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Research Article

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Susceptibility of oral bacteria to antimicrobial agents and virulence factors

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ABSTRACT

Tooth decay is considered the most widespread infectious disease in the world. This study aims to isolate and identify the important bacteria related to tooth decay, determine the sensitivity of bacteria in certain types of antimicrobial agents, and study the effect of heavy metals on bacterial isolates. A total of 50 swabs were collected from the mouths of patients from both sexes, with ages ranging from 1–60 years. The patients were advised to consult with dental clinics and specialized centers to isolate and identify the causative agents associated with oral diseases. Results showed that infection rates in younger age groups (1–20 and 20–40) are higher than the elder group (40–60), with percent incidence of 44% and 32%, respectively. Antibiotic sensitivity test against the isolates showed that chloramphenicol had the highest sensitivity effect with 83.2% followed by rifampicin and gentamicin with 81.35%, penicillin G with 64.40%, and streptomycin with 16.94%. In addition, 100% resistance was recorded against seven heavy metals, including silver nitrate, iron chloride, zinc chloride, and lead acetate. The sensitivity to mercury, cadmium, and copper sulfate were 100%, 86.44%, and 1.69%, respectively. Hemolysin had the highest ability to produce virulence factors (72.88%), followed by lecithinase (42.37%) and protease (25.42%). Lipase and urease had the lowest virulence factor production (10.16%).

Key words: Bacteria; Antibiotic; Heavy metals, Virulence factors.

INTRODUCTION

Tooth decay is one of the most common infectious diseases affecting millions of people globally [1]. One of the occasional factors for the disease is dental biofilm, which is the bacterial charge that forms permanently on the tooth surfaces [2]. Hazard factors include unsuitable salivary flow, low quality of salivary buffer, incomplete fluoride exposure, and increased consumption of sugar [3]. Caries indicates the centralized removal of susceptible dental hard tissues by acidic products from the bacterial fermentation of dietary carbohydrates [4]. Tooth decay is a chronic disease that is slowly developing in people. Tooth decay presents as smooth holes and fissured surfaces on the crown and root of a tooth. According to the World Health Organization, 60–90% of school children worldwide have dental cavities[5]. This decay is the result of the interaction of the oral microflora plaque, the tooth surface, nourishment, and the oral environment over time, causing destruction of the tooth enamel [6]. Recently, disease incidence for cavities is decreasing in industrialized nations but is increasing in developing nations [7]. The spread of caries is uneven across the population and communities. The highest incidence is in the lower socioeconomic groups, having limited access to adequate oral health care [8]. Despite the decline in incidence of caries, the United States of America is spending 10 billion USD each year on tooth decay treatment [9]. In other industrialized nations, such as the United Kingdom and China, caries prevalence in the past has been over 50% in children. In developing

countries, where oral health care is low, caries are increasing in an alarming rate. Previous studies done in Peru, Mexico, the Philippines, and Taiwan found caries in 75–90% of children [10].

Mutants Streptococci, a group of cariogenic bacteria, is associated in the initiation of dental caries [11]. Another group of bacteria that is substantial in the development of caries is Lactobacillus. Lactobacillus does not usually colonize the tooth surface, but is commonly found in the oral cavity including the dorsum of the tongue[1]. Although it could have a significant role in the caries advancement, Lactobacillus is not essential in the initiation of dental caries [12]. Positive association between salivary levels and bacterial caries is relevant to carbohydrate exhaustion. The presence of Streptococcus and Lactobacillus may potentially indicate the occurrence of not only caries but also of carbohydrate consumption [13]. *Streptococcus mutans* is commonly accepted as one of the most substantial etiologic agents in caries development and has been shown to directly cause caries in germ-free and specific pathogen-free rat models. However, the presence of caries has been found even in the absence of *S. mutans*. Although a high percentage of *S. mutans* has been recovered from teeth without caries, *S. mutans* remains the species that is most associated with caries. In gnotobiotic and specific germ-free rodent models, *S. mutans* has the potential to generate caries [14]. Despite the various properties in *S. mutans* that raises its cariogenicity, strong biofilm indicating the presence of dietary sucrose is a stringent component in the development of caries.

Thus, this study aims to isolate and partially identify important bacteria related to tooth decay and diseases of the mouth, determine the susceptibility of bacteria to certain types of antimicrobial agents, study the effect of some heavy metals for bacterial isolates, and study the ability of bacterial isolates in producing some of the virulence factors.

EXPERIMENTAL SECTION

Isolation of microbial isolates from patients

Collection of samples: With the assistance of dentists, specimens in this study have been collected from the dental units in health centers and dental clinics in Gambang, Pahang, Malaysia. Sterile swabs were used for the patients of both genders, with ages ranging from 1–60 years. Collected samples were transferred to the laboratory of Universiti Malaysia Pahang.

Microbial culture

Samples from the mouth of patients were cultured on nutrient agar plates and were incubated at 37° for 24 h. The samples were then purified and cultured on agar slants. These were kept in the chiller until use.

Antimicrobial activity test using disc diffusion method

Antibiotic sensitivity test

All antibiotics used in this study were from Mast disc^{tm}, Mast Diagnostics, Mast group, Mersey side, except for penicillin G, which was from Oxoid, Basingstoke, Hampshire, England. Streptomycin was prepared in the laboratory. Antibiotic discs (amoxicillin 10 µg, neomycin 10 µg, ampicillin 10 µg, tetracycline 10 µg,gentamicin 10 µg, chloramphenicol l10 µg, penicillin G 10 µg, streptomycin 10 µg, and rifampicin 5 µg) used Muller–Hinton agar from Hardy Diagnostics. According to the manufacturer's recommendations, the antibiotic discs were prepared and were autoclaved at 12 °C for 15 min. The medium was then cooled to 45–50 °C and poured onto the plates. The antibiotic discs were allowed to set on a level surface to a depth of approximately 4 mm. Inoculums from primary culture plates were prepared by touching 3–5 colonies with a swab and transferring them into a plate. The inoculums were mixed with two drops of sterile distilled water and were spread in three plates. The nine antibiotic discs prepared were placed onto the inoculated plates. Subsequently, they were placed in the chiller for 15 min and were incubated at 37 °C. After an overnight incubation, the diameter of each inhibition zone was measured and recorded in mm [15].

Heavy metals activity test

Preparation of concentration: Concentration was prepared by using 10 µg/mL for the seven heavy metals (i.e., silver nitrate, iron chloride, zinc chloride, lead acetate, copper sulfate, cadmium, and mercury). The stock solution was prepared for the concentration. Filter paper disc was used and was laden with 25 µl of heavy metal [16].

Virulence factors

Haemolysin

Hemolysin test was used to investigate the production of blood enzyme. The hemolytic activity of bacteria was assayed by using nutrient agar containing 5% blood. Bacterial isolate cultures were incubated at 37 °C for 24 h on blood agar plates. The appearance of a transparent zone around the bacteria indicates a positive result for hemolysin [17].

Protease

Skim milk agar medium was used to investigate the production of protease enzyme. The medium was prepared by mixing 100 ml of nutrient agar and 1 ml of sterile skim milk. The mixture was autoclaved to make it sterile and then poured into sterile dishes [18] Inoculums from primary culture plates were prepared by brushing 3–5 colonies via loop and transferring them onto the plates. The inoculums were incubated for 24 h at 37 °C. Decomposition on areas was observed

Lipase and Lecithinase

Egg yolk agar was prepared by mixing 100 ml of nutrient agar, which was sterilized via autoclave and was left to cool to 45 °C, with 5 ml of egg yolk. The agar was poured into sterile dishes. The agar was used to distinguish the bacteria that produce lipase or lecithinase enzyme [19]. Egg yolk agar was inoculated with colonies of pure isolated bacteria and was incubated at 37 °C for 24–48 h. Egg yolk agar is inferred to be effective on inhibiting lecithinase enzyme around the developing colonies. Egg yolk agar is also used to detect the effectiveness of lipase enzyme. Egg yolk agar test was conducted by immersing the dish in sufficient quantity of a saturated copper sulfate for 20 min. After the removal of excess solution, the dish was dried using the incubator for 30 min. Decomposition of fat by lipase enzyme was indicated by the emergence of greenish blue color in growth areas.

Urease Test

This test was done to investigate the ability of bacteria to produce urease enzyme and to analyze the urea of ammonia and carbon dioxide content. Urea agar was inoculated and incubated at 37 °C for 18–24 h. Positive result was considered to be indicated by the change in color of the media to pink[20].

RESULTS AND DISCUSSION

Patient's isolates

Data on bacterial and yeast (59) isolates during the primary isolation of samples are shown in table 1 and figure. 1. Data were obtained from the mouths of 50 patients of different ages and genders, composed of 54% males and females. The 20–40 and 1–20 years age group were the more infected, with 44% incidence, compared with the elder age group (40–60 years), with 32% incidence. This study confirmed that children and younger individuals are more susceptible to mouth infection compared with other age groups. This finding may be due to the low immunity and low health consciousness of these age groups, as well as due to other factors related to nutrition and public health that increases the rates of infection among them. In another study,[21] stated that children are more susceptible to decay-causing bacteria than other age groups are. Infected children who have malformed teeth showed high mortality rates. The frequent sugar consumption of children plays an important role in infections. Mothers can also transfer diseases from their infected teeth to their children. In such case, the levels of bacteria found at the children are similar with that of the mothers.

Table 1: Primary isolation of samples and percentages

Patients Samples & age (year)	Isolate number	Percentage (%)
Single isolate	33	55.93
Mixed isolate	26	44.07
1-20	16	32
20-40	22	44
40-60	12	24

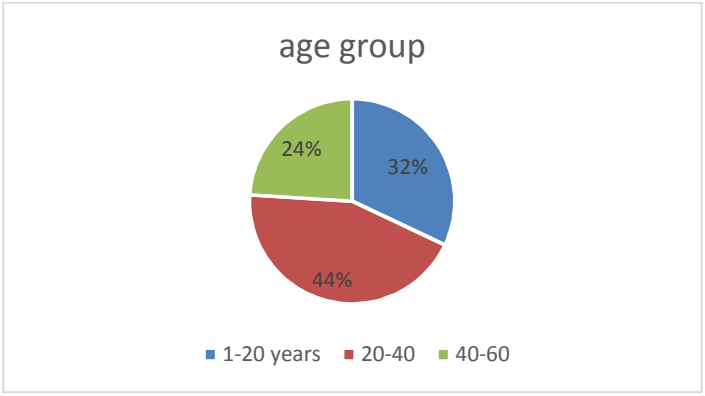

Figure 1. Percentage of isolates according to age group

Sensitivity of bacteria to antibiotics

Data presented in figure. 2 show the percentage sensitivity of bacterial isolates against nine antibiotics. The highest sensitivity to antibiotics was observed in chloramphenicol (83.05%), followed by gentamicin and rifampicin (81.35%). Streptomycin and penicillin G had 16.94% and 64.40% sensitivity, respectively.

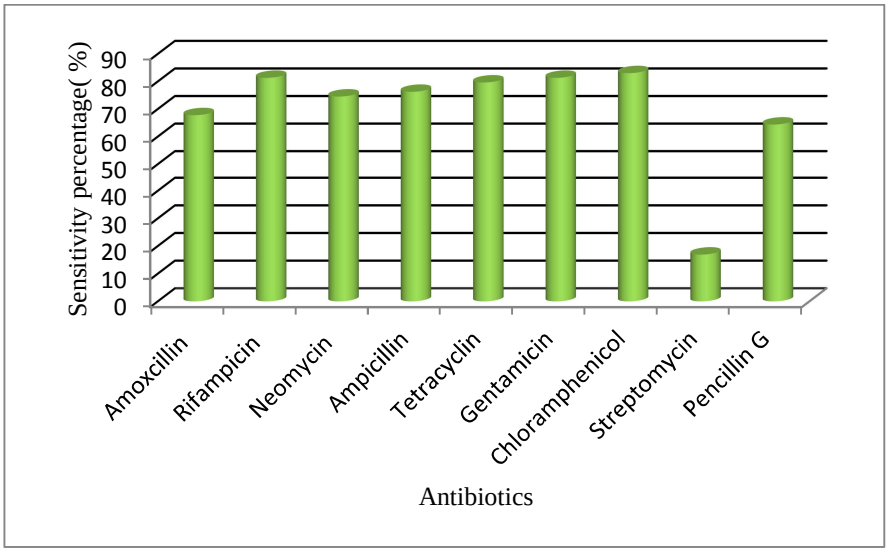

Figure 2. Percentage sensitivity of bacterial isolates antibiotics

This study showed that chloramphenicol had the most significant influence on the bacterial isolates taken from the mouth of the patients, followed by gentamicin and rifampicin. Tetracycline, ampicillin, neomycin, and amoxicillin were found less effective compared with other antibacterial drugs. One noticeable result was that the bacterial isolates showed variation in their resistance to the aminoglycoside antibiotic group. They had a sensitivity ratio of 74.57% to neomycin and 81.35% to gentamicin. Recently, a notable increase in the resistance to aminoglycoside antibiotics was noticed Livermore and Winstanley (2001) presented in their study the relationship between antibiotic and mechanism, giving a comprehensive discussion for those testing wide panels of drugs versus isolates. Resistance caused by the formation of the enzyme by resistant bacteria modifies the antibiotic, which in turn loses its effectiveness. Loss of outer membrane proteins, which reduces the permeability of the antibiotic inside the bacterial cell, can also be a reason for the loss of drug effectiveness[22]. Evidence from the current study showed that the majority of bacterial isolates are highly resistant to the antibiotic *β*-lactam (ampicillin, amoxicillin, penicillin G). High bacterial resistance to the antibiotic *β*-lactam is attributed to several mechanisms. High bacterial resistance can be caused by the ability of bacteria to produce the enzyme *β*-lactamase, which breaks the bind in *β*-lactam. This phenomenon changes the permeability barrier intimacy between the antibiotic and the target penicillin-binding

protein. Such result is compatible with the study done by Cherian and Manjunath (2003). They extended spectrum *β*-lactamase to produce Enterobacteriaceae in a tertiary care hospital in Trinidad and Tobago[23]. Their results showed the high resistance of the bacterial isolates to streptomycin, explaining the mechanism of resistance to this antibiotic. Thus, the binding of the molecule to the 30S subunit interferes with 50S subunit association with the mRNA strand, thus resulting in an unstable ribosomal-mRNA complex. This phenomenon also leads to a frame shift mutation and defective protein synthesis, thereby leading to cell death. Syal et al. (2013) reported that the streptomycin's therapeutic concentration of 10 mg/mL interferes in the Jaffe reaction and acts as non-creatinine chromogen. In their study, the streptomycin's interference in Jaffe reaction can be possibly a false positive creatinine estimation because of excessive dose exposure [24]. The study also showed an increasing resistance to tetracycline. This resistance is believed to be caused by the presence of plasmids that encode resistance to the antibiotic. Koo and Woo (2011) conducted a study on the distribution and transferability of tetracycline-resistance determinants in *Escherichia coli* isolated from meat and meat products. They reported that the high prevalence of tetracycline-resistant *E. coli* in meat may be due to the high transferability of tetracycline determinants [25]. They observed that the lowest resistance showed by the bacterial isolates was to chloramphenicol, gentamicin, and rifampicin. Most of the bacterial isolates appeared to be sensitive to these antibiotics due to the presence of plasmids. As a response, such antibiotics are currently used limitedly at hospitals.

Sensitivity of bacteria to heavy metal

Figure 3 shows the resistance and sensitivity percentages of bacterial isolates to the seven heavy metals. In this study, 100% resistance to the heavy metals silver nitrate, iron chloride, zinc chloride, and lead acetate was recorded. By contrast, the bacterial isolates appeared to be 100% sensitive to mercury and 86.44% and 1.69% sensitive to cadmium and copper sulfate, respectively.

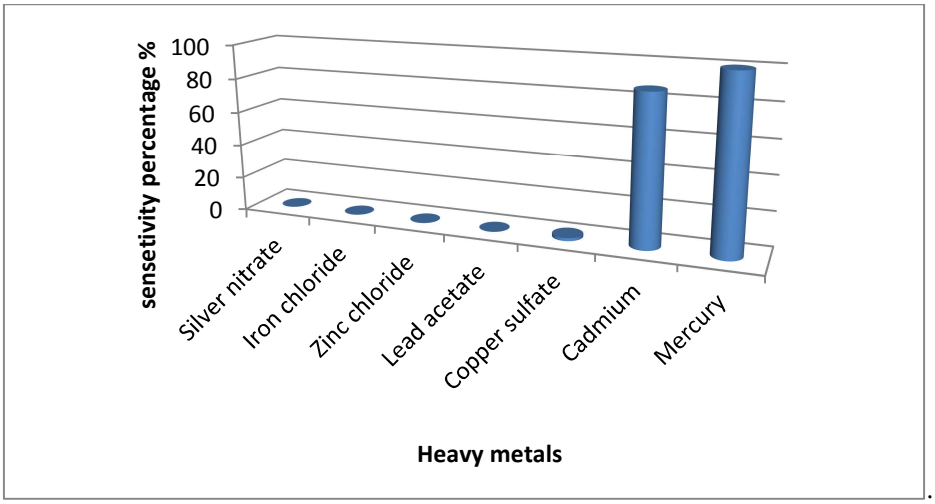

Figure 3. Percentage of bacterial isolates sensitive of heavy metal

Results from this study showed the high resistance of the bacterial isolates to silver nitrate. Starodub and Trevors (1990) reported a 39% resistance of *E. coli* to silver [26]. Therefore, the resistance of bacteria to silvers can be modified at the intervals of the microorganism's ordering. Silver resistance is stable at the intervals of microorganism population. Silver is also transmissible to sensitive recipient strains by conjugation or transformation in vitro. Silver (2003) found a link between bacterial silver resistance and molecular biology by using silver compounds [27] Results of the current study showed that the bacterial isolates presented resistance for lead. One of the mechanisms that microorganisms utilize to avoid the toxicity of heavy metals is to limit their movement across the cell envelope. Jarosławiecka and Piotrowska (2014) studied the main mechanisms of lead resistance, namely, cell exclusion and ion efflux to the cell exterior. The cytoplasm membrane is a natural barrier for lead. This role is opposed principally by lipopolysaccharide, a part of the outer membrane. Many macromolecules bind lead in Gram-negative microorganism. This role is contended principally by lipopolysaccharides, a large part of the outer membrane. In Gram-positive microorganisms, lead is bound by peptidoglycans as well as by teichoic and teichuronic acids [28] .The data obtained in this study show the sensitivity of microorganisms to cadmium. This

finding can attributed to the low concentration of cadmium, which increases the rates of sensitive isolates. Cohen et al. (1990) studied the effect of zinc and cadmium ions on *Escherichia coli* (*E. coli*). The exposure of *E. coli* to various concentrations of these ions resulted in an increase of the total protein and the presence of metal binding proteins in the cells. The activity of alkaline phosphates also increased in the presence of these ions [29]. By contrast, the heavy metal copper was found less effective on the bacterial isolates in the current study. Michels and Wilks (2005) reported that copper alloy surfaces have intrinsic properties, which can destroy a large variety of microorganisms. Copper alloys can cause an infectious human disease [30].

Virulence Factors

Figure 4 shows the percentage of bacterial isolates produced to five virulence factors. Hemolysin had the highest production to virulence factors with 72.88%, followed by lecithinase and protease with 42.37%, and 25.42% respectively. Less bacterial isolates were produced to virulence by lipase and urease (10.16%).

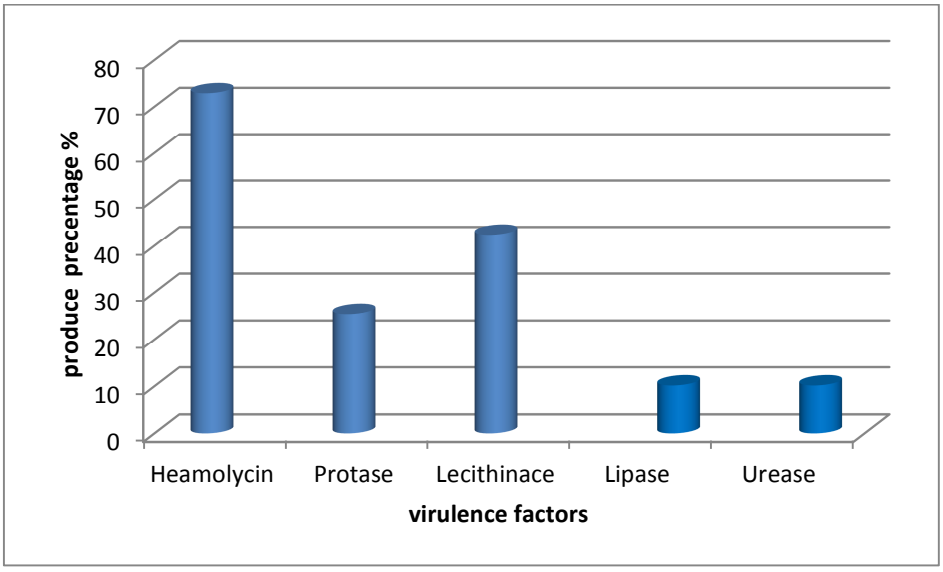

Figure 4. Percentage to bacterial isolate produced virulence factors

Virulence is the degree of pathogenicity exhibited by most pathogens and is a measure that effectively differentiates pathogenic and non-pathogenic strains. The degree of virulence depends on several virulence factors. In this study, the most significant result was that of hemolysin at 72.88%. A direct relationship between bacterial isolates and hemolysin was not observed. Bacterial isolate strains that are Gram-positive are noted to contain the highest number of Gram-positive bacteria with much hemolysin produced. Other authors have also shown that 89% of hemolysin produces clinical isolated strains [31]. Takahashi et al. (2014) showed that 80% of produced hemolysin from the human body is positive of *Aeromonas trota* [17]. Almost 95% of isolated human Streptococcus produces a characteristic hemolysin that is only among Streptococci. Hemolytic expression is always connected to the expression of a key virulence factor. Hemolysin is encoded by a single genetic locus known as the cyl operon[32]. Meanwhile, the second highest virulence factor produced in bacterial isolates was lecithinase at 42.37%. The phospholipid lecithin is one of the chief components of the cell membrane, which can be degraded by lecithinase enzyme, thus producing diglyceride and phosphorylcholine and causing toxicity. Sharaf et al. (2014) reported that 53 isolates from 60 bacterial isolates were positive of lecithinase when lecithinase-producing bacteria from commercial and homemade foods were studied. [33]. Bacterial proteases are recognized as virulence factors in a number of infectious diseases due to their cell and tissue damaging effects. In one study, in which the protease result was 25.42%, a connection was found between the increase in protease production by *Staphylococcus epidermidis* and the obscurity of *Staphylococcus aureus* in biofilms obtained from the same patient [34] Batra and Walia (2014) reported that 39 strains of bacteria-producing protease out of 57 strains were isolated from different soil samples from a cotton field [35]. The lowest percentage of virulence factors in the current study was recorded at 10.16% for both urease and lipase. Urease has a significant role in several biological processes. It is a virulence factor in many pathogenic organisms [36] . Morou et al 2011 reported that urease activity in plaque recorded a trend that remains

stable during the study period. Urease activity was negatively associated with sugar consumption. In addition, urease activity in saliva increased with age and was positively associated with the levels of *S. mutans* in saliva and with the educational level of the parents. Lipase is a triacylglycerol hydrolyzing enzyme that catalyzes the hydrolysis of water-insoluble free fatty acid and glycerols. Lipase also has a wide range of chemical reactions. The results of this study are similar to those of Thomas et al. (2003), in which they found that *Bacillus mycoides* showed a growth or production of lipase at temperatures below 10 °C or above 50 °C [37]. Joseph (2006) reported that sodium chloride increased lipase production, whereas the presence of metals in the media had an inhibitory effect. *S. epidermidis* immobilized cells in agar beads and increased lipase production by 3% compared with free cells. [38].

Results of the study showed that the rate of tooth caries was highest in the second age group 44%, The results of tests proved the antibiotic sensitivity , the optimal antibiotic for the tooth caries are chloramphenicol 83.05%, Gentamicin and Rifampicin (81.35%). The results of this study showed an increase in the proportion of resistance all heavy metals except mercury (100%), cadmium (86.44%) and copper sulfate (1.69%).The highest ability to produce virulence factors was hemolycin72.88% , lecithinase 42.37 and protease25.42%, lipase and urease were10.16%.

CONCLUSION

The higher infection was in younger age groups , chloramphenicol had the highest sensitivity effect, five of heavy metals were resistance bacterial isolates and hemolysin had the highest ability to produce virulence factors

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REFERENCES

[1] K Wongkamhaeng ;O Poachanukoon ; S Koontongkaew, *International J of pediatric Otorhinolaryngology*., **2014**, 78(5),860- 865.
[2] PE Petersen ;D Bourgeois ; H Ogawa; S Estupinan; C Ndiaye . *Bulletin of the World Health Organization.*, **2005**,83(9),661-9.
[3] I MejÀre; S Axelsson; G Dahlén; I Espelid; A Norlund; S Tranæus; et al. *Acta Odontologica Scandinavica.*, **2014**, (0),1-11.
[4] RH Selwitz; AI Ismail; NB Pitts. *The Lancet*., **2007**,369(9555),51-59.
[5] PE Petersen. *International dental J*. ,**2008**,58(3),115-121.
[6] DJ Lynch. USA,*University of Iowa*, **2010.**
[7] C Chu;E Lo. *Oral health & preventive dentistry*. ,**2008**,6(4) ,315–321.
[8] WH Bowen. *Critical Reviews in Oral Biology & Medicine*., **2002**,13(2),126-131.
[9] RM Benjamin. *Public Health Reports*. ,**2010**,125(2),158
[10] RA Bagramian ;F Garcia-Godoy ; AR Volpe. *Am J Dent.*, **2009**,22(1),3-8.
[11] WJ Loesche ; *Microbiological reviews*., **1986**,50(4),353.
[12] Takahashi ;B Nyvad . *J of Dental Research*., **2011**,90(3),294-303.
[13] J Van Houte . *Advances in dental research*., **1993**,7(2),87-96.
[14] N Takahashi ; B Nyvad . *Caries Research*. ,**2008**,42(6),409-418.
[15] J Vandepitte ;J Verhaegen ;K Engbaek ;P Rohner ;P Piot ;C Heuck . Basic laboratory procedures in clinical bacteriology: World Health Organization, **2003**.
[16] J Bakht ;A Islam ;H Ali ;M Tayyab ;M Shafi ..*African Journal of Biotechnology*, **2013**. 10(39),7658-7667.
[17] E Takahashi; H Ozaki ; Y Fujii ;H Kobayashi ;H Yamanaka ; S Arimoto. *PloS One*,**2014**, 9(3), 91149.
[18] PE Stukus. Investigating microbiology: a laboratory manual for general microbiology: saunders College Publishing; **1997**.
[19] R Cruickshank ; JP Duguid; BP Marmion; and RHA Swain., The Practical of medical microbiology 12th ed,Churchill livingstone,**1975**.
[20] AE Brown. Benson's Microbiological Applications: Laboratory Manual in General Microbiology, Short Version. ,11th ed. USA, McGraw-Hill Higher Education, **2009**.
[21] GG Rao. *Drugs*,**1998**,55(3),323-330.
[22] DM Livermore; TG Winstanley; KP Shannon. *Journal of Antimicrobial Chemotherapy*. ,**2001**,48(1),87-102.

[23] B Cherian, M Manjunath, PL Pinto, P Prabhakar. *The West Indian Medical Journal*. ,**2003**,52(1),3-31.
[24] K Syal ;A Srinivasan ;D Banerjee. *Clinical Biochemistry*. ,**2013**,46(1),177-179.
[25] H J Koo ; G J Woo . *International journal of food microbiology*. ,**2011**,145(2),407- 413.
[26] M Starodub; J Trevors .*Journal of Inorganic Biochemistry.*, **1990**,39(4), 317-325.
[27] S Silver ; *FEMS microbiology Reviews*., **2003,**27(2-3), 341-353.
[28] A Jarosławiecka; Z Piotrowska Seget,. *Microbiology*, **2014**,160(1),12-25.
[29] I Cohen; R Bitan;Y Nitzan, *Microbios*, **1990**. ,68(276-277),157-168.
[30] H Michel; S Wilks ;J Noyce ;C Keevil .,*Stainless Steel*, **2005**., 77000(55), 27-0.
[31] I Anacarso ; C Condò ; C Sabia ; P Messi ; S de Niederhausern S; M Bondi ., *Environmental and Humans in Italy* **2013**,1(1), 1-9.
[32] M Rosa; S Dramsi; B Spellerberg.*FEMS Microbiology Reviews*., 2014. **38**(5), 932– 946.
[33] EF Sharaf;W S El-Sayed ; R Abosaif. *Journal of Taibah University for Science*. ,**2014** ,8(3):207-215
[34] Vandecandelaere I, Depuydt P, Nelis HJ, Coenye T, *Pathogens and disease*.,**2014**,70(3),321-331.
[35] N Batra; M. Walia, *African Journal of Microbiology Research.*, **2014**, 8(7), 702-709.
[36] E Morou ;A Elias, R Billings ;R Burne ;V Garcia, V Brignoni, et al .*Archives of Oral Biology*, **2011**., **56**(11), 1282-1289.
[37] A Thomas ;M Mathew ; A Valsa ;S Mohan ;R Manjula. *Indian Journal of Microbiology.*, **2003**,43(1),67-69.
[38] B Joseph; PW Ramteke, PA Kumar. *J of General and Applied Microbiology.* ,**2006**,52(6),315-320