *Acinetobacter*, B- *E. coli*, C- *Klebsiella*, D- *Pseudomonas* sp.)

Table 2. Biochemical Characterization of Antibiotic Resistance Bacteria's

Test	K1	K2	K3	K4
Gram Stain	-	-	-	-
Shape	coccobacilli	rod shape	rod shape	rod shape
Motility at RT	nm	m	nm	m
MR	-	+	-	-
VP	-	-	+	-
Indole Production	-	+	-	-
Citrate	+	-	+	+
Urease	-	-	+	-
Catalase	+	+	+	+
Oxidate	-	-	-	+
Triple Sugar Iron Agar				
Butt	R	Y	Y	R
Slant	R	Y	Y	R
Gas	-	+	+	NA
H2S	-	-	-	-
Nitrate	-	+	+	+
Lactose Fermentation	-	+	+	-

RT, room temperature; (−), negative; (+), positive; m, motile; nm, nonmotile; Y, yellow; R: Red

Automated Sequencing of PCR Products

After sequencing the PCR products of the 16s rDNA the accuracy of the bacterial isolates was compared with the sequences obtained using the GenBank (BLAST) program. Based on the maximum identity results, bacterial isolates K1, K2, K3, and K4 were identified and submitted to GenBank and NCBI using the bank program for the benefit of other researchers, and accession numbers were obtained. Polymerase chain reaction (PCR) amplification of the 16s rDNA of the bacterial isolates resulted in an amplicon of 470 bp (Fig. 1). Bacterial sequence analysis and comparison showed that the isolates K1, K2, K3, and K4 were *Acinetobacter, E. coli, Klebsiella* and *Pseudomonas* sp., as they shared a homology of 100% with library, isolates were found to be *Acinetobacter, E. coli, Klebsiella* and *Pseudomonas* sp. The species-level identification of bacterial strains K1, K2, K3, and K4 was based on their maximum identity (90%), and their GenBank accession numbers were APK1, APK2, APK3, and APK4 (Table 3).

Table 3. Bacterial Species Identification and GenBank Accession No.

Sample ID	**Species Identification**	**Accession Number**
APK1	*Acinetobacter baumannii*	PP396733
APK2	*Escherichia coli*	PP396159
APK3	*Klebsiella pneumoniae*	PP396283

APK4	*Pseudomonas aeruginosa*	PP402697

Based on the NCBI BLASTn analysis, it was found that the sequences of isolates APK1, APK2, APK3 and APK4 exhibit a similarity of over 90% with the sequences of *Acinetobacter baumannii, E. coli, Klebsiella pneumoniae* and *Pseudomonas aeruginosa*, and, respectively, as documented in the GenBank database. Subsequently, similar sequences were retrieved from the NCBI GenBank and aligned using Clustal W2 for multiple sequence alignment (MSA). The Neighbor-Joining method was employed to construct the phylogenetic tree using default parameters in MEGA software. The 16S rRNA gene sequences of these isolates were deposited in the GenBank nucleotide database, and the corresponding accession numbers were obtained in Table 3. The sequence chromatograms of all four species are mentioned in Figures 3, 4, 5 & 6.

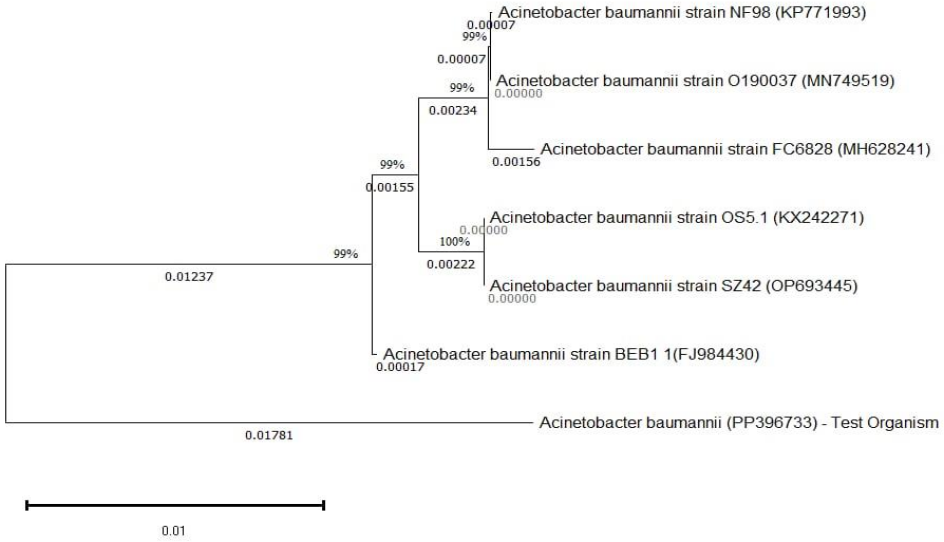

Figure 3. Phylogenetic Tree of *Acinetobacter baumannii*

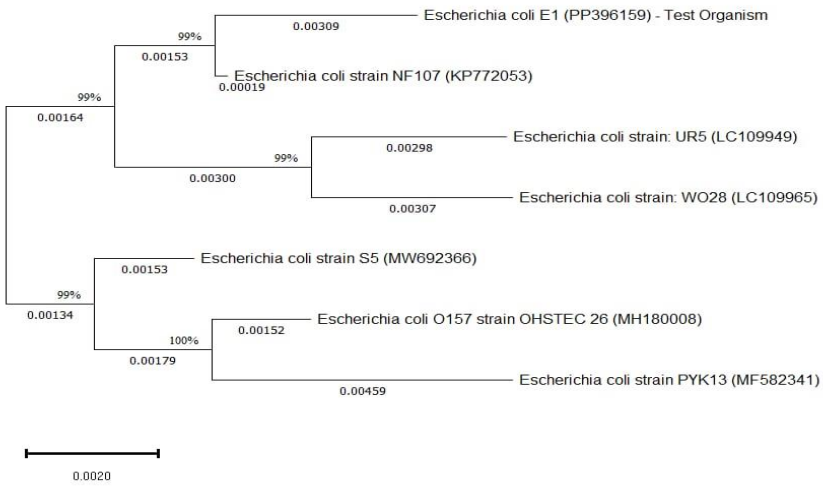

Figure 4. Phylogenetic Tree of *E. Solis*

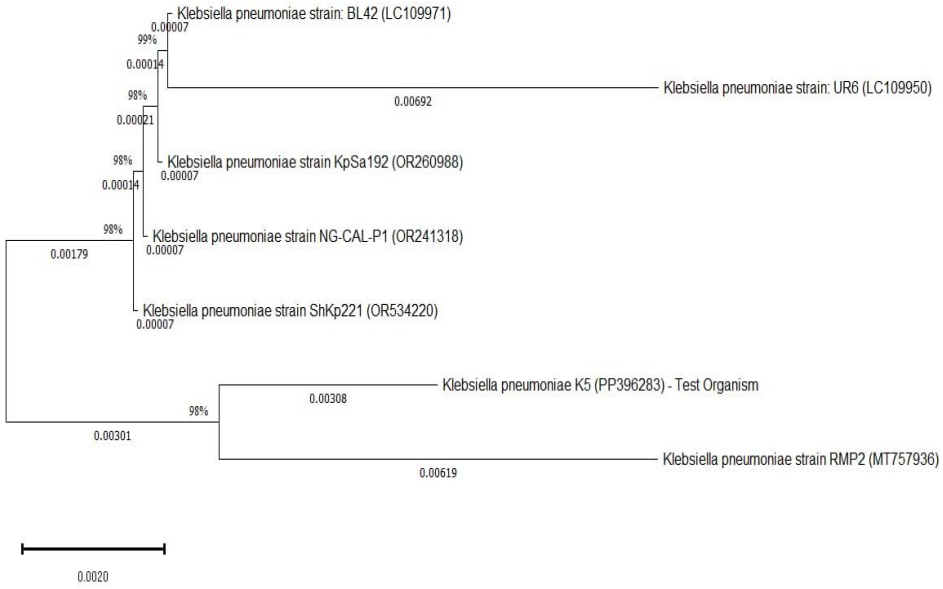

Figure 5. Phylogenetic Tree of *Klebsiella pneumonia*

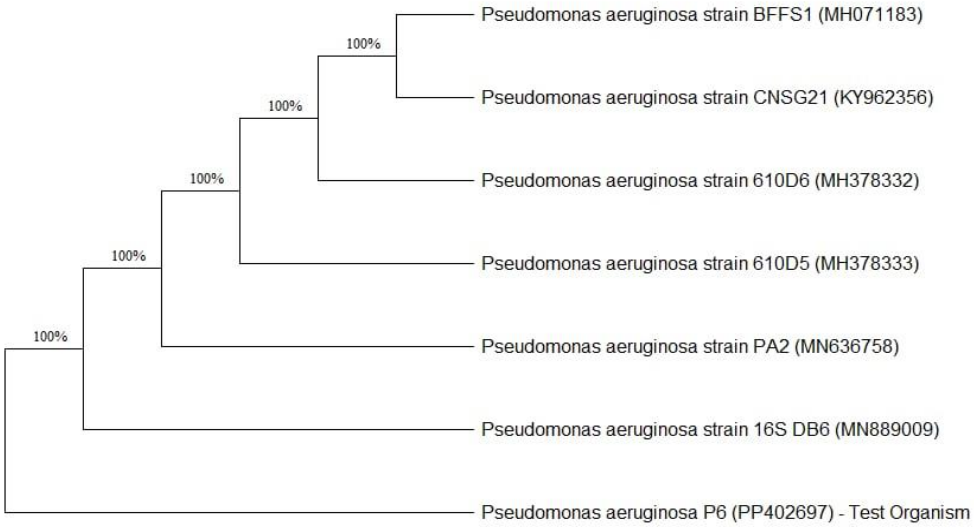

Figure 6. Phylogenetic Tree of *Pseudomonas aeruginosa*

Discussion

Morphological and biochemical data of pathogenic bacterial studies have been reported by various authors: the pathogenic organisms were *Pseudomonas* sp., *Acinetobacter* sp., *Candida* sp., *Escherichia coli,* and *Klebsiella* sp., isolated from various types of samples were determined by Barai et al. [16]. Amit et al. [17] reported that *Staphylococci, Klebsiella pneumoniae,* and *Acinetobacter baumannii* were isolated from the intensive care unit of a tertiary care teaching hospital. The profile of gram-negative bacteria isolated from pus includes *Klebsiella* sp., *E. coli*, *Pseudomonas* species, *Acinetobacter* species, *Proteus* species, *Citrobacter* species, and *Burkholderia* sp. isolated by Leema et al. [18]. In this study, we determined the types and distributions of antibiotic-resistant bacteria present in clinical samples and compared these results with those of bacteria present in other clinical settings. Similar findings were observed in previous

studies by Suma et al. [19], who showed antibiotic resistance patterns of *S. aureus* isolated from market milk in Tirupati, Andhra Pradesh. Identification of positive bacterial cultures revealed the presence of eight genes belonging to *Pseudomonas, Escherichia, Acinetobacter, Burkholderia, Klebsiella, Enterobacter, Proteus,* and *Staphylococcus* in the wound swabs of patients in the Burn Care Unit of a tertiary care hospital in Jharkhand, India [15].

Laghaa et al. [20] isolated and identified 30 *K. pneumoniae* isolates from the King Abdulaziz Specialist Hospital in Taif City, Saudi Arabia. Choushette and Rajendra [13] experimented with a total of six different types of bacteria (three gram-negative and three gram-positive): *K. pneumoniae, E. coli, Pseudomonas* sp., *S. aureus, E. faecalis,* and *S. pyogenes,* were obtained from the hospital sewage of Maharashtra, India. *Acinetobacter, E. coli, Klebsiella* and *Pseudomonas* sp. are opportunistic bacteria that cause serious diseases such as septicemia, (UTIs), fever, coughing, lung disorders, and nosocomial infections in normal and ICU patients. The present results suggest that the possibility and frequency of transfer of resistant pathogens among hospital staff, waste, high-range microbial contamination on door handles, bus surfaces, passengers touching seats, back doors, stanchions, low hygiene, continued exposure to antibiotics, and visitors may be chance to transmission were the predominant strains isolated from the tertiary care hospital Telangana. Direct detection of bacterial resistance in abscesses is possible and may be a potential method for rapid diagnosis and proactive therapy. Further studies evaluating the conclusion of antibiotic therapy dictated by the results of antibiotic-resistant bacteria detection are of great importance.

Antibiotics to the microbes that are most sensitive are drugs or medicines that might have developed low or high resistance owing to the routine use of older antibiotics, which probably eliminates resistance against equivalent antibiotics [21, 22]. There is a need for surveillance and management of antimicrobial resistance to commonly used antibiotics to determine their effectiveness, improve treatment and cure outcomes, and decrease the contamination of ward rooms with resistant strains of bacteria. In the present investigation, the species-level identification of bacterial isolates *Acinetobacter* (K1), *E. coli* (K2), *Klebsiella* (K3), and *Pseudomonas* sp. (K4) based on the maximum identity (90 %) and their GenBank accession numbers are APK1, APK2, APK3, and APK4. Similar findings have been reported previously. Hauhnar et al. [1] reported that the 16S rRNA gene was amplified in *Morganella morganii* strain HR *Bacillus cereus* (accession numbers MH285257 and MH327509). A 16s-rRNA sequence analysis was performed for *C. indologenes* bacterial identification (DSM 16777T158; GenBank accession no. LN681561) as reported by Zhang et al. [23]. Presently, the exact polymerase chain reaction and the extent to which the DNA gene plays a contributory role in the propagation of antimicrobial resistance is still unclear. More attention should be paid to the sequences as critical contributing factors to the link between resistance and propagation of resistant bacteria in clinical settings.

The presence of bacteria is usually a sign of contamination, and in addition to being characterized by genotypic antibiotic drug resistance, the ability of these bacteria to produce a biofilm complicates treatment, and low concentrations of antibiotics are not sufficient to inhibit the resistant bacterial population. The ability of resistant organisms to transfer their resistant genomic DNA to other pathogenic strains, which can cause severe infections, is a serious problem. The present study is the first to report the resistance profiles of *Acinetobacter, E. coli, Klebsiella* and *Pseudomonas* sp. isolates from the Telangana Tertiary Care Hospital. The variation in the isolated bacteria in unidentified samples has

been attributed to differences in the treatment practices at different regional locations. The level of detail obtained from 16s-rRNA analysis offers opportunities to use technologies for the development, monitoring, and management of 'health hospitals,' potentially linking town design with public health, for instance, in the design of hygienic rooms and public treatment systems.

The molecular identification of gram-negative bacteria in this study employed two main methods: direct PCR and 16S rDNA amplification and sequencing. The direct PCR approach involved colony PCR using species-specific primers targeting genes such as gad, khe, ecfX, and basC for identifying *Acinetobacter baumannii*, *E. coli*, *Klebsiella* pneumoniae, and *Pseudomonas* aeruginosa respectively. The results demonstrated a 100% correlation with the phenotypic identification, highlighting the reliability of these markers for molecular validation. Additionally, 16S rDNA amplification and sequencing were conducted on one isolate from each species, with DNA extracted from pure cultures and PCR amplification performed using primers targeting the V1-V9 region. Subsequent Sanger sequencing and sequence analysis confirmed the identity of the isolates, with NCBI BLASTn analysis showing over 90% similarity with sequences in the GenBank database. Phylogenetic analysis further supported the identification of these isolates, and the obtained 16S rRNA gene sequences were deposited in the GenBank nucleotide database with accession numbers PP396159, PP396283, PP402697, PP396733 respectively, providing valuable genetic data for future research and reference. Similar findings were observed in previous studies. Hauhnar et al. [1] showed that the 16S rRNA gene was amplified in the HC strain of *Morganella morganii*, with the HR strain *B. cereus* sequence accession no. MH285257 and MH327509. According to Zhang et al. [23]. a 16s-rRNA sequencing analysis was carried out for the identification of *C. indologenes* bacteria (DSM 16777T158; accession no. LN681561).

The study concluded the presence of antibiotic-resistant strains of *Acinetobacter*, *E. coli*, *Klebsiella*, and *Pseudomonas* sp. in different parts of the hospital, and their potential for easy transfer to patients, waste, and workers. This study highlights the importance of monitoring, identifying the use of antibiotics, and reducing the empirical treatment of bacterial infections. Therefore, there is a need to prepare effective guidelines for the careful use of antibiotics and direct release of clinical waste into the environment to avoid the spread of multiple bacterial antibiotic resistance. It is of utmost importance to analyze the molecular properties of multidrug-resistant (MDR) clinical isolates to comprehend the underlying genetic factors responsible for drug resistance.

Conclusion

In conclusion, this knowledge is essential for devising appropriate treatment approaches and adopting efficient measures to prevent infection. The findings derived from these investigations contribute to broader endeavours to address the escalating menace of antibiotic resistance in healthcare environments.

Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could influence the work reported in this study.

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Authors' Contribution

MKK: Performed the experiments; **CU & VP:** Provided technical assistance; **YM:** Analyzed the data and prepared the manuscript and **PS & PR:** Hypothesized, supervised, and finalized the manuscript.

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Research Content

The research content of This manuscript is original and has not been published elsewhere.

Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could influence the work reported in this study.

Ethical Approval

Not applicable.

Data Availability

Data will be available from the corresponding author on request.

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