ORIGINAL ARTICLE

In Vitro Assessment of Biofilm Formation by *Streptococcus pyogenes* Isolates From Invasive and Non-invasive Samples With Diverse *emm* Type Profiles

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ABSTRACT

Introduction: Biofilm is one of the important virulence factors that is responsible for the severity and progression of the *Streptococcus pyogenes* diseases. M-protein is involved in the irreversible attachment of *S. pyogenes* to surfaces during biofilm development. This study aims to determine the propensity of *S. pyogenes* to form biofilms and the molecular epidemiology of *S. pyogenes* isolates by *emm* typing. **Methods:** We screened 45 *S. pyogenes* isolates for the biofilm formation by Congo red agar (CRA) and quantified the biofilms by crystal violet microtiter-plate methods (CVMtP). The *emm* typing of all isolates was performed by conventional PCR with established primers according to the CDC protocol. **Results:** Majorities of *S. pyogenes* were isolated from non-invasive, 27 (60.0%) than invasive sources, 18 (40.0%). Regardless of invasiveness, 40 (88.9%) *S. pyogenes* isolates formed black colonies on CRA, while 43 (95.6%) of the isolates demonstrated various degrees of biofilm formation by CVMtP method. A total of 30 different *emm* types and subtypes were identified. No new *emm* types/subtypes were detected. The predominant *emm* types/subtypes were *emm*1, *emm*63, *emm*18.21, *emm*91, and *emm*97.4 which each gene accounted for 7.0%. All *emm* types/subtypes of *S. pyogenes* produced biofilms by CVMtP method except *emm*17.2 and *emm*57 which were isolated from non-invasive sources. **Conclusions:** Biofilm-producing *S. pyogenes* strains of various sources are genetically diverse and biofilm phenotypes are inherent to individual characteristic rather than specific *emm* type. Nonetheless, higher propensity of GAS to form biofilms warrants better management strategies to avoid treatment failures in the future.

Keywords: *Streptococcus pyogenes*, *emm* type, biofilm

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INTRODUCTION

Streptococcus pyogenes is one of the notorious pathogens affecting millions of people with significant morbidity and mortality every year. *S. pyogenes* diseases manifest in a wide variety of clinical presentations ranging from mild asymptomatic cases to suppurative and non- suppurative diseases such as acute pharyngitis, impetigo, necrotizing fasciitis, acute post- streptococcal glomerulonephritis and rheumatic heart disease (1,2).

Recently, an increased incidence of invasive Group A Streptococci (iGAS) among the general population in the United States and Canada was reported in 2017 and 2018, with the rates of 7–10 per 100,000 population (3, 4, 5). In addition, the number of laboratory-confirmed iGAS infections were also increased 1.5 times compared to the previous years (6). The reasons for the upsurge remain unexplained. However, expansion of specific *emm* types of GAS was postulated and it could be related to an increased virulence potential of those strains (5). Among these virulence properties is the ability of *S. pyogenes* strains to produce biofilms. Biofilms are responsible for a variety of GAS diseases and have a significant role in its pathogenesis and treatment failures (7).

Biofilm consists of polysaccharides, extrapolymeric components, nucleic acids and proteins, which is formed by a sessile community of microorganisms (8). The production of biofilms in GAS has been reported in *in-vitro* and *in-vivo* studies (9, 10, 11). In addition, several GAS *emm* types exhibit different degrees of biofilm formation in many studies (12, 13, 14), while a few studies have not (10, 15). In view of the scarcity of data on biofilm formation in GAS isolates in Malaysia, the present study aimed to investigate the propensity of GAS isolates to produce biofilms and their molecular epidemiology by *emm* typing method.

MATERIALS AND METHODS

Streptococcus pyogenes isolates

Forty-five non-duplicative *Streptococcus pyogenes* isolates were retrieved from our previous culture collection collected from Hospital Kuala Lumpur and Hospital Serdang from 2013 to 2015 (16). The isolates were stored at -70°C and gradually thawed for the subsequent procedures in the present study. The sources of the isolates were documented as follows: pus (n = 24), blood (n = 11), tissue (n= 7), wound (n= 2) and throat swab (n= 1) as shown on Table I. The isolates were grouped into invasive and non-invasive categories according to the previous study (16). The isolates were re- identified by using Gram staining technique, biochemical profiles and 16S rRNA gene sequencing (17). The ethical approval was obtained from the Ethics Committee for Research involving Human Subjects of Universiti Putra Malaysia (UPM/TNCPI/RMC/1.4.18.1).

Table I: The distribution of isolates according to the source of the clinical samples and its invasiveness

Sources	Invasiveness	No. of isolates	Percentage (%)
Pus	Non-invasive	24	53.3%
Wound	Non-invasive	2	4.4%
Throat	Non-invasive	1	2.2%
Tissue	Invasive	7	15.6%
Blood	Invasive	11	24.4%

Congo Red Agar Method

To screen for the biofilm formation, the Congo Red Agar (CRA) medium was prepared with brain heart infusion broth (Difco, USA) 52 g/L supplemented with 5% (w/v) sucrose and 0.08% (w/v) Congo red dye (Sigma Aldrich, Germany) according to an established protocol described in a previous study (18). *S. pyogenes* isolates were inoculated onto the CRA plates and incubated at 37 °C for 24 h under 5% CO2 (19). The production of biofilms was determined by the formation of black colonies with rough surface and edges as strong biofilm producers, while slightly black colonies with smooth surface and edges were considered intermediate-biofilm producers. Whereas pink colonies were considered non-biofilm producers (20). *S. epidermidis* ATCC 35984 strain (slime producer) was used as a positive control, whereas *S. epidermidis* ATCC 12228 strain (non-slime producer) was used for a negative control. The screening test was performed three times whereby each experiment was conducted in triplicate.

Crystal Violet Microtiter-Plate Method

To quantify the biofilm formation, crystal violet microtiter-plate methods (CVMtP) was performed with slight modifications according to the established protocol (21). Briefly, overnight bacterial cultures were diluted 1:100 with fresh brain heart infusion broth (Difco, USA). Then, aliquots of diluted bacterial suspension (200 µL) were transferred to each well of the 96-well microtiter plate and incubated for 48 h at 37°C. Following the incubation, the 96-well microtiter plate was carefully washed with Phosphate Buffer Saline (PBS) three times to eradicate the free-floating bacteria and heat-dried for 1 h at 60°C. Then, each well was stained with 150 µL of 0.1% crystal violet for 15 min. The dye in each well was washed and rinsed three times with deionized water. Lastly, the dye was solubilized with 150 µL of 95% ethanol and the absorbance was measured at 550 nm using an ELISA microplate reader (Dynex Technologies MRX II, USA). The experiment was repeated three times. *S. epidermidis* ATCC 12228 strain (non-biofilm producer) and *S. epidermidis* ATCC 35984 strain (biofilm producer) were used as negative and positive controls, respectively. The degrees for biofilm formation were calculated based on the formula according to the previous protocol (21) as follows:
non-adherent isolates = OD ≤ ODc, weakly adherent isolates = ODc < OD ≤ (2 x ODc), moderately adherent isolates = (2 x ODc) < OD ≤ (4 x ODc) and strongly adherent isolates = OD > (4 x ODc).

Establishment of *emm* typing

All GAS was subjected to *emm* typing protocol described in a previous study (16). Conventional PCR method was used for *emm* typing according to the Centers for Disease Control and Prevention (CDC) (22). One loopful of bacteria was collected from a 24-hour culture and was suspended in 300 µL of 0.85% NaCl. Then, the bacterial suspension was heated at 70°C for 15 minutes. Later, it was centrifuged at 13,000 rpm using Centrifuge 5425 (Eppendorf, USA) for three minutes and resuspended in 50 µL TE buffer (10 mM Tris, 1 mM EDTA, pH 8). Two µL of hyaluronidase (30 mg mL-1) and 10 µL of mutanolysin (3000 U mL-1) were added. The mixture was incubated for 30 minutes at 37°C. After that, it was heated at 100°C for 10 minutes to deactivate the enzymes. Finally, it was centrifuged and the supernatant was used as a template for PCR.

Briefly, bacterial DNA of *S. pyogenes* isolates were prepared and *emm* genes were amplified using a Bio-Rad MyCycler Thermal Cycler (Bio-Rad Laboratories Ltd., Watford, Hertfordshire, UK). *emm* genes were detected by using the forward (5'-TATT(CG)GCTTAGAAAATTAA-3') and reverse (5'-GCAAGTTCTTCAGCTTGTTT-3')

primers (16). The PCR amplification cycle is as follows: 94°C for 15 s, 46°C for 30 s, and 72°C for 75 s for the first 10 cycles, and later 94 oC for 15 s, 46°C for 30 s, 72°C for 75 s (with a 10 s increment for each of the subsequent 19 cycles). The procedure was repeated for 20 cycles and the forward primer was used as a sequence reaction for sequencing. The translated DNA sequences result of 5′ region of *emm* gene was aligned and compared with the sequence from the Blast-*emm* database, Centre for Disease Control and Prevention, Atlanta, GA, USA (3) and edited accordingly using Bioedit software version 7.0. The *emm* type and subtype of GAS were determined prior to the 90 bases encoding the N terminal 30 residues of the processed M protein and the exact 150 base sequences encoding the N terminal 50 residues of the mature M protein, respectively. Thirty bases that encode for the last 10 residues of the M protein signal sequence were used to identify the start sequence encoding the mature M protein. The sequence is considered to have *emm* gene allele (or sequence type) if it has greater than or equal to 95 % identity over at least the first 240 bases with the corresponding *emm* gene in the CDC database (23).

Statistical analysis

The correlation between the types of invasiveness with biofilm formation were analyzed using Fisher's Exact test. *P*-value less than 0.05 was considered as significant. All statistical tests were performed by using SPSS 22.0.

RESULTS

Biofilm formation by Congo red agar, crystal violet microtiter-plate methods and invasiveness

Of 45 *S. pyogenes* isolates, 27 (60.0%) and 18 (40.0%) were from non-invasive and invasive samples, respectively. A total of 40 (88.9%) isolates demonstrated the production of biofilms by CRA method. Among these biofilm producers, 25 (62.5%) and 15 (37.5%) of them were obtained from non-invasive and invasive samples, respectively. With regard to the biofilm formation categories, a total of 10 (22.2%) and 30 (66.7%) *S. pyogenes* isolates exhibited strong and intermediate-biofilm producers, respectively. Only five (11.1%) isolates were non-biofilm producers. Figure 1 shows the representative *S. pyogenes* isolates on Congo red agar plates with positive and negative controls in the present study.

The capability of *S. pyogenes* to produce biofilm was evaluated by using a CVMtP. Table II shows the classification of biofilm production based on optical density (OD) values from crystal violet assay for 48 hours at OD550 nm (Dynex MRX Revelation and Revelation TC 96 Well Microplate Reader, USA). The value of the absorbance indicates the degree of thickness of the biofilm produced. The degree of absorbance was classified into four different values based on the optical density cut-off value (ODc). The majority of *S. pyogenes* isolates as much as 43 (95.56%) produced biofilms and most of the isolates, 23 (51.1%) were categorized as moderately adherent. Seventeen isolates (37.78%) exhibited weakly adherent and only three (6.67%) had strong adherent biofilm formation. Two (4.44%) isolates did not produce biofilms.

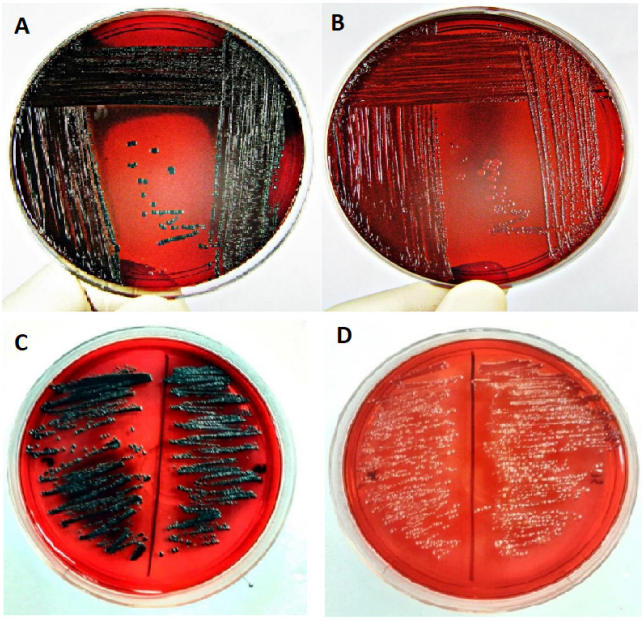

Figure 1: The morphology of the colonies of selected GAS isolates on Congo red agar (CRA) with different degrees of biofilm production based on the colour change. (A) black colonies of GAS with rough surface and edges indicate strong biofilm producers; (B) slightly black colonies with smooth surface and edges indicate intermediate-biofilm producers; (C) A positive control of *S. epidermis* ATCC 35984 strain (biofilm producer); (D) A negative control of *S. epidermidis* ATCC 12228 strain (pink colonies as non-biofilm producers).

Table II: Classification of biofilm production based on OD values from crystal violet assay for 48 hours at OD550 nm using ELISA auto reader

Biofilm formation	OD value	Number of isolates
Non-adherent	OD ≤ 0.14002	2 (4.44%)
Weakly adherent	0.14002 < OD ≤ 0.28004	17 (37.78%)
Moderately adherent	0.28004 < OD ≤ 0.56008	23 (51.11%)
Strongly adherent	0.56008 < OD	3 (6.67%)

Figure 2 shows the distribution of biofilm-producing *S. pyogenes* strains according to the source of samples. A total of 43 (95.3%) *S. pyogenes* isolates exhibited biofilm producers by CVMtP method. Interestingly, 18 (100%) of *S. pyogenes* isolates from all invasive sources exhibited biofilm producers compared to that of 25 (92.6%) non-invasive sources. With regard to the quantitation of biofilms, approximately half of GAS isolates 23 (51.1%) demonstrated moderate adherence. This is followed by 7 (37.8%) and 3 (6.7%) of the isolates were weakly and strongly adherent. Only 2 (4.4%) were categorized as non-adherent.

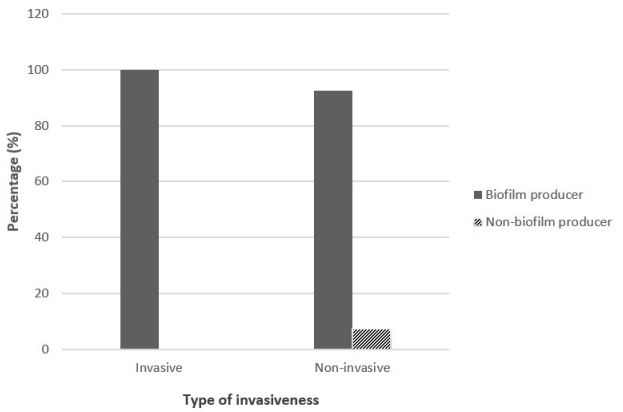

Figure 2: Distribution of biofilm-producing *S. pyogenes* strains according to source of sample

Biofilm formation and *emm* types

emm typing method revealed 30 different *emm* types and subtypes. A total of 28 *emm* types/subtypes exhibited biofilm-producing *S. pyogenes* strains which were represented by 43 (95.6%) of all isolates. Only two *emm* types (*emm*17.2 and *emm*57) exhibited non-biofilm producing strains (4.4%). There were no new *emm* types and subtypes detected. Table III shows the distribution of *emm* types and subtypes of *S. pyogenes* isolates in relation to biofilm formation.

Table III: The relationship between the detection methods of biofilm production and invasiveness

Detection method of biofilm production		Invasiveness, n (%)		*P* value*
		Invasive	Non-invasive	
Congo Red Agar	Positive and intermediate	15 (33.3)	25 (55.6)	0.375
	Negative	3 (43.5)	2 (4.3)	
Crystal violet microtiter-plate	Adherent	18 (40.0)	25 (55.6)	0.509
	Non adherent	0 (0.0)	2 (4.3)	

*Fisher's Exact Test
(*p* values < 0.05 were considered significant)

DISCUSSION

Streptococcus pyogenes is known as a "flesh-eating" bacterium for its ability to cause necrotizing soft tissue and fascia infections. Therapeutic failure in managing this invasive group A streptococcal (iGAS) disease is mainly due to in situ formation of *S. pyogenes* biofilms (13). The formation of biofilms among *S. pyogenes* isolates is associated with the dominancy of specific serotypes and strains (9, 24). However, there is a scarcity of data regarding the ability of GAS to form biofilms and their molecular epidemiology in Malaysia.

In the present study, high percentages of biofilms were detected among *S. pyogenes* isolates by both CRA (88.9%) and CVMtP (95.3%) methods. Higher yield of GAS biofilm formation via CVMtP method in the present study is not surprising as it has higher sensitivity rates than CRA as documented by other studies in Gram-positive bacteria (25, 26). For example, among 94 *Staphylococcus aureus* isolates that were screened for biofilms *in-vitro*, MtP yielded higher sensitivity rate compared to CVA (100% versus 89%) (25). In another study by Jain and colleagues (26), the sensitivity rate of MtP was higher than CVA (69.1% versus 65.5%) in 100 invasive isolates of *S. aureus*. CVMtP method utilizes spectrophotometric assessments and the degree of bacterial adherent to the inner surface of the wells can be semi-quantitatively measured (21).

To the best of our knowledge, there is no data available for us to compare GAS biofilm formation via CRA method. Nonetheless, CRA method has been commonly used to screen biofilm formation among many Gram-positive and negative bacteria for its rapidity and hassle-free procedure in most laboratories (27). Moreover, the colonies are viable for additional tests to be carried out (28). Nonetheless, CRA is not a quantitative assay and has less accuracy as it only relies on the chromatic assessment for the color change (27). In the present study, three *S. pyogenes* strains did not demonstrate biofilm formation via CRA method but exhibited moderate and weak-adherent categories via CVMtP method as shown in Table IV. Similar observations were also reported in other Gram-positive bacteria (25, 26). For example, biofilm formation was not observed in 13 *S. aureus* isolates by CRA method, however exhibited weak and strong adherent categories via CVMtP method (25). In addition, nine *S. aureus* isolates were grouped as non-biofilm producers by CVA methods but exhibited various degrees of adherent by CVMtP method (26).

With regard to CVMtP method, 75.9% and 89.5% of 29 and 172 GAS isolates, respectively demonstrated the ability to develop biofilms in studies from Jordan and Germany (29, 30). In another study in Italy, 90% of 289 GAS isolates demonstrated biofilm formation of different categories (9). Interestingly, most of the GAS isolates in previous studies exhibited weak to moderate adherence properties to the microtiter plate regardless of the type of sources (9, 29). In addition, the incubation periods of 72 hours until Day 6 are reported to be crucial for the development of strong biofilm formation in *S. pyogenes* according to Al-kafaween and colleagues (31). In the present study, the microtiter plates were only incubated up to 48 hours, which could possibly explain that most of the GAS (88.9%) exhibited moderate to weak adherence. Nonetheless, the first step of biofilm formation on biotic and abiotic surfaces by bacteria is adherence, which requires the bacteria to undergo an intricate process of switching from planktonic bacterial cells to biofilm phenotypes (32). This dynamic and complex process is influenced by several environmental factors including pH, temperatures, nutrients and others (33). The properties of microtiter plate such as the materials used to produce the plates and its chemical components may also influence the initial step of adherence by the bacteria (34).

In the present study, 100% of invasive GAS strains produced biofilms compared to 92.6% of invasive strains. In contrast, 77.8% (56/72) and 98% (98/100) of GAS from invasive and non- invasive sources produced biofilms in a study in Germany (29). In another study, almost 90% of 289 GAS isolates from invasive and non-invasive samples produced biofilms (9). Nonetheless, non-invasive strains were reported to be better biofilm formers compared to invasive strains (9, 29). It has been postulated that the ability of non-invasive strains to produce biofilms is part of protective mechanism for their survival to escape host immune response and antimicrobial treatment (9, 35). However, based on the statistical data in Table V shows that invasiveness does not have a strong correlation with biofilm production.

In biofilm formation, several virulence factors are involved. The M protein, which is highly expressed on the surface of all *S. pyogenes* isolates, has been shown to play an important role during the initial attachment of bacteria to epithelial cells. The 5′ end of the hypervariable region of M protein is encoded by the *emm* gene. Sequence analysis of *emm* genes (*emm* typing) has helped scientists to characterize GAS isolates according to their *emm* types. Interestingly, some GAS serotypes according to M-typing such as M2, 6, 14 and 18 were associated with the ability to produce biofilms compared to other serotypes (13). M-typing is an old method of GAS serotyping method, and because of the difficulty in obtaining the specific antisera, it has been replaced by *emm* typing method (36). In the present study, there was no dominancy of a single *emm* type among biofilm-producing GAS strains. Several *emm* types (*emm*63, *emm*18.21, *emm*1, *emm*91 and *emm*97.4) were predominantly involved in biofilm formation among our GAS isolates. In Italy, GAS strains of *emm*6 type demonstrated a greater preponderance for biofilm production than other *emm* types (9). Similarly, GAS strains with *emm*6 type exhibited more biofilm formation than others as well (14). However, a study has concluded that adherence and biofilm formation are not related to specific *emm* types but rather the individual traits of *S. pyogenes* strains (29).

Our study has several limitations. Although both in vitro methods used in the present study are cost effective and relatively easy to perform, they could not mimic the formation of GAS biofilms by in vivo settings. It would be interesting to conduct the antibiotic susceptibility testing and investigate associated virulence factors as biofilm-forming GAS strains would have some degree of resistance to common antibiotics and potential virulence determinants could be up/down regulated during biofilm formation. Lastly, the number of samples is relatively small for us to evaluate the performance parameters of these two methods such as their specificity, sensitivity and others.

CONCLUSION

Despite lack of involvement of *Streptococcus pyogenes* isolates in device-related infections, these isolates demonstrate remarkably high propensity of biofilm formation by Congo red agar and crystal violet microtiter-plate methods. Characterization of these biofilm-producing strains and evaluation on their interactions with other virulence factors may improve a greater understanding of its role in *S. pyogenes* pathogenesis. In turn, findings from such research works will provide potential platforms for better management strategies and treatment options in the future.

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REFERENCES

1. Bisno AL, Brito MO, Collins CM. Molecular basis of group A streptococcal virulence. The Lancet Infectious Diseases. 2003;3(4):191–200. PubMed PMID: 12679262.
2. Cunningham MW. Pathogenesis of group A streptococcal infections. Clinical Microbiology Reviews. 2000;13(3):470–511. PubMed PMID: 10885988.
3. Centres for Disease Control and Prevention. Active Bacterial Core Surveillance Report, Emerging Infections Program Network, Group A Streptococcus. Atlanta, US. 2020. Available from: https://www2.cdc.gov/vaccines/biotech/strepblast.asp;
4. Public Health England. Group A streptococcal infections: third report on seasonal activity in England, 2017/18. Health Protect Rep. 2018;12(13):5–7.
5. Tyrrell GJ, Fathima S, Kakulphimp J, Bell C. Increasing rates of invasive group a streptococcal disease in Alberta, Canada; 2003-2017. Open Forum Infect Dis. 2018;5(8):177.
6. Public Health England. Group A streptococcal infections: fourth update on seasonal activity, 2015/2016. May 6, 2016. https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/521550/hpr1616_SF-GAS2. pdf (accessed April 17, 2019).
7. Siemens N, Chakrakodi B, Shambat SM, et al. Biofilm in group A streptococcal necrotizing soft tissue infections. JCI Insight. 2016; 1(10) e87882. http://dx.doi.org/10.1172/jci.insight.87882.

8. Costerton JW. Cystic fibrosis pathogenesis and the role of biofilms in persistent infection. Trends Microbiol. 2001;9: 50–52.
9. Baldassarri L, Creti R, Recchia S, Imperi M, Facinelli B, Giovanetti E, et al. Therapeutic failures of antibiotics used to treat macrolide-susceptible *Streptococcus pyogenes* infections may be due to biofilm formation. J Clin Microbiol. 2006;44: 2721– 2727.
10. Manetti AG, Zingaretti C, Falugi F, Capo S, Bombaci M, Bagnoli F, et al. *Streptococcus pyogenes* pili promote pharyngeal cell adhesion and biofilm formation. Mol Microbiol. 2007;64: 968–983.
11. Riani C, Standar K, Srimuang S, Lembke C, Kreikemeyer B, Podbielski A. Transcriptome analyses extend understanding of *Streptococcus pyogenes* regulatory mechanisms and behavior toward immunomodulatory substances. Int J Med Microbiol. 2007;297: 513–523.
12. Conley J, Olson ME, Cook LS, Ceri H, Phan V, Davies HD. Biofilm formation by group A streptococci: is there a relationship with treatment failure? J Clin Microbiol. 2003; 41(9): 4043-8. http://dx.doi.org/10.1128/JCM.41.9.4043-4048.2003.
13. Lembke C, Podbielski A, Hidalgo-Grass C, Jonas L, Hanski E, Kreikemeyer B. Characterization of biofilm formation by clinically relevant serotypes of group A streptococci. Appl Environ Microbiol. 2006; 72(4): 2864-75. [http://dx.doi.org/10.1128/AEM.72.4. 2864-2875.2006] [PMID: 16597993].
14. Ogawa T, Terao Y, Okuni H, et al. Biofilm formation or internalization into epithelial cells enable *Streptococcus pyogenes* to evade antibiotic eradication in patients with pharyngitis. Microb Pathog. 2011; 51(1-2): 58-68. http://dx.doi.org/10.1016/j.micpath. 2011.03.009.
15. Thenmozhi R, Balaji K, Kumar R, Rao TS, Pandian SK. Characterization of biofilms in different clinical M serotypes of *Streptococcus pyogenes*. J Basic Microbiol. 2011;51:196–204.
16. Kalgo HM, Jasni AS, Hadi SRA, Umar NH, Hamzah SNA, Hamat RA. Extremely low prevalence of erythromycin-resistant *Streptococcus pyogenes* isolates and their molecular characteristics by M protein gene and multilocus sequence typing methods. Jundishapur J Microbiol. 2018;115.
17. Creti R, Gherardi G, Imperi M, Huolstein CV, Baldassarri L, Pataracchia M, et al. Association of group A streptococcal *emm* types with virulence traits and macrolide- resistance genes is independent of the source of isolation. J Med Microbiol. 2005;54:10:913-917.
18. Mathur T, Singhal S, Khan S, Upadhyay DJ, Fatma T, Rattan A. Detection of biofilm formation among isolates of staphylococci: an evaluation of three different screening methods. Indian J Med Microbiol. 2006;24(1):25–9.
19. Cotter JJ, O'gara JP, Mack D, Casey E. Oxygen-mediated regulation of biofilm development is controlled by the alternative sigma factor σβ in *Staphylococcus epidermidis*. Appl Environ Microbiol, 2009;75(1):261–4.
20. Freeman DJ, Falkiner FR, Keane CT. New method for detecting slime production by coagulase negative staphylococci. Journal of clinical pathology. 1989;42(8): 872-4.
21. Stepanovic S, Vukovic D, Daki I, Savic B, Vlahovic-Svabic M. A modified microtiter- plate test for quantification of staphylococcal biofilm formation. J Microbiol Methods. 2000;40:175-179.
22. Centres for Disease Control and Prevention. Active Bacterial Core Surveillance Report, Emerging Infections Program Network, Group A Streptococcus. Atlanta, US. 2020. Available from: http://www.cdc.gov/ncidod/biotech/strep/protocols.htm.
23. Facklam R, Beall B, Efstratiou A, Fischetti V, Johnson D, Kaplan E, et al. *emm* typing and validation of provisional M types for group A streptococci. Emerging Infectious Diseases. 1999;5(2):247–253.
24. Maddocks SE, Wright CJ, Nobbs AH, Brittan JL, Franklin L, Strumberg N, Kadioglu A, Jepson MA, Jenkinson HF. *Streptococcus pyogenes* antigen I/II-family polypeptide AspA shows differential ligand-binding properties and mediates biofilm formation. Mol Microbiol. 2011;81(4):1034-49. doi: 10.1111/j.1365-2958.2011.07749.x. Epub 2011 Jul 15. PMID: 21736640; PMCID: PMC3178794.
25. Melo PC, Ferreira LM, Nader Filho A, Zafalon LF, Vicente HIS, Souza V. Comparison of methods for the detection of biofilm formation by *Staphylococcus aureus* isolated from bovine subclinical mastitis. Brazilian Journal of Microbiology. 2013;44: 119-124. doi:10.1590/S1517-83822013005000031.
26. Jain A, Agarwal A. Biofilm production, a marker of pathogenic potential of colonizing and commensal staphylococci. J Microbiol Methods. 2009;76:88-92.
27. Hassan A, Usman J, Kaleem F, Omair M, Khalid A, Iqbal M. Evaluation of different detection methods of biofilm formation in the clinical isolates. Braz J Infect Dis. 2011;15(4):305-311.
28. Ruchi T, Sujata B, Anuradha D. Comparison of Phenotypic Methods for the Detection of Biofilm Production in Uro-Pathogens in a Tertiary Care Hospital in India. Int J Curr Microbiol App Sci. 2015;4: 840-849.
29. Aleksandra , Ina G, LJiljana B, Lazar R. Adherence and Biofilm Production of *Streptococcus pyogenes* [Internet]. IntechOpen Limited. 5 Princes Gate Court, London; 2016. Available from: https://www.intechopen.com/books/microbial-biofilms-importance-and-applications/adherence-and-biofilm-production-of-streptococcus- pyogenes.
30. Ismael NF. "Vinegar" as anti-bacterial biofilm formed by *Streptococcus pyogenes* isolated from recurrent tonsillitis patients, in vitro. Jordan J Biol Sci. 2013;6(3):191– 197.

31. Al-kafaween MA, Mohd Hilmi AB, Jaffar N, et al. Determination of optimum incubation time for formation of *Pseudomonas aeruginosa* and *Streptococcus pyogenes* biofilms in microtiter plate. Bull Natl Res Cent. 2019;43: 100. https://doi.org/10.1186/s42269-019-0131-9.
32. Kostakioti M, Hadjifrangiskou M, Hultgren SJ. Bacterial biofilms: development, dispersal, and therapeutic strategies in the dawn of the postantibiotic era. Cold Spring Harb Perspect Med. 2013; 3(4)a010306 [http://dx.doi.org/10.1101/cshperspect.a010306] [PMID:23545571].
33. Donlan RM. Biofilms: microbial life on surfaces. Emerg Infect Dis. 2002; 8(9): 881- 90. [http://dx.doi.org/10.3201/eid0809.020063] [PMID: 12194761].
34. Lemos M, Borges A, Teodysio J, Araьjo P, Mergulhro F, Melo L, et al. The effects of ferulic and salicylic acids on *Bacillus cereus* and *Pseudomonas fluorescen*s single-and dual-species biofilms. Int Biodeterior Biodegradation. 2014;86:42–51.
35. Marks LR, Reddinger RM, Hakansson AP. Biofilm formation enhances fomite survival of *Streptococcus pneumoniae* and *Streptococcus pyogenes*. Infect Immun. 2014; 82(3): 1141-6. [http://dx.doi.org/10.1128/IAI.01310-13] [PMID: 24371220].
36. Beall B, Facklam R, Thompson T. Sequencing *emm*-specific PCR products for routine and accurate typing of group A streptococci. J Clin Microbiol. 1996;34(4):953-8. [PubMed: 8815115]. [PubMed Central: PMC228924].