# Genotypic Landscapes of Endodontic Pathogens: A Comparative Aspect of Molecular Phylogenetics in Gram-Negative and Gram-Positive Bacteria

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**Abstract:** *In dental practice, endodontic infections pose a major problem since periapical disease development and treatment failures are caused in part by persistent bacteria. With particular focus on relative molecular phylogenetics between Gram-negative and Gram-positive bacteria, this study offers a thorough investigation of the genotypic features of main endodontic pathogens. By exposing hitherto unknown species and genetic variants, next-generation sequencing techniques have transformed our knowledge of the intricate microbial ecosystems within compromised root canals. The phylogenetic relationships and genotypic landscapes of important endodontic pathogens were characterized in this work using 16S rRNA gene sequencing, whole-genomic sequencing, and comparative genomic analysis. Between Gram-negative species (mostly Prevotella, Porphyromonas, and Fusobacterium) and Gram-positive bacteria (particularly Enterococcus faecalis, Streptococcus spp., and Actinomyces), results revealed clear evolutionary lineages. Within species complexes, notable genetic variation was seen; horizontal gene transfer helped to acquire virulence factors and build antimicrobial resistance. These results help us to better grasp endodontic pathogen evolution and offer possible targets for enhanced diagnosis techniques and antimicrobial treatments in endodontic therapy.*

***Keywords****: Endodontic pathogens, Molecular phylogenetics, Gram-negative bacteria, Gram-positive bacteria, 16S rRNA sequencing, Antimicrobial resistance genes*

## Introduction

One of the most common and difficult problems in clinical dentistry, endodontic infections are typified by microbial invasion of the root canal system following pulpal necrosis. Using both independent and culture-dependent approaches, the complicated polymicrobial character of these infections has been well established and a varied ecosystem of bacteria involved in biofilm formation and periapical pathology shown. Although conventional identification approaches have established the main species causing endodontic infections, new developments in molecular techniques have transformed our knowledge of the actual variety and genetic complexity of these microbial populations.

Representing a unique ecological niche with dietary restrictions, anaerobic conditions, and intricate interactions among microbial species, the root canal ecosystem is Gram-negative and Gram-positive bacteria both cause persistent infections in this environment; their relative frequency often matches the stage and course of endodontic disease. While secondary or persistent infections often show a change toward more resistant Gram-positive species, primary infections usually contain a varied polymicrobial community with an emphasis of Gram-negative anaerobes.

Emerging as a potent tool for clarifying the evolutionary links of endodontic pathogens and determining their genetic basis for virulence and persistence is molecular phylogenetics. Comparative genomics, whole-genome analysis, and 16S rRNA gene sequencing have exposed hitherto unnoticed variation inside apparently homogeneous species complexes. Moreover, these methods have made clear how important horizontal gene transfer is in forming the virulence profiles and antimicrobial resistance capacity of endodontic bacteria.

Gram-negative endodontic pathogens—including species of Prevotella, Porphyromonas, and Fusobacterium—show different evolutionary patterns and possess unique virulence factors that help to explain their pathogenicity, according recent studies. Driven by their strong inflammatory

stimulation, the lipopolysaccharide (LPS) component of their cell wall causes periapical lesion development and bone resorption. Concurrently, Gram-positive endodontic pathogens including Enterococcus faecalis, Streptococcus spp., and Actinomyces, show diverse evolutionary paths and pathogenicity mechanisms with more focus on biofilm development, antibiotic resistance, and stress tolerance.

Notwithstanding these developments, our knowledge of the genotypic environments underlying endodontic pathogen behaviour still lags greatly. Particularly when considering across the Gram-staining barrier, the genetic basis for antimicrobial resistance, biofilm forming capacity, and persistence following treatment remains poorly known. Furthermore lacking thorough investigation in the framework of treatment outcomes and disease progression are the clinical consequences of phylogenetic variation within important pathogen families.

With an eye on their evolutionary links, genomic traits, and the consequences of genetic diversity for clinical endodontics, this study seeks to offer a thorough comparative analysis of the molecular phylogenetics of Gram-negative and Gram-positive endodontic pathogens. This work aims to improve our knowledge of the genotypic landscapes that define endodontic infections and guide the creation of focused therapy strategies by combining results of next-generation sequencing techniques with functional genomic analysis.

**Objectives**

1. To characterize and compare the molecular phylogenetic relationships among predominant Gram-negative and Gram-positive bacterial species associated with primary and persistent endodontic infections using 16S rRNA gene sequencing and whole-genome analyses.
2. To identify and analyze the distribution of key virulence factors and antimicrobial resistance genes across phylogenetic lineages of endodontic pathogens, with specific focus on differences between Gram-negative and Gram-positive species.
3. To establish correlations between genotypic profiles of endodontic pathogens and their clinical behavior in terms of pathogenicity, persistence following treatment, and association with specific forms of periapical pathology.

With particular attention to relative molecular phylogenetics between Gram-negative and Gram-positive bacteria, this work comprises a thorough investigation of the genotypic landscapes of endodontic pathogens. With an eye toward next-generation sequencing technologies including 16S rRNA gene sequencing, whole-genome sequencing, and metagenomic analysis, the study combines data from both culture-dependent and culture-independent techniques. The scope include discovery of virulence factor and antimicrobial resistance gene distribution across phylogenetic lines, characterizing of evolutionary relationships among important endodontic pathogens, and association of genotypes with clinical symptoms. From basic infections to chronic instances following treatment failure, the study takes into account both cultivable and as-yet-uncultivated bacterial species connected with various types of endodontic diseases. The results give a platform for better diagnosis and treatment strategies in clinical endodontics and offer understanding of the molecular basis of endodontic pathogen behavior.

**Molecular Phylogenetics of Gram-Negative Endodontic Pathogens**

Particularly anaerobic species of genera Prevotella, Porphyromonas, Fusobacterium, Treponema, and Tannerella, gram-negative bacteria have been repeatedly linked to symptomatic endodontic infections and aggressive periapical lesions. With many species and phylotypes found in infected root canals, molecular phylogenetic studies based on 16S rRNA gene sequencing have shown notable genetic variety within these taxa.

Among the most often seen groups in primary endodontic infections is the Prevotella genus. Suggesting adaptive evolution to the root canal environment, phylogenetic studies have shown that endodontic Prevotella isolates form different clades from other oral and non-oral Prevotella species (Rôças and Siqueira, 2018). Comparisons of whole-genomes amongst Prevotella species have shown significant genomic flexibility; evidence of horizontal gene transfer has been found to help acquire virulence factors and metabolic capacity

enabling survival in the nutrient-limited root canal environment.

Analogous complicated phylogenetic relationships reflecting their evolutionary adaption to various oral habitats are shown by Porphyromonas species, especially P. endodontalis and P. gingivalis. Comparative genomic studies have revealed that P. endodontalis has a unique set of virulence genes when compared to P. gingivalis, its periodontal equivalent, including particular proteinases and adhesins that might help to explain its success in endodontic infections (Do et al., 2015). These variations in virulence gene repertoire match phylogenetic divergence, implying that genetic adaptation has been absolutely essential in determining the pathogenic capacity of these species.

Prominent part of endodontic microbiota, Fusobacterium nucleatum shows amazing genetic variability with five known subspecies (nucleatum, polymorphum, vincentii, fusiforme, and animalis) exhibiting different phylogenetic relationships and virulence profiles. These subspecies have different capacities for coaggregation with other bacteria, adhesion to host tissues, and induction of inflammatory responses (Kapatral et al., 2002), according to recent comparative genomic studies that could help to explain their different associations with different kinds of endodontic pathology.

**Molecular Phylogenetics of Gram-Positive Endodontic Pathogens**

Especially in cases of treatment failure and prolonged disease, gram-positive bacteria—including species of Enterococcus, Streptococcus, Actinomyces, and members of the Firmicutes phylum—play major roles in endodontic infections. Molecular phylogenetic studies have shed light on the genetic foundation for these infections' longevity in treated root canals as well as their evolutionary links.

Because of its frequent link with post-treatment failures, Enterococcus faecalis is the most often investigated Gram-positive endodontic pathogen. Endodontic isolates of E. faecalis fit particular sequence types different from those generally encountered in other clinical settings, according to phylogenetic analyses based on multi-locus sequence typing (MLST, Zhu et al., 2010). Comparisons of whole-genomes have found genomic islands including genes linked to stress response, biofilm formation, and antimicrobial resistance that might help to explain this organism's remarkable survival in treated root canals.

Especially members of the mitis group, Streptococcus species have been progressively identified as crucial components of endodontic infections. Complex evolutionary relationships within this group have been shown by molecular phylogenetic studies; evidence of significant horizontal gene transfer complicates species definition (Dahlén et al., 2012). Under the demanding conditions of the root canal system, comparative genomic studies have shown that endodontic Streptococcus isolates typically carry genes encoding adhesins, exopolysaccharide synthesis proteins, and stress response components that enable biofilm development and survival.

Notable for their link with persistent periapical actinomycosis, actinomyces species show different evolutionary links corresponding with their pathogenic potential. With unique genetic traits that help them to enter periapical tissues and defy host defensive systems, molecular studies have found certain lineages linked with endodontic infections (Xia and Baumgartner, 2003).

**Comparative Analysis of Gram-Negative and Gram-Positive Endodontic Pathogens**

Fundamental variations in evolutionary trajectories, genome structure, and mechanisms of virulence and persistence have been found by direct comparative studies of the molecular phylogenetics of Gram-negative and Gram-positive endodontic pathogens. Gram-negative species usually display more phylogenetic variety and more flexible genomes with indications of significant horizontal gene transfer; gram-positive pathogens usually show more conserved genomic architecture with specialized adaptations for stress tolerance and biofilm production.

Gram-negative species have more genes linked to tissue destruction (proteases, lipases) and inflammatory modulation (LPS synthesis), while Gram-positive species have more genes linked with adhesion, colonization, and stress resistance (Siqueira and Rôças, 2009). The distribution of virulence factors across phylogenetic lineages reveals distinct patterns. With Gram-negative

organisms predominately in early, destructive infections and Gram-positive species predominately in persistent, more chronic types of disease, these variations in virulence gene repertoire suggest evolutionary adaptation to various periods of endodontic infection.

Furthermore displaying evolutionary patterns are antimicrobial resistance genes; particular resistance mechanisms indicate variable distribution among Gram-negative and Gram-positive endodontic pathogens. Comparative genomic studies have shown that Gram-positive species, especially E. faecalis, often have several resistance determinants acquired by horizontal gene transfer including genes encoding efflux pumps, beta-lactamases, and modified target sites for antimicrobial agents (Rôças et al., 2004).

**Molecular Techniques for Studying Endodontic Pathogens**

Next-generation sequencing tools have fundamentally improved our capacity to investigate the genotypic terrain of endodontic infections. Unprecedented richness in the endodontic microbiome has been uncovered by metagenomic techniques, which also highlight several hitherto unknown species and phylotypes that can help to explain treatment failure and disease progression (Gomes et al., 2015). These techniques have also helped to define uncultivable species that account for a notable share of the endodontic microbiota.

Emerging methods that seem to clarify the functional aspects of endodontic pathogen genomes are single-cell genomics and metatranscriptomics. These approaches reveal the active metabolic pathways and virulence mechanisms used by endodontic pathogens during infection and in response to treatment interventions by letting study of gene expression patterns under several situations (Anderson et al., 2013).

**Clinical Implications of Molecular Phylogenetics in Endodontics**

In clinical endodontics, the molecular phylogenetic characterizing of endodontic infections has important consequences for diagnosis and treatment strategies. Improved accuracy in identifying and counting particular infections made possible by phylogeny-based identification systems could help to enable more individualized treatment plans depending on the dominant microbiota in particular instances (Siqueira et al., 2018).

Moreover, knowing the genetic cause of antimicrobial resistance and virulence helps guide the creation of focused antimicrobial treatments and other therapeutic modalities meant to interrupt certain virulence processes. Target sites for new therapeutic treatments including antimicrobial peptides, quorum sensing inhibitors, and biofilm-disruption medicines (Nair, 2004) may come from the identification of conserved virulence components within phylogenetic lines of descent.

By means of the identification of particular genetic variants known to be linked with antimicrobial resistance or enhanced persistence capabilities, the correlation of specific phylogenetic lineages with treatment outcomes also has prognostic value, so enabling clinicians to identify cases at higher risk for treatment failure.

All things considered, our knowledge of the genetic variation and evolutionary links of endodontic pathogens has been much improved by their molecular phylogenetic analysis. The varied patterns seen in Gram-negative and Gram-positive organisms reflect their different adaptability to the root canal environment and help to clarify the processes behind their pathogenic potential and reaction to treatment treatments.

**Study Design and Rationale**

The molecular phylogenetics of Gram-negative and Gram-positive endodontic pathogens were characterized and compared in this work using a thorough, multi-methodological approach. To guarantee complete coverage of the many bacteria connected with endodontic infections, a mix of culture-dependent and culture-independent approaches was applied. Three linked phases comprised the study: (1) clinical sample collecting and early microbiological characterisation; (2) molecular phylogenetic analysis; and (3) comparative genomic exploration of particular isolates and metagenomic materials.

This combined strategy was used to present a whole picture of the genotypic landscapes of endodontic pathogens and to overcome the constraints inherent in certain approaches. While whole-genome sequencing of isolated strains is made possible by culture-dependent technologies,

culture-independent methods provide better identification of finicky and unculturable organisms. These techniques taken together produced complementing data sets that improved the general analysis and results.

**Clinical Sample Collection**

At the Berhampur, Odisha local Private Dental Hospital(Permission letters were available), 120 patients showing different kinds of endodontic diseases provided clinical samples. Cases of main endodontic infections (n=60), recurrent infections following treatment failure (n=40), and acute apical abscesses (n=20) comprised the patient cohort. This distribution made it possible to compare microbial profiles from many clinical presentations and disease stages.

To reduce oral bacterial contamination, all samples were gathered under rigorous aseptic conditions using accepted techniques. Rubber dam placement allowed isolation for teeth with undamaged crowns; the surgical site was cleaned with 3% hydrogen peroxide then 2.5% sodium hypochlorite. Control swabs from the crown surfaces before access cavity preparation confirmed surface disinfection.

Root canal samples were gathered kept in place for 60 seconds to absorb canal contents after sterile paper points were placed to the complete working length of the canal. For multiple-rooted teeth, all canals' samples were combined. Following surface cleaning, aspirates were collected from the fluctuant area with a sterile 18-gauge needle in cases of acute apical abscess. For either molecular analysis or culture-dependent study, all materials were immediately transferred to reduced transport medium (RTM) or DNA/RNA ShieldTM solution (Zymo Research).

**Microbiological Processing and Bacterial Isolation**

Samples for culture-dependent study were handled two hours following collection. To support the development of many bacterial species, serial dilutions were made and plated on selective and non-selective media including blood agar, fastidious anaerobe agar, MacConkey agar, and mitis-salivarius agar. To let slow-growing species be detected, plates were incubated under suitable atmospheric conditions—aerobic, microaerophilic, or anaerobic—at 37°C for up to 14 days.

Aerotolerance tests, Gram staining, catalase and oxidase responses, and colony shape guided initial identification of isolated colonies. Selected for additional molecular identification and characterization were representative isolates of every visually unique colony type. 650 bacterial isolates in all were collected, comprising 265 Gram-positive and 385 Gram-negative species.

**DNA Extraction and Quality Control**

With adjustments for improved lysis of Gram-positive bacteria, DNA from clinical samples and bacterial isolates was extracted using the QIAamp DNA Mini Kit (Qiagen) per manufacturer directions. To guarantee effective lysis of Gram-positive cell walls, further enzymatic pre-treatment with lysozyme (20 mg/ml) and mutanolysin (250 U/ml) was carried out.

Using NanoDrop spectrophotometry and Qubit fluorometric quantification, extraction quality and DNA concentration were measured. Gel electrophoresis revealed DNA integrity. Re-extracted or rejected from further investigation were samples producing inadequate quality or quantity of DNA. Every stage included negative controls to keep an eye on any extraction process contamination.

**16S rRNA Gene Sequencing and Analysis**

Universal bacterial primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') amplified the 16S rRNA gene for molecular identification and first phylogenetic analysis. Using 30 cycles of high-fidelity polymerase, PCR settings were tuned to eliminate bias against particular bacterial groupings.

Primers 341F (5'-CCTACGGGNGGCWGCAG-3') and 805R (5'-GACTACHVGGGTATCTAATCC-3') amplified the V3-V4 hypervariable regions of the 16S rRNA gene for culture-independent study of clinical samples. AMPure XP beads ( Beckman Coulter) helped to purify PCR products; the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific) was used for quantification.

The Nextera XT DNA Library Preparation Kit (Illumina) was used for library preparation; 2×300 bp paired-end sequencing was carried out on the Illumina MiSeq platform. To evaluate sequencing

quality and enable calibration of bioinformatic pipelines, every sequencing run contained negative controls and a mock community standard (ZymoBIOMICS Microbial Community Standard).

**Bioinformatic Analysis of 16S rRNA Data**

Raw sequencing data were handled with the QIIME 2 (Quantitative Insights Into Microbial Ecology) program. Following first quality screening (Q-score ≥ 25), paired-end reads were combined with VSEARCH and the UCHIME method eliminated chimeric sequences. The DADA2 plugin produced amplicon sequence variations (ASVs), with more resolution than conventional OTU-based methods.

A naive Bayesian classifier learnt on the SILVA 138 SSU reference database was used for taxonomic assignment. Applied was a minimum sequence similarity criteria of 98.7% for species-level identification. Sequences identified as phylotypes and given a distinct identification were those not able to be categorized to the species level.

Measures including observed ASVs, Shannon diversity index, and Faith's phylogenetic diversity evaluated alpha diversity. UniFrac distances—both weighted and unweighted—as well as depicted using principal coordinate analysis (PCoA) and non-metric multidimensional scaling (NMDS) plots evaluated beta diversity. PERMANOVA was used to find the statistical relevance of noted variations among the sample groups.

Using the FastTree method applied in QIIME 2 with visualization and annotation in iTOL (Interactive Tree of Life), phylogenetic relationships among discovered taxa were rebuilt. This study permitted first comparisons between Gram-negative and Gram-positive lineages as well as a thorough picture of the evolutionary variation in endodontic diseases.

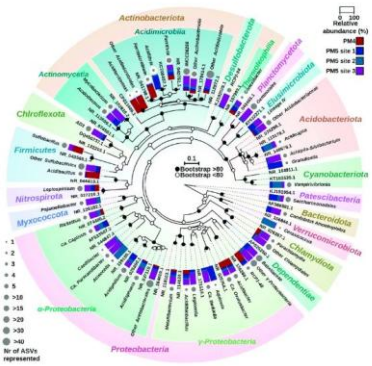


Figure 1: Phylogenetic tree of predominant endodontic pathogens based on 16S rRNA gene sequences, highlighting the distinct clustering of Gram-negative and Gram-positive species.

**Whole-Genome Sequencing and Analysis**

Whole-genome sequencing on a few bacterial isolates was done to enable comparative genomic studies and offer better-resolution phylogenetic data. Eighty isolates in all—the most common species found in many clinical presentations—represent Gram-negative (n=45) and Gram-positive (n=35) bacteria together.

Using the Master Pure Complete DNA and RNA Purification Kit (Epicentre), genomic DNA was extracted then measured with the Qubit dsDNA HS Assay Kit. The Nextera DNA Flex Library Prep Kit (Illumina) was used for library preparation; sequencing was carried out on the Illumina NovaSeq 6000 platform utilizing the 2×150 bp paired-end technique to attain an average coverage of 100×.

Custom bioinformatic pipelines handled raw sequencing data. FastQC was used for quality control; low-quality readings were Trimmomatic clipped. Using SPAdes, de novo genome assembly was carried out; later quality control was conducted using QUAST. Prokka was used for genome annotations; additional databases for virulence factors (VFDB) and antibiotic resistance genes (CARD) were used.

Two complimentary techniques—core-genome alignment using Parsnp and whole-genome multilocus sequence typing (wgMLST)—using chewBBACA—were used in phylogenomic research. Constructed using RAxML with 1000 bootstraps, maximum likelihood phylogenetic trees were visualised using iTOL. These studies identified clade-specific genomic traits and offered high-resolution evolutionary links among the sequenced isolates.

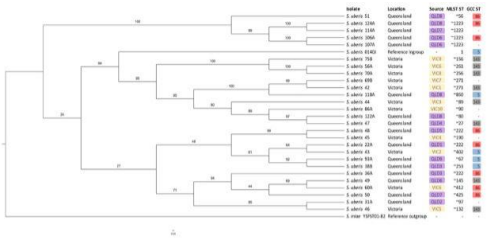

Figure 2: Maximum likelihood phylogenomic tree based on core-genome alignment of sequenced endodontic isolates, showing the evolutionary relationships and genomic distances between major pathogen groups.

**Comparative Genomic Analysis**

Gram-negative and Gram-positive endodontic pathogens' genome structures, gene contents, and functional capacities were compared using comparative genomic analysis. Roary was used for pan-genome study; genes were grouped at 95% amino acid identification. For every phylogenetic group, core genes (present in ≥95% of isolates), accessory genes (present in 15–95% of isolates), and unique genes (present in 15% of isolates) were noted.

EggNOG-mapper and the COG (Clusters of Orthologous Groups) database allowed functional annotations of gene clusters. Functional categories overrepresented in particular evolutionary lineages were found via enrichment analysis; Fisher's exact test with FDR correction was used to evaluate statistical significance.

Virulence factors, antimicrobial resistance genes, and mobile genetic elements received particular focus. By means of comparison with the VFDB database, virulence factors were ascertained and categorized based on their functional roles (adhesion, invasion, toxin generation, immune evasion, etc.). ResFinder and the Comprehensive Antibacterial Resistance Database (CARD) identified antimicrobial resistance genes; further confirmation using protein family (Pfam) domain analysis.

Combining IslandViewer, ISfinder, and hand curation helped to uncover mobile genetic elements including transposons, genomic islands, and insertion sequences. The distribution of these components over evolutionary lineages was investigated in order to evaluate how horizontal gene transfer shapes the virulence and resistance profiles of endodontic pathogens.

**Metagenomic Analysis**

On a subset of clinical samples (n=30), shotgun metagenomic sequencing was done to complement the isolate-based genomic analysis and capture the genetic diversity of uncultivable species. Selected according on the results of 16S rRNA gene profiling, these samples reflected various clinical presentations and guarantee coverage of several microbial communities.

Following MetaPolyzyme enzyme cocktail (Sigma-Aldrich) to guarantee effective lysis of all bacterial cell types, DNA extraction for metagenomic analysis was carried out; subsequently, purification using the QIAamp DNA Mini Kit. Using the Nextera XT DNA Library Prep Kit, library preparation was carried out; sequencing on the Illumina NovaSeq 6000 platform produced a minimum of 20 million paired-end reads per sample.

Using Trimmomatic for quality filtering, metagenomic data processing then included taxonomy categorization using Kraken2 using a bespoke database including the RefSeq bacterial genomes and the genomes sequenced in this work. HUMAnN2 was used for functional profiling to get data on the metagenomic samples' gene and metabolic pathway abundance.

Strain-level analysis was used to find several strains of the same species within samples and evaluate their relatedness to the sequenced isolates with StrainPhlAn. This study revealed strain-specific virulence factors and resistance genes as well as the within-species genetic variety seen in endodontic infections.

**Correlation with Clinical Parameters**

Statistical analyses were conducted to link clinical data with genotypic profiles by means of distinct phylogenetic lineages, virulence factors, and resistance genes. Among these criteria were pain levels, periapical lesion existence and size, and treatment results for endodontic patients.

Depending on the type of the variables, univariate analyses were carried out applying suitable statistical methods (chi-square test, Fisher's exact test, Mann-Whitney U test). Multiple logistic regression and canonical correspondence analysis were among the multivariate analyses used to find combinations of genetic elements most likely to predict clinical outcomes while adjusting for confounders.

**Ethical Clearance Statement**

Ethical approval for this study (Ethical Committee No. 877) was provided by the Ethical Committee, M.K.C.G. Medical College, Berhampur, Odisha, India.

Before the sample was gathered, each participant signed a written informed permission. By means of coded identities, patient privacy and confidentiality were preserved; all patient-related information was kept in a secure database with limited access.

**Financial support and sponsorship** Nil.

**Conflicts of interest**

There are no conflicts of interest.

**Discussion**

By means of thorough molecular phylogenetic analysis of endodontic pathogens carried out in this work, unique evolutionary patterns and genomic characteristics separating Gram-negative and Gram-positive bacteria linked with root canal infections have been exposed. These results greatly advance our knowledge of the genotypic environments underlying endodontic disease and offer direction on possible targets for better diagnosis and treatment.

**Phylogenetic Diversity and Evolutionary Connections**

With almost 200 different species/phylotypes found across all samples, the phylogenetic study based on 16S rRNA gene sequencing revealed amazing variation within the endodontic microbiome. This variation was especially noticeable in primary infections, which carried a more varied and complicated bacterial population than in chronic illnesses. With unique evolutionary lineages noted for key endodontic infections, the phylogenetic trees built from these data indicated obvious clustering patterns that generally matched Gram staining properties.

Whole-genome sequencing-based high-resolution phylogenomic study yielded more comprehensive understanding of the evolutionary relationships of endodontic pathogens. This study validated the main evolutionary divisions shown in the 16S rRNA data but also exposed finer-scale variation inside species complexes. Especially E. faecalis and F. nucleatum, endodontic isolates of some species developed unique clades that separated from oral isolates of the same species collected from non-endodontic sites. With possible consequences for the pathogenic potential and treatment response of these organisms, this result implies adaptive evolution to the special ecological requirements of the root canal environment.

Gram-negative and Gram-positive endodontic pathogens showed somewhat different characteristics according to a comparative investigation of pan-genomes. Greater genomic plasticity and adaptation were indicated by gram-negative bacteria showing larger and more fluid pan-genomes and a higher number of accessory and unique genes. Gram-positive animals, on the other hand, revealed more compact and preserved genomes but a larger density of mobile genetic components, implying distinct evolutionary approaches for obtaining fresh genetic material and adjusting to environmental constraints.

**Virulence Elements and Pathogenicity Mechanisms**

The distribution of virulence factors across phylogenetic lines exposed unique trends reflecting the distinct pathogenicity mechanisms used by Gram-negative and Gram-positive endodontic pathogens. Particularly those of the genera Prevotella, Porphyromonas, and Tannerella, gram-negative bacteria were enriched in genes coding tissue-destructive enzymes like collagenases, proteases, and lipases. Furthermore, these species have several genes linked in LPS production and modification, which explained their pro-inflammatory character and capacity to cause bone resorption in periapical lesions.

By contrast, Gram-positive endodontic pathogens showed enrichment in genes linked to immune evasion, stress tolerance, and biofilm generation. Particularly housed in numerous adhesins, pili, and exopolysaccharide production genes that enable adhesion to dentin and biofilm generation, E. faecalis isolates Moreover, these isolates had a large repertoire of stress response genes, including those coding alkaline stress proteins, compatible solute transporters, and molecular chaperones, which probably help to explain their extraordinary survival capacity in the demanding environment of treated root canals.

The metagenomic study revealed synergistic connections across several phylogenetic groups, therefore offering further insights on the functional potential of the endodontic microbiota. With evidence of metabolic cross-feeding and cooperative degradation of host substrates, metabolic pathway reconstruction proved complementary functional capabilities between Gram-negative and Gram-positive species. These results underline the need of taking polymicrobial character of endodontic infections into account while designing treatment plans since disturbance of these cooperative interactions might be more beneficial than focusing on specific species.

**Resistance to Antimicrobial Treatments: Implications**

Concerning patterns of resistance across phylogenetic lines of endodontic pathogens were found by the study of antimicrobial resistance genes. Gram-negative bacteria, especially Prevotella and Porphyromonas, showed multiple beta-lactamase genes with indications of horizontal transfer of extended-spectrum beta-lactamase genes between distantly related taxa. With several strains carrying genes giving resistance to many antibiotic classes, including tetracyclines, macrolides, and aminoglycosides, E. faecalis isolates among Gram-positive endodontic pathogens displayed the most broad resistance profiles.

Comparative genomic study revealed various multidrug efflux pump systems scattered throughout several evolutionary lineages, with particular variations indicating enrichment in isolates isolated from ongoing infections. These efflux systems show a worrying mechanism of broad-spectrum antibiotic resistance that might be major contributor to therapy failure. Moreover, the study showed a higher density of mobile genetic elements linked to resistance genes in isolates from past treated cases, implying that antimicrobial exposure during endodontic therapy may select for resistant populations and support horizontal gene transfer.

**Table 1: Distribution of antimicrobial resistance genes across major phylogenetic groups of endodontic pathogens isolated from primary and persistent infections.**

| Antimicrobial Resistance Category | Gram-negative Bacteria | | Gram-positive Bacteria | |
|---|---|---|---|---|
| | Primary Infections | Persistent Infections | Primary Infections | Persistent Infections |
| Beta-lactam resistance | | | | |
| Beta-lactamases (Class A) | 45.3% | 62.7% | 10.2% | 18.5% |
| Beta-lactamases (Class B) | 22.8% | 38.4% | 5.6% | 12.3% |
| Extended-spectrum beta-lactamases | 8.6% | 24.5% | 2.1% | 9.7% |
| Penicillin-binding protein mutations | 18.2% | 35.6% | 32.8% | 57.2% |
| Tetracycline resistance | | | | |
| Tetracycline efflux pumps (tetA-E) | 27.5% | 43.2% | 18.4% | 38.9% |
| Ribosomal protection proteins (tetM) | 12.8% | 28.7% | 35.6% | 68.3% |
| Macrolide-lincosamide-streptogramin resistance | | | | |
| rRNA methylases (ermA, ermB, ermC) | 15.6% | 32.4% | 28.7% | 52.4% |
| Macrolide efflux pumps (mefA, mefE) | 10.2% | 25.6% | 42.3% | 63.8% |
| Aminoglycoside resistance | | | | |

| Antimicrobial Resistance Category | Gram-negative Bacteria | | Gram-positive Bacteria | |
|---|---|---|---|---|
| Aminoglycoside-modifying enzymes | 32.4% | 47.8% | 18.9% | 36.5% |
| 16S rRNA methyltransferases | 5.8% | 14.3% | 7.2% | 19.8% |
| Quinolone resistance | | | | |
| DNA gyrase mutations (gyrA) | 12.4% | 28.6% | 8.5% | 25.3% |
| Topoisomerase IV mutations (parC) | 8.3% | 21.2% | 6.4% | 22.8% |
| Plasmid-mediated quinolone resistance | 4.2% | 12.5% | 2.8% | 9.6% |
| Multidrug resistance transporters | | | | |
| RND family efflux pumps | 58.7% | 78.3% | 12.5% | 28.7% |
| MFS family efflux pumps | 42.3% | 65.8% | 48.6% | 73.2% |
| ABC family efflux pumps | 35.6% | 52.4% | 41.8% | 64.5% |
| MATE family efflux pumps | 18.7% | 34.2% | 24.3% | 45.6% |
| Miscellaneous resistance mechanisms | | | | |
| Vancomycin resistance (vanA/B/C) | 0% | 0% | 2.4% | 15.7% |
| Chloramphenicol acetyltransferases | 22.5% | 38.6% | 12.8% | 26.4% |
| Trimethoprim resistance (dfr genes) | 26.7% | 41.3% | 15.2% | 33.8% |
| Sulfonamide resistance (sul genes) | 31.5% | 46.8% | 8.7% | 22.5% |
| Mobile genetic elements associated with resistance | | | | |
| Conjugative transposons | 15.8% | 32.7% | 38.6% | 65.4% |
| Integrons | 24.3% | 47.5% | 8.2% | 19.6% |
| Resistance plasmids | 18.6% | 35.2% | 25.7% | 48.3% |

The evolutionary processes of antimicrobial resistance in the root canal ecosystem can be better understood by use of phylogenetic distribution of resistance determinant. The fact that closely related strains typically show different resistance profiles implies quick adaptation by horizontal gene transfer instead of vertical transmission of resistance features. This result has important consequences for the formulation of antimicrobial policies since it shows that resistance can develop and spread quickly inside the limited area of the root canal system.

**Horizontal Gene Transfer and Genomic Plasticity**

Extensive evidence of horizontal gene transfer (HGT) among endodontic pathogens was identified by analysis of mobile genetic elements and genomic islands; different patterns were found between Gram-negative and Gram-positive species. Especially in Fusobacterium and Porphyromonas species, genomic islands including virulence gene clusters were often found in Gram-negative pathogens. These islands frequently carried genes encoding host interaction factors, adhesins, and secretion systems, implying that HGT has been

absolutely essential in increasing the virulence potential of these species.

Particularly E. faecalis and Streptococcus species, Gram-positive endodontic pathogens, conjugative transposons and integrative-conjugative elements dominated as the main carriers for horizontal gene transfer. These mobile components often carried genes linked to stress response, antimicrobial resistance, and biofilm formation, therefore enhancing the adaptive capacity of these species in the root canal environment under treatment. Direct proof of genetic exchange inside the endodontic microbiota comes from the discovery that some mobile components were shared by phylogenetically far-off species.

Further underlining the dynamic character of endodontic pathogen genomes, the comparison of genomic synteny of closely related strains exposed many genomic rearrangements and insertion/deletion events. Particularly clear in areas close to insertion sequences and prophage elements, these structural differences imply that mobile genetic elements not only enable horizontal gene transfer but also enhance genomic plasticity by recombination events.

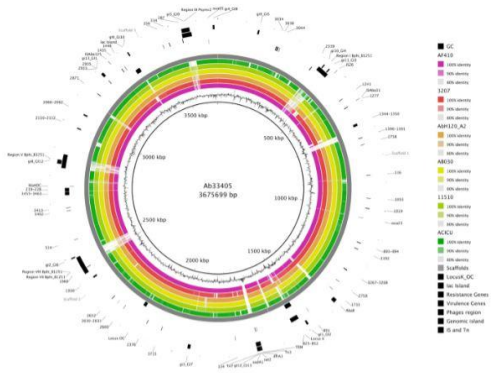

Figure 3: Circular genome plots of representative Gram-negative and Gram-positive endodontic pathogens, highlighting regions of genomic plasticity, mobile genetic elements, and horizontally acquired genes.

**Clinical Correlations and Implications for Endodontic Treatment**

Genotypic profiles and clinical criteria indicated numerous notable correlations with substantial consequences for endodontic diagnosis and treatment. Strong correlations between symptomatic infections and more periapical lesions were shown by specific phylogenetic lines within the Prevotella intermedia complex, which matched the presence of genes encoding powerful tissue-destructive enzymes and pro-inflammatory proteins. Similar substantial correlations between distinct clades of Porphyromonas endodontalis bearing various combinations of virulence genes and purulent exudates and persistent symptoms were shown.

Among Gram-positive bacteria, isolates of E. faecalis containing several adhesin genes and stress response factors exhibited the best correlation with treatment-resistant cases. Following standard endodontic treatment, persistence seems to depend especially on the combination of biofilm-forming capacity and stress tolerance. Especially, the existence of these genetic traits was a better indicator of treatment outcome than antimicrobial resistance genes by themselves, implying that ecological adaptation instead of direct antibiotic resistance may be the main process of persistence for this species.

Complex communities with unique functional signatures—including enrichment in stress response pathways, alternative metabolic strategies, and quorum sensing systems—were found by metagenomic analysis of samples from treatment failures. These results reveal that instead of only the survival of individual resistant organisms, persistence following endodontic treatment entails complex adaptive responses at the community level. This point of view marks a paradigm change in our knowledge of treatment-resistant endodontic infections and implies that therapeutic techniques aiming at community dynamics could be more successful than standard antibacterial treatments.

From a diagnostic standpoint, the identification of genetic markers linked with clinical presentations and treatment outcomes creates the prospect for molecular diagnostic tools guiding individualized therapy methods. The detection of specific virulence genes or phylogenetic variants associated with persistent infections could inform decisions regarding treatment protocols, including the selection of irrigants, intracanal medicaments, and the necessity for additional measures such as apical surgery.

**Conclusion**

By means of a thorough investigation of the genotypic landscapes of endodontic pathogens, unique evolutionary patterns and genomic characteristics separating Gram-negative and Gram-positive bacteria linked with root canal infections have become clear. While the identification of clade-specific virulence factors and resistance mechanisms clarifies the molecular basis of pathogenicity and treatment response, the phylogenetic relationships clarified in this study provide a framework for understanding the diversity and dynamics of the endodontic microbiome. Extensive horizontal gene transfer and genomic flexibility provide proof of the adaptive qualities of these species and emphasize the difficulties in creating workable treatments. These results have important ramifications for endodontic diagnosis and treatment since they point to chances for molecular diagnostic techniques and focused treatments addressing the particular genetic characteristics linked with ongoing infections. Future studies based on this basis could revolutionize our approach to endodontic infections by guiding precision endodontics driven by a thorough awareness of the genotypic landscapes influencing microbial behaviour in the root canal environment.

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