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Chapter 4

Adherence and Biofilm Production of *Streptococcus pyogenes*

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Additional information is available at the end of the chapter

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Abstract

Streptococcus pyogenes (group A streptococcus – GAS) can cause numerous human infections, varying from mild skin infections to life-threatening, e.g. necrotizing fasciitis. Adherence and biofilm production are important in streptoccocal pathogenesis. GAS adhesins are numerous and diverse, with the ability to bind to several different receptors at the same time, which leads to difficulties in their precise identification and classification. Biofilm production is one of the most probable explanation for therapeutic failure in the treatment of GAS infections. Most researchers agreed that biofilm formation is a trait of individual strains rather than a general serotype attribute. The aim of our study is to investigate differences in adherence to laminin and biofilm production between invasive and non-invasive isolates (NI) of GAS. In this study the correlation between adherence to laminin and invasiveness in GAS isolates is noticed. The strains isolated from GAS carriers and highly invasive (HI) GAS strains have excellent capacity for binding to laminin. When testing biofilm production, there was noticeable positive correlation between adherence and biofilm production among non-invasive isolates. Non-invasive isolates were stable biofilm productors. There was no correlation between adherence and biofilm production among invasive isolates. Invasive isolates were also unstable biofilm productors.

Keywords: *Streptococcus pyogenes*, Invasiveness, Adherence, biofilm production, hydrophobicity

1. Introduction

Streptococcus pyogenes (group A streptococcus – GAS) is one of the most frequent exclusively human pathogen. When speaking about human infections and clinical conditions, there are only

few bacteria like GAS showing so much different faces and such a wide spectra of different virulence factors. Even systematization of group A streptococcal diseases was not specified for a long time. Classification of GAS diseases has recently been successful, clarified, and systematized. Diseases caused by *S. pyogenes* are divided into pyogenic (superficial and invasive) and toxemic, with autoimmune complication such as post-infectious sequelae (acute rheumatic fever and acute post-streptococcal glomerulonephritis) [1]. Streptococcal carriage is a special clinical condition, during which, this strictly human pathogen became a not precisely opportunistic bacteria, but normal flora-like bacteria.

S. pyogenes is a successful human pathogen and is a cause of so many diseases by virtue of its numerous virulence factors, which in this high numbers are possessed only by few bacteria like *Staphylococcus aureus* and *Clostridium perfringens*. Therefore, it is quite incomprehensible that the bacteria with such numerous and different virulence factors is not expected to be the cause of life-threatening human diseases. Although it is so much discovered and known about GAS pathogenesis, there is still unknown why this bacteria so rare and unexpectedly activates his most powerful virulence factors such as toxins and hydrolytic enzymes, which are most active in necrotizing fasciitis and streptococcal toxic shock syndrome (STSS).

On the other hand, such invasive bacteria could become normal flora-like bacteria during pharyngeal or nasal carriage. Streptococcal carriage has been defined as the recovery of GAS from the nasopharynx or oropharynx in the absence of any evidence of acute infection [2]. Streptococcal carriers should not be treated with antibiotics, except in the cases of reappearance of disease or possible occurence of post-streptococcal sequelae. Genesis of streptococcal carriage was for a long time poorly understood. Nowadays, there are two theories which explain streptococcal carriage as the consequence of therapeutic failure happened after infection of strains capable to produce biofilm [3] or internalize into epithelial cells [4].

Considering that *S. pyogenes* is one of the few bacteria still sensitive to penicillin *in vitro*, and due to the development of new antibiotics, it would be expected that the incidence of streptococcal infections should decrease over time. But, epidemiological data suggest that percentage of streptococcal carriage has remained unchanged, and also the fact that frequency of the invasive GAS diseases have become more frequent, led to the establishment Strep-EURO study group for monitoring streptococcal invasive disease in 11 European countries. All these information and facts have led to actualizations of GAS, revelation of new virulence factors which contribute to new perspectives in the understanding of group A streptococcal pathogenesis.

2. Adherence and biofilm production of *Streptococcus pyogenes*

2.1. New insight into old problem of group A streptococcal adherence

Although sometimes not sufficiently emphasized, efficient adherence is the prime step in the pathogenesis of infective disease. Factors that influence adherence are diverse and can

originate from environment such as the substrate on which the biofilm creates, initial bacterial layer coating the substrate, and characteristics of bacteria multiplying in this medium [5].

Adherence is complex process that includes several different steps. In the first step, bacteria have to overcome the repulsive forces which are consequence of negative charge of the bacterial superficial adhesins and substrate. Afterwards, in the second step positively attracting forces (such as covalent, ionic, van der Waals, hydrophobic) are established between bacterial adhesin and compatible receptor on the human cell. These attractive forces act on the small distance and only after bacterial surpassing of the repulsive electrostatic forces. Most of these interactions are low affinity bonds, but acting together they turn out to be strong and high affinity. Van der Waals forces play crucial role in protein-protein recognition, when complementary lock-and-key shapes are involved [6]. Hydrophobic side chains on the proteins could be connected to each other also using the low affinity hydrophobic forces. This is very plain and simple observation of adherence, and we should highlighted here that in the same or similar environmental conditions even closely related species in genus *Streptococcus* could demonstrate very diverse attractive forces [7].

Nowadays, there is proposition of two-step adherence of *S. pyogenes* [6]. In the first step, lipoteichoic acid (LTA) as amphipathic molecule, enables overcoming of the repulsive electrostatic forces between bacteria and substrate. In the second step, microbial surface components recognizing adhesive matrix molecules (MSCRAMMS), such as M protein, fibronectin binding protein (SfbI), serum opacity factor (SOF), etc. adhere to specific receptor on the human cell. Besides of the MSCRAMMS, it is demonstrated that this second step of adherence could be provided by bacterial pili. The initial attachment is very dynamic process in that they demonstrate on-off kinetic effect and includes several chemical molecular interactions, such as hydrophobic, ionic and electrostatic forces. Second step of adherence probably involves more specific, complex and irreversible interactions with higher affinity between one or several different MSCRAMMS and human cells. Group A streptococcal ability to bind our cells with several different MSCRAMMS at the same time make difficulties in identification of streptococcal adhesins.

Bacterial adherence to human cells could be on the direct or indirect way. Direct way of adherence is displayed by binding of bacterial adhesion to specific receptor on the cell surface; e.g. capsular hyaluronic acid interacts with CD44 receptor on the surface of keratinocytes and induces reorganization of cytoskeletal actin and rupture of intercellular bridges enabling bacteria to penetrate the epithelium still staying extracellular and reaching deeper into the tisssue [8]. The other, indirect way of adherence is more common. Streptococcal adhesins first bind to proteins of extracellular matrix (ECM) such as fibrinogen, fibronectin, laminin, collagen as bridging molecules which than attach to cell membranes integrins [9].

2.1.1. Fibronectin binding proteins

Fibronectin (Fn) is a high-molecular weight glycoprotein that circulates free as a dimer in the soluble form in blood plasma or as a fibrillar form is assembled by cells as major component of the ECM. So far, fibronectin binding proteins are the best studied adhesins of *S. pyogenes* and currently 11 different such adhesins have been identified [10], divided in two types. First

type proteins are SfbI, PrtF2, SOF, SfbX, Fbp54, FbaA, and FbaB and they all contain Fn-binding repeats. Second type proteins are M1, Shr, Scl1, and GAPDH and they do not contain these repeats. It's estimated that 60% of initial attachment to epithelial cells is realized by streptococcal lipoteichoic acid, but afterwards MSCRAMMS, e.g. fibronectin binding proteins are the most important in the irreversible stage of adherence. Binding of these adhesins to Fn could result in irreversible attachment to the cell or biofilm production in tissue or bacterial internalization. Fibronectin acts as a bridge molecule for binding to $\beta 5\alpha 1$ integrins, with subsequently rearrangement of cytoskeletal actin and uptake of the invading bacteria [11].

Expression of Fn-binding proteins is regulated as response to the environmental conditions in which streptococci survive and multiply. Protein F/SfbI, which allows binding to epithelial cells of the dermis and Langerhans cells, show increased expression on bacterial surface with increasing pressure of oxygen, e.g. on the cell surface, thereby enabling a better adherence of the bacteria. When oxygen level is decreased, e.g. in deep tissue, expression of this protein is also diminished, allowing bacterial dissemination into deeper tissues [12]. SfbI expression could be diminished also by catalytic cleavage with serine protease streptococcal pyrogenic exotoxin B (SpeB) or by other bacterial surface proteases where infection occurs. Protein F2, detected in most SfbI negative- GAS strains, binds fibronectin with high affinity and is homologous to Fn-binding proteins of group C streptococci. Similarly as in protein F1/SfbI, F2 activity is also response to the environmental oxygen pressure [13]. Unlike these two proteins, M protein expression is enhanced in the deeper tissues with increased pressure of carbon dioxide, preventing phagocytosis and contributing to the dissemination of GAS [14].

2.1.2. Anchorless adhesins

Anchorless adhesins are attached to bacterial cell surface in the unknown mode, probably through hydrophobic interactions. Importance of these proteins is in their ability to separate from the cell surface, get away from the cell, detects environmental signals around streptococci and the information transferred back to *S. pyogenes* [6]. The anchorless adhesins are not grouped toghether because they are functionally and structurally diverse. Most of them have enyzmatic functions. For example, five anchorless adhesins are enyzmes in glycolytic pathway and they tipically are located in bacterial citosol: glyceraldehyde-3-phosphate dehydrogenase (GAPDH), α-enolase, phosphoglycerate kinase, phosphoglycerate mutase, and triose phosphate isomerase [15]. GAPDH, also designed as SDH and Plr, could bind to several human proteins (plasmin/plasminogen, fibronectin, and fibrinogen), cytoskeletal actin and myosin, acting as an important colonization factor. These five anchorless adhesins operate together as a complex in generating ATP molecules. As anchorless adhesins, they could produce extracellular ATP, which is attached to $P2X_7$ receptors on epithelial and immune cells, inducing apoptosis of these cells. In this way, *S. pyogenes* establishes control on the behavior of human cells and facilitates further progression of the infection [16].

2.1.3. Laminin binding adhesins

Laminin is high-molecular weight ECM protein and one of the major components of the basal lamina, which is part of the basement membrane in human cells. Although laminin is widely

distributed in our body, only a few laminin binding proteins are identified in GAS so far. Currently, proteins nominated as streptococcal hemoprotein receptor (Shr), laminin binding protein (Lbp), and streptococcal pyrogenic exotoxin B (SpeB) are identified as laminin binding proteins for *S. pyogenes*.

SpeB is anchorless adhesin, with enzymatic function as cysteine protease. SpeB was first identified as exotoxin, but this protein can be attached to the bacterial surface as adhesin. SpeB is synthesized during early stationary phase in nutritious poor media [15]. This protein, like M protein, has multiple functions (adhesin, proinflammatory effect, and enzymatic function). Besides his function as laminin binding adhesin, this protein can bind to fibronectin and vitronectin, allowing streptococcal dissemination in deep tissue [17] and activates metalloproteases included in remodeling and degrading of ECM [15].

Shr is probably the protein attached to cell membrane, because it contains nor LPXTG either QVPTG repeats, that recognize housekeeping or accessory sortases, enzymes which incorporate proteins in the cell wall [18]. Its membrane position corresponds to the primary role of Shr protein in uptaking of the heme and binding to its transporter in the cytoplasmic membrane [19, 20]. In addition to its metabolic role, and by the virtue of surface position, it has been shown that this protein have the ability to bind laminin and fibronectin, participating in this way in adherence and acting as MSCRAMMS [21].

Lbp belongs to the group of metal-binding receptors with modified accessory proteins. Lbp scavenges environmental zinc and transports it to carriers of the cell membrane, to which it is attached [22]. Also, Lbp is laminin binding adhesin [23]. This protein is not identified in the oral streptococci, but is present in all surveyed so far M serotypes of GAS [23]. Lbp is very short, even shorter than the thickness of the cell wall, and because of its location in the cell membrane, it is likely to have greater importance in the metabolism of metal than in the adherence to laminin [22].

2.1.4. Our experience with group A streptococcal adherence

Considering that the adherence of streptococci is still insufficiently examined process and that the streptococcal adhesins are numerous and irregularly and inadequately identified, in our study isolates were divided in three groups according to invasiveness of the disease they caused. The aim of our study was to investigate differences in adherence to laminin between invasive and non-invasive isolates (NI) of GAS.

2.1.4.1. Material and methods

2.1.4.1.1. Bacterial strains

In total, 172 GAS isolates were included in the study. They were divided into three groups: (1) 100 non-invasive isolates (NI) obtained from GAS carriers; (2) 50 low invasive (LI) isolates obtained from patients with tonsillopharyngitis; and (3) 22 highly invasive (HI) recovered from blood of patients with sepsis and STSS. All the isolates are part of the national collection of GAS strains formed at the National Reference Laboratory for Streptococci, Institute of

Microbiology and Immunology, Faculty of Medicine, University of Belgrade. The NI and LI isolates were collected during 2012, while HI isolates had been collected over the last two decades.

2.1.4.1.2. Laminin coating of microtiter plates

We investigated adherence of GAS strains to uncoated and laminin-coated microtiter plates. Laminin coating of the polystyrene microtiter plates (Kartell, Italy) was performed by using 0.5 mg/ml laminin (Sigma Aldrich, USA), in accordance with the manufacturer's instructions. The plates were coated for 2 hours at 37°C with laminin previously diluted in Hanks balanced salt solution (Sigma Aldrich, USA) to achieve final concentration of 5 μg/ml, and afterwards were washed three times with Hanks balanced salt solution.

2.1.4.1.3. Capsule removal by hyaluronidase

Prior to adherence testing, all isolates were treated with bovine testicular hyaluronidase, type VI-S, (Sigma-Aldrich, USA) diluted in enzyme diluent (20 mM Sodium Phosphate, 77 mM Sodium Chloride, 0.01% Bovine Albumin, pH 7.0 at 37°C) in order to remove their capsules, as previously described [24].

2.1.4.1.4. Quantification of adherence to laminin by GAS strains

Quantification of adherence of GAS strains to uncoated and laminin-coated microtiter plates was based upon the protocol described by Stepanovic *et al.* [25]. The strains were incubated overnight in THY at 37°C, and then diluted in fresh THY medium to achieve final concentration of 10^6 CFU/ml. Aliquots of bacterial suspension (100 μL) were transferred to each well of the 96-well microtiter plate and incubated for 30 minutes at 37°C. The content of each well was then aspirated and wells were washed three times with sterile phosphate buffer solution (PBS). The plates were left overnight at room temperature for drying and air fixation. The plates were stained with 100 μL of 2% (w/v) crystal violet and, afterwards, the dye bound to the adherent cells was solubilized with 100 μL of 33% (v/v) glacial acetic acid. The negative control wells contained Todd Hewitt broth supplemented with 1% yeast extract (THY) broth only. *Staphylococcus epidermidis* ATCC 14990 was used as the positive control. The optical density (OD) of each well was measured at 570 nm using an automated microtiter plate reader. The cut-off optical density OD (ODc) was defined as three standard deviations above the mean OD of the negative control. Strains with OD above ODc were considered adherent to microtiter plates. Strains were classified as follows:

$OD \leq ODc$ = non-adherent isolates, $ODc < OD \leq (2 \times ODc)$ = weakly adherent isolates (+), $(2 \times ODc) < OD \leq (4 \times ODc)$ = moderately adherent isolates (++) and $OD > (4 \times ODc)$ = strongly adherent isolates (+++). All analyses were performed in triplicate and repeated at least two times.

2.1.4.1.5. Statistical analysis

Student's *t*-test was used to measure the differences in adherence to uncoated and laminin-coated plates as well as the differences in adherence to laminin before and after the penicillin and erythromycin treatment within each group of GAS strains tested. ANOVA was used to determine the differences in adherence to laminin among different groups of GAS strains. Data analyses were done with the SPSS version 20. The differences were considered significant if $p < 0.05$, and highly significant if $p < 0.01$.

2.1.4.2. Results and discussion

To determine correlation between invasiveness of tested GAS strains and their ability to bind to laminin, we investigated adherence of NI, LI, and HI isolates to uncoated and laminin-coated microtiter plates. All isolates were treated with hyaluronidase in order to eliminate the interference of hyaluronic acid capsule on adherence. The proportions of NI, LI, and HI isolates that displayed adherence to uncoated microtiter plates were 98%, 71%, and 91%, respectively. In all adherent isolates the level of adherence was estimated as weak, but adherence of NI and HI isolates to uncoated plates was significantly higher than adherence displayed by isolates of the LI group ($p \leq 0.001$). All isolates tested displayed adherence to laminin-coated microtiter plates (**Table 1**).

	No (%) of weakly adherent isolates (+)	**No (%) of moderately adherent isolates (++)**	**No (%) of strongly adherent isolates (+++)**
NI group (total 100 isolates)	13 (13)	40 (40)	47 (47)
LI group (total 50 isolates)	13 (26)	27 (54)	10 (20)
HI group (total 22 isolates)	0	5 (23)	17 (77)

Table 1. Adherence to laminin of invasive and non-invasive GAS isolates.

The overall results showed significantly higher adherence ($F = 6.952$, $p \leq 0.001$) of GAS strains tested to laminin-coated microtiter plates than adherence to uncoated plates. This was noted in all three groups of strains, and the ratios of adherence to laminin-coated vs. uncoated plates were as follows: 1.86 in HI group ($t = 15.603$, $p \leq 0.001$), 1.36 in NI ($t = 19.730$, $p \leq 0.001$) and 1.24 in LI group ($t = 13.355$, $p \leq 0.001$). However, the level of adherence was different in different groups of strains, as shown in **Table 1**. Majority (54%) of LI isolates were moderately adherent, while most of NI (47%) and, in particular, HI isolates (77%) were strongly adherent. To our knowledge, this is the first study evaluating differences in adherence to laminin between invasive and non-invasive isolates of GAS, and, thus, there are no other previously reported results for direct comparison. Musumeci *et al*. [26] showed that the proportion of *S. pyogenes* strains carrying the *prtF2* gene, encoding internalization-associated fibronectin binding protein F2, was significantly higher among asymptomatic carriers than among children with pharyngitis. This suggests significant contribution of this adhesion to the ability of *S. pyo-*

genes to persist in the throat of asymptomatic carriers. Similarly, we established high adherence ability in isolates obtained from GAS carriers. As far as highly invasive isolates are concerned, positive correlation between invasiveness and adherence to laminin found in our study was also shown for group B streptococcus (GBS) [27]. The surface laminin-binding protein (Lmb) was significantly more expressed in invasive GBS strains isolated from cerebrospinal fluid of the neonates with meningitis than in non-invasive strains isolated from feces or vaginal swabs of colonized asymptomatic pregnant women [27].

In conclusion, this study showed correlation between adherence to laminin and invasiveness in GAS isolates. The strains isolated from GAS carriers and highly invasive GAS strains have excellent capacity for binding to laminin.div4close

2.2. Biofilm

The greatest importance of the effective bacterial adherence is in the attachment to host cells and the aggregation of bacteria, which then create a signal for the biofilm production. In a collective way of existence bacteria gain a protective matrix layer, which in planktonic lifestyle does not exist and is responsible for the most of mechanisms that bacteria avoid eradication from the infection site.

According to literature, *S. pyogenes* is also capable for biofilm production. Researchers have noticed GAS microcolonies in skin lesions of patients with impetigo [28], and after that the same was observed in experimental zebrafish skin infection [29]. Also, three-dimensional communities resembling biofilm were detected in pediatric tonsillar samples after tonsillectomy of patients with adenotonsillar hypertrophy, contributing to the theory that biofilm formation is one of the probable explanations for the GAS persistence and carriage [30]. Besides these *in vivo* experiments, several authors have been proven *S. pyogenes* biofilm production also *in vitro* in static or flow conditions, e.g. in polystyrene microtiter plates, plastic coverslips or flow chambers [31, 32].

Biofilm production is one of the most probable explanation for therapeutic failure in the treatment of infections with this bacteria, *in vitro* sensitive to the tested antibiotic. Several authors have noticed better biofilm production in non-invasive streptococcal strains compared to invasive strains [33, 34], and also in erythromycin-sensitive isolates compared to erythromycin-resistant isolates of *S. pyogenes* [35]. These results strongly indicate that biofilm production is protective mechanism enabling bacterial survival of antibiotic treatment and immune system reaction. Also, collective lifestyle allows *S. pyogenes* easier horizontal gene transfer by transformation, generating virulent clones and biofilm phenotype [36].

Although Baldassari *et al*. [35] have proven biofilm production in 90% of tested invasive and non-invasive isolates, still most researchers agreed that biofilm formation is a trait of individual strains rather than a general serotype attribute. GAS biofilm production is complex process influenced not only by environmental conditions such as ECM proteins, incubation temperature and medium, but also by many bacterial virulence factors such as capsule, SpeB, M protein, pili, etc. Manetti *et al.* [37] assumed that on this multifactorial process also affect antigenic variation of M protein and pili, in addition to a variety known and poorly surveyed

virulence factors, thus further complicating the already insufficiently elucidated process of biofilm assembling. Doern *et al*. [38] suggested significance of in the timely manner production of several enzymes, such as SpeB, which in addition to his role in adhesion, is also one of the crucial factors in biofilm formation. SpeB expression is very high during both planktonic and biofilm lifestyle of GAS, that is almost a unique and uncommon phenomenon, because genetic expression is very different during this two distinct bacterial life stages. In planktonic lifestyle SpeB is highly synthesized in the early stationary phase. In the early phase of collective lifestyle, in order to begin with biofilm formation, it is necessary to prevent SpeB production. In the later stages of biofilm dispersion, production of SpeB is extensive, leading to protein degradation in the biofilm matrix. SpeB expression and disintegration of matrix protein are activation signal for other secreted proteases and nucleases, like Sda1, which degrade DNA and proteins in biofilm, contributing to biofilm dispersal and dissemination of bacteria throughout the body.

Fibronectin-collagen-T antigen (FCT) classification is only partially managed to link biofilm production with certain FCT groups. In FCT region of *S. pyogenes* genome are placed genes encoding several virulence factors important for regulation of matrix production: fibronectin binding proteins F1 and F2 (prtF1, prtF2), pilus – associated proteins (Cpa, Fca, Fcb) and RofA/Nra regulator [39–42]. Koller *et al*. [43] have shown correlation between FCT type 1, 2, 5, 6, and 9 and homogenous biofilm production. FCT type 9 isolates were poor biofilm producers, while FCT type 3 and 4 isolates were unequally and irregular biofilm producers. Manetti *et al.* [44] demonstrated association between biofilm formation among FCT types 2, 3, 5, 6 and FCT subset 4 in acidic surroundings.

2.2.1. Our experience with biofilm production of Streptococcus pyogenes

Considering that biofilm production, like adherence, is still not sufficiently explained virulence factor, we supposed that dividing isolates according to invasiveness would be interested. Like in adherence experiments, we also divided isolates in three groups in order to show correlation between biofilm production and invasiveness of strains tested.

Our goal was to find out whether biofilm production as virulence factor is correlated with specific disease/clinical condition. Considering that adherence and hydrophobicity are in relationship with biofilm production, we also wanted to show possible association between them.

2.2.2. Material and methods

2.2.2.1. Determination of hydrophobicity

Hydrophobicity was measured by two different methods described previously by Rosenberg *et al.* [45] and by Lindhal *et al*. [46]. Microbial adhesion to hydrocarbons (MATH) test described by Rosenberg *et al*. [45] was measured with determination of percentage of bacteria adhered to xylene and hexadecane. The salt aggregation test (SAT) described by Lindhal *et al*. [46] is based on the principal of salting out of the surface proteins and bacterial precipitation with a

series of ammonium sulfate solutions with various molarities (0.008 M to 4 M). The highest molarity of solution giving the visible aggregation was scored as numerical value of bacterial surface hydrophobicity or SAT value.

2.2.2.2. Biofilm production

Biofilm production was determined by the same methodology [25] as adherence testing with modification in incubation period of 12, 24, and 48 hours. As in adherence testing, according to ODc isolates were designed as non-producers = OD < ODc, weak biofilm producers = ODc < OD ≤ (2 × ODc) (+), (2 × ODc) < OD ≤ (4 × ODc) = moderate biofilm producers (++) and OD > (4 × ODc) = strong biofilm producers (+++).

2.2.2.3. Statistical analysis

ANOVA was used to determine the differences in hydrophobicity and biofilm production among different groups of GAS strains. Correlation between adherence, hydrophobicity, and biofilm production was determined by Pearson test. Data analyses were done with the SPSS version 20. The differences were considered significant if $p < 0.05$, and highly significant if $p < 0.01$.

2.2.3. Results

2.2.3.1. Adherence

Adherence results are shown in Section 2.1.4.2.

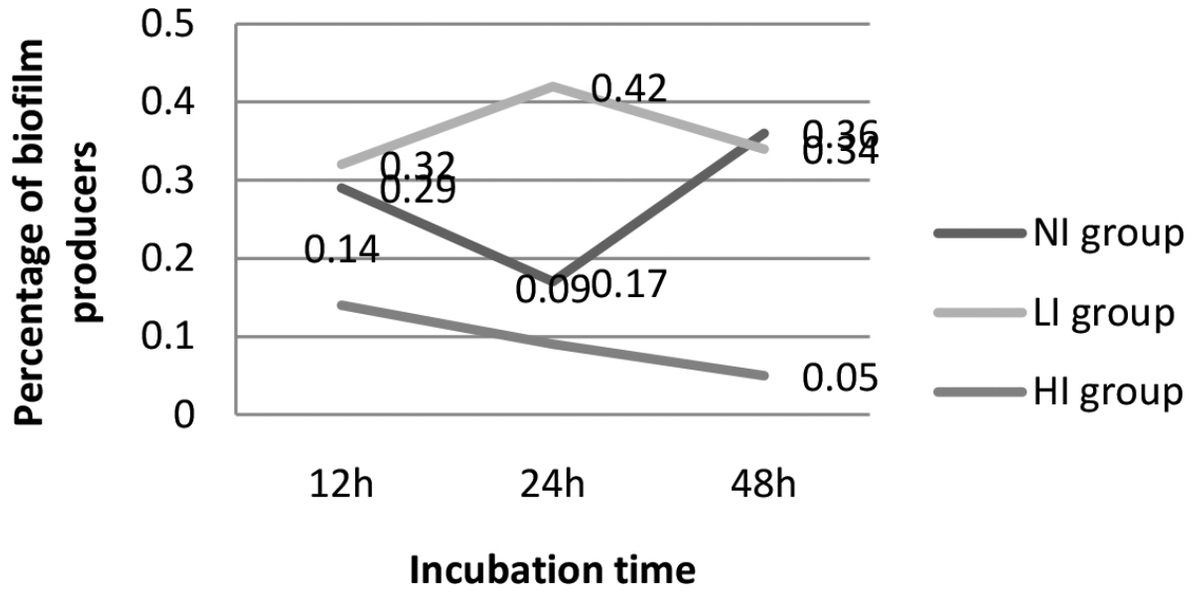
2.2.3.2. Hydrophobicity

Measurement of bacterial hydrophobicity was first performed by MATH test using hexadecane as hydrocarbon after removal of the capsule, which hinders superficial hydrophobic proteins. Adherence to hexadecane was very low and with no statistical difference between groups. After that we tested GAS adherence to xylene. In our assay adherence to xylene were 48.49, 22.78, and 36.09 for NI, LI, and HI group, respectively. It was noticed statistically significant difference between groups, particularly NI group isolates were more hydrophobic in relation to other two groups ($p < 0.001$, $p = 0.041$) and HI group isolates in relation to LI group ($p = 0.044$).

2.2.3.3. Biofilm production

When we did dynamic analysis of biofilm production during specified incubation periods (12–48 hours) all three groups have shown different pattern ($p = 0.040$). Although NI and LI groups started and finished at similar percentages, and as well as that NI group increased biofilm production, and LI group decreased during the time, there was no difference between groups

($p = 0.262$). HI group of isolates constantly were low biofilm producers, which was significantly different from two other groups ($p = 0.026$, $p = 0.001$), as it is shown in **Graph 1**.



Graph 1. Percentage of biofilm producers during tested periods.

2.2.3.4. Analysis of correlation between adherence, hydrophobicity and biofilm production

Adherence and hydrophobicity are very important in process of the biofilm formation. We used various measurement methods to establish possible connection between these three traits of bacteria.

When non-invasive group of isolates was analyzed, positive correlations were noticed between adherence and biofilm formation after 48 hours of incubation ($r = 0.205$, $p = 0.040$), and between biofilm production after 12 and 24 hours ($r = 0.166$, $p = 0.03$), and after 12 and 48 hours of incubation ($r = 0.255$, $p = 0.001$). These results indicated that isolates which adhered efficiently and establish biofilm after 12 hours will also be good biofilm producers after 24 and 48 hours of incubation. In this group of strains, negative correlation was noticed between hydrophobicity measured with xylene and biofilm production after 12-hours ($r = -0.236$, $p = 0.018$) and 24 hours of incubation ($r = -0.201$, $p = 0.045$), i.e. more hydrophobic strains were worse biofilm producers. In NI group of strains, early biofilm producers were stabile producers during entire examined period.

When low invasive group of strains was explored, no correlation was noticed between adherence, hydrophobicity, and biofilm production ($p > 0.05$). Positive correlation was noticed in biofilm production between 24 and 48 hours incubation ($r = 0.166$, $p = 0.03$). In LI group of strains, late biofilm producers were stabile producers.

When highly invasive group of isolates was studied, no correlation was noticed between adherence, hydrophobicity, and biofilm production ($p > 0.05$) or in biofilm production during different incubation periods ($p > 0.05$). Isolates of HI group were non-stable biofilm producers, i.e. they formed and rapidly disbanded their biofilm and matrix.

2.2.3. Discussion

In this study it is demonstrated correlation between adherence, hydrophobicity and biofilm production for non-invasive isolates, while for low and highly invasive isolates no correlation was noticed.

Group A streptococcal adherence is still unrevealed process depending on unspecific hydrophobic bonds and on specific protein-protein or protein-carbohydrate interactions. Hydrophobic interactions are weak non-covalent interactions between water and hydrophobe (non-polar low-water soluble molecules). Hydrophobic interactions are stronger than other weak intermolecular forces (van der Waals or Hydrogen bonds) and depend on several factors: temperature, number of carbon atoms on hydrophobe and shape of hydrophobe.

According to Rosenberg *et al.* [45] MATH test should be performed with phosphate buffer molarity higher than 150 mM, because only under these conditions hydrophobic bonds are stronger than electrostatic. We used PUM buffer containing phosphate, potassium, urea and magnesium with recommended 150 mM molarity and pH 7.1. Even in this recommended conditions, our isolates have not adhered to hexadecane, even after capsule removal, as suggested by Offek *et al*. [47]. According to experiment performed by Nagao and Benchetritt [48], we used xylene instead of hexadecane, afterwards our isolates adhered to this new hydrocarbon. Hexadecane is low-reactive saturated alkane hydrocarbon in contrast to xylene, which is more reactive non-saturated aromatic hydrocarbon. This structural modifications and differences in molecular polarization could be probable explanations for low ability of GAS strains to adhere to hexadecane. In this study it was noticed differences between groups in hydrophobicity measured with xylene, and it was also observed that groups which were more hydrophobic also better adhered to laminin, as expected.

Since *S. pyogenes* is not associated with indwelling device-infections, it was assumed that isolates tested will not at all or will have weak ability for adherence to uncoated microtiter plate. Because of *S. pyogenes* strains weak ability to adhere we also supposed that biofilm production will be at low level. Our assumption was approved in this study, especially for highly invasive isolates, which were low and unstable biofilm producers during tested periods of incubation. Similar results for invasive isolates were observed for *S. pneumoniae* [33, 34]. According to these results we could assume that for invasive isolates biofilm production is not crucial virulence factor.

Non-invasive isolates from streptococcal carriers have shown direct, positive relationship between adherence to uncoated microtiter plate and late stage biofilm production. These isolates were also the most stable biofilm producers during all three incubation intervals, confirming the latest theory that biofilm production could be possible explanation for phar-

yngeal carriage [3]. Marks *et al*. [36] supposed that biofilm producing bacteria down-regulate genes associated with dissemination and invasive disease to adapt to asymptomatic oropharyngeal colonization in mice and the tonsil and adenoid tissue are less toxic to epithelia cells and inducing less inflammation. Somewhat surprising result in this study is the negative correlation of the hydrophobicity with biofilm production in non-invasive isolates tested. This could be explained by the fact that GAS posses abundance of surface adhesins, most of them are inadequate identified and tested, but all of them could affect the hydrophobicity and subsequent formation of biofilm. The most hydrophobic streptococcal surface adhesins are M protein and LTA [49]. Therefore one possible explanation of our findings could be the quantification of LTA in cell envelops of tested isolates.

In our study, we did not find any relationship between different methods of adherence and hydrophobicity measurements for low and highly invasive isolates. Also, we showed that highly invasive isolates have been unstable biofilm producers, contributing to previous findings of other researchers that biofilm production is not crucial virulence factor for invasive strains.

According to literature, this was first work about determination of the relationship between adherence, hydrophobicity and biofilm production for *S. pyogenes*. Surprisingly when were compared adherence and biofilm production, no direct correlation was found for isolates of *S. epidermidis* [50] and *Acinetobacter baumannii* [51], indicating that adherence and biofilm production are not always and explicitly linked.

In conclusion, it is obvious that adherence and biofilm production are not phenotypic traits of all species, but rather individual characteristic of every strain. It is important to emphasize that our experiments have been conducted *in vitro*, so in order to define the role of these three tested virulence factors *in vivo*, complex interactions between various streptococcal adhesins with ECM proteins and host cells should be considered. Due to the numerous virulence factors GAS has excellent adherence capacity, which enables the occurrence of infections, usually not as serious as expected.

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References

[1] Cunningham MW: Pathogenesis of group A atreptococcal infection. Clin Microbiol Rev. 2000;13:470–511. doi: 10.1128/CMR.13.3.470-511.2000.

[2] DeMuri GP, Wald ER: The group A streptococcal carrier state reviewed: still an enigma. J Ped Infect Dis. 2014;3:336–342. doi: 10.1093/jpids/piu030.

[3] Ogawa T, Terao Y, Okuni H, Ninomiya K, Sakata H, Ikebe K, *et al.*: Biofilm formation or internalization into epithelial cells enable *Streptococcus pyogenes* to evade antibiotic eradication in patients with pharyngitis. Microb Pathog. 2011;51:58–68. doi: 10.1016/j.micpath.2011.03.009.

[4] Sela S, Neeman R, Keller N, Barzilai A: Relationship between asymptomatic carriage of *Streptococcus pyogenes* and the ability of the strains to adhere to and be internalised by cultured epithelial cells. J Med Microbiol. 2000;49:499–502. doi: 10.1099/0022-1317-49-6-499.

[5] Donlan RM: Biofilms: microbial life on surfaces. Emerg Infect Dis. 2002;8:881–890. doi: 10.3201/eid0809.020063.

[6] Nobbs AH, Lamont RJ, Jenkinson HF: *Streptococcus* adherence and colonization. Microbiol Mol Biol Rev. 2009;73:407–450. doi: 10.1128/MMBR.00014-09.

[7] Busscher HJ, van de Belt-Gritter B, Dijkstra RJ, Norde W, van der Mei HC: *Streptococcus mutans* and *Streptococcus intermedius* adhesion to fibronectin films are oppositely influenced by ionic strength. Langmuir. 2008;24:10968–10973. doi: 10.1021/la8016968.

[8] Cywes C, Wessels MR: Group A streptococcus tissue invasion by CD44- mediated cell signalling. Nature. 2001;414:648–652. doi: 10.1172/JCI10195.

[9] Kreikemeyer B, Klenk M, Podbielski A: The intracellular status of *Streptococcus pyogenes*: role of extracellular matrix-binding proteins and their regulation. Int J Med Microbiol. 2004;294:177–188. doi: 10.1016/j.ijmm.2004.06.017.

[10] Yamaguchi M, Terao Y, Kawabata S: Pleiotropic virulence factor- *Streptococcus pyogenes* fibronectin-binding proteins. Cell Microbiol. 2013;15:503–511. doi: 10.1111/cmi.12083.

[11] Cue D, Southern SO, Southern PJ, Prabhakar J, Lorelli W, Smallheer JM, *et al.*: A nonpeptide integrin antagonist can inhibit epithelial cell ingestion of *Streptococcus pyogenes* by blocking formation of integrin alpha 5 beta 1-fibronectin-M1 protein complexes. Proc Natl Acad Sci USA. 2000;97:2858–2863. doi: 10.1073/pnas.050587897.

[12] Gibson C, Fogg G, Okada N, Geist RT, Hanski E, Caparon M: Regulation of host cell recognition in *Streptococcus pyogenes*. Dev Biol Stand. 1995;85:137–144. PMID:8586164.

[13] Jaffe J, Natanson-Yaron S, Caparon MG, Hanski E: Protein F2, a novel fibronectin-binding protein from *Streptococcus pyogenes*, possesses two binding domains. Mol Microbiol. 1996;2:373–384. doi: 10.1046/j.1365-2958.1996.6331356.x.

[14] Caparon MG, Geist RT, Perez-Casal J, Scott JR: Environmental regulation of virulence in group A streptococci: transcription of the gene encoding M protein is stimulated by carbon dioxide. J Bacteriol. 1992;174:5693–5701. PMCID: PMC206517.

[15] Collin M. *Streptococcus pyogenes* secreted enzymes interacting with the human host [thesis]. Department of Cell and Molecular Biology, Lund University, Sweden; 2001.

[16] Yilmaz O, Yao L, Maeda K, Rose TM, Lewis EL, Duman M, *et al.:* ATP scavenging by the intracellular pathogen *Porphyromonas gingivalis* inhibits P2X7-mediated host-cell apoptosis. Cell Microbiol. 2008;10:863–875. doi: 10.1111/j.1462-5822.2007.01089.x.

[17] Bisno LA, Brito MO, Collins CM: Molecular basis of group A streptococcal virulence. Lancet Infect Dis. 2003;3:191–200. doi: 10.1016/S1473-3099(03)00576-0.

[18] Barnett TC, Patel AR, Scott JR: A novel sortase, SrtC2, from *Streptococcus pyogenes* anchors a surface protein containing a QVPTGV motif to the cell wall. J Bacteriol. 2004;186:5865–5875. doi: 10.1128/JB.186.17.5865-5875.2004.

[19] Bates CS, Montanez GE, Woods CR, Vincent RM, Eichenbaum Z: Identification and characterization of a *Streptococcus pyogenes* operon involved in binding of hemoproteins and acquisition of iron. Infect Immun. 2003;71:1042–1055. doi: 10.1128/IAI.71.3.1042-1055.2003.

[20] Zhu H, Liu M, Lei B: The surface protein Shr of *Streptococcus pyogenes* binds heme and transfers it to the streptococcal heme-binding protein Shp. BMC Microbiol. 2008;23:8–15. doi: 10.1186/1471-2180-8-15.

[21] Fisher M, Huang YS, Li X, McIver KS, Toukoki C, Eichenbaum Z: Shr is a broad-spectrum surface receptor that contributes to adherence and virulence in group A streptococcus. Infect Immun. 2008;76:5006–5015. doi: 10.1128/IAI.00300-08.

[22] Linke C, Caradoc-Davies TT, Young PG, Proft T, Baker EN: The laminin-binding protein Lbp from *Streptococcus pyogenes* is a zinc receptor. J Bacteriol. 2009;19:5814–5823. doi: 10.1128/JB.00485-09.

[23] Terao Y, Kawabata S, Kunitomo E, Nakagawa I, Hamada S: Novel laminin-binding protein of *Streptococcus pyogenes*, Lbp, is involved in adhesion to epithelial cells. Infect Immun. 2002;70:993–997. doi: 10.1128/IAI.70.2.993-997.2002.

[24] Šmitran A, Vučković-Opavski N, Erić-Marinković J, Gajić I, Ranin L: Adherence and biofilm production of invasive and non-invasive isolates of *Streptococcus pyogenes* after hyaluronidase treatment. Arch Biol Sci. 2013;65:1353–1361. doi: 10.2298/ABS1304353S

[25] Stepanovic S, Vukovic D, Dakic I, Savic B, Svabic-Vlahovic M: A modified microtiter-plate test for quantification of staphylococcal biofilm formation. J Microbiol Methods. 2000;40:175–179. doi: 10.1016/S0378-1135(01)00377-7.

[26] Musumeci R, Bue CL, Milazzo I, Nicoletti G, Serra A, Speciale A, Blandino G: Internalization-associated proteins among *Streptococcus pyogenes* isolated from asymptomatic carriers and children with pharyngitis. Clin Infect Dis. 2003;37:173–179. doi: 10.1086/375589.

[27] Al Safadi R, Amor S, Hery-Arnaud G, Spellerberg B, Lanotte P, Mereghetti L, et al.: Enhanced expression of lmb gene encoding laminin-binding protein in *Streptococcus agalactiae* strains harboring IS1548 in scpB-lmb intergenic region. PLoS One. 2010;5:e10794. doi: 10.1371/journal.pone.0010794.

[28] Akiyama H, Morizane S, Yamasaki O, Oono T, Iwatsuki K: Assessment of *Streptococcus pyogenes* microcolony formation in infected skin by confocal laser scanning microscopy. J Dermatol Sci. 2003;32:193–119. doi: 10.1016/S0923-1811(03)00096-3.

[29] Neely MN, Pfeifer JD, Caparon M: Streptococcus-zebrafish model of bacterial pathogenesis. Infect Immun. 2002;70:3904–3914. doi: 10.1128/IAI.70.7.3904-3914.2002.

[30] Roberts AL, Connolly KL, Kierse DJ, Evans AK, Poehling KA, Peters PR, Reid SD: Detection of group A Streptococcus in tonsils from pediatric patients reveals high rate of asymptomatic streptococcal carriage. BMC Pediatrics. 2012;12:3. doi: 10.1186/1471-2431-12-3.

[31] Cho KH, Caparon MG: Patterns of virulence gene expression differ between biofilm and tissue communities of *Streptococcus pyogenes*. Mol Microbiol. 2005;57:1545–1556. doi: 10.1111/j.1365-2958.2005.04786.x.

[32] 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;72:2864–2875. doi: 10.1128/AEM.72.4.2864-2875.2006.

[33] Sanchez CJ, Kumar N, Lizcano A, Shivshankar P, Dunning Hotopp JC, *et al.*: *Streptococcus pneumoniae* in biofilms are unable to cause invasive disease due to altered virulence determinant production. PLoS ONE. 2011;6:e28738. doi: 10.1371/journal.pone.0028738.

[34] Mayanskiy AN, Chebotar IV, Lazareva AV, Mayanskiy NA: Biofilm formation by *Streptococcus pneumoniae*. Mol Gen Microbiol Virusol. 2015;30:124–131. doi: 10.3108/S0891416815030040

[35] 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. doi: 10.1128/JCM.00512-06.

[36] Marks LR, Mashburn-Warren L, Federle MJ, Hakansson AP: *Streptococcus pyogenes* biofilm growth in vitro and in vivo and its role in colonization, virulence and genetic exchange. J Infect Dis. 2014;210:25–34. doi: 10.1093/infdis/jiu058.

[37] Manetti AGO, Zingaretti C, Falugi F, Capo S, Bombaci S, Bagnoli F, *et al.*: *Streptococcus pyogenes* pili promote pharyngeal cell adhesion and biofilm formation. Mol Microbiol. 2007;64:968–983. doi: 10.111/j.1365-2958.2007.05704.x.

[38] Doern CD, Roberts AL, Hong W, Nelson J, Lukomski S, Swords WE, Reid SD: Biofilm formation by group A streptococcus: a role for the streptococcal regulator of virulence (Srv) and streptococcal cysteine protease (SpeB). Microbiology. 2009;155:46–52. doi: 10.1099/mic.0.021048-0

[39] Bessen DE, Kalia A: Genomic localization of a T serotype locus to a recombinatorial zone encoding extracellular matrix-binding proteins in *Streptococcus pyogenes*. Infect Immun. 2002;70:1159–1167. doi: 10.1128/IAI.70.3.1159-1167.2002.

[40] Ramachandran V, McArthur JD, Behm CE, Gutzeit C, Dowton M, Fagan PK, *et al.*: Two distinct genotypes of prtF2, encoding a fibronectin binding protein, and evolution of the gene family in *Streptococcus pyogenes*. J Bacteriol. 2004;186:7601–7609. doi: 10.1128/JB.186.22.7601-7609.2004.

[41] Nakata M, Podbielski A, Kreikemeyer B: MsmR, a specific positive regulator of the *Streptococcus pyogenes* FCT pathogenicity region and cytolysin-mediated translocation system genes. Mol Microbiol. 2005;57:786–803. doi: 10.1111/j.1365-2958.2005.04730.x.

[42] Mora M, Bensi G, Capo S, Falugi F, Zingaretti C, Manetti AG, *et al.*: Group A streptococcus produce pilus-like structures containing protective antigens and Lancefield T antigens. Proc Natl Acad Sci USA. 2005;102:15641–15646. doi: 10.1073/pnas.0507808102.

[43] Koller T, Manetti AGO, Kreikemeyer B, Lembke C, Margarit I, Grandi G, Podbielski A: Typing of the pilus-protein-encoding FCT region and biofilm formation as novel parameters in epidemiological investigations of *Streptococcus pyogenes* isolates from various infection sites. J Med Microbiol. 2010;59:442–452. doi: 10.1099/jmm.0.013581-0.

[44] Manetti AGO, Köller T, Becherelli M, Buccato S, Kreikemeyer B, Podbielski A, *et al.*: Environmental acidification drives *S. pyogenes* pilus expression and microcolony formation on epithelial cells in a FCT-dependent manner. PLoS One. 2010;5:e13864. doi: 10.1371/journal.pone.0013864

[45] Rosenberg M, Gutnick E, Rosenberg M: Adherence of bacteria to hydrocarbons: a simple method for measuring cell- surface hydrophobicity. FEMS Microbiol Lett. 1980;9:29–33. doi: 10.1111/j.1574-6968.1980.tb05599.x.

[46] Lindhal M, Faris A, Wadstrom T, Hjerten S: A new test based on "salting out"to measure relative surface hydrophobicity of bacterial cells. Biochim Biophys Acta. 1981;77:471–476. doi: 10.1016/0304-4165(81)90261-0.

[47] Offek I, Whitnack E, Beachey E: Hydrophobic interaction of Group A streptococci with hexadecane droplets. J Bacteriol. 1983;154:139–145. PMCID: PMC217440.

[48] Nagao PE, Benchetritt LC: Virulent and avirulent strains of group B streptococci from Rio de Janeiro, Brazil. Relationship between differences in surface hydrophobicity, sialic acid content and macrophage interaction. Mem Inst Oswaldo Cruz. 1999;94:497–498. doi: 10.1590/S0074-02761999000400012.

[49] Courtney HS, Ofek I, Penfound T, Nizet V, Pence MA, Kreikemeyer B, *et al.*: Relationship between expression of the family of M proteins and lipoteichoic acid to hydrophobicity and biofilm formation in *Streptococcus pyogenes*. PloS ONE. 2009;4:e4166. doi: 10.1371/journal.pone.0004166

[50] Cerca N, Pier GB, Vilanova M, Oliviera R, Azeredo J: Quantitative analysis of adhesion and biofilm formation on hydrophilic and hydrophobic surfaces of clinical isolates of *Staphylococcus epidermidis*. Res Microbiol. 2005;156:506–514. doi: 10.1016/j.resmic.2005.01.007

[51] McQueary CN, Actis LA: *Acinetobacter baumanii* Biofilms: variations among strains and correlation with other cell properties. J Microbiol. 2011;49:243–250. doi: 10.1007/s12275-012-1555-1.