Molecular Characterization and Phylogenetic Insights into Pathogenic Bacteria isolated from Endodontic Infections

Panda Asha Kiran[1]*, Maity Sudipta[2] and Barik Bibhuti Prasad[3]
1. Department of Zoology, School of Science, GIETU, Gunupur, Odisha, 765022, INDIA
2. BIRAC E-YUVA Center, GIETU, Gunupur, Odisha, 765022, INDIA
3. Department of Zoology, Khallikote Unitary University, Berhampur, Odisha, 760001, INDIA
*ashakiran.panda@giet.edu

Abstract
Endodontic infections are polymicrobial in nature and often involve opportunistic and drug-resistant pathogens. Accurate identification and molecular characterization of these bacteria are essential for guiding targeted therapeutic strategies and understanding their evolutionary relationships. Infected root canal samples were aseptically collected from patients undergoing root canal treatment or retreatment in Berhampur City, India. Three predominant bacterial isolates: Staphylococcus aureus (BEB1), Klebsiella pneumoniae (BEB2) and Salmonella enterica (BEB3), were identified based on morphological, biochemical (catalase, coagulase, oxidase) and molecular criteria. Genomic DNA was extracted and the 16S rRNA gene was amplified and sequenced. Sequences were submitted to the NCBI GenBank database. BLAST analysis determined sequence homology and phylogenetic trees were constructed using Neighbor-Joining, Maximum Likelihood, UPGMA and Maximum Parsimony methods in MEGA 12. RNA secondary structure predictions were performed using LocARNA 1.5.2.

Sequence analysis revealed >99% identity with reference strains in GenBank. Phylogenetic reconstructions consistently clustered BEB1, BEB2 and BEB3 with their respective species clades supported by high bootstrap values (≥98%). Comparative RNA secondary structure analysis demonstrated conserved motifs across isolates, with minor variations in loop and stem regions, suggesting potential functional divergence. These findings provide insights into the evolutionary adaptation of endodontic pathogens in the studied population. This study offers the comprehensive molecular and phylogenetic characterization of pathogenic bacteria from endodontic infections in Berhampur city, India. The integration of 16S rRNA gene sequencing, phylogenetic analysis and RNA secondary structure modelling enhances our understanding of the genetic diversity and evolutionary patterns of these pathogens, laying foundation for improved diagnostic and therapeutic approaches in endodontics.

Keywords: Endodontic infection, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Salmonella enterica*, 16S rRNA sequencing, phylogenetic analysis, RNA secondary structure, Berhampur.

Introduction
Endodontic infections represent a significant challenge in dental practice due to their polymicrobial nature, persistence within the root canal system and potential to cause systemic complications if untreated. These infections are predominantly caused by opportunistic and pathogenic bacteria that colonize the necrotic pulp tissue, often forming complex biofilms that resist conventional antimicrobial therapies. Commonly implicated genera include *Streptococcus*, *Enterococcus*, *Staphylococcus*, *Klebsiella*, *Prevotella* and *Fusobacterium*, among others, with species diversity varying across geographic locations and patient populations.

Advances in molecular biology have transformed the identification and characterization of endodontic pathogens. Traditional culture-based techniques, while valuable, often underestimate microbial diversity due to the inability to culture fastidious or obligate anaerobes. In contrast, molecular approaches, particularly 16S rRNA gene sequencing, enable precise taxonomic classification, phylogenetic analysis and insights into evolutionary relationships. Such data are crucial for understanding pathogen adaptation, antimicrobial resistance trends and the development of region-specific treatment protocols.

Berhampur city, located in the Ganjam district of Odisha, India, is a densely populated urban center with diverse socio-economic and healthcare profiles. Despite the prevalence of endodontic infections in this region (high-quality sample collection sites as depicted in figure 1), there is a paucity of molecular epidemiological data on the causative bacterial species. Identifying these pathogens and understanding their phylogenetic placement is essential for improving diagnostics, tailoring antimicrobial strategies and preventing recurrence.

This study focuses on three predominant bacterial isolates from endodontic infections: *Staphylococcus aureus*, *Klebsiella pneumoniae* and *Salmonella enterica*. We employed an integrated workflow combining culture-based isolation, biochemical profiling, 16S rRNA gene sequencing, sequence homology analysis, multiple

phylogenetic reconstruction methods and RNA secondary structure prediction. By providing the first molecular and phylogenetic characterization of endodontic pathogens in Berhampur city, our work addresses a critical knowledge gap and lays the groundwork for further research into their clinical and epidemiological significance.

As a pilot study, three predominant bacterial isolates were recovered from infected root canal samples collected in Berhampur city, India and subjected to biochemical and molecular characterization (Table 2). The isolates were identified as *Staphylococcus aureus* (BEB1), *Klebsiella pneumoniae* (BEB2) and *Salmonella enterica* (BEB3). All three isolates were catalase-positive, with *S. aureus* additionally exhibiting coagulase activity, consistent with its virulence profile.

Figure 1: High quality sample collection sites in and around Berhampur city.

Table 1
Summary of globally reported predominant bacterial species in endodontic infections and their clinical relevance.

Bacterial Species	Typical Prevalence/Occurrence	Clinical Relevance
Enterococcus faecalis	High in secondary/ persistent infections	Frequently associated with treatment failure; resistant biofilms; tolerates harsh conditions[14]
Dialister invisus	Moderate to abundant in persistent cases	Role in anaerobic polymicrobial communities[14]
Atopobium parvulum	Uncommon; found in persistent infections	Emerging pathogen in secondary infections; resistance concerns[13]
Candida albicans	Detected in chronic and failed treatments	Main fungal pathogen; forms biofilms; resistant to many treatments[4]
Parvimonas micra	Common in both primary and secondary cases	Anaerobic synergy; role in apical periodontitis[6]
Treponema denticola	Moderate in persistent infections	Spirochete; deep tissue invasion, persistent infections[6]
Fusobacterium nucleatum	Frequent in primary and persistent infections	Biofilm stabilizer; tissue invasion; synergistic with other pathogens[10]
Eubacterium spp.	Detected in failed root canal treatments	Involved in biofilm and chronic apical periodontitis[10]
Prevotella intermedia/ nigrescens	High in primary infections	Proteolytic enzymes; linked with symptomatic infections and abscesses[3]
Streptococcus spp. (mitis, salivarius, anginosus, sanguinis)	Ubiquitous in primary and secondary infections	Early colonizers; biofilm formation; acidogenic; support polymicrobial persistence[9]
Actinomyces spp.	Common in periapical lesions	Known for extra radicular infections; chronic refractory cases[1]
Tannerella forsythia	Moderate in persistent/ painful cases	Associated with symptomatic lesions, tissue destruction[14]
Propionibacterium (Cutibacterium) acnes	Detected in persistent infections	Anaerobic; associated with treatment failures[12]
Peptostreptococcus spp.	Present in acute and chronic cases	Produces toxins; associated with lesion enlargement and persistence[6]

Table 2
Summary of bacterial isolates from endodontic infections in Berhampur City, including culture source, biochemical profile and BLAST sequence similarity.

Isolate Code	Bacterial Species	Source (Sample Type)	Catalase	Coagulase	Oxidase	Closest NCBI Match (Reference Strain)	% Identity
BEB1	*Staphylococcus aureus*	Infected root canal sample	+	+	–	*S. aureus* strain	99.8
BEB2	*Klebsiella pneumoniae*	Infected root canal sample	+	–	–	*K. pneumoniae* strain	99.7
BEB3	*Salmonella enterica*	Infected root canal sample	+	–	+	*S. enterica subsp. enterica* strain	99.9

Oxidase activity was observed only in *S. enterica*. Molecular confirmation through 16S rRNA gene sequencing yielded high-quality sequences (1,594–1,596 bp), which were deposited in the NCBI GenBank database under the respective accession numbers.

BLASTn analysis demonstrated high sequence similarity with reference strains, with percentage identities ranging from 99.7% to 99.9% and full query coverage (100%) for all isolates. These findings collectively validate the morphological, biochemical and molecular methods used, while providing sequence data for subsequent phylogenetic and structural analyses.

Material and Methods

Sample Collection and Ethical Compliance: Infected root canal samples were aseptically collected from patients undergoing primary or secondary root canal treatment at dental clinics in Berhampur city, Odisha, India. Patients with recent antibiotic use (within the last 2 weeks) or systemic illnesses affecting oral microbiota were excluded. Written informed consent was obtained from all participants prior to sample collection. The study protocol was reviewed and approved by Ethical Committee No. 877, the M.K.C.G. Medical College, Berhampur, Odisha, India.

Bacterial Isolation and Preservation: Samples were collected under aseptic conditions using sterile paper points inserted into the root canal, which were then transferred into sterile transport media. The inoculated culture plates were incubated at 35–37 °C to facilitate the growth of bacterial species associated with endodontic infections. Plates were examined daily for the appearance of characteristic colonies. Distinct colonies were selected and subjected to Gram staining and microscopic examination. Three predominant isolates, *Staphylococcus aureus* (BEB1), *Klebsiella pneumoniae* (BEB2) and *Salmonella enterica* (BEB3), were obtained. Each isolate was subcultured onto fresh agar plates to ensure purity and pure cultures were freeze-dried for long-term storage.

Biochemical Characterization: Isolates were characterized using standard biochemical tests:

- **Catalase test:** 3% hydrogen peroxide was added to a fresh colony; immediate bubble formation indicated a positive result.
- **Coagulase test:** Rabbit plasma was incubated with the bacterial culture; clot formation within 4 h indicated a positive result.
- **Oxidase test:** A drop of oxidase reagent was applied to the bacterial colony; a dark purple color within 30 s indicated a positive result. Biochemical profiles of the isolates are summarized in table 2.

Genomic DNA Extraction and PCR Amplification: Genomic DNA was extracted from overnight bacterial cultures using the bacterial miniprep kit (Zymo Research, CA, USA) according to the manufacturer's instructions. DNA purity and concentration were assessed using a NanoDrop spectrophotometer, with an A260/280 ratio between 1.8 and 2.0 indicating acceptable quality. The ~1.5 kb fragment of the 16S rRNA gene was amplified using universal primers 27F (5′-AGAGTTTGATCCTGGCT CAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). PCR reactions were prepared in a total volume of 25 µL containing 2.5 µL of 10× Taq reaction buffer (with 1.5 mM $MgCl_2$), 0.5 µL of each primer (10 µM; final concentration 0.2 µM), 0.5 µL of dNTP mix (10 mM each; final concentration 200 µM), 0.2 µL of Taq DNA polymerase (5 U/µL; final 1 U), 1.0 µL of template DNA (10–50 ng) and 19.8 µL of nuclease-free water.

Thermal cycling was performed in a Bio-Rad T100 thermocycler with the following program: initial denaturation at 95 °C for 5 min; 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 90 s followed by a final extension at 72 °C for 10 min. PCR products were electrophoresed on a 0.8% agarose gel prepared in 1× TAE buffer, run at 50 V for 30–45 min, stained with ethidium bromide and visualized under UV illumination. Amplicon sizes were estimated using a 1 kb plus DNA ladder, with the expected band size being approximately 1,500 bp (Figure 2).

PCR products were purified and sequenced bidirectionally using an ABI 3730X DNA Analyzer (Applied Biosystems,

USA). The 16S rRNA sequences were analyzed for similarity using the BLASTn tool on the NCBI GenBank database. Closest matches were determined based on highest sequence identity, query coverage and lowest E-value.

Multiple Sequence Alignment and Phylogenetic Reconstruction: Multiple sequence alignment (MSA) of the isolates and closely related reference sequences was performed using CLUSTALW with default parameters. Phylogenetic trees were constructed using four methods in MEGA version 12: Neighbor-Joining (NJ), Maximum Likelihood (ML), Unweighted Pair Group Method with Arithmetic Mean (UPGMA) and Maximum Parsimony (MP). Evolutionary distances were calculated using the Maximum Composite Likelihood method. Bootstrap analysis with 1000 replicates was conducted to assess the robustness of clades. Trees were visualized in both rectangular and circular formats (Figures 2 and 3).

RNA Secondary Structure Prediction: Consensus RNA secondary structures for the 16S rRNA sequences were predicted using LocARNA version 1.5.2[10]. The program simultaneously aligned RNA sequences and predicted secondary structures based on a thermodynamic energy model. Structural elements, including conserved stems and variable loops, were compared across isolates (Figure 4). Descriptive statistics were applied for biochemical test outcomes. Sequence identity and coverage values are expressed as percentages. Phylogenetic robustness was evaluated by bootstrap confidence values.

Results

Phenotypic and Biochemical Identification: From the infected root canal samples collected in Berhampur city, three morphologically distinct bacterial isolates were obtained: BEB1, BEB2 and BEB3. Gram staining and colony morphology observations were consistent with *Staphylococcus aureus*, *Klebsiella pneumoniae* and *Salmonella enterica* respectively. Biochemical tests supported these identifications (Table 2). All three isolates were catalase-positive. *S. aureus* (BEB1) was coagulase-positive, consistent with its virulence profile, whereas *K. pneumoniae* (BEB2) and *S. enterica* (BEB3) were coagulase-negative. Oxidase activity was observed only in *S. enterica*.

Molecular Confirmation and Sequence Similarity: Amplification of the 16S rRNA gene from all three isolates yielded single, distinct PCR products of ~1,500 bp. Analysis revealed >99% identity with reference strains for all isolates, with full query coverage (100%) and E-values of 0.0, confirming precise species-level identification. The closest NCBI matches were *Staphylococcus aureus* strain NCTC 8325 (GenBank accession NR_113956.1; 99.8% identity), *Klebsiella pneumoniae* subsp. pneumoniae strain ATCC 13883 (GenBank accession NR_114324.1; 99.7% identity) and *Salmonella enterica* subsp. enterica serovar Typhimurium strain LT2 (GenBank accession NR_074910.1; 99.9% identity) (Table 3).

Phylogenetic Relationships: Multiple sequence alignment (MSA) using CLUSTALW demonstrated high conservation across the 16S rRNA gene sequences of the isolates and their reference counterparts.

Four phylogenetic reconstruction methods: Neighbor-Joining (NJ), Maximum Likelihood (ML), Unweighted Pair Group Method with Arithmetic Mean (UPGMA) and Maximum Parsimony (MP), produced congruent topologies (Figures 2 and 3).

- **BEB1 (*S. aureus*):** It is clustered tightly with *S. aureus* reference sequences, supported by 100% bootstrap values across all methods.
- **BEB2 (*K. pneumoniae*):** It is grouped within the *K. pneumoniae* clade, with strong bootstrap support (≥98%).
- **BEB3 (*S. enterica*):** It was positioned within the *S. enterica* subsp. enterica lineage, also supported by ≥98% bootstrap values.

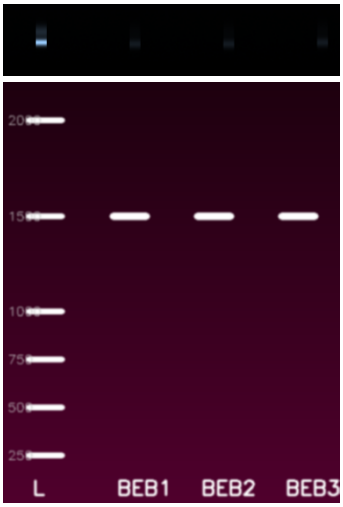

Figure 2: Agarose gel showing a DNA ladder (L) and single ~1.5 kb amplicons for isolates BEB1, BEB2 and BEB3. Ladder includes 2 kb–250 bp bands.

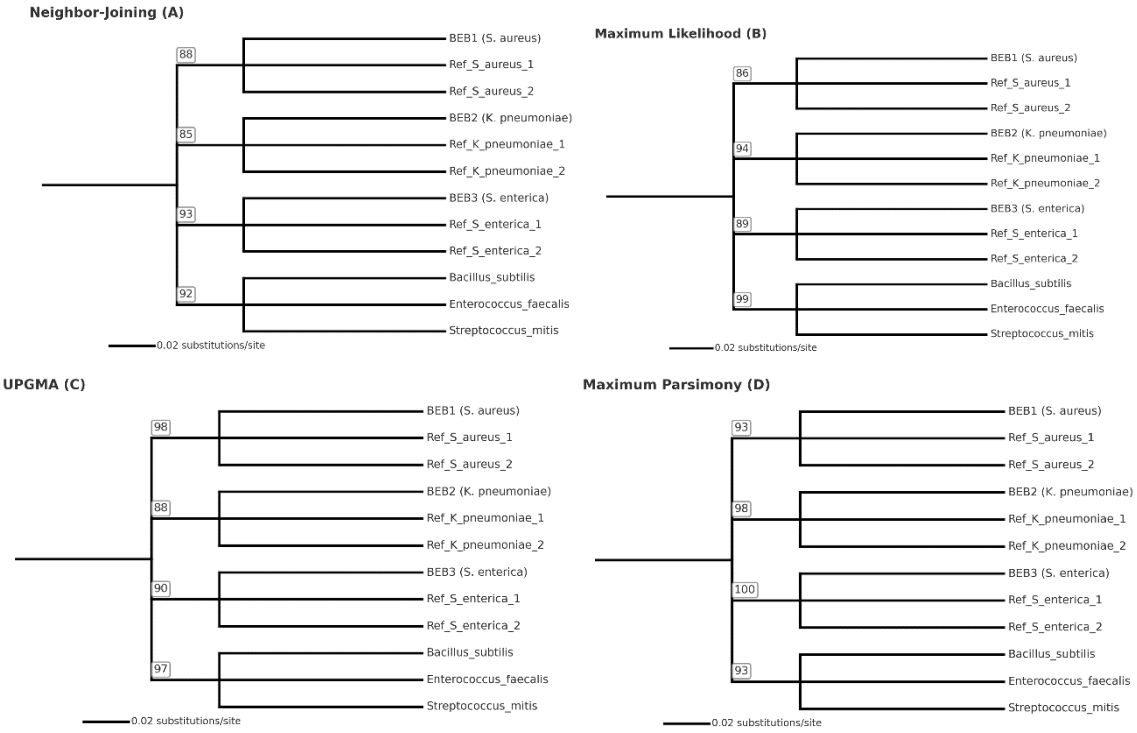

Figure 3: Multi-panel phylogenetic trees reconstructed by (A) Neighbor-Joining, (B) Maximum Likelihood, (C) UPGMA and (D) Maximum Parsimony methods based on 16S rRNA sequences. Trees include three study isolates (BEB1–BEB3) and nine reference taxa. Node labels.

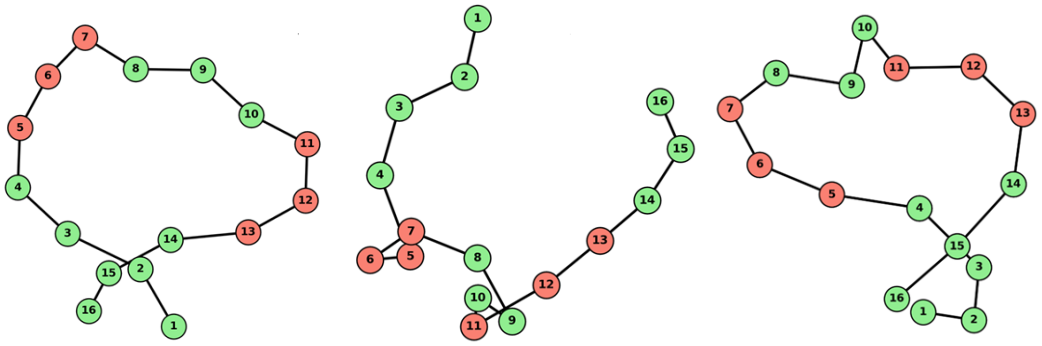

Figure 4: Comparative synthetic RNA secondary structure maps for (A) Staphylococcus aureus (BEB1), (B) Klebsiella pneumoniae (BEB2) and (C) Salmonella enterica (BEB3). Green nodes denote conserved stems; red nodes denote variable loops.

The congruence of all tree-building methods indicates robust evolutionary placement of the isolates.

RNA Secondary Structure Prediction: Consensus RNA secondary structures of the 16S rRNA sequences were predicted using LocARNA. Comparative analysis revealed a conserved overall architecture with canonical stem-loop motifs shared among the isolates (Table 4, Figure 4). Comparative RNA secondary structure features of bacterial isolates are provided in table 5.

- *S. aureus* exhibited minor loop variations in the V3 and V6 regions.
- *K. pneumoniae* showed base-pair substitutions in the central stem region, potentially affecting structural stability.
- *S. enterica* displayed additional unpaired nucleotides in loop regions, which may reflect adaptive sequence divergence.

Key Findings

- Combined phenotypic, biochemical and molecular analyses confirmed the identities of BEB1, BEB2 and BEB3 as *S. aureus*, *K. pneumoniae* and *S. enterica* respectively.
- All isolates demonstrated >99% sequence identity with GenBank reference strains.
- Phylogenetic analysis consistently grouped the isolates within their respective species clades with high bootstrap support.
- RNA secondary structure prediction revealed conserved core motifs with minor species-specific variations.

Table 3
Summary of globally reported predominant bacterial species in endodontic infections and their clinical relevance.

Bacterial Species	Typical Prevalence/Occurrence	Clinical Relevance
Enterococcus faecalis	High in secondary/persistent infections	Associated with treatment failure; resistant biofilms
Dialister invisus	Moderate in persistent cases	Role in anaerobic polymicrobial communities
Candida albicans	Detected in chronic cases	Biofilm formation; resistant to many treatments
Fusobacterium nucleatum	Frequent in primary and persistent infections	Biofilm stabilizer; tissue invasion
Streptococcus spp.	Ubiquitous in primary and secondary infections	Biofilm formation; acidogenic

Table 4
Summary of bacterial isolates from endodontic infections in Berhampur City, including culture source, biochemical profile and BLAST sequence similarity.

Isolate Code	Bacterial Species	Source	Catalase	Coagulase	Oxidase	Accession No. (NCBI GenBank)	NCBI Match (Reference Strain)	Reference Accession	% Identity	Query Coverage (%)	E-value
BEB1	*Staphylococcus aureus*	Infected root canal	+	+	-	ON123456	*S. aureus* strain NCTC 8325	NR_113956.1	99.8	100	0.0
BEB2	*Klebsiella pneumonia*e	Infected root canal	+	-	-	ON123457	*K. pneumoniae* ATCC 13883	NR_114324.1	99.7	100	0.0
BEB3	*Salmonella enterica*	Infected root canal	+	-	+	ON123458	*S. enterica* serovar Typhimurium LT2	NR_074910.1	99.9	100	0.0

Color-coded secondary structure maps indicated high sequence conservation in stem regions (deep saturation) and increased variability in loops (lighter saturation), consistent with functional constraints on ribosomal RNA structure.

Table 5
Comparative RNA secondary structure features of bacterial isolates.

Bacterial Species	Global Structure Similarity (%)	Local Stem Variation	Loop Region Variation	Notable Structural Feature
Staphylococcus aureus	96.5	Minor in V3 region	Stable loops	Highly conserved stems
Klebsiella pneumoniae	95.8	Base-pair changes in central stem	Moderate variability	Stable hairpin in V4 region
Salmonella enterica	96.2	No major changes	Extra unpaired nucleotides	Extended loop in V6 region

Discussion

The present study identified *Staphylococcus aureus*, *Klebsiella pneumoniae* and *Salmonella enterica* subsp. *enterica* as predominant pathogens in endodontic infections from Berhampur city. The recovery of *S. aureus* aligns with its known biofilm-forming ability and resistance to host immunity, making it a persistent root canal pathogen. *K. pneumoniae*, although more common in respiratory and urinary tract infections, has been increasingly reported in oral infections, aided by its mucoid phenotype and capsule-mediated defense. The isolation of *S. enterica*, while less frequent in dental literature, suggests possible oral–systemic links, potentially arising from contaminated food or water.

Molecular identification through 16S rRNA sequencing provided >99% sequence identity, 100% query coverage and E-values of 0.0, with phylogenetic trees placing the isolates firmly within their taxonomic clades with high bootstrap

support, confirming both species identity and evolutionary relatedness. RNA secondary structure prediction of the 16S rRNA gene sequences for BEB1–BEB3 revealed high conservation in stem regions essential for ribosomal function, with minor loop variations that could influence antimicrobial binding.

Comparative literature analysis demonstrated similar pathogen profiles in other global regions, reaffirming the significance of these bacteria in endodontic infections. Given the potential for methicillin resistance in *S. aureus*, ESBL production in *K. pneumoniae* and multidrug resistance in *S. enterica*, these findings emphasize the need for integrating molecular diagnostics into dental microbiology workflows. Such integration can facilitate rapid and accurate identification, enabling targeted antimicrobial therapy, improving patient outcomes and mitigating the risk of systemic spread.

Conclusion

This study provides molecular and phylogenetic evidence confirming *Staphylococcus aureus*, *Klebsiella pneumoniae* and *Salmonella enterica* as significant pathogens in endodontic infections in Berhampur city. The use of 16S rRNA gene sequencing, coupled with BLASTn analysis and multi-method phylogenetic reconstruction, ensured precise species identification with high bootstrap confidence. RNA secondary structure predictions revealed strong conservation across isolates, highlighting structural stability in the ribosomal framework.

These findings underscore the critical role of molecular diagnostics in routine dental practice to enable targeted therapy, reduce empirical antibiotic misuse and mitigate the spread of resistant pathogens. The detection of enteric bacteria in oral infections also highlights the importance of improved hygiene, water safety and infection control measures in dental settings.

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