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IMPACT OF ANTIMICROBIAL AGENTS ON BACTERIAL ISOLATES FROM DENTAL DECAY

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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 factores on bacterial isolates. A total of 50 swabs were collected from the mouths of patients from both gender, with ages ranging from 1–60 years. 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.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%).

Keywords: bacteria, dental caries, heavy metals, virulence factors.

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 indicates 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 and Lo, 2008). 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 (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*., 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 and Nyvad, 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 (Van Houte, 1993). 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 and Nyvad, 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, study the effect of some heavy metals for oral 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 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 (Bakht *et al*., 2013).

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 heavy metals 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 two plates. The seven heavy metals discs prepared were placed onto the inoculated plates. After an overnight incubation, the diameter of each inhibition zone was measured and recorded in mm (Ali *et al.,* 2015).

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 (Ali *et al*., 2015). 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 h. Positive result was considered to be indicated by the change in color of the media to pink (Brown, 2009).

RESULTS AND DISCUSSION

Patient's isolates

Data on bacterial and yeast (59) isolates during the primary isolation of samples are shown in table 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 stag 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,(Rao, 1998) 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 years	16	32
20-40	22	44
40-60	12	24

Sensitivity of bacteria to heavy metal

Figure-1 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.

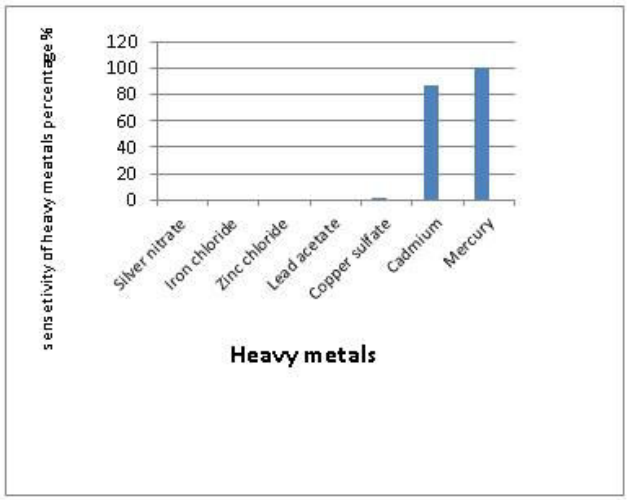

Figure-1. Percentage of bacterial isolates sensitive of heavy metal.

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 (Starodub and Trevors, 1990). 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 (Silver, 2003) 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 (Jarosławiecka and Piotrowska-Seget, 2014) .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 (Cohen *et al.*, 1990). 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 (Michels *et al.*, 2005).

Virulence factors

Figure-2 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 oral bacterial isolates were produced to virulence by lipase and urease (10.16%).

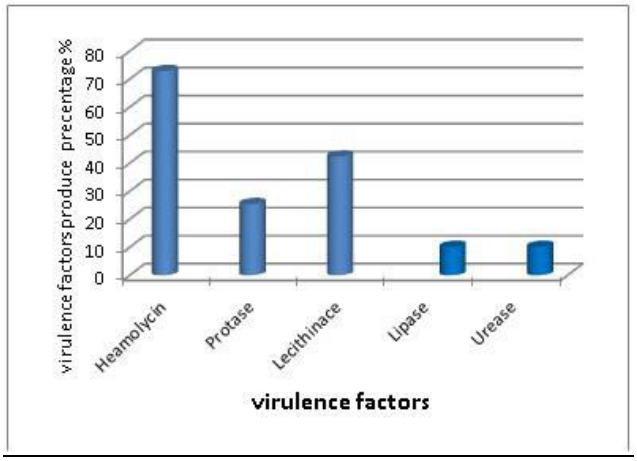

Figure-2. Percentage to bacterial isolate produced virulence factors.

Virulence is the degree of pathogenicity exhibited by most pathogens and is a measure that effectively differentiates pathogenic and nonpathogenic 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. (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, 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. (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. 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 (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 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 protease 25.42%, lipase and urease were10.16%.

CONCLUSIONS

The higher oral infection was in second age stage groups, five of heavy metals were resistance to oral microbial isolates and hemolysin had the highest ability to produce virulence factors for microbial oral isolates, the heavy metals resistance and hemolysin produced help oral microorganisms to increase dental caries infection.

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