ORIGINAL RESEARCH

Microbiological Profile of Primary Teeth with Irreversible Pulpitis and Pulp Necrosis with/without Abscess and their Susceptibility to Three Antibiotics as Intracanal Medication

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Abstract

Context: The ineffective disinfection potential of conventional intracanal medicaments to eliminate enteropathogens from root canal systems leads to their persistence contributing to endodontic treatment failures. Hence, the use of appropriate intracanal medicament becomes the essential phase to accomplishing comprehensive decontamination of the root canal system. When applied topically as an intracanal medicament, antibiotics eradicate residual microorganisms from tortuous endodontic spaces, minimizing the risk of systemic toxicity.

Aims and objectives: To evaluate the prevalence of various bacterial species associated with signs of irreversible pulpitis and pulp necrosis with/without abscess in primary teeth root canals and their susceptibility against three antimicrobial agents.

Materials and methods: The pulp tissue and organic debris were retrieved from deciduous teeth (n = 50) from children between the age of 3–10 years and cultured. The bacterial identification and antibacterial profiling of isolated bacteria were done against clindamycin, metronidazole, and doxycycline through minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) assay. The MIC and MBC of each antibiotic were expressed as mean ± standard deviation (SD), range, and standard error (SE of the mean). The intergroup comparisons were done by the Kruskal–Wallis test, while intragroup pair-wise comparisons were done using the Wilcoxon signed-rank test. The confidence level will be set at 95%.

Results: Aerobic bacteria were found in 54%, microaerophilic bacteria in 76%, facultative anaerobes in 26%, and obligatory anaerobes were isolated from 30% of teeth. The intragroup and intergroup comparisons of test agent MIC revealed a nonsignificant difference ($p > 0.05$). The intragroup MBC comparisons of all the test agents revealed statistically nonsignificant ($p > 0.05$), while intergroup comparisons demonstrated nonsignificant ($p > 0.05$) to highly significant difference ($p < 0.001$).

Conclusion: Clindamycin demonstrated promising antibacterial activity against most of the isolated bacteria, while against metronidazole and doxycycline, most of the bacteria were moderate to highly resistant.

Clinical significance: Determining the antibacterial agents' efficacy along with modifications can help to target maximum pathogenic microbes and reduce catastrophic endodontic therapy failures.

Keywords: Antibacterial agents, Dental pulp cavity, Prevalence, Pulpitis, Tooth deciduous.

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Introduction

The exposure of the pulp-dentin complex to physical, chemical, or biological insults in the form of caries, trauma, or inadvertent clinical procedures often jeopardizes the functional lifespan of a tooth, subsequently leading to diseases of the pulp and periradicular tissues.[1] Irreversible pulpitis and pulp necrosis with/without abscess are frequently encountered conditions in pediatric dental practice. There have been instances of deciduous pulps showing combined signs of irreversible pulpitis and pulp necrosis. However, the presence of accessory canals complicates the scenario by disseminating infective agents into the osseous medullary spaces and around the developing tooth,[2] thus compromising the basic objectives of successful endodontic therapy. The persistence of residual microorganisms with their byproducts secondary to incomplete mechanochemical cleansing becomes the major contributing factor to endodontic treatment failure.[1]

First predominant bacteria isolated from primary root canals were alpha-hemolytic *Streptococci*, followed by *Streptococcus salivarius*.[3] Since then, various studies have identified multiple bacterial species in primary root canals,[4] few of which have mentioned the prevalence of different microbial species responsible for endodontic conditions like pulpitis, pulp necrosis, or apical periodontitis.[5] Although used for a long time for root

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canal disinfection and/or obturation, the nonantibiotic agents have shown inconstant outcomes either singly or in combinations,[6] with few disadvantages, like cytotoxicity and mutagenicity by phenolic compounds, loss of potency of aldehydes and biguanides on direct contact with organic remnants and nonionic surfactants, altered inflammatory cell function by corticosteroids, and rapid attrition of calcium hydroxide efficacy due to dentinal protein buffering; with low diffusion coefficient through microbial biofilms.[7]

Antimicrobial agents have been found effective as single-agent or multidrug combinations to eliminate vegetative as well as nonvegetative forms of microorganisms left inside the complex root canal space.[8] Hence, the use of appropriate intracanal medicament becomes the buttressing phase for accomplishing the comprehensive decontamination of the root canal system. When applied topically as an intracanal medicament, such agents eradicate infective microorganisms from tortuous endodontic spaces, which are otherwise difficult to eliminate by conventional procedures, along with minimal systemic toxicity.[9] Few studies have mentioned the efficacy of various antimicrobial agents as have been tested, but the exact doses of agents to eliminate pathogenic microorganisms responsible for endodontic infections have not been determined.

There is extensive data available for microbiological species associated with apical periodontitis and abscess, pulp necrosis, and irreversible pulpitis of primary teeth.[4] But, the data about microbial prevalence, signs of irreversible pulpitis, and pulp necrosis with/without abscess as well as their susceptibility against antimicrobial agents, is limited. Hence, the present experimental study aimed to evaluate the prevalence of various bacterial species associated with signs of irreversible pulpitis and pulp necrosis with/without abscess in primary teeth root canals and their susceptibility against three antimicrobial agents.

Materials and Methods

The present *in vitro* study was conducted at the Department of Pediatric and Preventive Dentistry in association with the Department of Microbiology after gaining approval from the Institutional Ethical Committee letter, Ref. No, DMIMS (DU)/IEC/2015-16/1744, dated 31/12/2015.

Patient Sampling Procedure

All the participants and parents/guardians were informed about the study, and a fully informed written consent form was obtained from them. The samples for microbiological evaluation were retrieved from pulp chambers of deciduous teeth of children between 3 and 10 years (males and females) and referred to the Department of Pediatric and Preventive Dentistry for endodontic therapy. A total of 50 teeth (16 maxillary incisors, 18 mandibular molars, and 16 maxillary molars) with signs of irreversible pulpitis and pulp necrosis with/without abscess were included.

Inclusion and Exclusion Criteria

During case selection, care was taken to select carious teeth without direct endodontic exposure to the intraoral environment. The cases included the teeth showing intact roots or less than two-thirds of physiological root resorption; no periodontal pockets; irreversible pulpitis and pulp necrosis with or without abscess; radiographic evidence of bone loss around periradicular areas and no endodontic intervention of the root canals.[4,5] Only the coronal structures allowing rubber dam isolation were included. Children with unhealthy conditions, presenting any systemic disease, using antimicrobial mouthwash, or receiving antibiotics 3 months before participating in the study were excluded.[10]

Specimen Sample Collection

The procedure for bacterial sample collection was followed as mentioned by da Silva et al.,[10] and Ito et al.[4] For the mandibular molars, distal root canals while for the maxillary molars and palatal root canals were mapped for microbial sampling.[10] The area of interest was disinfected with 2% chlorhexidine gluconate (Hexedine® mouthwash, 2% v/w, ICPA Health Products Ltd, Mumbai, Maharashtra, India) for 1 minute. Local anesthesia (Lignox® 2% A, lignocaine, and adrenalin injection, 1:80,000, Indoco Remedies Ltd, Mumbai, Maharashtra, India) was administered, and after achieving signs of anesthesia, the dental dam (GDC Fine Crafted Dental Pvt. Ltd., Hoshiarpur, Punjab, India), was secured on carious teeth. The carious tooth structure was removed, and access cavity preparation was made with a high-speed air rotor (NSK Pan-Max Plus, Nakanishi International, Japan) and spherical diamond burs (BR 26, Mani Dia-Burs, Mani Inc., Tochiji, Japan), and few drops of saline (Infutec Healthcare Ltd. Mumbai, Maharashtra, India), were dripped into the pulp chamber and root canals. Baseline bacterial and tissue debris samples were first collected by introducing sterile 15 no. endodontic K files (Mani Inc., Tochiji, Japan) with discrete up-and-down filing motions, 1 mm short of predetermined radiographical apex or root length. Bacterial samples were recovered by inserting four absorbent paper points (0.02 and 0.04 taper, Meta Biomed Co Ltd, Korea) for 30 seconds compatible with root canal dimensions till the predetermined length sequentially. The paper points were then removed from root canals and dipped immediately into the test tube containing 2.5 mL of reduced transport fluid, followed by instant transportation to the microbiology lab for further processing.[10,11] After sample collection, teeth were treated endodontically.

Laboratory Procedures

In the laboratory, each bacterial sample was converted to direct colony suspension with the vortex mixing device (SPINWIN Centrifuge, Korea) in Brain Heart infusion (BHI) broth (BHI, HiMedia Laboratories Pvt. Ltd, Mumbai, Maharashtra, India).[4,10] A 0.05 mL of pure samples from every dilution were streaked onto the BHI agar plates (HiMedia Laboratories Pvt. Ltd, Mumbai, Maharashtra, India) with an inoculation loop in laminar flow biological safety cabinet (Bio-Clean Air Devices, Chennai, Tamil Nadu, India). All the agar plates were incubated in an aerobic and anaerobic environment for primary bacterial culture. Colonies appearing at this stage were again subcultured on specific media (HiMedia Laboratories Pvt. Ltd, Mumbai, India) as follows:

- Blood agar supplemented with 5.0 µg/mL hemin and 1.0 µg/mL menadione.
- Thioglycolate agar with hemin and vitamin K.
- Sabouraud agar.
- Tryptic soy agar supplemented with 5% defibrinated sheep blood, 5.0 µg/mL hemin, malachite green, and bacitracin.
- Modified mitis salivarius-bacitracin (MSB) sucrose agar prepared as per Davey and Rogers was seeded up to 10^{-1} dilutions.[12]
- Sucrose-bacitracin agar (SB20).[13]

The BHI agar and Sabouraud agar plates were grown in the aerobic incubator (Adarsh International, Haryana, India) at 37°C for 24–48 hours.[4] While for anaerobic culture, blood agar with

hemin and menadione, thioglycolate agar with hemin and vitamin K, and tryptic soy agar were incubated at 37°C in an anaerobic incubator (Cerelab Technology, Howrah, West Bengal, India) in an environment with 10% H_2, 10% CO_2, and 80% N_2 for 48–72 hours. For modified MSB sucrose agar plates, incubation was done under a micro-aerobic environment in the candle jar (HiMedia India, Pvt. Ltd. Mumbai, Maharashtra, India) at 37°C for 48–72 hours. After incubation, each plate was examined in daylight, and the number of colonies was calculated in the form of CFU/mL under the electronic colony counter (Labmatrix manufacturing LLP, Bengaluru, Karnataka, India). These microbial colonies were further selected for microbiological characterization through various confirmatory tests like oxygen tolerance, catalase production, and the gaseous requirement for 48 hours in aerobic, microaerobic, and anaerobic environments to identify the bacterial and fungal species responsible for the endodontic infections. All microorganisms were gram-stained and characterized using identification kit Analytical Profile Index (API) 20A (API, Biomeriéux, France). For identifying and confirming *Streptococcus mutans (S. mutans)* and *Streptococcus sobrinus*, three to four colonies were grown on SB20 agar plates, followed by mannitol, sorbitol, raffinose fermentation, and H_2O_2 formation and evidence of bacitracin sensitivity.[10]

Preparation of Secondary Microbial Aliquots

The secondary microbial aliquots were prepared by diluting newly isolated microbial colonies from respective culture media into the freshly prepared BHI broths to obtain the suspensions of 0.5 McFarland standards (comprising of about 10^7 CFU/mL).[14,15]

Preparation of Antibacterial Agent Stock Solutions

All the analytical grade antibacterial agents were purchased for this study. Clindamycin HCL and doxycycline HCL were purchased from HiMedia Labs Pvt. Ltd, Mumbai, Maharashtra, India; while metronidazole HCL was purchased from MP Biomedicals LLC, France. The stock solutions of all antimicrobial agents were prepared by adding 1 mg of antibacterial agent in 10 mL of sterile water and converting to a concentration equivalent to 100 µg/mL.[16,17]

Determination of MIC and MBC

The MIC determination was done following Clinical and Laboratory Standard Institute guidelines using the double dilution method.[18] From each stock solution, 1 mL was added to the test tubes and diluted two-fold from 100 to 0.2 µg/mL, respectively. The last MIC tube without any test agent was maintained as the negative control. A total of 5 µL of microbial aliquots were added to all the MIC tubes and incubated at 37°C for 24–48 hours under aerobic, micro-aerobic, and anaerobic conditions, as mentioned previously. After the incubation, microbial growth was inspected visually and confirmed with spectrophotometry (optical $density_{600}$—0.6–0.7).[14] The MIC tube with the lowest drug concentration, without turbidity, was considered as the MIC of that agent against the particular microbial strain.[15] To determine MBC for all antimicrobial agents, 5 µL of the MIC broth was streaked onto the selective culture media plates as per each bacterial species and incubated for 48 hours at 37°C, as mentioned previously. The lowest concentration of the antimicrobial agent demonstrating no CFU growth on media was considered as the MBC for that particular agent and bacteria, respectively.[15] The breakpoint recommendations given by the National Committee for Clinical Laboratory Standards were used for interpretation purposes.[19] All the procedures were repeated in triplicates ($n = 3$) to average out the readings and minimize the errors.

Statistical Analysis

The isolated microbial strains from primary teeth root canals were categorized on oxygen requirement (aerobic, microaerophilic, and anaerobic), as well as staining characteristics (gram-positive/negative). The number of teeth showing microbial strains was expressed as a percentage while counted as CFU/mL. All the data on MIC and MBC of antibacterial agents against the isolated microbial species were entered in an Excel sheet and subjected to statistical analysis using IBM Statistical Package For The Social Sciences statistics for windows, version 22.0 (IBM Corp., Armonk, New York, United States of America) to check the normality of data distribution using Shapiro–Wilk (SW) test followed by descriptive statistics. The analyzed MIC and MBC data for each microorganism and drug were expressed as mean ± SD, range, and SE, for intergroup comparisons of the antibacterial agents' MIC and MBC, Kruskal–Wallis test, and intragroup pair-wise comparisons were done using the Wilcoxon signed-rank test. The confidence level will be set at 95% ($p < 0.05$).

Results

The overall prevalence of the isolated bacterial species from all the samples in primary teeth root canals with irreversible pulpitis and pulp necrosis with/without abscess was shown in Table 1. Aerobic and anaerobic microorganisms were present in all root canals of 50 teeth (100%), varying from 73 to 9300 CFU/mL. Aerobic bacteria were found in 27 teeth (54%), ranging from 76 to 9300 CFU/mL. Microaerophilic bacteria were isolated from 38 teeth (76%) ranging from 84 to 3025 CFU/mL. Facultative anaerobic bacteria were isolated from 13 teeth (26%). Obligatory anaerobic bacteria were isolated from 15 teeth (30%) ranging from 73 to 1067 CFU/mL. Aerobic bacteria like *Candida albicans* (*C. albicans)* and *Bacillus subtilis* (*B. subtilis)* were seen in 34 and 6% of cases, respectively. Aerobic and facultative anaerobic bacteria like *Pseudomonas aeruginosa* (*P. aeruginosa)* were isolated in 14% of cases. Facultative anaerobes like *Staphylococcus aureus (S. aureus)* were isolated in 12% of cases, *Actinomyces viscosus (A. viscosus)* were isolated in 6% of cases, and *Eikenella corrodens (E. corrodens)* in 8% of cases. Facultative micro-aerophilic bacteria were seen at the highest incidence in the present study that included bacteria like the genus *Streptococcus* in a total of 32 teeth (64%; range: 102–15307 CFU/mL), of which *S. mutans* were seen in seven teeth (14%), *S. mitis* was isolated from five teeth (10%) whereas *Streptococcus oralis (S. oralis)* in three teeth (6%). Other micro-aerophilic bacteria isolated were *Enterococcus faecalis (E. faecalis)* (12%), *Escherichia coli (E. coli)* (6%), and *Aggregatibacter actinomycetemcomitans (A. actinomycetemcomitans)* (8%) of the total cases. *Lactobacillus* species altogether were also isolated at relatively high incidence (20%) of total cases. It was quite surprising to observe the high occurrence of obligatory anaerobes like *Fusobacterium nucleatum (F. nucleatum)* (4%), *Prevotella intermedia* (*P. intermedia)* (10%), *Porphyromonas gingivalis (P. gingivalis)* (8%), *Veillonella parvula (V. parvula)* (6%), and *Tannerella forsythia (T. forsythia)* (2%) which accounted for around 30% of total case investigated here.

Table 2 shows the overall MIC of all the isolated microbial strains against all test agents. Among all the isolated strains, aerobic bacteria were found intermediate sensitive to resistance against clindamycin, metronidazole, and doxycycline, while aerobic and facultative anaerobes were found resistant against all the test agents. The facultative anaerobes were found moderately sensitive to clindamycin and doxycycline but resistant to metronidazole,

except for *S. aureus,* which was resistant against all agents. Similarly, all the facultative anaerobes and microaerophilic strains were also found to be moderately sensitive to clindamycin and doxycycline but resistant to metronidazole, except *E. faecalis*, which was resistant against all the agents. The obligate anaerobes were found highly sensitive to all three agents except *P. intermedia* which showed resistance to metronidazole. The value of the SW test (W) demonstrated the non-normal distribution of the MIC data ($W < 0.75$) and high statistical significance ($p < 0.05$). The intergroup comparisons (Kruskal–Wallis test) of MIC of all the test agents revealed the degree of freedom (df) at 20, and statistically nonsignificant ($p > 0.05$). While intragroup comparisons (Wilcoxon signed-rank test) demonstrated a nonsignificant difference between clindamycin vs metronidazole and clindamycin vs doxycycline ($p > 0.05$), but a significant difference between metronidazole vs doxycycline ($p < 0.05$) (Table 3).

The MBC of all the antibacterial agents against the isolated strains is displayed in Table 4. It was observed that the aerobic bacteria were eliminated by antimicrobial agents between the range 5.27–41.67 µg, while the aerobic and facultative anaerobes were eliminated by all the agents between the range 41.67–50 µg. The facultative anaerobes were eliminated between the range of 6.25–50 µg by all antibacterial agents. The facultative anaerobes and microaerophilic bacteria were eliminated by all the agents between the range of 3.20–50 µg. The obligate anaerobes were eliminated between the range 2.13–41.67 µg by all the agents. The value of the SW test (W) demonstrated the normal distribution of the MBC data ($W \geq 0.75$), with clindamycin showing significant ($p < 0.05$), metronidazole showing nonsignificant ($p > 0.05$), and doxycycline showing a high statistically significant difference ($p < 0.001$). The intragroup MBC comparisons (Kruskal–Wallis test) of all the test agents revealed df at 20, but statistically nonsignificant ($p > 0.05$). While intergroup comparisons (Wilcoxon signed-rank test) demonstrated a statistically significant difference for clindamycin vs metronidazole ($p < 0.05$), while clindamycin vs doxycycline demonstrated a nonsignificant difference ($p > 0.05$). The intergroup comparison of metronidazole vs doxycycline revealed a highly significant statistical difference ($p < 0.001$) (Table 5).

Discussion

The entry of pathogenic bacteria is responsible for endodontic infections causing irreversible pulpitis or pulp necrosis. These infections comprise mixed microbial populations involving commensals, pathogenic aerobic as well as anaerobic bacteria, of which the last one predominates the pulpal infections. The uneliminated bacterial leftovers with tissue debris can lead to catastrophic treatment failures and inadequate secondary entombment into the intraradicular ramifications.[1,10] Hence, identification of such bacteria is crucial for their elimination through locally delivered suitable mono or multidrug antimicrobial agent combinations.

Though the previous experiments evaluated microbial profiles of primary teeth with irreversible pulpitis,[20] pulp necrosis, apical periodontitis, and abscess,[4] periapical lesions[10] as well as pre and postendodontic therapy, the microbial prevalence of irreversible pulpitis and pulp necrosis with/without abscess along with their antimicrobial susceptibility against selected agents has not yet been attempted. The bacterial species isolated from the root canals of the sample teeth contained

Table 1: Prevalence of isolated bacterial strains from root canals of primary teeth

			Teeth		
Bacteria	*Type (aerobic/anaerobic)*	*Gram stain (±)*	*Number*	*(%)*	*CFU/mL*
C. albicans	Aerobic	Gm+	17	34	9300
B. subtilis subsp spizizenii	Aerobic	Gm+	03	06	76
P. aeruginosa	Aerobic and facultative anaerobic	Gm–	07	14	6423
S. aureus	Facultative anaerobic	Gm+	06	12	587
A. viscosus	Facultative anaerobic	Gm+	03	06	97
E. corrodens	Facultative anaerobic	Gm–	04	08	463
S. mutans	Facultative anaerobic and microaerophilic	Gm+	07	14	3025
S. mitis	Facultative anaerobic and microaerophilic	Gm+	05	10	1362
S. oralis	Facultative anaerobic and microaerophilic	Gm+	03	06	149
E. faecalis	Facultative anaerobic and microaerophilic	Gm+	06	12	1085
E. coli	Facultative anaerobic and microaerophilic	Gm–	03	06	503
A. actinomycetemcomitans	Facultative anaerobic and microaerophilic	Gm–	04	08	983
Lactobacillus acidophilus	Facultative anaerobic and microaerophilic	Gm+	03	06	317
Lactobacillus rhamnosus	Facultative anaerobic and microaerophilic	Gm+	04	08	2206
Lactobacillus fermentum	Facultative anaerobic and microaerophilic	Gm+	02	04	93
Lactobacillus delbrueckii	Facultative anaerobic and microaerophilic	Gm+	01	02	84
F. nucleatum	Obligate anaerobic	Gm–	02	04	81
P. intermedia	Obligate anaerobic	Gm–	05	10	1067
P. gingivalis	Obligate anaerobic	Gm–	04	08	548
V. parvula	Obligate anaerobic	Gm–	03	06	93
T. forsythia	Obligate anaerobic	Gm–	01	02	73

Aerobic, 20 (40%); aerobic and facultative anaerobic, 7 (14%); facultative anaerobic, 13 (26%); facultative anaerobic and microaerophilic, 38 (76%); obligate anaerobes, 15 (30%)

Table 2: MIC values of antimicrobial agents against the isolated microbial strains

	Clindamycin		*Metronidazole*		*Doxycycline*	
Bacteria	*Mean ± SD*	*MIC range (µg/mL)*	*Mean ± SD*	*MIC range (µg/mL)*	*Mean ± SD*	*MIC range (µg/mL)*
C. albicans	0.53 ± 0.23	0.4–0.8	5.22 ± 1.79	3.15–6.25	2.12 ± 0.90	1.6–3.15
B. subtilis subsp *spizizenii*	16.67 ± 7.22	12.5–25	20.83 ± 7.22	12.5–25	20.83 ± 7.22	12.5–25
P. aeruginosa	33.33 ± 14.4	25–50	20.7 ± 7.49	12.5–25	16.67 ± 7.22	12.5–25
S. aureus	10.42 ± 3.61	6.25–12.5	41.67 ± 14.43	25–50	8.33 ± 3.61	6.25–12.50
A. viscosus	0.67 ± 0.23	0.4–0.8	2.63 ± 0.90	1.6–3.15	2.13 ± 0.92	1.6–3.15
E. corrodens	0.53 ± 0.23	0.4–0.8	4.18 ± 1.79	3.15-6.25	1.07 ± 0.46	0.8–1.6
S. mutans	1.07 ± 0.46	0.8–1.6	8.33 ± 3.61	6.25-12.5	4.18 ± 1.79	3.15–6.25
S. mitis	1.33 ± 0.46	0.8–1.6	2.12 ± 0.90	1.6–3.15	1.33 ± 0.46	0.8–16
S. oralis	3.15 ± 0.0	3.15	6.25 ± 0.00	6.25	2.13 ± 0.92	1.6–3.15
E. faecalis	20.83 ± 7.22	12.5–25	41.67 ± 14.43	25–50	25 ± 0.00	12.5–25
E. coli	16.67 ± 7.22	12.5–25	10.42 ± 3.61	6.25–12.5	16.67 ± 7.22	12.5–25
A. actinomycetemcomitans	5.22 ± 1.79	3.15–6.25	16.67 ± 7.22	12.5–25	5.23 ± 1.76	3.15–6.25
Lactobacillus acidophilus	2.63 ± 0.89	1.6–3.15	2.63 ± 0.90	1.6–3.15	1.07 ± 0.46	0.8–1.6
Lactobacillus rhamnosus	2.12 ± 0.89	1.6–3.15	1.07 ± 0.46	0.8–1.6	2.67 ± 0.92	1.6–3.15
Lactobacillus fermentum	1.33 ± 0.46	0.8–1.6	0.67 ± 0.23	0.4–0.8	2.13 ± 0.92	1.6–3.15
Lactobacillus delbrueckii	0.40 ± 0.00	0.4	0.8 ± 0.00	0.8	0.67 ± 0.23	0.4–0.8
F. nucleatum	1.07 ± 0.46	0.8–1.6	0.67 ± 0.23	0.4–0.8	0.8 ± 0.00	0.8
P. intermedia	4.18 ± 1.79	3.15–6.25	5.22 ± 1.79	3.15-6.25	2.67 ± 0.92	1.6–3.15
P. gingivalis	4.18 ± 1.79	3.15–6.25	1.07 ± 0.46	0.8–1.6	4.22 ± 1.76	3.15–6.25
V. parvula	0.8 ± 0.0	0.8	2.12 ± 0.90	1.6–3.15	0.4 ± 0.00	0.4
T. forsythia	1.07 ± 0.46	0.8–1.6	2.12 ± 0.90	1.6–3.15	0.8 ± 0.00	0.8
Mean ± SD (Intermicrobial)	6.10 ± 8.71		9.38 ± 12.42		5.77 ± 7.37	
SE of Means	1.90		2.71		1.61	
SW test values (W)	0.69		0.70		0.70	
p-value of SW test	<0.001**		<0.001**		<0.001**	

The mean and SD of MIC values for individual bacteria in relation to each drug were calculated along with the range for the same; SD, standard deviation; SE of mean, standard error of means; SW test, Shapiro–Wilk test (W ≥ 0.75, normal data distribution); **highly significant ($p < 0.001$); *significant ($p < 0.05$); NS, not significant ($p > 0.05$)

Table 3: Intergroup and intragroup comparisons of MIC of antimicrobial agents against all bacteria

Kruskal–Wallis test (intragroup comparisons)			
	X^2	*df*	*p-value*
Antibacterial agent	20.00	20	0.458

Wilcoxon signed-rank test (intergroup comparisons)				
Test agent comparisons	*Statistics*	*p-value*	*Mean difference*	*SE difference*
Clindamycin vs metronidazole	55.00[a]	0.065 (NS)	−1.97	1.98
Clindamycin vs doxycycline	83.00[b]	0.643 (NS)	−0.14	0.89
Metronidazole vs doxycycline	164.50[a]	0.028 (S)	2.00	1.82

df, degree of freedom; SE, standard error; [a], one pair(s) of values were tied; [b], two pair(s) of values were tied

broadly aerobic and anaerobic bacteria like black-pigmented bacilli, *Streptococci*, *mutans Streptococci*, as well as gram-negative aerobic rods. Such occurrence can be attributed to inconsistent oral hygiene practices from patients leaving back food particles interred into dental plaque,[21] subsequently initiating and progressing caries and periodontal diseases. The detailed microbiological analysis identified the highest prevalence of facultative anaerobic and micro-aerophilic gram-positive bacterial isolates than other species, similar to the findings of Silva et al.[10] The smaller dimensions of primary teeth with less thickness of dentin, unlike permanent teeth, cause early pulp invasion to favor the growth of facultative and obligate anaerobic bacteria.[10] Black pigmented bacilli like *P. gingivalis*, *P. intermedia*, and *F. nucleatum* were isolated at a prevalence of 22% from primary teeth root canals with irreversible pulpitis and necrotic pulp with/without abscess, as compared to previous studies

Table 4: MBC values of antimicrobial agents against the isolated microbial strains

	Clindamycin		*Metronidazole*		*Doxycycline*	
Bacteria	*Mean ± SD*	*MBC range (µg/mL)*	*Mean ± SD*	*MBC range (µg/mL)*	*Mean ± SD*	*MBC range (µg/mL)*
C. albicans	5.27 ± 1.79	3.2–6.25	12.50 ± 0.00	12.5	20.83 ± 7.22	12.5–25
B. subtilis subsp spizizenii	41.67 ± 14.43	25–50	41.67 ± 14.43	25–50	25.00 ± 0.00	25
P. aeruginosa	50.00 ± 0.00	50	50.00 ± 0.00	50	41.67 ± 14.43	25–50
S. aureus	33.33 ± 14.43	25–50	50.00 ± 0.00	50	50.00 ± 0.00	50
A. viscosus	33.33 ± 14.43	12.5–25	20.83 ± 7.22	12.5–25	6.25 ± 0.00	6.25
E. corrodens	10.43 ± 3.58	6.25–12.5	12.50 ± 0.00	12.5	12.50 ± 0.00	6.25
S. mutans	8.37 ± 3.58	6.25–12.5	25.00 ± 0.00	25	12.50 ± 0.00	12.5
S. mitis	16.67 ± 7.22	12.5–25	33.33 ± 14.43	25–50	3.20 ± 0.00	3.2
S. oralis	10.43 ± 3.58	6.25–12.5	20.83 ± 7.22	12.5–25	5.27 ± 1.79	3.2–6.25
E. faecalis	33.33 ± 14.43	25–50	33.33 ± 14.43	25–50	50.00 ± 0.00	50
E. coli	25.00 ± 0.00	25	41.67 ± 14.43	25–50	50.00 ± 0.00	50
A. actinomycetemcomitans	33.33 ± 14.43	25–50	25.00 ± 0.00	25	12.50 ± 0.00	12.5
Lactobacillus acidophilus	20.83 ± 7.22	12.5–25	33.33 ± 14.43	25–50	8.37 ± 3.58	6.25–12.5
Lactobacillus rhamnosus	10.43 ± 3.58	6.25–12.5	25.00 ± 0.00	25	5.27 ± 1.79	3.2–6.5
Lactobacillus fermentum	10.43 ± 3.58	6.25–12.5	33.33 ± 14.43	25–50	10.43 ± 3.58	3.2–6.5
Lactobacillus delbrueckii	12.50 ± 0.00	12.5	20.83 ± 7.22	12.5–25	8.37 ± 3.58	6.25–12.5
F. nucleatum	12.50 ± 0.00	12.5	12.50 ± 0.00	12.5	4.23 ± 1.79	3.2–6.5
P. intermedia	17.17 ± 13.57	12.5–25	41.67 ± 14.43	25–50	3.20 ± 0.00	12.5
P. gingivalis	41.67 ± 14.43	25–50	25.00 ± 0.00	25	25.00 ± 0.00	25
V. parvula	10.43 ± 3.58	6.25–12.5	12.50 ± 0.00	12.5	5.27 ± 1.79	3.2
T. forsythia	20.83 ± 7.22	25	14.60 ± 9.53	25	2.13 ± 0.92	1.6–3.2
Mean ± SD (intermicrobial)	21.81 ± 13.13		27.88 ± 12.19		17.24 ± 16.68	
SE of means	2.87		2.66		3.64	
SW test values (W)	0.89		0.92		0.78	
p-value-SW test	0.025*		0.083 (NS)		< 0.001**	

The mean and SD of MBC values for individual bacteria in relation to each drug were calculated along with the range for the same; SD, standard deviation; SE of mean, standard error of mean; SW test, Shapiro–Wilk test; (W ≥ 0.75, normal data distribution); **highly significant ($p < 0.001$), *significant ($p < 0.05$); NS, not significant ($p > 0.05$)

Table 5: Intergroup and intragroup comparisons of MBC of antimicrobial agents against all bacteria

Kruskal–Wallis test (intragroup comparisons)			
	X^2	*df*	*p-value*
Antibacterial agent	20.00	20	0.458

Wilcoxon signed rank test (intergroup comparisons)				
Test agent comparisons	*Statistics*	*p-value*	*Mean difference*	*SE difference*
Clindamycin vs metronidazole	31.00[a]	0.033 (S)	−8.33	2.49
Clindamycin vs doxycycline	146.50[b]	0.125 (NS)	6.04	3.02
Metronidazole vs doxycycline	150.50[d]	0.005 (HS)	12.50	2.89

df, degree of freedom; SE, standard error; [a], four pair(s) of values were tied; [b], one pair(s) of values were tied; [d], three pair(s) of values were tied

demonstrating the prevalence of 30[10] and 36%[3]. While Assed et al.,[22] and Pantera et al.,[23] demonstrated an incidence of 49 and 60% of samples contaminated with anaerobes, respectively. Aerobic bacteria like *C. albicans* and *B. subtilis* were found at an incidence of 34 and 6%, respectively, which is in agreement with previous studies.[10,24] The lactobacillus species was isolated in 20% of cases, while *Streptococci* were isolated in 30% of cases, unlike the findings of Marsh and Largent, who found an 82% prevalence of the same.[17] The *mutans Streptococci* identified were *S. mutans*, *Streptococcus mitis (S. mitis)*, and *S. oralis*, with a similar methodology used in a previous study.[4] However, no literature supported such a high prevalence of *Lactobacilli* and *mutans Streptococci* in primary teeth root canals previously. The reason for such observation might be attributed to direct exposure of the root canals to the oral environment through caries lesions or error in the collection of microorganisms from the areas other

than the lesion being collected along with corresponding sites with disease progression.[25]

The results obtained in this study revealed that microbial ecology of the primary teeth with irreversible pulpitis and necrotic pulp with/without abscess comprises multiple bacterial species, which include anaerobic black-pigmented bacilli and cocci, as well as aerobic cocco-bacilli like *Lactobacilli* and *mutans Streptococci*. All the investigated endodontic samples presented a multi-microbial bacterial ecology, the most prevalent microorganisms of which were facultative anaerobes, microaerophiles, and anaerobes. This is in agreement with the findings of previous studies.[26] Usually, aerobic microbes prepare the environment for the installation of facultative and obligate anaerobes through the utilization of oxygen and enhancing the installation of CO_2-dependent micro-aerophilic species. Aerobic endo-pathogens such as *C. albicans*, *B. subtilis*, or *P. aeruginosa* utilize O_2 molecules from the local microenvironment developing low redox potential.[27] This gradually decreased oxygen in root canals, together with endotoxins, exotoxins, proteins, glycoproteins, and nutritional elements from the food chain, shift the microbial paradigm through natural selection, predominating micro-aerophilic and anaerobic microorganisms.[28]

The test antibacterial agents used in this experiment have been identified with numerous other properties apart from antibacterial ones, like clindamycin showing extended postadministrative potential preventing bacterial recolonization and new biofilm formation. It also disrupts bacterial protein synthesis causing bacterial damage beyond repairability.[29] Likewise, it deteriorates microbial toxin production and augments bacterial opsonization and phagocytosis, enhancing the intracellular killing capacity of polymorphonuclear neutrophils at subinhibitory doses.[30] Owing to high osseo-diffusibility, it demonstrates high osteo-penetration. Metronidazole demonstrates innate and adaptive immunomodulation. Its topical application to immune cells arrests pro-inflammatory cytokines production through interleukin (IL)—1β, IL-6, IL-8, IL-12, and LPS-induced tumor necrosis factor-α modulation.[31] It also synergizes the responses of other antibiotics by upregulating macrophage anti-inflammatory responses through enhanced IL-10 production and decreased extracellular matrix gene expression in the native tissues encouraging healing at the periapical tissue microenvironment.[32] However, owing to its rapid efficacy loss and high microbial resistance development on qualitative or quantitative dose alteration, its usage as a mono-drug therapy agent is more warranted than, in combination, to manage endodontic infections. Doxycycline, along with antimicrobial activity, also possesses the capacity to alter infectious inflammatory response by impeding the expression of monocytic triggering receptor expressed on myeloid cells 1.[33] At subinhibitory doses, it improves anti-inflammatory and host responses through the reduction in monocytic pro-inflammatory cytokines (IL-8), besides matrix metalloproteinases (MMP) 8, and MMP-9 activity, the key enzymes of tissue destruction. It halts osteoclastogenesis and bone resorption by minimizing receptor activator of nuclear factor-κβ ligand stimulated mononuclear cells and macrophage colony-stimulating factor.[34] On account of its broad-spectrum activity, it has been used successfully as a topical intracanal antibiotic, smear layer, pulp solvent, and dentine hardener.[35]

When the MICs of the test agents to inhibit 99% of the bacterial growth were evaluated, most of the bacterial strains were inhibited effectively between 12.5–25 µg/mL concentration. Among them, clindamycin demonstrated a promising role against most of the isolated bacteria strains. It was effective against all the obligate anaerobes and most of the facultative anaerobes as well as micro-aerophilic bacteria. However, *B. subtilis*, *P. aeruginosa*, *S. aureus*, *E. faecalis*, *E. coli*, and *A. actinomycetemcomitans* demonstrated resistance to it. Metronidazole was found to have limited efficacy against tested microbial species. Most of the aerobes, facultative anaerobes except *A. viscosus*, *E. corrodens*, obligate anaerobes except *P. intermedia*, as well as microaerophiles except *S. mitis*, and *Lactobacilli* were intermediate to resistant against it, which is in agreement with the reports by Baumgartner and Xia.[36] When the efficacy of doxycycline was evaluated, it was observed to be effective against the aerobes except for *B subtilis*, obligatory anaerobes except for *P. gingivalis*, facultative anaerobes except for *P. aeruginosa*, and *S. aureus* as well as micro-aerophilic bacteria *S. mutans*, *E. faecalis*, *E. coli*, and *A. actinomycetemcomitans* similar to the observations published in previous studies.[37,38] When the MBC of all the test agents to eliminate 99% of the bacterial strains was evaluated, it was observed that most of the bacterial strains were eliminated at 50 µg/mL. When individual bacteria were investigated, it was observed that aerobes like *B. subtilis* required a high concentration of all the test agents to eliminate. The facultative anaerobes *P. aeruginosa*, *S. aureus*, and *A. viscosus* required high concentrations of test agents for complete elimination, suggesting high resistance of these agents against individual bacteria. Among facultative anaerobes and microaerophiles, *E. faecalis*, *E. coli*, and *A. actinomycetemcomitans* were found highly resistant against test agents, while all *Lactobacilli* showed mild to moderate to clindamycin and doxycycline but high resistance against metronidazole.[39] Most of the obligate anaerobes were eliminated at low concentrations of all the individual test agents, which is in agreement with the previous studies.[40]

The possible explanation for the development of high resistance against various nonantibiotic as well as antibiotic agents by the above-mentioned endodontic pathogens can be justified through their high potential to form biofilms resulting in inadequate and slow penetration into the bacterial cells.[41] Additionally, the high water-solubility of such agents leads to easy deactivation and retarded penetration during biofilm matrix passage.[42] Adsorption of positively charged antibiotics onto the surface of negatively charged biofilm matrix augments faster inactivation than diffused ones.[43] Microbial capability to form a highly protected, phenotypic spore-like cellular state also increases their resistance against antibacterial agents.[44] Though the individual antimicrobial agent showed limited efficacy against the isolated bacterial strains, they can be used in combination to improve antimicrobial effects through high microbial eradication with successful endodontic therapy outcomes and least microbial resistance, as documented by Dahake and Baliga.[39] Such therapeutic modifications can present results like multilocation cellular damage of microbes, including cell walls, mitochondria, nucleic acids, protein synthesis cycle, and other cellular organelles, creating a possibility of reduced microbial cell survival at lower therapeutic doses.[40]

Conclusion

Within the limitations of the study, it can be concluded that the resistant microbial isolates have been identified from the root canal samples used in this study, most of which showed moderate to high resistance against the antibacterial agents used, *viz* clindamycin, doxycycline, and metronidazole. However, further studies are advocated to determine their efficacy with few modifications like altering drug delivery modes or combining to target maximum

pathogenic microbes and reduce catastrophic therapeutic failures. For antimicrobial agents, concentration and time-dependent activity may be better suited for their application than the only time-dependent one.

Consent for Publication

All authors give consent for the publication of the article.

Authors' Contributions

PTD and SK carried out the research work by gathering the articles for the study, thinking of the concept, and drafting the manuscript. PTD and SK participated in structuring the research, defined the intellectual content, performed the quality check, participated in its design and coordination, and helped to draft the final manuscript. All the authors read and approved the final manuscript.

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