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ORIGINAL ARTICLE

Plasmid Analysis of Resistant Bacteria Isolates from Human Orodental Specimens

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ABSTRACT

Background and Objective: Plasmids play a significant role in developing and spreading multidrug resistance genes in nosocomial pathogens, which is becoming a public health challenge. This study analyzed the plasmid of ten multi-drug resistant isolates identified among 163 bacterial strains obtained from human orodental specimens.

***Methodology*:** Isolates were subjected to plasmid content analyses and antibiogram before and after curing using standard agar disc diffusion and agar dilution methods. Plasmid DNA isolation and curing were carried out using standard alkaline lysis method and acridine orange as curing agent, while plasmid profiling of cured and wild strains was done on agarose gel electrophoresis.

Results: The bacterial isolates were multi-drug resistant and 80.0% of them harboured one plasmid with a molecular weight of 10.0 kb. The observed plasmid DNA was of type 1 colistins plasmids on comparison with plasmid databases. Minimum inhibitory concentrations of test antibiotics against cured isolates were reduced, corresponding to increased antibacterial effect. However, the cured isolates retained resistance to amoxicillin, which indicated a chromosomal amoxicillin resistance.

Conclusion: The presence of plasmid-borne multi-drug resistance gene in human orodental specimen as observed in our study underscores the need for antibiogram, rational antibiotic usage or stewardship to contain the public scourge of multi drug resistant antibiotic phenomenon.

Keywords: Plasmid, bacteria, antibiogram, resistant, infections.

INTRODUCTION

Plasmid profiling involves determining the presence, size, number, and type of extra-chromosomal DNA molecules capable of independent replication in a bacterial cell [1]. These molecules are different from the bacteria chromosomal DNA [2]. A relationship has been observed between multiple antibiotic resistance phenotypes and plasmid presence in resistant isolates [3].

Plasmid functions include translocation from one host cell to another, sometimes across broadly divergent phylogenetic boundaries. Plasmids known to be self-transmissible, transfer resistance genes through conjugation and transformation [4]. Mobilizable plasmids carry *operon tra* (transfer gene that code for pili on the cell surface) and *trb* (transfer mating bridge

operon, responsible for gene transfer) genes. These plasmids enables the transfer of acquired antibiotic resistance genes across cells, known as horizontal gene transfer. This transfer of genes have resulted in the development and spread of multi-drug resistant bacteria. Also, a variety of organisms have undergone crucial evolutionary processes due to horizontal gene transfer. In these processes, the self-transmissible plasmids ends up enlarging the gene pools responsible for gene transfer between various organisms [5].

Some other gene transfers can take place at the intracellular level among bacterial chromosomes, plasmids and transposons. This level of antimicrobial resistance transmission have been known to occur between microorganisms in animals and humans. Chromosomal mutation in genes coding for antibiotic targets is among established modes of development of resistance to antibiotics. However, the extent of the spread of these resistance genes to other bacteria is limited as it can only be passed from parents to offsprings (vertical transfer) [6].

Most of the current increase in antibiotic resistance has been due to the rapid spread of resistance plasmids through horizontal gene transfers [7]. An attractive strategy for removing antimicrobial resistance genes from a bacterial population would be through plasmid removal or disaggregation called plasmid curing. Plasmid curing compounds are basically DNA intercalating agents such as acridine orange, ethidium bromide, chlorpromazine, among others, which disrupt plasmid DNA replication by integrating into DNA [8].

Some agents like ascorbic acid cause DNA breakage, while the coumarins and quinolones affect plasmid DNA supercoiling [9]. Additionally, some other curing compounds, including poly-unsaturated fatty acids and TraE inhibitors prevent plasmid induced conjugation [9]. Exposure of microbial host strain to elevated temperature, exposure to ultra-violet radiation, treatment with crystal violet, sodium dodecyl sulphate and thymidine starvation are the other reported effective techniques [10]. This study presented antibiotic resistance profile data of some orodental bacterial isolates. The correlative roles of plasmid and chromosomal microbial DNA of these isolates, their multiple antibiotic resistance phenotype and impact on orodental infections management are highlighted.

METHODOLOGY

Bacterial isolates

Specimens were collected from 163 patients who visited the University of Benin Teaching Hospital's Dental Clinic, Benin City, Nigeria for tooth extraction from May 2017 to September, 2019 (Protocol number: ADM/E22/A/VOL.VII/14540). Deep socket culture specimen were collected with a sterile swab stick dipped into the socket of the extracted tooth. They were transported to the laboratory within 2.0 hours in 5.0 mL sodium thioglycolate broth. Each specimen was sub cultured in 10% blood agar plate and incubated at 37 °C for 24 hours. Resultant isolates were purified by further sub culturing based on colonial morphologies. In the instance where two colonies appeared different on blood agar plate both were sub cultured; whereas if two or more colonies on the plate were similar, only one colony was sub cultured. Identical colonies from the purified cultures were suspended in sterile broth for 12 hours and chromosomal extraction was undertaken by boiling the culture for onward identification using 16SrRNA. From this pool, ten bacterial isolates were randomly selected for plasmid profiling. These included: *Lysinibacillus fusiformis*, *Pseudomonas aeruginosa*, *Bacillus albus*, *Bacillus cereus*, *Bacillus thuringensis*, two strains of *Bacillus paramycoides*, *Bacillus anthracis, Paenibacillus quercus* and *Providencia alcalifaciens*.

Identification of isolates using 16SrRNA gene sequencing

Sequencing reaction

A total reaction volume of 7.0 µL, containing 1.0 µL of cleaned DNA, 0.7 µM universal primer and 2.0 µL big dye DNA sequencing kit (Applied Biosystems Ltd, UK) was diluted with 4 parts 5x sequencing buffer.

Using a thermal cycler, the following reactions were carried out with lid heat at 110 °C, initial denaturation at 95 °C for 10 seconds followed by denaturation at 50 °C for 5.0 seconds and annealing at 60 °C for 4.0 minutes. The reaction was looped back to stage 2, 99 times and the lid heat turned off at 4.0 °C.

The polymerase chain reaction (PCR) products of 16SrRNA gene fragment was obtained for each isolate using 1500bp universal 16SrRNA primers. Additional reagents included genomic DNA (50-100 ng), Tris-KCl reaction buffer, dNTP mix (0.2 mM), Taq DNA polymerase 5.0 µL and $MgCl_2$. PCR cycling

conditions used were; initial denaturation at 94 °C for 2 minutes, denaturation at 94 °C for 1.0 minute, annealing at 55 °C for 2 minutes, elongation at 72 °C for 2 minutes and final extension at 72 °C for 2 minutes. PCR amplification was performed for 30 cycles. The resultant amplicon was sequenced and data obtained were evaluated using bioinformatics computational analysis. The nucleotide sequences obtained were compared to available sequences in gene bank for identification of each isolate. Using the gene package program (version 9.0.5), phylogenetic tree was generated by the neighbour joining method. The numbers at the forks show the numbers of occurrence of the repetitive groups to the right out of the 100 boot strap samples.

Antibiogram test

Isolates were subjected to antimicrobial susceptibility test using the disc diffusion technique on Mueller-Hinton agar as described by Baur and co-worker [11]. Test antimicrobial agents used were selected based on treatment guidelines of orodental infections at the study centre and these included; amoxicillin, amoxicillin-clavulanic acid combination, metronidazole, clindamycin and perfloxacin. Antibiotic used in this study were Oxoid products manufactured in England. For the disc diffusion test, a sterile swab stick was applied into each standardized inocula and used to evenly streak the entire surface of an already prepared Mueller Hinton agar plate. Then each disc of test antibiotics were applied on the culture media and incubated at 37 °C for 24 hours. From a previously prepared 10.0 mg/mL stock solution (S/S) of each test antibiotic, the following amount were dispensed and mixed with appropriate volume of molten double strength (D/S) Mueller Hinton agar to give a concentration range of 0.32-10.5 mg/mL for MIC (Minimum Inhibitory Concentration) determination; 640 µL of S/S into a Petri dish plus 19.36 mL of D/S Mueller-Hinton agar to give 0.32 mg/mL; 1.28 mL of S/S into a Petri dish plus 18.72 mL of D/S Mueller-Hinton agar to give 0.64 mg/mL; 2.50 mL of S/S into a Petri dish plus 17.50 mL of D/S Mueller-Hinton agar to give 1.28 mg/mL; 5.12 mL of S/S into a Petri dish plus 14.88 mL of D/S Mueller-Hinton agar to give 2.56 mg/mL and 10.25 mL of S/S into a Petri dish plus 9.76 mL of D/S Mueller-Hinton agar to give 5.12 mg/mL.

All plates were allowed to set and then dried at 50 °C for 10 minutes in a hot air oven. Positive control Petri dish had no antibiotic incorporated into the set Mueller Hinton agar plate. While the negative control dish contained only 10% dimethyl sulphoxide (Sigma, Germany) used as diluent for the stock solution. A sterile wire loop was applied into each standardized inocula and then streaked into the surface of corresponding plates. All plates were incubated at 37 °C for 24 hours in triplicates. Inhibition zone diameters and minimum inhibitory concentrations (MICs) obtained were recorded in millimetres and mg/mL, respectively. These were interpreted as susceptible, or resistant by comparison with published guidelines for antimicrobial susceptibility testing for commonly occurring pathogens obtained from clinical isolates [12].

Plasmid DNA isolation and quantification

Standardized pure colonies of each strain were transferred into Mueller-Hinton broth and incubated for 16-20 hours at 35 °C. The broth cultures were harvested after 20 hours, with each strain divided into 2 aliquots. First aliquot (untreated with acridine orange) had it plasmid DNA extracted; while the second aliquot was treated with acridine orange as a curing agent. The treated bacterial strains were subjected to susceptibility tests as previously described.

First aliquot of harvested cells of each bacterial strain had their plasmid DNA extracted with alkaline lysis method using ZymoPure™ Plasmid Miniprep Kit system [13]. Bacterial cells of each strain was transferred into Eppendorf tube containing 250 µL ZymosPure™ P1 (Red) buffer and vortexed at a speed of 8000 rpm. The resultant homogenized bacterial buffer solution and 250 µL ZymosPure™ P2 (Green) buffer were gently mixed for about 6 to 8 times and allowed to stand at room temperature for 3 minutes. Approximately 250 µL of ice-cold ZymosPure™ P3 (Yellow) buffer was added to the above solution, mixed thoroughly until a yellow coloured precipitate indicative of a neutralized lysate was obtained. This lysate was incubated in ice for 5 minutes and thereafter centrifuged for 10 minutes at 14,000 rpm until the solution separated into supernatant and pellet. About 275 µL ZymosPure™ binding buffer was added to 600 µL of supernatant previously decanted into an empty Eppendorf tube. The resultant mixture, which was transferred into the Zymos-Spin™ 11-P column in a collection tube, was incubated for 2 minutes and then centrifuged at 5000 rpm for 2 minutes. After spinning, the flow-through was discarded, and the membrane containing the

extracted DNA plasmid was washed 3 consecutive times with 800, 800 and 200 µL of ZymosPure™ wash. Each wash was followed by centrifugation at 5000 rpm for 1.0 minute. Lastly, the Zymos-Spin™ 11-P column containing the washed plasmid DNA was transferred into 1.5 mL tube and about 25 µL of ZymosPure™ elution buffer was added to the column. This was incubated at room temperature for 2 minutes and then centrifuge at 10,000 rpm for 1.0 minute in a micro-centrifuge. The eluted plasmid DNA was stored at 20 °C prior to loading onto the gel electrophoresis tank.

Electrophoresis analysis of plasmid DNA

The extracted plasmid DNA was subjected to agarose gel electrophoresis with 1.6 g of agarose gel in 200 mL of water. The mixture was homogenised by heating for 3 minutes in a microwave oven and allowed to cool to 56 °C. After which 5.0 µL of ethidium bromide was transferred into the cooled agarose solution. The agarose-ethidium bromide mixture was poured onto a casting tray with a comb placed across its rim to form wells. The gel was allowed to set for 45 minutes and the comb was carefully removed. Exactly 8.0 µL of the plasmid DNA samples were then loaded into the wells after mixing with 2.0 µL of bromophenol blue in an Ependoff tube. A DNA molecular weight marker was also loaded into one of the wells. The gel was then electrophoresed in a horizontal tank at a constant voltage of 120 V for 40 minutes [14].

At the end of the process, plasmid DNA bands were viewed by fluorescence of bound ethidium bromide under a short wave ultraviolet light trans-illuminator and the photographs were taken using a Samsung 12.2 mega pixel, digital camera (Germany). Plasmid DNA bands were matched with DNA ladder standard molecular weight marker in the range of 1.0-10 kb (New England Biolab).

Plasmid curing

Second aliquot of harvested cells were each treated with acridine orange according to the method of Brown [15]. Nutrient broth was prepared and supplemented with 0.1 mg/mL acridine orange. A 20 µL volume of the overnight culture of the bacteria was sub-cultured into 5.0 mL of the nutrient broth containing acridine orange. The samples were then incubated at 37 °C for 72 hours in a shaker bath at a revolution of 150 rpm. After 72 hours incubation, the isolates were sub-cultured onto Mueller Hinton agar to determine susceptibility of treated isolates to the antibiotics by the measurement of inhibition zone diameters and MICs as previous described Plasmid DNA extraction was repeated on the treated strains to determine success or otherwise of the treatment process.

RESULTS

Table **1** shows the inhibition zone diameters (IZD) in millimetres and the resistant pattern of the wild and cured bacterial isolates to test antibiotics. *L. fusiformis* had an IZD of 12 mm with perfloxacin, increasing to 24 mm in the cured form. *P. aeruginosa* had IZD of 26/28 mm with perfloxacin and 0/27 mm with clindamycin. An IZD range of 19-27 mm was observed for perfloxacin for wild in the *Bacillus genera* except for *B. thurigenesis* that was resistant to perfloxacin, clindamycin and metronidazole before curing, with IZD of 0 mm as against 25 mm, 26 mm and 24 mm, respectively, post DNA curing treatments.

A similar trend was observed by *B. anthracis* which was resistant to amoxicillin-clavulanic acid, clindamycin and metronidazole with pre-curing zone diameter of 0 mm as against 15 mm, 21 mm and 21 mm post curing treatment, respectively. It should be noted that all the test isolates cured and wild were resistant to amoxicillin. *P. alcalifaciens* had IZD of 32 mm with perfloxacin for the wild/cured variants of the isolates but the isolates were completely resistant to amoxicillin-clavulanic acid, clindamycin and metronidazole in their wild forms as against 32 mm, 24 mm and 22 mm, respectively for the cured isolates.

Table **2** shows the MICs of all test antibiotics against the wild and cured bacterial isolates. The MICs were higher in the wild isolates with a range of 0.32 mg/mL to greater than 10.5 mg/mL. On the other hand a lower MIC values of 0.04 - 5.12 mg/mL were obtained for cured isolates.

Figures **1** and **2** shows the plasmid profiles of wild and cured multi-drug antibiotic resistant bacterial isolates, respectively. From the electrophoresis results wild bacterial isolates contained one plasmid band each with the same molecular weight of 10 kb except for two isolates with no visible band (*L. fusiforms* and *B. paramycoides*), while the electrophoresis results of the cured bacterial isolates had no visible plasmid band, consistent with successful plasmid curing process.

Table 1. Inhibition zone diameters (mm) and multi-drug resistance pattern of wild and cured isolates to different antibiotics.

Isolate	Code	PEF	CDC	AMC	MET	AML	MDR patterns
L. fusiformis	1U	12	-	-	-	-	CDC, AMC, MET, AML
	1C	24	-	-	-	-	CDC, AMC, MET, AML
P. aeruginosa	2U	26	-	-	-	-	CDC, AMC, MET, AML
	2C	28	27	-	-	-	AMC, MET, AML
B. albus	3U	19	-	-	-	-	CDC, AMC, MET, AML
	3C	30	26	22	29	-	AML
B. cereus	4U	23	-	-	-	-	CDC, AMC, MET, AML
	4C	23	22	21	19	-	AML
B. thuringensis	5U	-	-	-	-	-	PEF, CDC, AMC, MET, AML
	5C	25	26	-	24	-	AMC, AML
B. paramycoides	6U	22	-	-	-	-	CDC, AMC, MET, AML
	6C	22	-	41	-	-	CDC, MET, AML
B. paramycoides	7U	19	-	16	-	-	CDC, MET, AML
	7C	30	23	23	13	-	AML
B. anthracis	8U	21.5	-	-	-	-	CDC, AMC, MET, AML
	8C	31	21	15	29	-	AML
P. quercus	9U	27	18	-	23	-	AMC, AML
	9C	32	23	-	24	-	AMC, AML
P. alcalifaciens	10U	32	-	-	-	-	CDC, AMC, MET, AML
	10C	32	24	32	22	-	AML

PEF = perfloxacin, CDC = clindamycin, AMC = amoxicillin-clavulanic acid, MET = metronidazole, AML = amoxicillin, MDR = multi-drug resistance

Table 2. Minimum inhibitory concentration (mg/mL) and multi-drug resistance pattern of wild and cured isolates to test antimicrobial agents.

Isolate	Code	PEF	CDC	AMC	MET	AML	MDR patterns
L. fusiformis	1U	> 0.32	> 10.5	4	> 10.5	2.56	CDC, AMC, MET, AML
	1C	0.04	> 10.5	4	> 10.5	2.56	CDC, AMC, MET, AML
P. aeruginosa	2U	0.32	> 10.5	1	> 10.5	0.32	CDC, AMC, MET, AML
	2C	0.32	5.12	1	> 10.5	0.32	AMC, MET, AML
B. albus	3U	0.32	> 10.5	4	4	0.64	PEF, CDC, AMC, MET, AML
	3C	0.02	5.12	1	1	0.64	AML
B. cereus	4U	0.32	> 10.5	4	4	2.56	CDC, AMC, MET, AML
	4C	0.32	5.12	1	1	2.56	AML
B. thuringensis	5U	0.32	> 10.5	1	10.5	2.56	CDC, AMC, MET, AML
	5C	0.08	5.12	1	5.12	2.56	AMC, AML
B. paramycoides	6U	> 0.32	> 10.5	1	> 10.5	0.32	CDC, AMC ,MET, AML
	6C	> 0.32	> 10.5	0.32	> 10.5	0.32	CDC, MET, AML
B. paramycoides	7U	0.32	> 10.5	1	> 10.5	0.32	CDC, MET, AML
	7C	0.04	5.12	0.02	5.12	0.32	AML
B. anthracis	8U	0.32	> 10.5	1	10.5	4	CDC, MET, AML
	8C	0.04	5.12	0.08	5.12	4	AMC, AML
P. quercus	9U	0.32	> 10.5	4	> 10.5	4	AMC, AML
	9C	0.04	5.12	4	> 10.5	4	AMC, AML
P. alcalifaciens	10U	0.04	> 10.5	1	10.5	4	CDC, AMC, MET, AML
	10C	0.04	> 10.5	0.32	5.12	4	AML

PEF = perfloxacin, CDC = clindamycin, AMC = amoxicillin-clavulanic acid, MET = metronidazole, AML = amoxicillin, MDR = multi-drug resistance

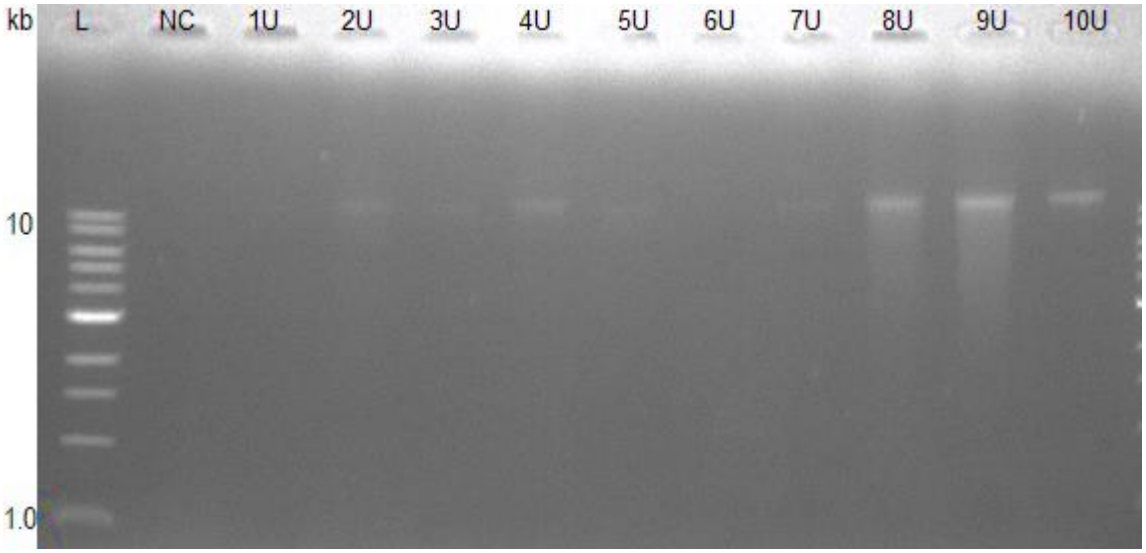

Figure 1. Electrophoresis result of wild multi-drug antibiotic resistant bacterial isolates.

(L is 1kb - 10kb DNA ladder (molecular marker). Isolates 2U, 3U, 4U, 5U, 7U, 8U, 9U and 10U are positive for plasmid DNA with bands at 10kb while isolates 1U and 6U are negative for plasmid DNA. NC is a no plasmid DNA template control)

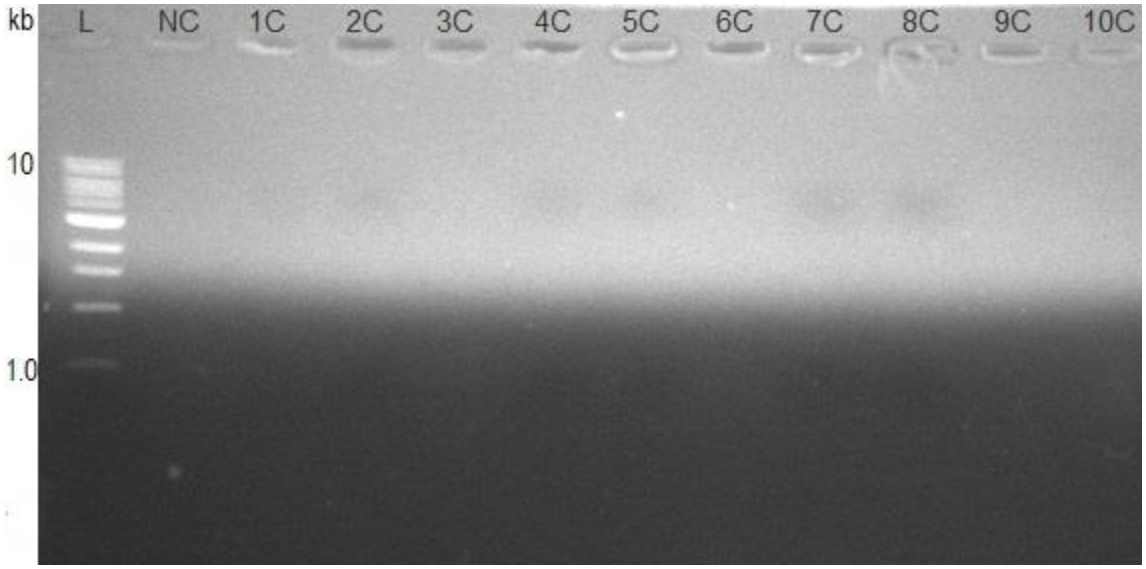

Figure 2. Electrophoresis result of cured multi-antibiotic resistant bacterial isolates.

(L is 1kb - 10kb DNA ladder (molecular marker). Isolates 1-10C are negative for plasmid DNA. NC is a no plasmid DNA template control)

DISCUSSION

The mouth harbours distinct habitats such as the mucosal surfaces of the cheeks, palate, periodontal region, tongue as well as the teeth, which provide different ecologic conditions that facilitate the growth of pathogenic bacteria. *Enterobacteria* do not usually occur in the oral cavity as recorded in this study, but reports exists implicating *P. aeruginosa* isolates as endodontic infectious agents [16]. This organism has been recovered from periapical lesions following endodontic treatment and from periodontal pockets of patients with refractory periodontal diseases [17]. In this study, this rare orodental pathogen was resistant to clindamycin, metronidazole and the β-lactams (amoxicillin-clavulanic acid and amoxicillin) prior to curing. The observed multi-drug resistance pattern

may be due to the presence of 10 kb molecular weight plasmid in the cell as observed from the plasmid DNA profile. The ability of this bacterium to grow as biofilm may be another determinant factor for the observed bacterial resistance to these antimicrobial agents [18]. However, this study showed susceptibility of *P. aeruginosa* to perfloxacin prior to curing. This is in line with existing literature where fluoroquinolones were successfully used in the treatment of periapical *P. aeruginosa* infections [19].

Perfloxacin and other quinolones have been reported to have antibacterial and antiplasmid activities. Weisser and Wiedemann reported that five fluoroquinolones and two quinolones cured four plasmids in *E. coli* [20]. In another study, sub-inhibitory levels of quinolones had cured *E. coli* of large clinical plasmids [9]. Additionally, quinolones have been documented as agents capable of curing and reducing the copy number of several plasmids [21]. Agents such as these have been shown to reverse antibiotic resistance in several bacterial species through the inhibition of efflux pump [22]. These anti-plasmid activities of perfloxacin may have accounted in part for the observed susceptibility of all test isolates to the antimicrobial agent in this study.

This study isolated many species of *Bacillus* which have not been previously associated with orodental infections, including *B. paramycoides, B. thuringensis and B. albus*. All the wild *Bacillus* species were resistant to multiple antibiotics (perfloxacin, metronidazole, clindamycin and β-lactams). However, after curing, all the test isolates became sensitive to test antibiotics except amoxicillin indicative of chromosomal resistance. This observation correlates with some previous studies where the bulk of *Bacillus* species used in these studies showed variable susceptibilities to the penicillins but were susceptible to clindamycin and fluoroquinolone [23,24]. In an *in vitro* susceptibility study, clindamycin, fluoroquinolones and combination were relatively effective against *Bacillus species* as single agents [25]. This agrees with our finding, as *B. cereus* was susceptible to clindamycin, perfloxacin and amoxicillin-clavulanic acid after curing. However, the observed resistance of all the isolates to amoxicillin even after curing could be suggestive of chromosomally-mediated mechanism of resistance [26]. The isolation of *Paenibacillus quercus* from human orodental specimen in this study is novel as this species had only been previously associated with soil rather than humans [27]. The observed susceptibility to the fluoroquinolones and resistance to the β-lactams of this genus has been demonstrated in previous studies with many species of Paenibacillus [28]. Isolation of *B. cereus* also agreed with existing literature which documented that treatment mediated damage to the buccal mucosa could expedite adherence and colonization of the oral cavity by *B. cereus* [29].

Detailed analysis of the cured isolates showed reduced MIC values in synergy with increased antibacterial effects of test antibiotics in the plasmid free isolates. This observation highlighted the fact that plasmid genes might have encoded resistance in the wild isolates. Isolation *of L. fusiformis* from human orodental specimen as observed, contrasted with its natural soil habitat, despite the recently reported isolation from temperate air in Saudi Arabia and Singapore [30].

The observed increase in antibacterial effects of perfloxacin and amoxicillin-clavulanic acid against plasmid free *L. fusiformis* (1U) and *B. paramycoides* (6U) after curing may be attributable to the ability of acridine orange to revert antibiotics resistance via possible inhibition of efflux pump in various bacterial species [31]. The obtained plasmid size of 10 kb was comparable to *type 1 colistin plasmids*. These are small, mobilizable plasmids with molecular weight range of 6-10 kb often numbering about 20 copies per bacterial cell [32]. They harbour colistin genes *mcr-4* and *mcr-5*, known for their unique role in the spread of antimicrobial resistance of the quinolones and β-lactam antibiotics [33-37] as well as sulphonamide and tetracycline resistance [38,39]. Possible coexistence of this novel colistin resistant genes in a plasmid (with molecular weight 10 kb) within the same bacterial host as observed may explain in part the multi-drug resistance phenotypes among the isolates prior to curing [40].

CONCLUSIONS

Presence of plasmids in bacterial isolates obtained from human orodental specimen as seen in our study is of great concern as this could be implicated in the spread of multidrug resistant pathogens to other focal-oral sites in the human body. Results derived from plasmid curing assay can provide information that would influence better antibiotic management policy in the study centre. It is therefore

recommended that antibiogram and molecular plasmid typing could provide insight in the choice of antimicrobial agents used for the successful therapeutic management of orodental infections.

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