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Plasmid Profile of Plaque Bacterial Isolates from Patients of a Teaching Hospital

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ABSTRACT

The accumulation of dental plaque plays a crucial role in the proliferation and dissemination of orodental pathogens exhibiting multidrug resistance. Within the biofilm, small DNA molecules known as plasmids play a pivotal role in transferring resistance genes among bacteria, thereby promoting the widespread transmission of antimicrobial resistance. The study, conducted at the University of Benin Teaching Hospital (UBTH), involved 105 patients from paediatric and periodontal departments. Plaque samples were evaluated for antimicrobial susceptibility using standard disc diffusion method before and after plasmid curing. Plasmid curing was carried out using acridine orange. The bacterial isolates displayed multidrug resistance, and after plasmid curing treatment, a significant increase in zones of inhibition (p-value < 0.05) against antimicrobials was noted specifically for anaerobically cultured *Clostridium* and *Prevotella.* The presence of plasmid-mediated multidrug resistance genes in plaque emphasizes the importance of antibiograms and judicious antibiotic utilization in the management of orodental diseases to address the challenge posed by the development of multidrug-resistant orodental pathogens.

Keywords: Dental plaque, orodental pathogens, plasmid curing, demographics, bacteria, antimicrobial resistance

Introduction

Dental plaque is a dynamic and diverse ecosystem, influenced by various factors such as diet and oral hygiene practices. Fermentable carbohydrates, particularly sugars, facilitate the growth of dental plaque (Astasov-Frauenhoffer and Kulik, 2021). The bacteria in the mouth feed on sugars when sugary foods and drinks are consumed and as a consequence, acids are produced that demineralize the tooth enamel resulting in cavities (Giacaman, 2018). Plaque production can also be influenced by the pH balance in the mouth. Acidic environments encourage the growth of microorganisms that are known to cause tooth decay (Krzyściak *et al*., 2014). Over time, this microbial succession may result in the emergence of increasingly diverse collection of microbial cells that may metamorphosize overtime into plaques, tartar or dental calculus (Sedghi *et al*., 2021). This heterogeneous mixture of microbial population can serve as a reservoir for the exchange of genetic materials among pathogenic microbes through quorum sensing and plasmid transfer mechanisms (Stalder and Top, 2016).

The major mechanism of acquisition of resistance in plaque bacteria is believed to be mediated by the presence of plasmids, an extra-chromosomal genetic element, capable of transferring and conferring antibiotic resistance among different classes of microbial cells (Rodríguez-Beltrán *et al*., 2021).

Plasmid is a circular DNA molecule with the ability to replicate independently of the host bacterial chromosome. Its replication is rapid owing to its small size DNA (Carattoli, 2011). This can range from a few base pairs to several kilos of base pairs (Ongkudon *et al*., 2013). The genes carried by plasmids are not always relevant to the bacterial cell, but they can benefit the host cell (San Millan and MacLean, 2017). Plasmid genes can encode antibiotic resistance, which is referred to as resistance plasmid (R- plasmid) (Rozwandowicz *et al*., 2018).

When a resistance plasmid enters a new host, it imposes a fitness cost, putting the plasmid-bearing bacteria at a competitive disadvantage in the absence of antibiotics (San Millan and MacLean, 2017).

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However, in the presence of antibiotics, positive selection can result in a beneficial plasmid gene migrating into the bacterial chromosome, enhancing the clone's survival. Horizontal gene transfer aided by mobile genetic elements has an impact on practically every aspect of bacterial evolution as certain plasmid-bacterium associations become extraordinarily successful, leading to the emergence of "superbugs" that proliferate uncontrollably in healthcare settings (Lipworth *et al*., 2021). These particular linkages between plasmids and bacterial clones are widespread, with specific plasmid, showing strong connections to distinct bacterial lineages. Illustrative instances include the connections between *Klebsiella pneumoniae* sequence type 11 (ST11) or ST405 and the plasmid pOXA-48 (housing the carbapenemase gene blaOXA-48) (Chiu *et al*., 2018), as well as the association between *Escherichia coli* ST131 clade C2/H30Rx and IncFII plasmids carrying blaCTX-M (Lipworth *et al*., 2021). Plasmid mediated gene transfer has impacted on several areas of bacterial evolution including acquisition of genes involved in virulence, ecological interaction and antimicrobial resistance (Schroeder *et al*., 2017). Therefore, determining the plasmid profile of plaque associated bacteria in this antimicrobial resistant agent is strategic for the removal of antimicrobial resistant genes from bacterial populations. The goal of this study was to investigate the plasmid profile of bacterial isolates obtained from dental plaque of patients and their multiple resistant phenotypes.

Materials and Methods

Bacterial Isolates

The study conducted between April and August 2023 involved the evaluation of 105 specimens obtained from plaque deposits in the mouths of participants seeking scaling and polishing at a designated centre. Institutional review board approval (PROTOCOL NUMBER: ADM/E 22/A/VOL.VII/148301144) was obtained. Informed consent and, for minors, assent forms were obtained. Plaque was collected using a mouth mirror and sterile dental tweezer, immediately placed in a thioglycolate broth, and transported to the lab within 2 hours. Sub-culturing onto blood agar plates followed, with aerobe plates incubated for 24 hours and anaerobe plates incubated initially for 12 hours in Robinson cooked-meat broth followed by an additional 24 hours of incubation in blood agar. Bacterial colonies which developed on the cultured plates were observed and colonies were selected based on morphology, and isolates were identified using colonial characteristics, Gram staining, and standard biochemical tests.

Antimicrobial susceptibility test

Isolates were subjected to antimicrobial susceptibility testing using the disc diffusion method on Mueller-Hinton agar as highlighted by Baur and co-worker (1966). The selection of test antimicrobial agents (Pefloxacin 10µg, Gentamicin 10µg, Ampicillin-cloxacillin 30µg, Cefuroxiume 20µg, Amoxicillin 30µg, Ceftriaxone 30µg, Ciprofloxacin 10µg, Streptomycin 30µg, Cotrimoxazole 30µg, Erythromycin 19µg) was based on treatment guidelines for orodental infections at the dental clinic. All plates were allowed to set and then dried in a hot air oven at a controlled temperature of about 50 ºC for 30 minutes with the lid separated from the plate and both inverted with their inner surfaces facing downwards. The positive control Petri dishes did not have antibiotics incorporated into the Mueller-Hinton agar plate, while the negative control dishes contained a specific strength of a stock solution. A sterile swab stick was introduced into each standardized inoculum and used to evenly streak the entire surface of a prepared Mueller-Hinton agar plate. Plates were cultured in duplicates and incubated at 37°C for 24 hours. Inhibition zone diameters (mm) were obtained and appropriately recorded before and after treating with acridine. These were interpreted as susceptible or resistant by comparison with published guidelines for antimicrobial susceptibility testing for commonly occurring pathogens, as obtained from clinical isolates (EUCAST, 2015).

Plasmid Curing

Antimicrobial testing was carried out both before and after isolates underwent treatment with acridine orange following Brown's method (Brown, 2000). Double-strength nutrient broth, supplemented with 0.1 mg/mL acridine orange, was used to sub-culture 20 µL from overnight bacterial cultures and incubation at 37°C for 72 hours in a shaker bath (Gallenkamp-England) at 150 rpm. Afterward, sub-culturing onto Mueller-Hinton agar was performed, and antimicrobial susceptibility testing was reiterated to assess the plasmid cured isolates' susceptibility by measuring inhibition zone diameters (mm).

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Data Analysis

Inhibitory zone diameters were entered and defined as numeric data prior to the analysis with respect to EUCAST breakpoint. Statistical differences between variables were calculated using t-test (paired differences). Differences between groups were considered significant as P value < 0.05.

Results

Table 1 shows the macroscopic and microscopic characteristics of isolates with raised colonies (174) observed more often than flat (58) and the Gram staining inferences for each of the isolates.

Tables 2 and 3 present the inhibition zone diameter (IZD) and resistance patterns of wild and cured aerobic and anaerobic bacteria isolates to various antimicrobials. *Streptococcus* displayed increased IZD post-curing, particularly with fluoroquinolones (19.24mm to 21.63mm), while anaerobic incubation showed a notable change from no zone to 19.77mm against aminoglycosides. Similar trends were observed for beta-lactams and macrolides, except for aminoglycosides and antifolates, resulting in a consistent increase in IZD across isolates but varying resistance patterns among antimicrobial classes. Notably, a resistant pattern emerged for aminoglycosides and antifolates in both wild and cured isolates.

Table 1: Microscopic and Macroscopic Characteristics of Bacterial Isolates

Name of Bacterial Isolates	Gram's Reaction	Colonial Morphology		Biochemical Test
Aerobes		**Raised Colonies**	**Flat Colonies**	
Streptococcus spp (n=55)	GPC	39	16	Catalase test
Bacillus spp (n=19)	GPB	13	6	Oxidase test
Staphylococcus spp (n=18)	GPC	10	8	Coagulase test
Pseudomonas aeruginosa (n=14)	GNB	6	8	Oxidase test
Haemophilus spp (n=2)	GNB	1	1	Chocolate agar test
Klebsiella Pneumoniae (n=9)	GNB	6	3	Citrate test
Anaerobes				
Streptoccocus spp (n =48)	GPC	38	10	Catalase test
Prevotella (n =39)	GNB	35	4	Indole test
Staphylococcus spp (n = 18)	GPC	16	2	Coagulase test
Clostridium spp (n = 10)	GPB	10	0	Oxidase test
	Total	174	58	

Key: GNB = Gram Negative Bacilli, GPB = Gram Positive Bacilli, GPC = Gram Positive Cocci

Table 2: Inhibition zone diameter (mm) and multi-drug resistant characteristics of anaerobes after curing

Bacterila Isolates	Code	FLQ	AMG	BL	AF	MAC	Pattern of MDR	P-Value
Streptococcus spp	1U	23.05	-	13.67	13.70	14.33	AMG	0.80
	1C	24.62	19.77	20.54	16.70	20.78		
Prevotella	2U	13.23	16.29	13.23	16.29	15.45		0.01
	2C	20.92	18.54	20.92	18.54	22.27		
Staphylococcus spp	3U	22.17	-	14.44	13.00	-	M AC	0.07
	3C	23.28	21.89	19.89	17.33	17.00		
Clostridum spp	4U	19.20	15.80	15.70	17.00	-	MAC	0.03
	4C	24.20	19.80	18.60	18.33	-	M AC	

Key: FLQ = Fluoroquinolones, AMG= Aminoglycosides, BL= Beta-lactams, AF= Antifolates, MAC= Macrolides, C= Cured Isolates, U= Uncured Isolates, - = No zone, MDR= Multidrug resistance

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Table 3: Inhibition Zone Diameter (mm) and Multi-Drug Resistant characteristics of aerobes after curing

Bacterial Isolates	Code	FLQ	AMG	BL	AF	MAC	Pattern of MDR	P-Value
Streptococcus spp	1U	19.24	-	18.91	-	17.90	AMG, AF	0.06
	1C	21.63	-	22.06	-	20.62	AMG, AF	
Bacillus spp	2U	21.42	-	17.16	-	-	AMG, AF, MAC	0.23
	2C	25.42	-	19.84	-	21.14	AMG, M AC	
Staphylococcus spp	3U	17.68	15.11	22.63	-	18.00	AF	0.75
	3C	23.63	16.05	24.11	-	22.29	AF	
Pseudomonas spp	4U	17.64	21.79	-	-	-	BL, AF, MAC	0.27
	4C	24.07	22.36	-	-	22.60	BL,	
Haemophilus spp	5U	22.50	-	30.00	-	-	AMG, AF, M AC	0.39
	5C	23.50	-	30.00	-	19.00	AMG, AF	
Klebsiella spp	6U	26.44	15.11	23.33	-	-	AMG, MAC	0.22
	6C	27.44	20.89	24.89	-	21.33	AF,	

Key: FLQ= Fluoroquinolones, AMG= Aminoglycosides, BL= Beta-lactams, AF= Antifolates, MAC = Macrolides, C= Cured Isolates, U= Uncured Isolates, - = No zone, MDR= Multidrug resistance

Discussion

The investigation into the plasmid profile of bacteria within dental biofilms sheds light on the intricate interplay between microbial communities in dental plaque and the development of plasmid-mediated resistance among multiple bacterial species. This study evaluated the plasmid profile of bacteria in ten multiple resistant species identified among 117 bacterial isolates collected from patients' plaque samples. The distinction between raised and flat bacterial colonies underscores the diverse forms of bacterial growth, with factors such as bacterial species, isolates, growth conditions, and genetic elements contributing to the variations in colony morphology (Finkelstein *et al.*, 1997). The coexistence of both raised and flat isolates in the samples implies the presence of distinct bacterial populations, reflecting genetic diversity and adaptations to the environment (Kassen *et al*., 2013). The prevalence of Gram-positive cocci suggests their common occurrence in dental plaque, while the scarcity of Gram-positive bacilli indicates a lesser prevalence in the oral microbiota of dental plaque.

Among the findings, it was observed that 48 isolates of *Streptococcus* spp were initially susceptible to fluoroquinolones before curing, and this susceptibility improved after curing, as indicated by an increase in the mean inhibitory zone diameter (IZD) from 23.05mm to 24.62mm.

Similar trends were observed for other classes of antimicrobials tested against *Streptococcus* spp, with fluoroquinolones and macrolides demonstrating complete susceptibility post-curing.

The study suggested that plasmids might be associated with the observed multi-drug resistance pattern, aligning with previous research by Cherazard *et al*. (2017) on Streptococcus resistance mechanisms.

Interestingly, not all *Streptococcus* spp isolates were cured for most of the antimicrobials, suggesting the presence of chromosomal-encoded resistance, consistent with findings by Kaufhold and Potgieter (1993) regarding *Streptococcus mitis* resistance to gentamicin. For isolates obtained from aerobic culture, there was an overall increase in susceptibility to all antimicrobials tested after curing, possibly indicating the involvement of plasmids carrying resistance traits, as suggested by Cherazard *et al*. (2017).

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In the case of *Prevotella*, all resistant isolates against fluoroquinolones became susceptible after curing. A significant increase ($p<0.05$) in mean IZDs of isolates susceptible to other antimicrobials after curing was also noted. This aligns with a study by Leung *et al*. (2002) where chimeric plasmids were observed in native *P. intermedia* plasmids. Conversely, isolates of *S. aureus* displayed a reduction in the frequency of cured isolates compared to wild isolates. However, mean IZDs increased across all antibiotic classes, with fluoroquinolones showing the highest zones of inhibition, likely due to their ability to target both rapidly dividing and dormant spp cells. Resistance to macrolides suggested plasmid-mediated mechanisms, consistent with Becker *et al*. (2018).

For *Clostridium*, there was a significant increase ($p<0.05$) in mean IZDs against all antimicrobials post-curing, indicating potential plasmid-encoded resistance, except for macrolides, implying a chromosomal-encoded pattern, as reported by Farrow *et al*. (2001). The reduced number of isolates susceptible to beta-lactams may result from chromosomally-mediated resistance via altered penicillin-binding proteins (PBPs), as discussed by Ender *et al*. (2004).

Isolates of *Bacillus* spp from aerobic culture showed increased susceptibility to all antimicrobials after curing, suggesting a role for resistance plasmids in conferring multidrug resistance. *Pseudomonas* initially displayed resistance to beta-lactams, antifolates, and macrolides, which improved after curing, except for beta-lactams. This supported the notion of chromosomal-encoded altered penicillin binding proteins, as indicated by previous research (Ender *et al*., 2004). *Klebsiella* remained resistant to antifolates after curing, suggesting chromosomal-mediated resistance.

Finally, *Haemophilus* spp, although not typical colonizers of the oral cavity, were found to be resistant to aminoglycosides, antifolates, and macrolides before and after curing. Mechanism of resistance to aminoglycoside includes enzymatic modification**,** producing enzymes called aminoglycoside-modifying enzymes (AMEs) (Labby and Garneau-Tsodikova, 2013). These enzymes chemically modify the aminoglycoside antibiotics, rendering them inactive by adding an acetyl, phosphoryl or adenyl groups to the antibiotics, preventing them from binding to the bacterial ribosomes and inhibiting protein synthesis. Increasing the production of the target enzyme; dihydrofolate reductase to compensate for the inhibitory effect of antifolates (Heinberg and Kirkman, 2015). This overproduction can overcome the inhibition and allow the bacteria to continue synthesizing folate. The ability of these bacteria to grow as biofilm may be another determinant factor for the observed bacterial resistance to these antimicrobial agents (Hall and Mah, 2017).

The variability in the maximum efficient concentration of a particular curing agent, as reported by Carlton and Brown (1981), spanning from 100 to 1000 times, as noted by Haque (2017), underscores the intricate nature of curing processes. The effectiveness of a curing substance hinges on several critical factors, including the specific species targeted and the mechanism of action of the curing agent. These variables collectively influence the outcome of the curing process. Different microorganisms may respond differently to the same curing agent due to variations in their cell structures, metabolic pathways, and susceptibility to the agent's mode of action. Additionally, the curing agent's mechanism of action plays a pivotal role. Some curing agents may disrupt cell membranes, inhibit essential enzymes, or interfere with DNA replication, making them effective against certain microorganisms but ineffective against others. This phenomenon underscores the need for precise and tailored curing strategies to mitigate the risk of resistance development, emphasizing the importance of a comprehensive understanding of curing agent interactions with microbial species to achieve successful outcomes in various applications, including antibiotic susceptibility testing and microbial control. In conclusion, the detection of plasmid-borne multidrug resistance genes within the dental plaque of study participants underscores the critical importance of implementing measures such as antibiograms and rational antibiotic usage. These findings highlight the urgent need for antimicrobial stewardship practices in the treatment of orodental diseases. By carefully selecting antibiotics based on the susceptibility profiles of these resistant genes, healthcare providers can enhance treatment efficacy while minimizing the risk of antibiotic resistance development.

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