ASM Sc. J. 19, 2024

https://doi.org/10.32802/asmscj.2023.1345

# Hyaluronidase Involvement in *Streptococcus pneumoniae* Biofilm Activity

M.A.A. Suaidan, N.M.M. Amin, M.I.E. Othman, N.A.M. Nayian, A.Z.A. Mansor and H.A. Yusof*

*Department of Biomedical Science, Kulliyyah of Allied Health Sciences, International Islamic University Malaysia, Pahang, Malaysia*

*Streptococcus pneumoniae* causes numerous severe infections and results in a significant number of mortality cases due to various virulence factors, including its ability to form a biofilm. The antimicrobial resistance and the challenges the host immune system faces in combating the biofilm make treatment against *S. pneumoniae* increasingly difficult. Consequently, investigations into pneumococcal biofilm are of paramount importance. The formation and enhancement of pneumococcal biofilm have been found to be influenced by various molecules, including hyaluronic acid. The degradation of hyaluronic acid by its specific enzyme, hyaluronidase, is believed to amplify the pathogenicity of pneumococci through increased colonisation. To delve deeper, the roles of hyaluronidase and hyaluronic acid in pneumococcal biofilm formation were investigated. The wild-type and Δ*hyl S. pneumoniae* strains were cultivated in Brain Heart Infusion (BHI) media, both with and without the addition of hyaluronic acid, for six days. The formation of the biofilm at each incubation interval was identified using a crystal-violet (CV) microplate biofilm screening assay. These readings were analysed using the non-parametric Mann-Whitney test. The Δ*hyl* mutant strain generally exhibited lower biofilm production during the initial four days but significantly surpassed the wild-type strain on days 5 and 6. No notable differences in biofilm measurements were observed between the two strains when cultured in media enriched with hyaluronic acid. Hyaluronidase appears to play a role in the biofilm cycle of *S. pneumoniae,* whereas additional hyaluronic acid in the culture medium seems to have no marked impact on biofilm activities. Further research is necessary to gain a clearer understanding of hyaluronidase's function in *S. pneumoniae* biofilm activities.

**Keywords**: *Streptococcus pneumonia*; biofilm; hyaluronidase; hyaluronic acid

## I. INTRODUCTION

*Streptococcus pneumoniae* is a Gram-positive, facultative anaerobic diplococcus often found in the human upper respiratory tract. In healthy individuals, it usually remains asymptomatic. However, this opportunistic organism can become pathogenic in susceptible individuals, particularly those with weakened immune systems, such as the elderly and young children. In addition to causing pneumonia and otitis media, *S. pneumoniae* can lead to other invasive pneumococcal diseases, including meningitis and septicaemia (Henriques-Normark & Tuomanen, 2013). It is estimated that each year, there are 14.5 million cases of severe pneumococcal disease among children under five years of age worldwide. Moreover, it results in approximately half a million deaths in low- and middle-income countries (CDC, 2017) despite the availability of vaccines and antibiotics. The ability of pneumococci to form biofilms exacerbates the situation, as antimicrobial agents and the host immune system struggle to penetrate these pneumococcal biofilms (Domenech *et al.*, 2011).

*Corresponding author's email: hanani@iium.edu.my

Biofilms are a potent mechanism for bacteria to shield themselves from antimicrobial agents and the host immune system (Moscoso *et al.*, 2009). They provide a protective environment for bacteria to proliferate before dispersing to other regions as the biofilm matures and detaches from its initial surface (Fleming & Rumbaugh, 2017). Numerous studies have identified many regulatory factors that contribute to both the formation and detachment of biofilms (Pecharki *et al.*, 2008; Hart *et al.*, 2013; Yadav *et al.*, 2013; Ibberson *et al.*, 2016; Fleming & Rumbaugh, 2017), which are triggered by different environmental conditions. These factors are often influenced by varying environmental conditions. The role of biofilms in bacterial pathogenicity has been extensively explored in various bacterial species, but it has been less so in pneumococci, especially the factors influencing the biofilm activity in this pathogen (Yadav *et al.*, 2013; Blumental *et al.,* 2015). A study by Ibberson *et al.* (2016) found that the pneumococcal biofilm was enhanced by several substances, including hyaluronic acid. Notably, hyaluronic acid also serves as a substrate for the hyaluronidase enzyme (Hyl), produced by various organisms, including humans, animals, and microorganisms such as bacteria and fungi (Starr & Engleberg, 2006; Todar, 2012). Hyaluronidase has been reported to successfully degrade hyaluronic acid-containing biofilms on both, biotic and abiotic surfaces (Moscoso *et al.*, 2009; Domenech *et al.*, 2011). Hyaluronic acid, derived from bacterial capsules, supports pneumococcal growth in biofilms. The upregulation of the hyaluronidase gene when hyaluronic acid is present in the culture medium suggests a potential relationship between hyaluronic acid and hyaluronidase in *S. pneumoniae* biofilm formation (Yadav *et al.*, 2013). While hyaluronidase has been shown to play a role in bacterial invasion into host body, its contribution to biofilm activity remains unclear. Therefore, this fundamental study aims to investigate the role of pneumococcal hyaluronidase and its substrate, hyaluronic acid, in *in vitro* pneumococcal biofilm activities.

## II. MATERIALS AND METHODS

### *A. Reviving Wild-type and Δhyl mutant S. pneumoniae Strains*

The sample used in the study was *S. pneumoniae* strain serotype 23F, selected from the pneumococci culture stock (Yusof *et al.*, 2015). This strain has been previously identified as a hyaluronidase producer through the hyaluronic acid turbidity reduction assay (Yusof *et al.*, 2015). The Δ*hyl* mutant was developed from this wild-type strain by deleting the entire hyaluronidase-encoding gene (hyl gene) using polymerase chain reaction (PCR)-based approach (unpublished data). The construction of the mutant was verified through DNA sequencing analysis, and its hyaluronidase activity was confirmed as negative via the hyaluronic acid turbidity reduction assay. All the strains were stored in a culture medium supplemented with glycerol at -50$^{o}$C before use.

The frozen glycerol stocks of the wild-type and Δ*hyl* mutant strains were thawed at room temperature. After thawing, one loopful of each culture was inoculated into 25 ml of Brain Heart Infusion (BHI) broth (Oxoid Ltd, Hampshire) and then incubated for 18 hours at 37$^{o}$C in a $CO_2$-enriched atmosphere. Subsequently, a loopful of these cultured strains was streaked onto Blood Agar plates (BAP) (Isolac, Malaysia) and BHI agar plates for further used.

### *B. Crystal-Violet Microplate Biofilm Screening Assay*

The biofilm of both wild-type and Δ*hyl* mutant *S. pneumoniae* strains grown in BHI broth, both with and without the addition of 2 μg/ml hyaluronic acid (Acrose Organics, Belgium), was measured. The measurement was conducted using the crystal-violet microplate assay, following methods adapted from Yadav *et al.* (2013) and Stepanović *et al.* (2000). Three independent experiments were performed, with triplicate assays for each. Assays were conducted in 96-well round-bottom polystyrene plates. The experimental setup included both a positive and negative control. The positive control comprised bacteria grown in broth supplemented with 2 μg/ml glucose (Merck Kgaa, France), and the negative control was the broth without bacteria. Initial inoculums were prepared and standardised to

an optical density ($OD_{600}$) between 0.008 to 0.012. The plates were then incubated at 37°C in a $CO_2$-enriched atmosphere for six days, with daily biofilm measurements (Yusof, 2015).

After incubation, the BHI broth was discarded, and the plates were gently washed three times with 200 µl of sterile phosphate-buffered saline (PBS) (Sigma Life Science, St. Luois). Subsequently, the plates were air-dried and stained with 50 µl of 0.1% crystal violet (Sigma-Aldrich, USA) for 15 minutes. Excess stain was removed, and the plates were washed three times with sterile distilled water. Finally, the stained biofilms were dissolved in 200 µl of 95% ethanol (Hmbg Chemical, Johor), and OD at 570 nm wavelength was measured using a microplate reader.

### *C. Statistical Analysis*

Data were presented as mean±standard deviation (SD) and analysed using Statistical SPSS for Windows, version 12.0.1. The Mann-Whitney test was employed to compare biofilm activity between the wild-type and Δ*hyl* mutant strain of *S. pneumoniae*, as well as within each strain across different culture mediums. Results were considered statistically significant when $p<0.05$.

## III. RESULTS AND DISCUSSION

The biofilm of pneumococci has gained importance in healthcare due to its resistance to antibodies and antimicrobial treatments that are typically effective against planktonic cells. Additionally, its ability to initiate pneumococcal colonisation plays a pivotal role in the onset of pneumococcal diseases such as otitis media, pneumonia, meningitis and invasive pneumococcal diseases (Oggioni *et al.*, 2006; Domenech *et al.*, 2011). Studying the behaviours of pneumococcal biofilm can offer valuable insights into the development of vaccines and new treatments that prevent biofilm formation, disrupt existing biofilms, or enhance the efficacy of standard antibiotics against pathogen associated with biofilms.

Hyaluronic acid, a component in extracellular matrix in human connective tissues, has been identified play a role in biofilm matrix formation. It either directly contributes as a component of the biofilm matrix or indirectly by acting as the primary carbon source for the cell (Ibberson *et al.*, 2016; Marion *et al.*, 2012). In Group A Streptococcus, hyaluronic acid is broken down by hyaluronidase, transforming it into smaller components that provide carbon energy for biofilm growth (Starr & Engleberg, 2006). Considering the intricate relationship between hyaluronidase and hyaluronic acid, it is postulated that they might be involved in similar activities. While the involvement of hyaluronic acid in biofilm formation is well-documented, the role of its enzyme, hyaluronidase, in biofilm processes remains less defined. Gaining insight into its function within the biofilm cycle could identify it as a potential target for future antibiofilm treatments in healthcare.

To ascertain the role of hyaluronidase in pneumococcal biofilm activities, this study cultivated both the wild-type and Δ*hyl* mutant strains to compare their biofilm formation capabilities. Biofilm measurements over a six-day period revealed a steady increase in biofilms in BHI broth for both the wild-type and mutant strains (Figure 1). During the first four days, the Δ*hyl* mutant strain formed fewer biofilms compared to wild-type strain, with a notable difference on day 2 (OD wild type = 0.13±0.01 / OD Δ*hyl* mutant = 0.10±0.01, $p=0.025$). However, by the end of the incubation time, especially on the fifth and sixth day, the measured biofilm of the Δ*hyl* mutant strain significantly surpassed that of the wild-type strain.

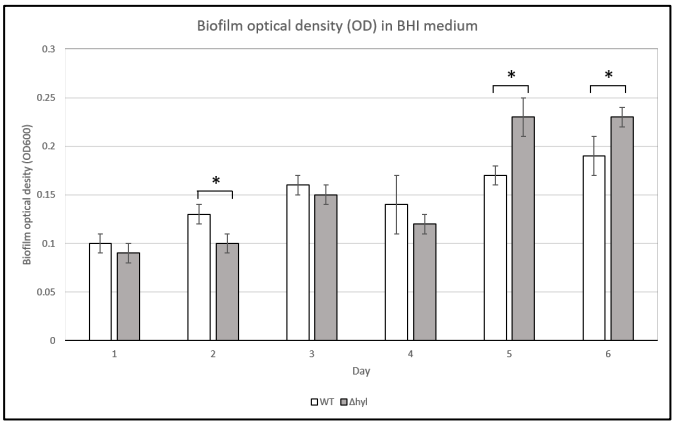


Figure 1. Biofilm formation of wild-type and Δ*hyl* mutant strains of *S. pneumoniae* grew in BHI media. All data are presented as means±SD, with the asterisk (*) indicating values that are statistically significant at $p<0.05$.

The notably low biofilm observed in the Δ*hyl* mutant strain on the day 2, coupled with its weak adherence level, suggests that the biofilm cycle at this point might be in the initial attachment phases. The adherence level of the biofilm was classified based on the following ranges:

OD < ODc (non-adherence)
ODc < OD < 2x ODc (weak adherence)
2x ODc <OD < 4x ODc (moderate adherence)
4x ODc < OD (strong adherence)

Here, "OD" referred to the optical density of the samples, while "ODc" is defined as the optical density of negative control, which contains only the broth (Nyenje *et al*., 2013). Given the weak adherence of biofilm on day 2, it seems to reflect the initial attachment phase in the biofilm cycle. This weak adherence is characteristic of the early stages of the biofilm cycle, where a cell's attachment to the surface is reversible, potentially influenced by van der Waals bonds (Deev *et al*., 2021). The precise mechanism through which pneumococcal hyaluronidase facilitates adherence remains unclear. However, its enzymatic degradation capability might be used to expose adhesins on the cell's surface, thus promoting better binding interactions. For instance, the degradation of capsular polysaccharides (CPS) in *Pasteurella multocida* by hyaluronidase led to increased biofilm formation. This is hypothesised to result from the exposure of more surface proteins, including adhesins, that facilitate attachment to the host surface (Petruzzi *et al*., 2017).

The significantly marked increase in biofilm production by the Δ*hyl* mutant strain (OD day 5 = 0.23±0.02 / OD day 6 = 0.23±0.01) compared to the wild-type strain (OD day 5 = 0.17±0.01 / OD day 6 = 0.19±0.02) towards the end of the incubation period (days 5 and 6) implies an alternative function of this enzyme in the biofilm life cycle. Without hyaluronidase, the mutant cells appear capable of generating a more substantial biofilm than their wild-type counterparts. Based on the adherence capacity, the biofilm readings for the Δ*hyl* mutant strain on days 5 and 6 indicated moderate adherence. Given the adherence levels and trajectory of the biofilm readings on days 5 and 6 for the Δ*hyl* mutant strain, it can be inferred that these cells might be in the maturation phase. The role of hyaluronidase in the specific maturation process is infrequently addresses; its presence in cells mostly noted for its function in assisting the adhesion and dispersal stages of biofilm across various bacterial species (Petruzzi *et al*., 2017; Starr & Engleberg, 2006; Yadav *et al*., 2013). Yet, in the present study, a contrasting observation was made wherein greater biofilm formation occurred in the absence of this enzyme. This suggests that hyaluronidase may act as a suppressor of biofilm formation during this phase. Nonetheless, this postulation should be approached with caution, as the observed biofilm reading patterns might not comprehensively represent a complete biofilm cycle encompassing all three phases: adhesion, maturing and dispersal.

Consistent with a suppressive role, the increase in biofilm readings in the absence of hyaluronidase seems more closely aligned with the biofilm dispersal mechanism. The hyaluronidase-deficient strain appears incapable of detaching the biofilm from the well's abiotic surface, a process typically managed by the wild-type strain. In a prior study by Pecharki *et al*. (2008), the role of hyaluronidase in *Streptococcus intermedius* during biofilm dispersal was suggested. Their research indicated that the mutant strain, lacking the hyaluronidase enzyme, produced 30% more biofilm than the wild-type strain when cultured in media enriched with hyaluronic acid. Speziale and Geoghegan (2015) reported that one of the primary components identified in the biofilm matrix of staphylococci is poly-β(1,6)-N-acetylglucosamine, which intriguingly is a precursor for hyaluronic acid. Considering the activity of hyaluronidase, which cleaves hyaluronic acid between acetylglucosamine and glucuronic acids, one can also hypothesise that an alternative degradation mechanism of hyaluronidase in biofilm detachment might involve acting on this compound.

This inference is strengthened by the negligible difference observed in biofilm formation between culture medium with and without added hyaluronic acid. The incorporation of hyaluronic acid into the biofilm culture medium aimed to elucidate its role in pneumococcal biofilm formation, shedding light on the hypothesised function of its degradative enzyme,

hyaluronidase, in the biofilm process. However, biofilm measurements demonstrated consistent results for the wild-type strain in media both with and without hyaluronic acid (Figure 2a). The inclusion of hyaluronic acid did not seem to significantly affect biofilm formation. This diverges from previous studies where neither enhancement nor inhibition of biofilm formation was observed. Several studies have shown an increase in biofilm readings when bacteria were cultured in a media enriched with hyaluronic acid (Yadav *et al.*, 2013; Pecharki *et al.*, 2008; Ibberson *et al.*, 2016). It was postulated that the added hyaluronic acid was posited to serve as large disaccharide energy molecules which, when broken down by hyaluronidase, transform into simpler sugars that support bacterial growth and subsequently biofilm development (Liu *et al.,* 2011; Ibberson *et al.*, 2016; Marion *et al.*, 2012). Conversely, other findings suggest that the introduction of hyaluronic acid might hinder biofilm formation by disrupting bacterial adherence to the cellular substrate (Alharbi *et al.*, 2023; Drago *et al.*, 2014).

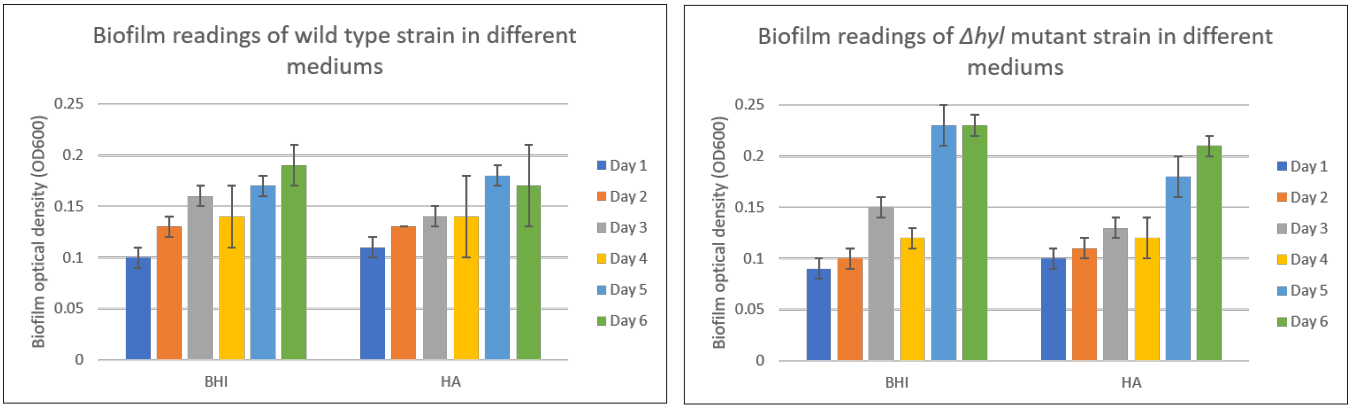


Figure 2. Biofilm readings in BHI and hyaluronic acid-enriched culture medium, a) wild-type strain b) Δ*hyl* mutant strain. All data are presented as means±SD.

In a comparable experiment, the Δ*hyl* mutant strain was cultured in hyaluronic acid-enriched medium, aiming to delve deeper into the interplay between this enzyme and its substrate in biofilm processes. It was found that the growth pattern of the biofilm in the hyaluronic acid-enriched medium was analogous to that in the BHI medium (Figure 2b). This observation aligns with the initial hypothesis that the biofilm readings for the Δ*hyl* mutant strain in the hyaluronic acid-enriched medium would likely mirror those in the BHI medium, given that the hyaluronidase enzyme, which acts on hyaluronic acid, is absent. Yet, this observation should be considered alongside results from other experimental conditions to fully understand the role of hyaluronidase in the biofilm life cycle and its dependence on its substrate, hyaluronic acid.

The present study was able to show a possible role of hyaluronidase in the biofilm cycle that might be independent of its interaction with hyaluronic acid. While much of the literature concerning the biofilm process frequently discusses this enzyme and its substrate in tandem, an alternative mechanism could be at play. Thus, it is crucial to further investigate its role, taking additional factors into consideration. This is because the biofilm process in *in vitro* conditions is influenced by several elements, such as nutrient availability, temperature, pH, and virulence genes expression. Moreover, *in vivo* conditions present even more complex interactions, including host-specific interaction and quorum sensing (Chao *et al.*, 2014; Preda & Sandulescu, 2019). Ultimately, understanding the role of hyaluronidase in the biofilm cycle could have significant implications for healthcare, especially since biofilm-associated infections are often more challenging to treat than standard infections.

## IV. CONCLUSION

In conclusion, this study suggests that pneumococcal hyaluronidase might play a role in *in vitro* biofilm formation, though the exact mechanism remains elusive. Interestingly, this study did not identify a significant contribution of hyaluronic acid to biofilm formation in *S. pneumoniae*, in contrast to findings from other studies. Further research is essential to better understand the involvement of hyaluronidase in biofilm processes, be it in the formation or detachment stages, within this pathogenic bacterium.

## V. ACKNOWLEDGEMENT

This project was funded by Fundamental Research Grant Scheme Government of Malaysia [FRGS/1/2016/SKKII/UIAM/02/1].

## VI. REFERENCES

Alharbi, MS, Alshehn, FA, Alobaidi, AS, Alrowis, R, Ashibani, N & Niazy, AA 2023, 'High molecular weight hyaluronic acid reduces the growth and biofilm formation of the oral pathogen *Porphyromonas gingivalis'*, The Saudi Dental Journal, vol. 35, no. 2, pp. 141-146.

Blumental, S, Granger-Farbos, A, Moïsi, J, Soullié, B, Leroy, P, Njanpop-Lafourcade, BM, Yaro, S, Nacro, B, Hallin, M & Koeck, JL 2015, 'Virulence factors of *Streptococcus pneumonia*. Comparison between African and French invasive implication for future vaccines', PLoS One, vol. 10, no. 7, e0133885.

Chao, Y, Marks, LR, Pettigrew, MM & Hakansson, AP 2014, '*Streptococcus pneumoniae* biofilm formation and dispersion during colonization and disease', Frontiers in Cellular and Infection Microbiology, vol. 4, p. 194. doi: 10.3389/fcimb.2014.00194.

Deev, D, Rybkin, I, Rijavec, T & Lapanje, A 2021, 'When beneficial biofilm on materials is needed: electrostatic attachment of living bacterial cells induces biofilm formation', Frontiers in Materials, vol. 8, p. 624631.

Domenech, M, García, E & Moscoso, M 2011, 'Biofilm formation in *Streptococcus pneumonia'*, Microbial Biotechnology, vol. 5, no. 4, pp. 455-465.

Drago, L, Cappelletti, L, Vecchi, ED, Pignataro, L, Torretta, S & Mattina, R 2014, 'Antiadhesive and antibiofilm activity of hyaluronic acid against bacteria responsible for respiratory tract infections', APMIS, vol. 122, no. 10, pp. 1013-1019.

Fleming, D & Rumbaugh, K 2017, 'Approaches to dispersing medical biofilms', Microorganisms, vol. 5, no. 2, p. 15. doi: 10.3390/microorganisms5020015.

Hart, M, Tsang, L, Deck, J, Daily, S, Jones, R, Liu, H, Hu, H, Hart, MJ & Smeltzer, MS 2013, 'Hyaluronidase expression and biofilm involvement in *Staphylococcus aureus* UAMS-1 and its *sarA*, *agr* and *sarA agr* regulatory mutants', Microbiology, vol. 159, pp. 782-791.

Henriques-Normark, B & Tuomanen, E 2013, 'The pneumococcus: epidemiology, microbiology, and pathogenesis', Cold Spring Harbor Perspective in Medicine, vol. 3, no. 7, a010215. doi: 10.1101/cshperspect.a010215.

Ibberson, C, Parlet, C, Kwiecinski, J, Crosby, H, Meyerholz, D & Horswill, A 2016, 'Hyaluronan modulation impacts *Staphylococcus aureus* biofilm infection', Infection and Immunity, vol. 84, no. 6, pp. 1917-1929.

Liu, L, Liu, Y, Li, J, Du, G & Chen, J 2011, Microbial production of hyaluronic acid: current state, challenges, and perspectives', Microbial Cell Factories, vol. 10, no. 1, p. 99.

Marion, C, Stewart, J, Tazi, M, Burnaugh, A, Linke, C, Woodiga, S & King, S 2012, '*Streptococcus pneumoniae* can utilize multiple sources of hyaluronic acid for growth', Infection and Immunity, vol. 80, no. 4, pp. 1390-1398.

Moscoso, M, Garcia, E & Lopez, R 2009, 'Pneumococcal biofilms', International Microbiology, vol. 12, pp. 77-85.

Nyenje, ME, Green, E & Ndip, RN 2013, 'Evaluation of the effect of different growth media and temperature on the suitability of biofilm formation by *Enterobacter cloacae* strains isolated from food samples in South Africa', Molecules, vol. 18, no. 8, pp. 9582-9593.

Oggioni, MR, Trappetti, C, Kadioglu, A, Cassone, M, Iannelli, F, Ricci, S, Andrew, PW & Pozzi, G 2006, 'Switch from planktonic to sessile life: a major event in pneumococcal

pathogenesis', Molecular Microbiology, vol. 61, no. 5, pp. 1196-1210.

Pecharki, D, Petersen, F & Scheie, A 2008, 'Role of hyaluronidase in *Streptococcus intermedius* biofilm', Microbiology, vol. 154, no. 3, pp. 932-938.

Petruzzi, B, Briggs, RE, Swords, WW, De Castro, C, Molinaro A & Inzana, TJ 2017, 'Capsular polysaccharide interferes with biofilm formation by *Pasteurella multocida* serogroup A', mBio: e01843-17.

Preda, VG & Sandulescu, O 2019, 'Communication is the key: biofilms, quorum sensing, formation and prevention, Discoveries, vol. 7, no. 3, e100. doi: 10.15190/d.2019.13

Speziale, P & Geoghegan, JA 2015, 'Biofilm formation by staphylococci and streptococci: structural, functional, and regulatory aspects and implications for pathogenesis', Frontiers in Cellular and Infection Microbiology, vol. 5, no. 31. doi: 10.3389/fcimb.2015.00031.

Starr, CR & Engleberg, NC 2006, 'Role of hyaluronidase in subcutaneous spread and growth of Group A streptococcus', Infection and Immunity, vol. 74, no. 1, pp. 40-48.

Stepanovic, S, Vukovic, D, Dakic, I, Savic, B & Svabic-Vlahovic, M 2000, 'A modified microtiter-plate test for quantification of staphylococcal biofilm formation', Journal of Microbiological Methods, vol. 40, no. 2, pp. 175-179.

Todar, K 2012, Mechanisms of bacterial pathogenicity, Retrieved from http://textbookofbacteriology.net/pathogenesis_4.html

Yadav, M, Chae, S, Park, K & Song, J 2013, 'Hyaluronic acid derived from other streptococci supports *Streptococcus pneumoniae* in vitro biofilm formation', BioMed Research International, 2013, pp. 1-7.

Yusof, HA 2015, 'Molecular characterization and enzymatic activity of hyaluronate lyase among *Streptococcus pneumoniae* Malaysian isolates', PhD thesis, Universiti Putra Malaysia, Malaysia.

Yusof, HA, Desa, MNM, Masri, SN, Malina, O & Jamal, F 2015, 'Hyaluronatelyase production by *Streptococcus pneumoniae* isolated from patients and carriers', Tropical Biomedicine, vol. 32, no. 3, pp. 413-418.