International Journal of ChemTech Research
CODEN (USA): IJCRGG, ISSN: 0974-4290, ISSN(Online):2455-9555
Vol.11 No.10, pp 84-94, 2018

Biofilm Formation andMultiple Antibiotic Resistance Index of Bacteria Isolates from Saliva, Teeth and Necrotic Roots Canals of Teeth of Dental Patients

Merriam Ghadhanfar Alwan[1,]*, Ashraf Abbas Drais[1]and Asmat Ahmad[1]

[1]School of Bioscience and Biotechnology,Faculty of Science and Technology, UniversitiKebangsaan Malaysia, 43600 Bangi, Selangor, Malaysia.

Abstract : Globally, antibiotic resistance among oral microbiota has constituted an increasing health challenge, and limited information regarding such resistance is available. This study was designed to isolate both aerobic and anaerobic bacteria from different sources of oral samples and screen the isolates for biofilm forming and antibiotics resistance abilities. A total of 72 samples were collected, 21, 31 and 20 were from saliva, teeth and necrotic roots canals respectively. In general, among 267 total isolates, 16.2% were identified as *Enterococcus* sp. and it was considered as the most prevalence genus, followed by *Streptococcus* sp. (15.8), *Staphylococcus* sp. (13.5%), *E. coli* (7.5%), *Bacillus* sp. (6.4%), *Enterobacter* sp. (5.6%), *Pseudomonas* sp. (4.5%), *Proteus* sp. (4.5%), *Clostridium* sp. (4.1%), *Actinomyces* sp. (3%), *Peptostreptococcus* sp. (3%), *Klebsiella* sp. (2.6%), *Bacteroides* sp. (2.6%), *Lactobacillus* sp. (2.3%) , *Fusobacterium* sp. (1.9%), *Micrococcs* sp. (1.5%), *Salmonella* sp. (1.1%), *Provotella* sp. (1.1%), *Shigella* sp. (0.8%), *Eubacterium* sp. (0.8%), *Aerococcus* sp. (0.8%), *Chromobacterium* sp. (0.4%).Three methods were used to detect the biofilm formation ability of the isolates. The results showed that the percentages of strong biofilm formation of the isolates for each method were 11.9%, 27.7%and 39% for Congo red agar method, Tube method and Microtitre plate methodrespectively. The highest multiple antibiotic resistance index (MAR)was among the isolates from necrotic roots canals (0.82) followed by teeth (0.71) and saliva (0.69). According to isolate's genera, *Enterococcus* sp. showed the highest MAR indices among the isolates, which recorded 0.97, 0.96, and 0.89 for the isolates from saliva, necrotic roots canals and teeth respectively.

Introduction

The oral cavity is the first part of the gastrointestinal tract and it has several features that make it a distinct microbial habitat. The various surfaces in the oral cavity are continuously bathed with saliva and they represent different ecological niches in which distinct inhabitants exist within this complex environment[1]. The diverse features of the different surfaces found in the oral cavity, each with different key ecological factors such as adhesion ligands, pH, nutrients, redox potential, oxygen tension, and temperature, make it a unique microbial habitat in the human body [1].Oral diseases are considered as one of the major public health challenges owing to

DOI= http://dx.doi.org/10.20902/IJCTR.2018.111012

their high prevalence and incidence across the globe. These diseases include dental caries, teeth loss, oro-dental trauma, oral mucosal lesions and oropharyngeal cancers [2].The demolition of the calcified tissue of the teeth is called dental caries,and it occurs as a result of the presence of diverse oral bacteria that can use the fermentable carbohydrates which surround the tooth for the protracted period[3].Based on both culture-dependent and culture-independent methods conducted by several scientists all over the world, it has been estimated that about 700 species of bacteria are capable of inhabiting the human oral cavity. Fewer novel bacterial species are now being discovered from the oral cavity [4], [5].The oral cavity is an environment where diverse bacterial species can be investigated through routine culture methods,and several studies have estimated that cultured oral bacterial species account for approximately 50% of the microorganisms identified by culture-independent methods [6], [7].Several Gram-positive and Gram-negative bacterial genera are found in the oral cavity. Among the Gram-positive ones are *Enterococcus*, *Peptostreptococcus*, *Streptococcus*, *Staphylococcus*, *Actinomyces*,*Corynebacterium*, *Eubacterium*, and *Lactobacillus* species, whereas Aggregatibacter (formerly *Actinobacillus*), *Haemophilus*, *Bacteroides*, *Campylobacter*, *Leptotrichia*, *Prophyromonas*, *Capnocytophaga*, *Prevotella*, *Tannerella*, *Eikenella*, *Treponema*, *Fusobacterium*, and *Wolinella* species are among the Gram-negative ones [1].Biofilms on the other hand, are defined as microbially derived sessile communities characterized by the cells that are irreversibly attached to a substratum or to each other. These bacteria are usually embedded in a matrix of extracellular polymeric substances (EPS) synthesized by the bacterial community and they exhibit an altered phenotype with respect to growth rate and gene transcription [8].Within a biofilm community, bacteria communicate with each other through the synthesis of chemotactic particles or pheromones, a phenomenon referred to as quorum sensing [9]. Microorganisms growing in a biofilm community have been reported to be intrinsically more resistant to antimicrobial agents compared to planktonic cells. High antimicrobial concentrations are required to inactivate organisms growing in a biofilm, as antibiotic resistance can increase 1,000 fold [10].Biofilms are associated with numerous medical conditions such as indwelling medical devices, dental plaque, upper respiratory tract infections, peritonitis, and urogenital infections[11]. Both Gram-positive and Gram-negative bacteria can form biofilms. Bacteria commonly involved in biofilm formation include *Enterococcus faecalis*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus viridans*, *Escherichia coli*, *Klebsiella pneumonia*, *Proteus mirabilis* and *Pseudomonas aeruginosa*[11].There are various methods of detecting biofilm production have been reported,and these include the Tissue Culture Plate (TCP) [12], Tube method (TM) [13],Congo Red Agar method (CRA) [14]. Multiple antibiotic resistance (MAR) indexing has been shown to be a cost-effective and valid method of bacteria source tracking. MAR index values higher than 0.2 are considered to have originated from high-risk sources of contamination such as human beings, commercial poultry farms, swine and dairy cattle farms where antibiotics are frequently employed[15]. The global problem of antimicrobial resistance is particularly pressing in developing countries, where infectious disease burden is usually high,and cost constraints mitigate the common application of newer, more expensive therapeutic agents [16]. Even though treatment failure resulting from antimicrobial resistance is common, information on the biofilm forming ability and MAR ability of bacteria isolates from the oral cavity is still limited, especially in Malaysia. Understanding these bacteria dynamics could unravel vital information that could be useful in treating, preventive and control measures targeted against pathogenic oral microbiota.Hence this study aimed to isolate aerobic and anaerobic bacteria from different oral samples from saliva, teeth and necrotic roots canals of teeth and to determine the biofilm formation and multi antibiotics resistance (MAR) indices of these clinical isolates of bacteria.

Experimental

Subject Groups

A total of 72 oral specimens (21 saliva, 31 teeth, and 20 root canal) were collected from patients attending Dental Clinic, health clinic Seri Kembangan / Selangor / Malaysia and Dental Clinic, UKM/ Bangi/ Malaysia. The patients (males and females) were chosen randomly, with age ranging from 5 to 55, who did not use antibiotics for the last 3 months and all patients have various dental and periodontal problems. The patients were instructed not to drink, eat, smoke, or clean their teeth for 2h before the sampling. All samples were collected by special dentist. Each patient was given a printed paper explaining the aim of this study and their consents were obtained.

Microbial Samples Collection

Saliva samples :

This study was conducted on unstimulated saliva samples. Subjects were instructed not to drink, eat, smoke, or clean their teeth for 2h before the sampling. Saliva samples were collected in sterilized tubes which were then transported to the Laboratory within 2h [17].

Teeth Samples:

Clinical specimens (teeth) were aseptically collected from each patient and transported in Stuart's transport mediumfor processing in the laboratory [18].

Root canal samples:

Following the removal of the paper point from the root canals, they were each placed into sterile 2 ml Eppendorf tubes containing Stuart transport medium which preserves strict anaerobes for 40 minutes and facultative anaerobes for up to 2 hours [19].

Isolation and Identification of the isolates

Isolation and identification of aerobic, anaerobic and facultative organisms were performed using standard bacteriologic techniques. Peptone water was used for the Ten-fold serial dilutions of the samples,and the diluted samples were subsequently spread (0.1 ml) on Brain Heart Infusion agar (BHIA) (Oxoid) plates. The plates were then incubated aerobically and anaerobically for 1-5 days at 37 °C. Following incubation, isolation and purification of the bacterial colonies were performed using the same medium [20].Morphological characteristics, cultural characteristics and biochemical properties of the isolates were determined and subsequently analyzed to confirm the Genera of each isolate. For the Cultural characteristics of the isolates, selective media were used (MacConkey Agar, Bile Esculin Agar, Enterococcsel Agar, Manitol Salt Agar, S-S agar, Eosin Methylene Blue Agar, TCBS, MRS agar, Actinomysetes isolation agar, Staphylococcus agar, Pseudomonase agar and Slanetz and Bartley medium). For the biochemical identification, several biochemical tests were employed to determine the genera of each isolate. These tests which were performed following gram stain include catalase, oxidase, acid production from carbohydrates, motility test, TSI test (triple sugar-iron agar), IMVC test (indole methyl red voges- proskauer citrate) and Simmon citrate test.

Detection of biofilm formation

Three methods were employed for screening biofilm forming ability of the isolates. Two of methods (Congo red agar method and tube method) are considered qualitative methods and the one (Microtiter plate method) is considered as a quantitative method. For Congo Red Agar (CRA) method, the medium was prepared by mixing 47 g/L Brain heart infusion agar (Oxoid, UK) with 50 g/L sucrose, and 8 g/L Congo red indicator (Oxoid, UK). Test isolates were cultured on CRA plates and subjected to incubation for 24 h at 37 ^{o}C. Black colonies with dry crystalline consistency which is an indication of biofilm formation were considered positive [11]. For Tube Method, the test isolates were inoculated in 10 mLtrypticase soy broth (TSB) containing 1% glucose. The mixture was subsequently subjected to incubation for 24 h at 37^{o}C. Following incubation, the mixture was decanted and washed with phosphate buffer saline (PBS) (pH 7.3). The tubes were air-dried and stained with 0.1% crystal violet. Excess stain on the tubeswere washed with deionized water and air-dried in an inverted position. The tubeswere considered positive for Biofilm formation when a visible film lined the wall and the bottom of the tube[13].For the Microtiterplate method**,** the test isolates were subjected to overnight incubation on brain heart infusion broth (BHIB) at 37 ^{o}C. Subsequently, 200 µL of the suspension was diluted with the same broth to 1:40. The tests were performed in triplicate by introducing them into sterile 96-well polystyrene microtiter plates (Sigma Aldrich, USA). Following incubation at 37^{o}C for 24 h period, the wells were washed with 200 µL PBS and then air-dried in an inverted position for 1 h. The wells were subsequently stained with 1% crystal violet (CV) for 15 min at room temperature. After washing off excess stain with PBS, CV was extracted from adhering bacterial cells using 200 µL of 80:20 (v/v) ethyl alcohol/acetone. The OD of the wells was read at 570 nm (OD570) using microplate ELISA reader (BioRad, USA)[21].

Multi Antibiotic Resistance index (MAR Index)

Antibiotic resistance of the strong biofilm forming isolates (saliva n=55, teeth n= 32 and necrotic roots canals n= 17) were determined on Muller & Hinton Agar (Oxoid) using Kirby-Bauer disk diffusion method [22]. The antibiotics selected for the study comprised of: kanamycin K (30µg), vancomycin VA (30µg), amoxycillin AML (10µg), erythromycin E (30µg), ampicillin AMP (10µg), cephalothin KF (30µg), chloramphenicol C (30µg), tetracycline (30µg), ciprofloxacin CIP (10µg), nalidixic acid NA (30µg), gentamicin CN (10µg), novobiocin NV (30µg), carbenicillin CAR (100µg), oxacillin OX (5µg), penicillin G (10 UI) and streptomycin (10µg). All the antibiotic discs were procured from Oxoid (UK). *Enterococcus faecalis* ATCC 33186 was used as control strains. Multiple Antibiotic Resistance (MAR Index) Index of the samples was calculated by the formula[23].MAR Index = Total number of resistance scored/ (number of isolates* Total number of antibiotics tested).

Statistical analysis

The MTP method was considered the gold-standard for this study and compared with data from TM and CRA methods. Parameters like sensitivity, specificity, negative predictive value, positive predictive value and accuracy were calculated. True positives were biofilm producers by MTP, TM and CRA methods. False positive were biofilm producers by TM and CRA methods and not by MTP method. False negative were the isolates which were non-biofilm producers by TM and CRA but were producing biofilm by MTP method. True negatives are those which were non biofilm producers by all the three methods[24].

Results and Discussion

Culture findings

The number of total samples collected during this study and the summary of the culture findings following culturing of the samples on non-selective enrichment medium (Brain heart infusion agar) is presented in Table (1). A total of 72 clinical samples were collected (21, 31 and 20 from saliva, teeth and necrotic roots canals respectively). Following culturing of the samples, all samples showed positive culture except two samples from necrotic root canals which did not give any growth either aerobically or anaerobically. Based on the results of the culture, positive culture from necrotic roots canals was 90% (18/20 cases). This result is compatible with [25]who reported 86.7% positive cultures (26/30 cases) of aerobic and anaerobic microorganisms from necrotic roots canals of teeth. The percentage of pure cultures (having one type of bacteria on the selected plate) of the samples were 0%, 0% and 38.9% to for saliva, teeth and necrotic roots canals respectively. The number of isolates per sample was 6.5, 2.8 and 2.3 for saliva, teeth and root canals respectively, and this is an indication of the polymicrobial nature of oral samples. Our finding agreed with those of [26] and[25]where 2.43 and 2.65 isolates per tooth were reported.

Table (1) Summary of Culture Findings

Sample source	No. of total samples	No. of positive samples (%)	No. of negative samples (%)	No. of pure culture (%)	No. of mix culture (%)	No. of isolates per sample	No. of isolates
Saliva	21	21 (100%)	0 (0%)	0 (0%)	21 (100%)	6.5	137
Tooth	31	31 (100%)	0 (0%)	0 (0%)	31 (100%)	2.8	88
NRC*	20	18 (90%)	2 (10%)	7 (38.9%)	11 (61.1%)	2.3	42
Total	72	70 (97.2 %)	2 (2.8%)	7 (10%)	63 (90%)	3.7	267

*NRC= necrotic root canal

In the present study, the percentage of gram positive isolates from the 267 isolates was 70% while that of gram negative isolates was 30%. The highest percentage of gram positive isolates (83%) was found in Necrotic roots canals isolates, followed by saliva isolates (65%) and then Teeth isolates (63%). This result showed that Gram negative bacteria are the predominant bacterial isolates from oral samples of patients with dental caries problems. According to O_2 growth requirements, 75% of the total isolates were facultative

anaerobic bacteria and this was the highest, which was followed by anaerobic bacteria (16%) and aerobic bacteria (8%). Interestingly, the highest percentage of the facultative anaerobes (97%) were obtained from teeth isolates, followed by saliva isolates (72%) and the least percentage (57%) was from necrotic roots canals isolates. Among the isolates from necrotic roots canals, facultative anaerobic isolates were found to be the most predominant (57%) followed by anaerobic (31%), and the least percentage (12%) was aerobic microbes. Based on cell morphology, 54% of the total isolates were found to be cocci while 46% of the isolates were Rod-shaped. Surprisingly, the highest percentage of cocci isolates were found among necrotic roots canals isolates (60%), followed by teeth isolates (53%), while saliva isolates on the contrary were predominantly rod shape (52%). All the phenotypic characteristics were showed in figure (1). Several studies have shown that primary and persistent endodontic infections vary in their microflora. Primary infections are generally induced mostly by anaerobic, Gram-negative organisms, while Gram-positive facultative bacteria are associated with persistent infection. In total, the predominant bacteria from saliva were gram +, facultative and rod shaped anaerobes isolates while the predominant microbes from teeth and necrotic roots canals isolates were found to be gram +, facultative and cocci microbes. These findings corroborate with the findings reported in an earlier study where facultative anaerobes and gram positive bacteria were found to be the most predominant microbes in canals with failed endodontic treatment[27].

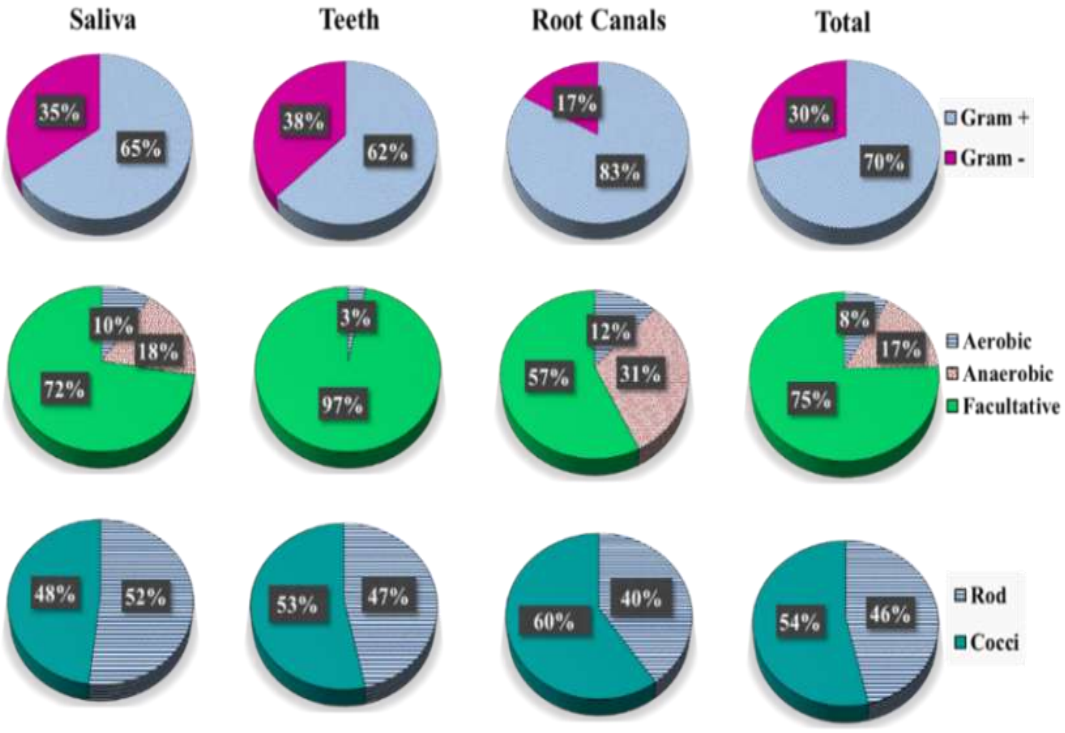

Figure (1)Phenotypic characteristics of the bacterial isolates from different oral samples

Several studies carried out to determine the relationship between dental caries and saliva microflora have reported that there is variations in the number and types of microbial organisms found in saliva between patients with different DMFT indexes (DMFT Index Decayed, Missing, and Filled Index) [28][29][30]. From the 72 oral samples collected, 267 bacterial isolates were obtained and designated as S1 to S137 for saliva isolates, H1 to H88 for teeth isolates and M1 to M42 for root canal isolates. From the 267 isolates, 43 (16.2%) were identified as *Enterococcus* sp. and it was found to be the most prevalent genus. *E. faecalis*has been reported as a leading cause of endodontic failures for over 30 years. However, this claim became a subject of debate as recent studies have documented that other species, such as streptococci, may be the most predominant pathogens associated with persistent endodontic infections. The difference may be as a result of differences in methodological approaches for sampling and detection, differences in clinical conditions or socio-geographical differences in the subjects considered in the studies [22] [23] [24]. The numbers and percentages for each genus as obtained in this study is shown in figure 3. Several genera were found to be present in all samples types (saliva, teeth and necrotic roots canals) and these genera include *Enterococcus* sp., *Streptococcus* sp., *Staphylococcus* sp., *Bacillus* sp. and *Actinomyces* sp., while others found to be present in only one source of samples and they

include *Salmonella* sp. (saliva), *Shigella* sp. (saliva),*Micrococcs* sp. (saliva)*Chromobacterium* sp. (necrotic roots canals),*Eubacterium* sp. (saliva) and*Aerococcus* sp. (saliva). On the other hand, the highest percentage of *Enterococcus* sp. was in necrotic roots canals samples (21.4%) followed by teeth samples (15.9%) and the least was from saliva samples (14.6%). These findings are in agreement with the findings reported in an earlier related study where *E. faecalis* was reported to be more frequent in the root canals compared to the saliva[31].For *Streptococcus* sp., the highest percentage was found in teeth samples (19.3%) followed by samples from necrotic roots canals (16.6%) and in the least was from saliva samples (13.1%). The third most prevalent genus was *Staphylococcus* sp. and its prevalence percentages was (18.2%), (11.6%) and (9.5%) in the teeth, saliva and necrotic roots canals respectively. To our knowledge, this study represents the first in which *Salmonella* sp., *Shigella* sp. and *Chromobacterium* sp. have been isolated from oral samples. The role of these bacterial species in oral flora is also unknown and needs to be further investigated.Figure (2) shows the prevalence of the isolates from saliva, teeth and necrotic canals of teeth according to the genera.

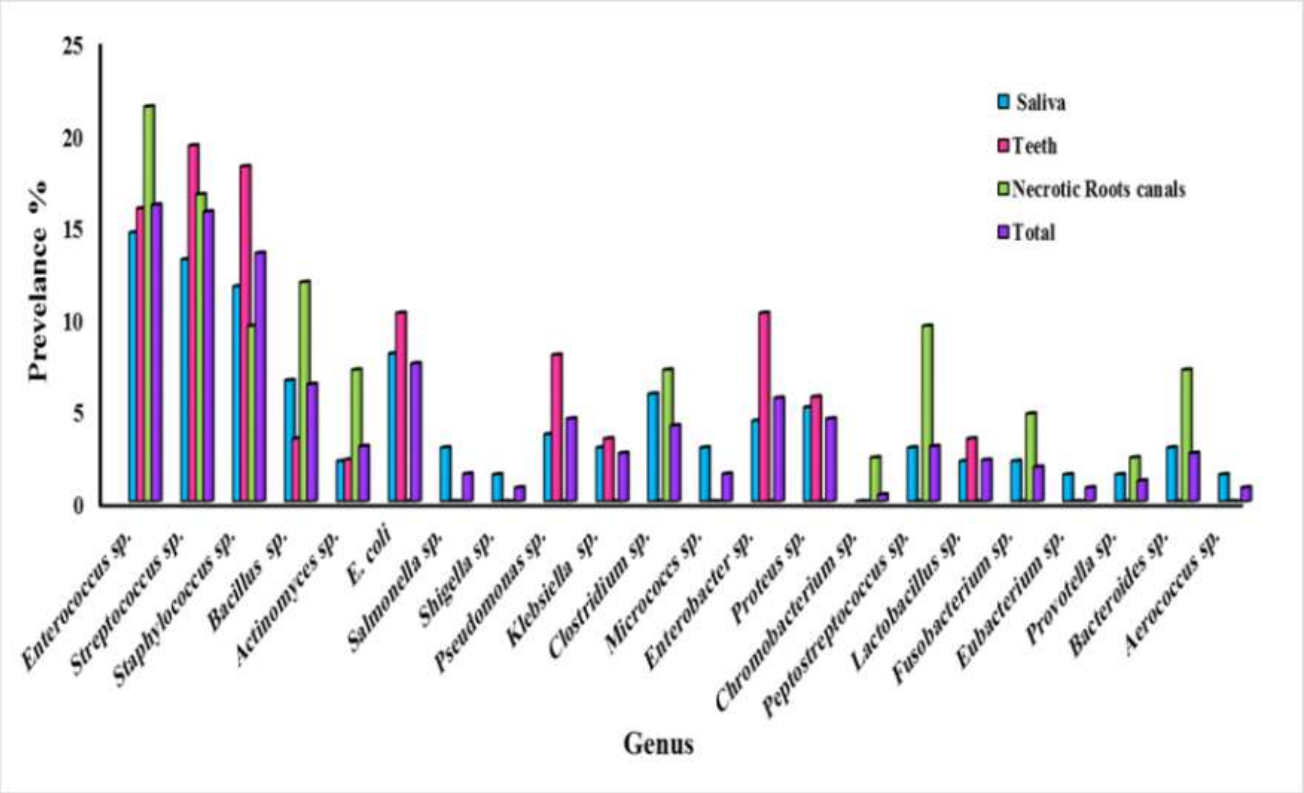

Figure (2) the prevalence of the isolates from saliva, teeth and necrotic canals of teeth according to the genera

Biofilm formation

From the 267 isolates obtained in this study, CRA method showed that only 32 (11.9%) are capable of forming strong biofilm, while the tube method demonstrated that 74 (27.7%) isolates are strong biofilm formers. On the other hand, MTP method showed that 104 (39%) of the isolates are strong biofilm formers. Based on the results obtained in our study, it could be concluding that CRA method is not the most reliable screening method for detection of biofilm formation since it detects low percentages of strong isolates from all samples sources. Our findings corroborate with the findings of Knobloch et al who reported that CRA method was not reliable for biofilm detection in their study. In the said study, out of 128 isolates of *S. aureus*, CRA detected only 3.8% of the isolates as biofilm producers as compared to MTP which detected 57.1% as biofilm producing bacteria[32].In another related study, Ruzickaet al. which 147 isolates of *S. epidermidis*were obtained, TM detected biofilm formation in 79 (53.7%) of the isolates while CRAdetected 64 (43.5%) isolates as biofilm producers. They demonstrated that TM is better for biofilm detection compared to CRA[33]. Baqai et al. The efficiency of TM in detecting biofilm formation among uropathogens has also been evaluated and it was found that 75% of the isolates exhibited biofilm formation While for CRA method, only 11 isolates were found to be biofilm producing bacteria and 99 as non-biofilm producers [34].Figure (3)showed the percentages of biofilm

formation ability of the isolatesfrom saliva teeth and necrotic roots canal by different screening methods (CRA, TM and MTP). The highest percentage of strong biofilm forming bacteria was found to be the necrotic roots canals isolates (40.5%) using MTP method while the lowest percentage of strong biofilm forming bacteria were the necrotic roots canals isolates using CRA method. Based on the analysis of our results, TM methods was 81% sensitive, 87% specific and 82.8% accurate for detection of biofilm forming bacteria, while CRA method was 58% sensitive, 75.8% specific and 62% accurate (Table 2). Based on our findings, we suggest that TM method should be given preference over CRA in the detection of strong biofilm producing bacteria since it correlated well with MTP for identifying strong biofilm producers. However, it is hard to differentiate between moderate, weak and non-biofilm producers due to the changeability in the results detected by different observers, hence TM cannot be suggested as general screening test to identify biofilm producing isolates[13],[35]. Figure (4) show the colors of different bacterial isolates cultured on CRA.

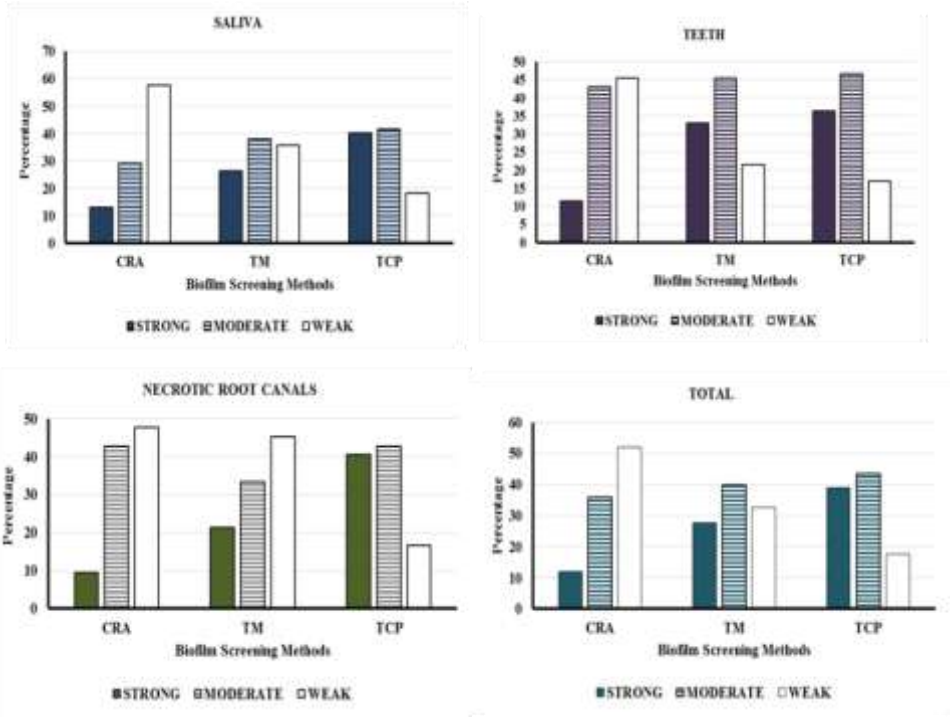

Figure (3) Screening of biofilm ability of the isolates from saliva, teeth, necrotic roots canals and the total of the isolates by different methods

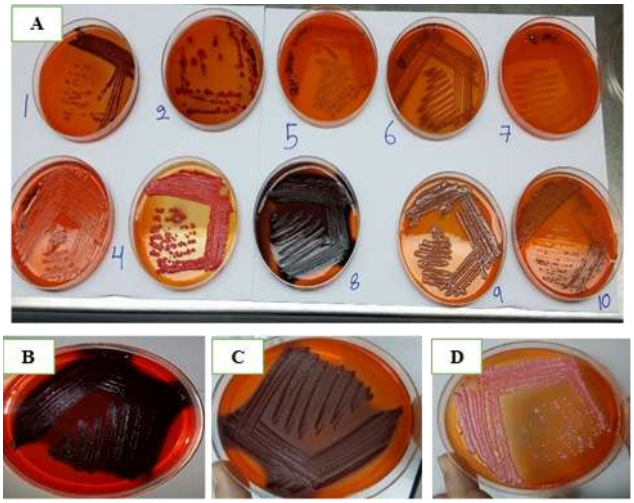

Figure (4) Culture colors with CRA. (A) different bacterial isolates; (B) black colonies, strong biofilm producers; (C) gray colonies, moderate biofilm producers; (D) pink colonies, non-biofilm producers.

Table (2) Diagnostic parameters of Tube method and Congo Red Agar method for biofilm detection

Diagnostic parameters	Screening Method	
	CRA	TM
Sensitivity (%)	58%	81%
Specificity (%)	75.8%	87%
Positive predictive value (%)	89.5%	96%
Negative predictive value (%)	33%	54%
Accuracy	62%	82.8%

Multi Antibiotic Resistance index (MAR Index)

In this study, antibiotic susceptibility profiles of strong biofilm forming isolates examined in order to establish the MAR index for isolates from different oral sources. MAR index obtained in this study indicates that multi resistance to drugs are predominant in the all isolates and this resistance is related to the strong biofilm formation by these isolates. Figure 5show the MAR index of the total isolates from saliva, teeth and necrotic roots canals, which it was 0.69, 0.71 and 0.82 respectively. The highest MAR index was among necrotic roots canals isolates followed by teeth and saliva. MAR index values greater than 0.2 indicate high risk source of contamination where antibiotics are often used [15]. These findings showed that a greater proportion of the isolates are likely to be from high risk source, originating from an environment where several antibiotics are used [36].Based on the genera of the isolates, *Enterococcus* sp. showed the highest MAR index among the isolates, with values of 0.97, 0.96, 0.89 for isolates from saliva, necrotic roots canals and teeth respectively. These results are consistent with the results presented in a similar study where it was reported that the multiple antibiotic resistance index (MAR) of *E. faecium* strains isolated from tenderloin beef samples ranged from 0.5 to 0.9. Hence, a MAR index of 0.89–0.97 is an indication that the *Enterococcus* sp. used in this study originated from high risk sources. In general, most of other genera from teeth and necrotic roots canals recorded higher MAR index compared to saliva isolates. Three genera from saliva samples *Bacillus* sp. (0.16), *E .coli* (0.19) and *Pseudomonas* sp. (0.15) were found to have MAR index below 0.2, MAR index values of less than or equal to 0.2 is an indication that the strain originated from animals where antibiotics are seldom or never used [29].The higher level of resistance to antibiotics among oral samples isolated in this study, especially necrotic roots canals isolates is alarming and this could be associated with cases of failure of the root canal filling. There have also been associations made between specific species in endodontic infections and antibiotic resistance and multidrug resistance found in *Enterococcus faecalis* associated with persistent endodontic disease[37],[38]. Figure (6) shows the MAR indices of strong biofilm forming isolates from saliva, teeth and necrotic roots canals of teeth.

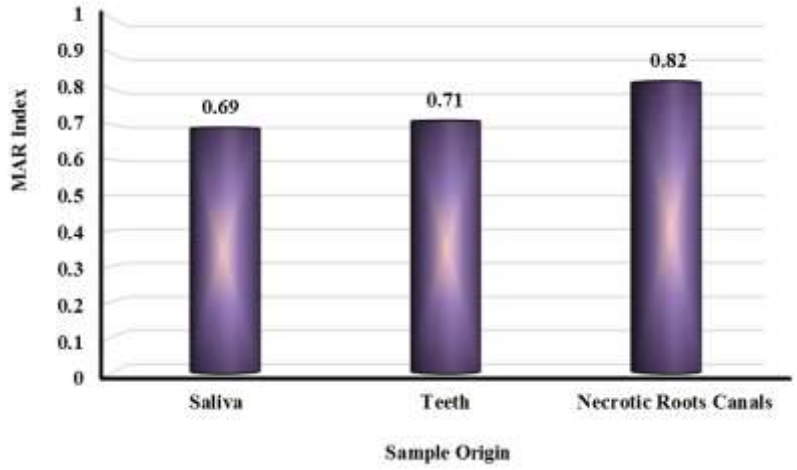

Figure (5) Multiple antibiotic resistance index (MAR) at different oral sites

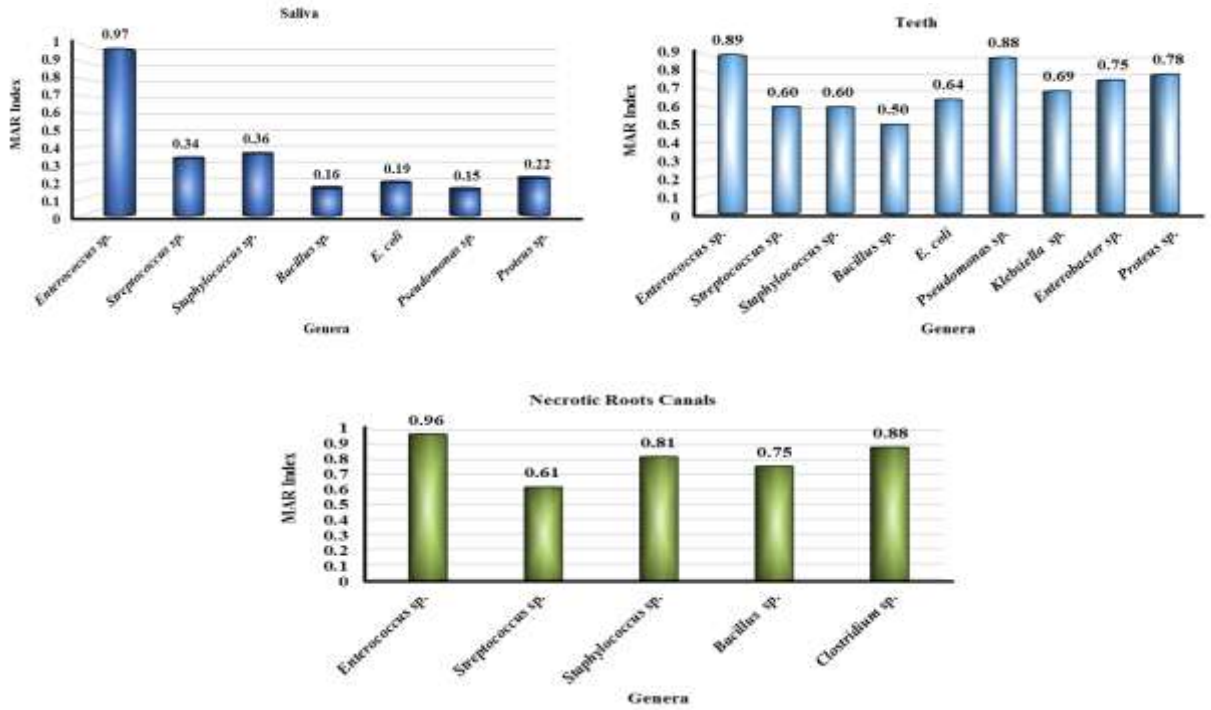

Figure (6) MAR indices of strong biofilm forming isolates from saliva, teeth and necrotic roots canals of teeth

Conclusion

In conclusion, this study appears to be the first to report the isolation of *Salmonella* sp., *Shigella* sp. and *Chromobacterium* sp. from oral samples. Gram positive bacteria were found to be the predominant bacteria isolates constituting 70% of the isolates and the Necrotic roots canals were found to harbour higher percentage of Gram positive bacteria isolates compared to the teeth and saliva isolates. Additionally, TM and MTP were found to be the most preferred methods for screening biofilm producing bacteria. Using the isolates obtained, this study also established a strong correlation between biofilm formation and MAR. Based on the findings of this study, MTP method is the best method for screening biofilm forming ability of oral microbiota and necrotic root canal bacteria isolates have the highest MAR resistance ability.

Acknowledgments

The authors are grateful to the School of Biosciences and Biotechnology and the Faculty of Science and Technology, UniversitiKebangsaan Malaysia for providing financial and moral support by grants 04-01-02-SF1244 from Ministry of Science, Technology and Innovation (MOSTI) of Malaysia. The authors also appreciate the stuff of the Dental Clinic, Health Clinic Centre, Serikembangan, Selangor and Dental Clinic, UKM/Bangi in Malaysia.

References

1. Marsh, P.D., *Role of the oral microflora in health.* Microbial Ecology in Health and Disease, 2000. 12(3): p. 130-137.
2. Petersen, P.E., *The World Oral Health Report 2003: continuous improvement of oral health in the 21st century–the approach of the WHO Global Oral Health Programme.* Community Dentistry and oral epidemiology, 2003. 31(s1): p. 3-24.
3. Lamont, R.J. and H.F. Jenkinson, *Oral microbiology at a glance*. Vol. 24. 2010: John Wiley & Sons.
4. Botero, J.E., et al., *Occurrence of periodontopathic and superinfecting bacteria in chronic and aggressive periodontitis subjects in a Colombian population.* Journal of periodontology, 2007. 78(4): p. 696-704.

5. Jenkinson, H.F. and R.J. Lamont, *Oral microbial communities in sickness and in health.* Trends in microbiology, 2005. 13(12): p. 589-595.
6. Kolenbrander, P.E., et al., *Communication among oral bacteria.* Microbiology and molecular biology reviews, 2002. 66(3): p. 486-505.
7. Paster, B.J., et al., *The breadth of bacterial diversity in the human periodontal pocket and other oral sites.* Periodontology 2000, 2006. 42(1): p. 80-87.
8. Donlan, R.M. and J.W. Costerton, *Biofilms: survival mechanisms of clinically relevant microorganisms.* Clinical microbiology reviews, 2002. 15(2): p. 167-193.
9. Danhorn, T. and C. Fuqua, *Biofilm formation by plant-associated bacteria.* Annu. Rev. Microbiol., 2007. 61: p. 401-422.
10. Stewart, P.S. and J.W. Costerton, *Antibiotic resistance of bacteria in biofilms.* The lancet, 2001. 358(9276): p. 135-138.
11. Reid, G., *Biofilms in infectious disease and on medical devices.* International journal of antimicrobial agents, 1999. 11(3): p. 223-226.
12. Christensen, G.D., et al., *Adherence of coagulase-negative staphylococci to plastic tissue culture plates: a quantitative model for the adherence of staphylococci to medical devices.* Journal of clinical microbiology, 1985. 22(6): p. 996-1006.
13. Christensen, G.D., et al., *Adherence of slime-producing strains of Staphylococcus epidermidis to smooth surfaces.* Infection and Immunity, 1982. 37(1): p. 318-326.
14. Freeman, D., F. Falkiner, and C. Keane, *New method for detecting slime production by coagulase negative staphylococci.* Journal of clinical pathology, 1989. 42(8): p. 872-874.
15. Osundiya, O., R. Oladele, and O. Oduyebo, *Multiple antibiotic resistance (MAR) indices of Pseudomonas and Klebsiella species isolates in Lagos University Teaching Hospital.* African journal of clinical and experimental microbiology, 2013. 14(3): p. 164-168.
16. Okeke, I.N., et al., *Antimicrobial resistance in developing countries. Part I: recent trends and current status.* The Lancet infectious diseases, 2005. 5(8): p. 481-493.
17. ÖZDABAK, N., et al., *Identification of aerobic bacterial flora in saliva of subjects who apply to the Faculty of Dentistry in Atatürk University by using microbial identification system.* Atatürk Üniversitesi Diş Hekimliği Fakültesi Dergisi, 2012. 2012(1).
18. Gbolahan, O., et al., *Bacteriologic features and antimicrobial resistance of organisms associated with extracted teeth: findings from a Nigerian university teaching hospital.* West African journal of medicine, 2010. 30(6): p. 436-441.
19. Guimarães, N.L.S.d.L., et al., *Microbiological evaluation of infected root canals and their correlation with pain.* RSBO (Online), 2012. 9(1): p. 31-37.
20. Prescott, L., J. Harley, and D. Klein, *Isolation of pure cultures.* Microbiology, 5th Edition, The McGraw-Hill Companies, New York, 2002: p. 106-110.
21. Elhadidy, M. and A. Elsayyad, *Uncommitted role of enterococcal surface protein, Esp, and origin of isolates on biofilm production by Enterococcus faecalis isolated from bovine mastitis.* Journal of Microbiology, Immunology and Infection, 2013. 46(2): p. 80-84.
22. Bauer, A., et al., *Antibiotic susceptibility testing by a standardized single disk method.* American journal of clinical pathology, 1966. 45(4): p. 493.
23. Hinton, M., A. Hedges, and A. Linton, *The ecology of Escherichia coli in market calves fed a milk-substitute diet.* Journal of Applied Microbiology, 1985. 58(1): p. 27-35.
24. Hassan, A., et al., *Evaluation of different detection methods of biofilm formation in the clinical isolates.* The Brazilian Journal of Infectious Diseases, 2011. 15(4): p. 305-311.
25. Rani, A. and A. Chopra, *Isolation and Identification of Root Canal Bacteria from Symptomatic Nonvital Teeth with Periapical Pathosis.* Endodontology, 2006. 18(1): p. 112-7.
26. Brook, I., E.H. Frazier, and M.E. Gher, *Aerobic and anaerobic microbiology of periapical abscess.* Molecular Oral Microbiology, 1991. 6(2): p. 123-125.
27. Gomes, B., et al., *Microbiological examination of infected dental root canals.* Molecular Oral Microbiology, 2004. 19(2): p. 71-76.
28. Gabris, K., et al., *Associations between microbiological and salivary caries activity tests and caries experience in Hungarian adolescents.* Caries research, 1999. 33(3): p. 191-195.
29. Llena-Puy, M.C., C. Montañana-Llorens, and L. Forner-Navarro, *Cariogenic oral flora and its relation to dental caries.* ASDC journal of dentistry for children, 2000. 67(1): p. 42-6, 9.

30. Gudkina, J. and A. Brinkmane, *Caries experience in relation to oral hygiene, salivary cariogenic microflora, buffer capacity and secretion rate in 6-year olds and 12 year olds in Riga.* Stomatologija, 2008. 10(2): p. 76-80.
31. Wang, Q.-Q., et al., *Prevalence of Enterococcus faecalis in saliva and filled root canals of teeth associated with apical periodontitis.* International journal of oral science, 2012. 4(1): p. 19-23.
32. Knobloch, J.K.-M., et al., *Evaluation of different detection methods of biofilm formation in Staphylococcus aureus.* Medical microbiology and immunology, 2002. 191(2): p. 101-106.
33. Růžička, F., et al., *Biofilm detection and the clinical significance ofStaphylococcus epidermidis isolates.* Folia microbiologica, 2004. 49(5): p. 596-600.
34. Baqai, R., M. Aziz, and G. Rasool, *Urinary tract infections in diabetic patients and biofilm formation of uropathogens.* Infect. Dis. J. Pak, 2008. 17(1): p. 21-24.
35. Mathur, T., et al., *Detection of biofilm formation among the clinical isolates of staphylococci: an evaluation of three different screening methods.* Indian journal of medical microbiology, 2006. 24(1): p. 25.
36. Ehinmidu, J.O., *Antibiotics susceptibility patterns of urine bacterial isolates in Zaria, Nigeria.* Tropical Journal of Pharmaceutical Research, 2003. 2(2): p. 223-228.
37. Dahlen, G., et al., *Identification and antimicrobial susceptibility of enterococci isolated from the root canal.* Molecular Oral Microbiology, 2000. 15(5): p. 309-312.
38. Pinheiro, E., et al., *Antimicrobial susceptibility of Enterococcus faecalis isolated from canals of root filled teeth with periapical lesions.* International endodontic journal, 2004. 37(11): p. 756-763.

*****