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# Microbiological Study Against Bacterial Isolates From Different Patient

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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 and virulence factors on oral bacterial isolates. A total of 50 swabs were collected from the mouths of patients from both gender, 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% Also, these differences were found have lower effect for isolates against (10) heavy metals, where it showed resistance to Iron 3.38%, then nickel, aluminum, copper, lead to 20.33%, 22.03%, 27.11%, 28.81% respectively, also sliver shown 57.62%. And, this similarity were found have sensitive to antimony and chromium 61.01%.while appeared sensitivity to mercury and cadmium by 100, 86.44. 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%).

## INTRODUCTION

Tooth decay is one of the most common infectious diseases affecting millions of people globally (Wongkamhaeng *et al*., 2014). One of the occasional factors for the disease is dental biofilm, which is the bacterial charge that forms permanently on the tooth surfaces (Petersen *et al*., 2005). Hazard factors include unsuitable salivary flow, low quality of salivary buffer, incomplete fluoride exposure, and increased consumption of sugar (MejÀre *et al*., 2014). Caries

indicate the centralized removal of susceptible dental hard tissues by acidic products from the bacterial fermentation of dietary carbohydrates (Selwitz *et al*., 2007). 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 (Petersen, 2008). 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 (Lynch, 2010). Recently, disease incidence for cavities is decreasing in industrialized nations but is increasing in developing nations (Chu andLo, 2008). The spread of caries is uneven across the population and communities. The highest



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incidence is in the lower socioeconomic groups, having limited access to adequate oral health care (Bowen, 2002). Despite the decline in incidence of caries, the United States of America is spending 10 billion USD each year on tooth decay treatment (Benjamin, 2010). 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 (Bagramian *et al*., 2009).

Mutants Streptococci, a group of cariogenic bacteria, is associated in the initiation of dental caries(Ali*et al*.MakkyYusoff, 2015). 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 (Wongkamhaeng *et al*., 2014). Although it could have a significant role in the caries advancement, Lactobacillus is not essential in the initiation of dental caries (N. Takahashi andNyvad, 2011). 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(Ali*et al*.Makky*et al*.Batool*et al*. *et al*., 2015). *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 (N. Takahashi andNyvad, 2008). 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.

## MATERIALS AND METHODS

***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 hour, 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, were autoclaved at 121 °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 (Vandepitte *et al*., 2003).

***Heavy metals activity test:***

Prepare concentration: Prepared concentration was prepared by using 10 milligram /liter for the ten heavy metals (i.e., Iron, copper, aluminum, antimony, nickel, lead, Silver, chromium, cadmium and mercury) the stock solution was prepared for concentration. Filter paper disc was used and laden with 25 µl of heavy metal (Bakht *et al*., 2013). For Antimicrobial activity test using disc diffusion method

*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 (E. Takahashi *et al*., 2014).

*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 (Stukus, 1997) 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 (Cruickshank *et al*., 1975). 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 hour. Positive result was considered to be indicated by the change in color of the media to pink(Brown, 2009).

## RESULTS AND DISCUSSION

*Patients isolates:*

In this study, has been obtained from the mouths of 50 patients in different ages and both of genders with percentage of 54% males and 46%females, as shown bacteria and yeast (59) isolates in the Table 1 and Figure. 1, 2. It has been shown the primary isolation of samples. The impact of age on the infection rates of the tooth caries showed the age groups of (20-40 years) and (1-20 years) were the most of the infected compared to elder group (40-60 years), as was the incidence of 44% and 32% respectively.

**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 |

The study confirmed that children and the younger are more susceptible to mouth infection. This may be due largely to reasons related to immune shortages of the infected people in these age groups as well as consciousness of health or other factors related to nutrition and public health that increase the rates of infection in patients of children and the younger (Rao, 1998) stated that children are more susceptible to the bacteria that cause decay. That necrosis of the infected children appears to have teeth mutans with its different kinds and with high rates. Also, the frequent consumption of sugar play an important role in the infection with emphasis on the role of the mother as a source of transmission of disease from her infected teeth to her baby, where the levels of these bacteria with mothers are similar to those found at their children.

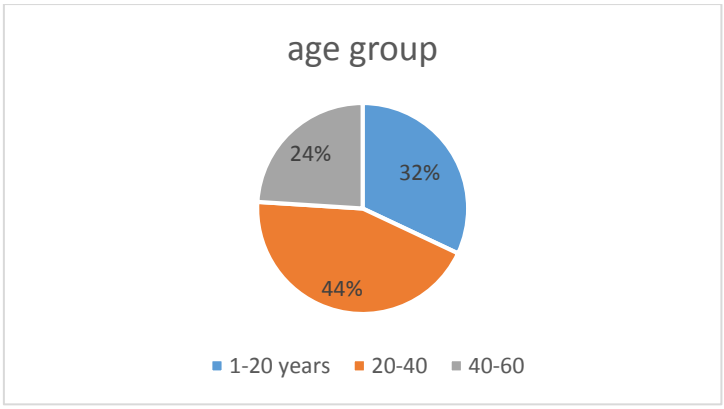


**Fig. 1:** Percentage of isolates according to age stage group.

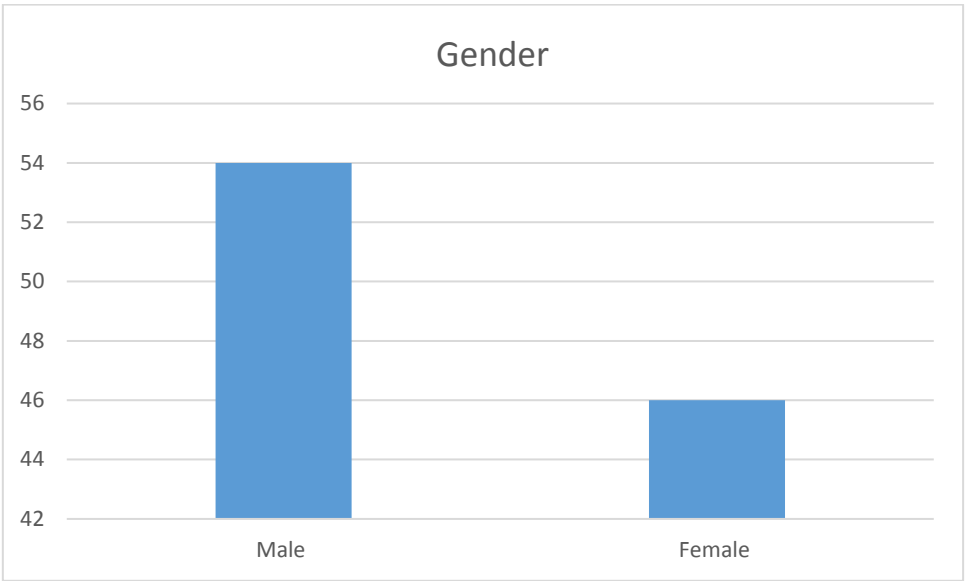


**Fig. 2:** Percentage of isolates according to gender

***The sensitivity of bacteria to antibiotics:***

Data represented in Figure 3 show the percentage sensitivity of bacterial isolates against nine antibiotics, where it showed they were streptomycin and penicillin G 16.94%, 64.40% respectively. While noted the highest sensitivity to antibiotics was chloramphenicol (83.05%), similarity sensitive for Gentamicin and Rifampicin with (81.35%).

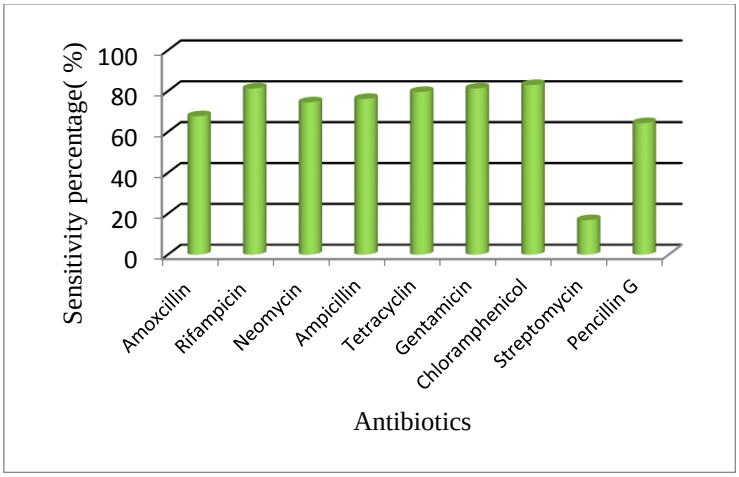


**Fig. 3:** Percentage sensitivity of bacterial isolates antibiotics.

The current study showed that the chloramphenicol is the best of antibiotic in its influence on the bacterial isolates taken from the mouth, followed by the antibiotic Gentamicin and Rifampicin. The antibiotic tetracycline, Ampicillin, Neomycin, Amoxcillin are less effective on the bacterial isolates. One of the results we have noticed that the bacterial isolates showed variation in their resistance to antibiotics of the group of aminoglycosidate, the ratio of sensitive to Neomycin 74.57%. The percentage of sensetive to Gentamicin by 81.35%. The resistance to aminoglycosidate antibiotics increased notably in recent times, Livermore and Winstanley, 2001 studied Relationships between antibiotic and mechanism are also presented to allow full interpretative reading for those testing wide panels of drugs versus isolates. This resistance which is due to the formation of the enzyme by resistant bacteria modifies the antibiotic and thus loses its effectiveness or because of loss of outer membrane proteins, which reduces the permeability of the antibiotic inside the bacterial cell (Livermore *et al*., 2001). Evidenced by the results of the current study, the majority of bacterial isolates possessed prescription relatively high resistance to antibiotics represented β-lactam (Ampicillin, Amoxicillin, Pencillin G). The high bacterial resistance to antibiotics β-lactam due to several mechanisms, most notably the ability to produce enzymes which β-lactamase the broken bind β-lactam, change the permeability barrier intimacy between the antbiotic and locations of the target Penicillin Binding Protein, came our results are compatible with Cherian and Manjunath, 2003 during their study that extended spectrum beta lactamase producing enterobacteriaceae in a tertiary care hospital in Trinidad and Tobago (Cherian *et al*., 2003). The results of this study also showed the high resistance shown by the bacterial isolates to streptomycin explains the mechanism of resistance to this antibiotic, Speculation on this mechanism indicates that the binding of the molecule to the 30S subunit interferes with 50S subunit association with the mRNA strand. This result in an unstable ribosomal-mRNA complex, leading to a frameshift mutation and defective protein synthesis leading to cell death. Syal, *et al* 2013, reported that streptomycin therapeutic concentrations of 10 mg/mL interfere in the Jaffe reaction and acted as non-creatinine chromogen during in their study that referred Streptomycin interference in Jaffe reaction Possible false positive creatinine estimation in excessive dose exposure (Syal *et al*., 2013). The study also shows an increasing resistance to tetracycline, it is believed that this resistance resulted from the presence of plasmids that encode resistance to the antibiotic which moves significantly. Koo and Woo, 2011 during their study that distribution and transferability of tetracycline resistance determinants in *E. coli* isolated from meat and meat products reported that the high prevalence of tetracycline resistant *Escherichia coli* in meat may be due to the high transferability of tetracycline determinants (Koo andWoo, 2011). It is noted during the study that the lowest resistance showed by the bacterial isolates was to chloramphenicol, Gentamicin and Rifampicin. It appeared that most of the bacterial isolates were sensitive to these adversaries and may be due to response to the majority of the isolates of these two adversaries to being of limited use antibiotics at the present time in hospitals, leading to increased resistance to antibiotics can be passed as determinants responsible for drug resistance to antibiotics by plasmids.

***Sensitivity of bacteria to heavy metal:***

The results as shown in Figure 4 that the resistance and sensitive percentages of bacterial isolates seven heavy metals, where it showed explain the resistance to heavy metals, Iron 3.38% , then nickel , aluminum ,copper, lead to 20.33%, 22.03%, 27.11%, 28.81% respectively, also sliver shown 57.62%. And, this similarity were found have sensitive to antimony and chromium 61.01%.while appeared sensitivity to mercury and cadmium by 100%, 86.44%

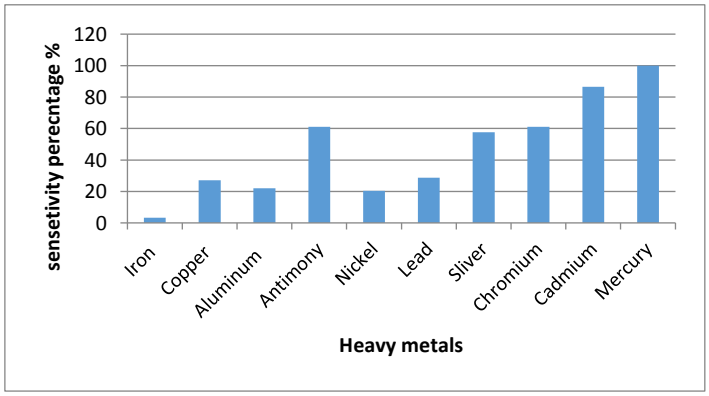


**Fig. 4:** Percentage of bacterial isolates sensitive of heavy metal

The results of this study also showed the high resistance shown by the bacterial isolates to iron Guohua Jiang *et al* studied reduction of iron in scrubbing solution by magnetic iron microspheres immobilized of iron reducing bacteria so reported that magnetic microspheres to immobilize iron reducing bacteria to improve the biological reduction of iron which was one of the key steps in nitrogen oxides removal by the integrated chemical absorption biological reduction process. The immobilized bacteria performed well iron reduction than free bacteria even

under unfavorable pH and temperatures(Jing *et al*., 2012). Evidenced by the results of the current study, the majority of bacterial isolates possessed prescription relatively high sensitive to mercury. Mercury is additionally the sole microorganism metal resistance system whose mechanism ends up in large-scale transformation of its target. The mechanisms of different ion to resistances are supported effluent pumps or living thing sequestration. Barkay and Miller (2003) studied bacterial mercury resistance from atoms to ecosystems so reported that one or more proteins apparently involved in transport genes conferring occur on chromosomes, plasmids, and transposons and their operon arrangements can be quite diverse, structural genes, several of which are modular. proteins protects host cells from this toxic metal (Barkay *et al*., 2003). The data obtained during this study clearly shows that with sensitive microorganism of cadmium. This may be due largely to reasons related to less concentration from cadmium increase the rates of sensitive isolates. Cohen, *et al*. (1990) studied that the effect of zinc and cadmium ions on Escherichia coli and they were noted that exposure of E. coli to various concentrations of these ions resulted in an increase of the total protein and the metal binding proteins amount in the cells. The activity of alkaline phosphates was raise in the presence of these ions (Cohen *et al*., 1990).

***Virulence Factors:***

Figure 5 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%).

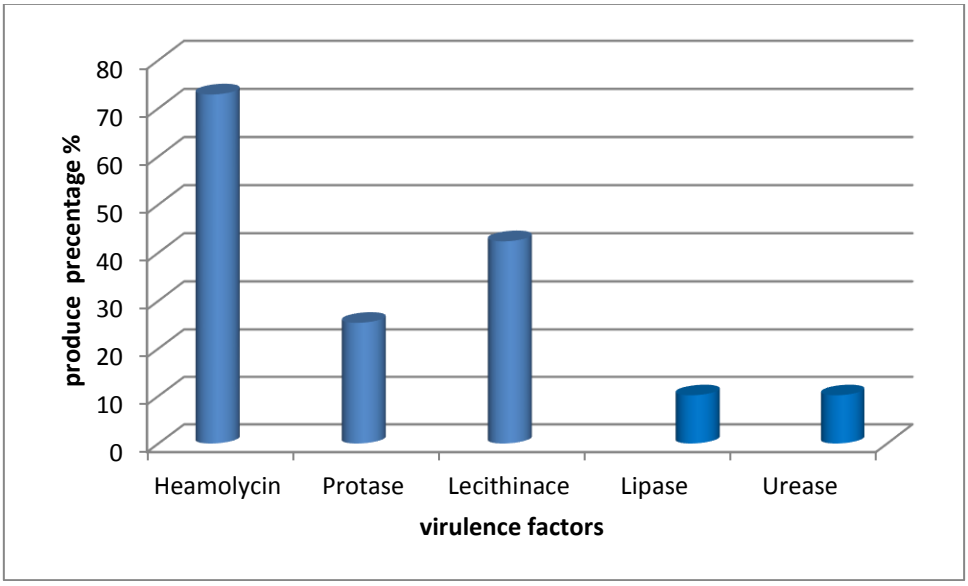


**Fig. 5:** Percentage to bacterial isolate produced virulence factors

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 (Anacarso *et al*., 2013). Takahashi *et al*. (2014) showed that 80% of produced hemolysin from the human body is positive of *Aeromonas trota* (E. Takahashi *et al*., 2014). 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. (Rosa-Fraile *et al*., 2014). 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. 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. (Sharaf *et al*., 2014). 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 (Vandecandelaere *et al*., 2014) 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 (Batra andWalia, 2014). 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 (Morou-Bermudez *et al*., 2011) . Morou *et al* 2011 reported that urease activity in plaque recorded a trend that remains stable during the study period. 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 (THOMAS *et al*., 2003). 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. (Joseph *et al*., 2006).

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%.

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