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Association of Oral Microbiota Properties on Hyaluronidase and Biofilm Formation

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Correlation between hyaluronidase (Hyl) activity and biofilm detachment in a few bacterial species was found. However, it is unclear if this association applies to bacterial species or for more general bacterial characteristics. This study determined the association between biofilm production and Hyl activity among bacterial isolates from the oral cavity of healthy subjects, and its association with Gram staining group, colony surface morphology and bacteria shape. The swab was taken from the tongue, cheek and entire teeth surfaces of 35 subjects, and tested for biofilm through modified microtiter plate assay while Hyl production was screened through HA rapid plate method. Forty-four isolates were found, each 50% are Gram-positive, and Gram-negative bacteria, with the majority are cocci and non-mucoid colony. More than 70% of isolates are moderate and strong; (n= 17, 38.6%) and (n=15, 34.1%) respectively for biofilm production; and 68.2% are Hyl producer. A significant association was found between Hyl and bacterial shape (p=0.018) and colony morphology (p=0.018), while other association is not significantly measured, including between Hyl and biofilm (p=0.659). This study showed that biofilm production is not affected by the characteristics of the bacteria to produce or not produce hyaluronidase. Meanwhile, Hyl production is prone in rod shape and mucoid isolates which need further investigations.

Keywords: biofilm; hyaluronidase; colony morphology; Gram-positive; Gram-negative; bacterial shape

I. INTRODUCTION

Bacterial hyaluronidase (Hyl) is a virulence factor that involves in pathogenesis of disease in certain bacteria, where the most established studied bacteria are *Staphylococcus aureus* and *Streptococcus pneumoniae* (Ibberson *et al.* 2014). This enzyme degrades hyaluronic acid (HA) which abundantly present in the human body including the skin, umbilical cord, synovial fluid, eyes, lungs and skeletal tissues (Meyer & Palmer 1934; Juhlin 1997; Armstrong & Bell 2002; Papakonstantinou *et al.* 2008). In the oral cavity, HA was found in all periodontal tissues, particularly in gingiva and periodontal ligament (Dahiya & Kamal 2013). It plays various roles as cell adhesion, migration and differentiation. Degradation of HA by Hyl enzyme reduces the viscosity of the tissue while increases the tissue permeability, where it can create a pathway for substance delivery and dispersion across the membrane (Necas *et al.* 2008). Previously, Pecharki *et al.* (2008) found the involvement of Hyl in biofilm detachment in *Streptococcus intermedius* isolated from the oral cavity. The findings were supported by Ibberson *et al.* (2016) who was found an increase of biofilm mass in the mutant *Staphylococcus aureus* strain lacking Hyl, which later was found to involve in biofilm detachment. At the moment, it is unclear if the association of Hyl inactivity is specific for different bacterial species, or to more general characteristics. It is because, most of the previous studies were conducted for specific bacterial species including *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus pneumoniae*, *Treponema pallidum* and *Clostridium perfringens* (Fitzgerald & Gannon 1983; Hynes & Walton 2000; Hart *et al.* 2009). Thus this study was conducted to reveal if Hyl

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activity and biofilm production are influenced by any specific bacterial characteristics, based on its Gram's classification, bacterial shape and colony morphology.

II. MATERIALS AND METHOD

A. Sample Collection

This study was obtained ethical approval from IIUM Research Ethics Committee (IREC) (IREC 2017-042). The swab samples were taken from the tongue, cheek and entire teeth surfaces of 37 healthy subjects. The swabs were immediately streaked on nutrient and MacConkey agar (MAC) (Oxoid, Hampshire) and incubated aerobically at 37°C for 24 hours. The colony morphology was recorded where each different colony was stained with Gram's reagent for microscopic observation.

B. Determination of Biofilm-Producing Isolates

Detection of biofilm production was performed following microtiter plate method by Christensen *et al.* (1985) modified by Stepanovic *et al.* (2000). The isolates were grown in trypticase soy broth (TSB) medium supplemented with 1% glucose and incubated for 18 hours for sufficient bacteria growth. Then 200µl of the cultures were inoculated further in individual 96 well plates. The well plates were incubated for 24 hours at 37°C in static condition. The content of wells plates was aspirated and washed three times with 200µl of sterile physiological saline before fixing with 200µl of 99% methanol for 15 minutes. The plate was emptied and left to dry. Next, the wells were stained with 200µl of crystal violet used for Gram staining for 5 minutes. The excess stain was removed by placing under running tap water. The content of the wells was resolubilized with 160µl of 33% glacial acetic acid for 30 minutes. Micro enzyme-linked immunosorbent assay (ELISA) auto reader with wavelength 570nm was used to measure the optical density (OD). The values are considered as the index of attachment to a surface. The classification of bacteria adherent category was done based on a formula introduced by Stepavonic *et al.* (2000).

C. Screening of Hyaluronidase Production

Each positive biofilm-producing bacteria isolates was screened for hyaluronidase production through rapid plate screening method (Smith & Willet 1968). A single colony of isolates was streaked on brain heart infusion agar (Oxoid, Hampshire) plates containing 2mg/ml hyaluronic acid or sodium hyaluronate 95% (HA) (ACROS Organic, USA) supplemented with 5% bovine serum albumin (Vivantis Technology, Oceanside) and incubated for 24 to 48 hours at 37°C. Then, the agar plate was treated with 2N acetic acid for 10 minutes. The halo zone surrounding the colony was observed. Commercial hyaluronidase was used as positive control and culture broth without inoculum for negative control.

D. Statistical Analysis

The association analysis was carried using Pearson or Fisher Exact test through SPSS version 12.0 software. Any p-value ≤ 0.05 are deemed as significant value.

III. RESULT AND DISCUSSION

In total, 44 isolates were successfully detected from the 35 subjects. To find the association of hyaluronidase production with specific characteristics of the organism, all the isolates were further grouped based on colony surface morphology (mucoid and non-mucoid appearance), Gram staining and its shape. Majority of the isolates were non-mucoid (n=34, 77.3%) while remaining 10 isolates (22.7%) were mucoid colony. Half of the isolates (n=22, 50%) were Gram-positive and Gram-negative bacteria, and 34 isolates (77.3%) were cocci while remaining 10 isolates (22.7%) were rod in shape (Figure 1).

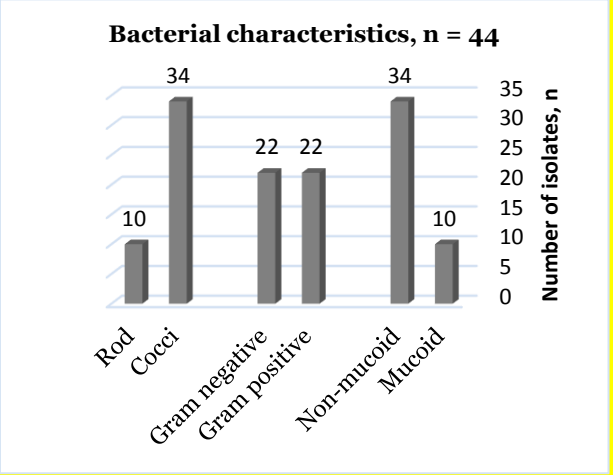

Figure 1. Distribution of *Streptococcus pneumoniae* isolates into bacteria shape, Gram's group and colony surface morphology

In the present study, the biofilm production was detected from 18 hours of incubated bacterial culture. In the previous study, biofilm formation was detected as early as 4 hours and reach maximum production after 8 hours incubation (Gutierrez *et al.* 2016). In this study, biofilm was detected in all isolates, which further classified as a weak, moderate and strong producer. In combination, 72.72% of the isolates are moderate and strong biofilm producer (Figure 2). The association analysis showed biofilm production has no significant association with any of bacterial characteristics (Table 1).

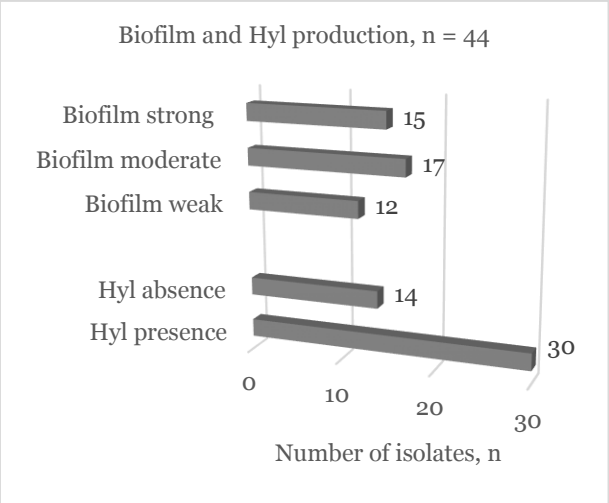

Figure 2. Biofilm and Hyl production in all isolates

While for Hyl production, the majority of the isolates were positive (n=30, 68.2%) (Figure 2). All isolates with a mucoid appearance on the agar plate presence with Hyl production (100%), where a significant association between Hyl production and colony morphology (p=0.018) was observed. Table 2 also shows a slight preponderance of Gram-negative bacteria to produce Hyl, without evidence of significant association value. Another significant value was observed for rod-shaped bacteria to produce Hyl (100%) (p=0.018) (Table 2).

Further analysis showed a majority of moderate biofilm producer are Hyl producer (43.3%). However, no significant association value was identified between these criteria (Table 3).

Table 1. Association of biofilm production and bacterial characteristics

	Biofilm producer, n (%)			*p*-value
	Weak	Moderate	Strong	
Mucoid	3 (30.0)	3 (30.0)	4 (40.0)	0.818
Non-mucoid	9 (26.5)	14 (41.2)	11 (32.4)	
Gram positive	7 (31.8)	8 (36.8)	7 (31.8)	0.863
Gram negative	5 (22.7)	9 (40.9)	8 (36.4)	
Cocci	3 (30.0)	3 (30.0)	4 (40.0)	0.818
Rod	9 (26.5)	14 (41.2)	11 (32.4)	

Table 2. Association of Hyl production and bacterial characteristics

	Hyl production, n(%)		p-value
	Presence	Absence	
Mucoid	10 (100.0)	0 (0.00)	**0.018***
Non-mucoid	20 (58.8)	14 (41.2)	
Gram positive	13 (59.1)	9 (40.9)	0.332
Gram negative	17 (77.3)	5 (22.7)	
Cocci	20 (58.8)	14 (41.2)	**0.018***
Rod	10 (100.0)	0 (0.0)	

*significant at $p \leq 0.05$

Table 3. Association between biofilm and Hyl production

	Biofilm producer, n (%)			*p*-value
	Weak	Moderate	Strong	
Hyl presence	8 (26.7)	13 (43.3)	9 (30.0)	0.659
Hyl absence	4 (28.6)	4 (28.6)	6 (42.9)	

A study conducted by Pecharki *et al.* (2008) found that Hyl promotes detachment of biofilm and the results of 18 h of exposure HA-grown biofilms to Hyl strongly reduced the

biofilm mass. In an earlier study, Yadav *et al.* (2013) observed increases in biofilm biomass when grown in medium supplemented with HA, and they demonstrated that the HA supports *Streptococcus pneumoniae* growth. However, no association of Hyl and biofilm production were investigated in their study. Ibberson *et al.* (2016), also reported that in an established HA-containing biofilm, the induction of Hyl resulted in biofilm dispersal compared to uninduced control and confirmed their findings that exogenous Hyl could detach HA-containing biofilm. The detachment of biofilm by Hyl enzyme is achieved through degradation of the HA substrates, which was supplemented in the biofilm growth media (Kaplan 2010).

Association between biofilm and Hyl activity was investigated in limited studies, among specific bacterial species. This valuable information could explain the pathogenesis of the bacteria, but a confirmation is needed for other bacterial species, especially in common biofilm producer in human. In human, biofilm is highly produced in the oral cavity. Therefore this study is specifically aimed to investigate the association of biofilm and Hyl activity in bacteria isolated from the oral cavity of a healthy individual. In addition, the study was also looking for any association of biofilm and Hyl with bacterial characteristics including their Gram's staining group, colony morphology and bacterial shape.

In the previous study, Hyl in *Streptococcus intermedius*, a common bacteria causing abscess formation (Whiley *et al.* 1992; Tran *et al.* 2008) was found to correlate with biofilm activity by dispersing the biofilm and facilitate new biofilm formation at a different site (Pecharki *et al.* 2008). In another study, the elevated amount of Hyl was detected in the mutant strain of *Staphylococcus aureus* lacking biofilm, but no significant correlation was declared (Hart *et al.* 2013). Most of the studies, albeit low in number, reports on Gram-positive bacteria, while information available for Gram-negative bacteria is lacking.

The present study found that Hyl production is not associated with biofilm production. Combination of biofilm and Hyl production level (e.g. Strong biofilm producer and Hyl producer) also not significantly associated with the aforementioned bacterial characteristics (data not shown). From this association analysis, this finding indicates a lack of contribution of Hyl in biofilm activity. However, further study should be done by looking at the involvement of Hyl production during biofilm formation (*in situ*), which may give a better understanding of the association between these two components.

Based on Table 1, almost equal distribution of biofilm level (weak, moderate, strong) found among Gram-positive isolates. However, a slight preponderance Gram-negative isolates to produce moderate and strong biofilm. Lee *et al.*, (2016) showed that the biofilm formation of Gram-positive bacteria is influenced by lipoteichoic acid available on cell wall meanwhile Gram-negative biofilm formation is influenced by lipopolysaccharide (LPS) found in the outer membrane. However, Table 1 showed no association was found between biofilm formation and Gram bacteria group which is similar to Mai-Prochnow *et al.* (2016) who observed similar biofilm biomass in Gram-positive and Gram-negative bacterial isolates and suggested that other factors are responsible for biofilm thickness and structure. In bacteria, Gram staining classification is based on the cell properties correlated with the cell wall. Although the differences of biofilm in both class not widely discussed, the thickness and structure of both classes are found similar (Mai-Prochnow *et al.* 2016). Differences of cell wall properties in Gram-positive and Gram-negative bacteria may affect the bacterial attachment to the surface, the first stage in biofilm formation (Renner & Weibel 2011). The other factors that may influence the formation of biofilm are the composition of the surrounding environment that may affect the bacterial attachment to the surface (Renner & Weibel 2011; Fysun *et al.* 2019). Marks *et al.* (2012) demonstrating higher biofilm formation in *Streptococcus pneumoniae* strain which lack of capsules. In that study, they found that lacking the capsules increase the bacterial binding to the host cells, which later increase the colonization, likewise the biofilm formation.

Similar findings were observed in other studies where the production of capsules block the formation of biofilm in *Prophyromonas gingivalis*, *Vibrio vulnificus* and *Neiserria meningitidis* (Joseph *et al.* 2004; Yi *et al.* 2004; Davey *et al.* 2006). Firm attachment of bacteria to host cells is a critical stage for biofilm development. Attachment of bacteria to the biotic or abiotic surface relies on many factors, including surface proteins which act as adhesin or adherence factors.

Attenuated glycosyltransferase in *Porphyromonas gingivalis* produced immature fimbriae which affect the ability of the bacteria to perform autoaggregation and gave a severe impact on its adherence capability (Narimatsu *et al.* 2004). In addition, surface protein gingipains play the main role in *P. gingivalis* adherence to other bacterial or human epithelial cells (Tokuda *et al.* 1996). Covering the surface protein by the capsule (Mesnage *et al.* 1998) might reduce the adherence capacity of the surface protein, further affect the biofilm formation. In the present study, no significant association was recorded between biofilm formation and mucoid and non-mucoid isolates. This finding needs further investigation, by considering other factors to be included, such as the species of the bacteria.

In term of Hyl production, a significant association was identified for colony morphology and bacterial shape, while no significant association for Gram's classification group. Although no significant differences were identified between Gram-positive and Gram-negative bacteria, it interesting to observe more than 77% of Gram-negative isolates possess Hyl activity (Table 2). In previous studies, various Hyl producer was reported among Gram-positive bacteria including *Streptococcus pneumoniae*, *Streptococcus agalactiae*, Streptococcus Group A and B and Streptomyces spp. (Ohta & Kaneko 1970; Calvinho *et al.* 1998; Sellin *et al.* 1998; Starr & Engleberg 2006; Yusof *et al.* 2015), while few reported on Gram-negative bacteria, including *Propionibacterium acnes* (Tyner & Patel 2015). Therefore, the authors are planning to identify the bacterial species further to add on more data on Hyl producer in Gram-negative bacteria. In Gram-negative bacteria, the production of enzyme is in periplasmic space, the zone between the cytoplasmic membrane and outer membrane that contain abundant proteins (Hynes & Walton 2000).

Meanwhile, in Gram-positive bacteria, this enzyme is retained in or near the cell membrane instead (Silhavy *et al.* 2010). Thus, the difference in locations of proteins responsible for Hyl secretion between Gram-positive and Gram-negative might influence the difference in Hyl activity. This assumption needs further analysis to confirm the differences.

Hyaluronidase production was found to associate with the mucoid appearance colonies on the agar plate. This is similar to Yusof *et al.* (2015) who also found high Hyl producer in mucoid colonies of *Streptococcus pneumoniae*. Mucoid colonies which appear like a water-glistening and most of the time become confluent on the agar plate are related to the production of slime and capsule by the cell (Kandi 2015). Capsulated bacteria represent more capsular materials and usually indicate higher virulence of the bacteria since the capsule could prevent the phagocytosis by host immune system (Kelly *et al.* 1994). One of the possible explanations is for high Hyl in capsulated bacteria is to degrade the capsule, thus enhance the bacterial adherence to the host tissues. This explanation is highly applicable for *Streptococcus pyogenes*, where the capsule are highly made of hyaluronic acids, the substrate for Hyl (Starr & Engleberg 2006). However, the significance of high capsulated materials and Hyl activity in bacteria producing non-HA capsules such as *S. pneumoniae* and other bacteria are currently unknown.

Another significant association found is all the rod shape bacteria producing Hyl. This finding contradicts to the previous study, which rarely found hyaluronidase producer among rod-shaped bacteria, except in Bacillus sp., *Clostridium perfringens* and *Propionibacterium acnes* (Hynes & Walton 2000; Guo *et al.* 2014). Since the present study does not identify the isolates up to species level, it is unclear if all the rod shape isolates represent one or more bacterial species. Thus further analysis is needed to confirm the association.

IV. CONCLUSION

In the current study, the data showed all oral cavity isolates produce biofilm in various amount, and the majority of them are Hyl producer. Although this number could not explain the exact role and mechanism of Hyl in bacterial pathogenesis, it may indicate the capability of the certain bacterial group to produce these two virulence factors. There is no significant association between biofilm and Hyl production, however, *in situ* tests for Hyl production during biofilm formation or at other stages is needed for better understanding. A significant association was found for mucoid colony and rod shape isolates which require further investigation to understand the relationship and its mechanism.

V. ACKNOWLEDGEMENT

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