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ADHERENCE AND BIOFILM PRODUCTION OF INVASIVE AND NON-INVASIVE ISOLATES OF *STREPTOCOCCUS PYOGENES* AFTER HYALURONIDASE TREATMENT

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Abstract - Biofilm represents a protected mode, which allows bacteria to survive and proliferate in a hostile environment. Little is known whether the ability to form biofilms is a characteristic of all groups of A streptococcal (GAS) strains and whether there is a relationship between biofilm formation and a clinical source of isolates. A capsule physically covers superficial adhesins and other proteins, essential in bacterial attachment, as the first step in biofilm formation. It is also possible that hyaluronic acid could form part of the complex extracellular polymer matrix of biofilms and contribute to the three-dimensional architecture of the biofilm. The aim of this study was to investigate if there are differences in adherence and biofilm production between GAS strains with different pathogenic potential, and the possible role of the capsule in this process. A total of 122 isolates were divided into three groups: noninvasive (NI), low invasive (LI) and highly invasive (HI). Adherence, SpeB and biofilm production were tested before and after hyaluronidase treatment. There was no difference in adherence between untreated GAS strains, but after capsule removal, NI and HI isolates adhered significantly better than the LI group. Before treatment, isolates of the HI group were the worst biofilm producers, but after capsule removal, they became the best biofilm producers. There was no difference in SpeB production among GAS isolates, regardless of the hyaluronidase treatment.

Key words: Streptococcus pyogenes, adherence, biofilm, capsule, hyaluronidase

INTRODUCTION

Biofilm is a microbially derived sessile community characterized by cells that are irreversibly attached to a substratum and embedded in a matrix of extracellular polymeric substances (Donlan and Costerton, 2002). Biofilm represents a protected mode, which allows bacteria to survive and proliferate in a hostile environment. Its structure allows sufficient nutrients to sustain growth and, at the same time, protect the bacteria from toxic compounds and reagents present in the environment. (Costerton et al., 1999).

Biofilms are of special significance in medicine. Many pathogenic and nosocomial bacteria with the ability to form biofilms are responsible for acute and chronic infections. It has been estimated by the National Institute of Health (United States) that more than 80% of persistent bacterial infections are likely to involve biofilms (Bjarnsholt et al., 2002) Examples of typical biofilm-associated diseases are caries, gingivitis, periodontitis, endocarditis and prostatitis. Such infections are extremely difficult to eradicate because of the resistance of biofilm to numerous antimicrobial agents and products of the immune sys-

tem (Donlan and Costerton, 2002; Costerton et al., 1999).

Streptococcus pyogenes is a frequent cause of bacterial diseases worldwide. Diseases can vary from relatively mild tonsillopharyngitis and pyoderma to severe forms such as necrotizing fasciitis and septicemia, which could be lethal (Cunningham, 2000). As far as biofilm is concerned, streptococcal species such as Streptococcus gordonii and Streptococcus mutans are well-known biofilm formers (Ellen et al., 1997; Jefferson, 2004), and recent observations suggesting that biofilm may have a role in S. pyogenes infections have been reported. Hidalgo-Grass et al. (2004) observed that structured communities appear to be present in necrotizing fasciitis lesions, and Neely et al. (2002) found similar characteristics in a model of *S. pyogenes* myositis in zebra fish. Recently, Ogava et al. (2011) proposed that biofilm formation or internalization into epithelial cells enable *Streptococcus pyogenes* to establish pharyngeal carriage in patients with pharyngitis. However, little is known about whether the ability to form biofilms is a characteristic of all group A streptococcal (GAS) strains and whether there is a relationship between biofilm formation and a clinical source of isolates.

Streptococcal pyrogenic exotoxin B, as other streptococcal pyrogenic exotoxins, was first well known for its pyrogenicity, enhancement of endotoxin shock and superantigenic effect on the immune system (Cunningham, 2000). Nowadays, SpeB is an extracellular cysteine protease capable of cleaving both host and bacterial proteins and contributes to tissue damage and dissemination (Chiang-Ni and Wu, 2008). Doern et al. (2009) have found that SpeB degrades GAS extracellular proteins and host proteins integral to the biofilm and contribute to biofilm dispersal. Dispersal of the GAS biofilm and the constitutive production of SpeB would result in increased virulence and tissue damage (Connolly et al, 2011). SpeB production was found to be essential for establishing a murine skin infection that ultimately resulted in systemic infection, reduced clearance by the innate immune response and increased mortality (Lukomski et al., 1998).

GAS capsule is made of hyaluronic acid, a high-molecular-weight polymer (Girish and Kemparaju, 2007). Besides its well known antiphagocytic role, the capsule attaches to CD44, a hyaluronic acid-binding protein (Schrager et al., 1998) on human epithelial cells, which induces cytoskeletal rearrangements and allows bacteria to stay extracellular as they penetrate the epithelium (Cywes et al., 2001). The capsule physically covers superficial adhesins and other proteins, essential in bacterial attachment, as the first step in biofilm formation (Schembri et al., 2004). It is also possible that hyaluronic acid will form part of the complex extracellular polymer matrix of biofilms and contribute to the three-dimensional architecture of biofilm (Pecharki et al., 2008).

The aim of this study was to investigate if there are differences in adherence and biofilm production between GAS strains with different pathogenic potential and the possible role of the capsule in this process.

MATERIALS AND METHODS

Bacterial strains

A total of 172 *Streptococcus pyogenes* isolates used for biofilm testing were divided into three groups: (1) a noninvasive group (NI) obtained from carriers (100 isolates); (2) a low invasive group (LI) isolated from patients with tonsillopharyngitis (50 isolates), and (3) a highly invasive (HI) group (22 isolates), isolated from the blood of patients with sepsis and necrotizing fasciitis obtained from the collection of the Institute of Microbiology and Immunology, School of Medicine, Belgrade.

Isolates were cultured on Columbia agar plates containing 5% sheep blood and stored at -80°C in Todd Hewitt broth (Biolife, Italy). Identification was based on colony morphology, beta hemolysis, Gram-stain morphology, bacitracin sensitivity, trimethoprim-sulphometoxasole resistance and slide agglutination test (Slidex Strepto-Kit, Bio-Merieux, France).

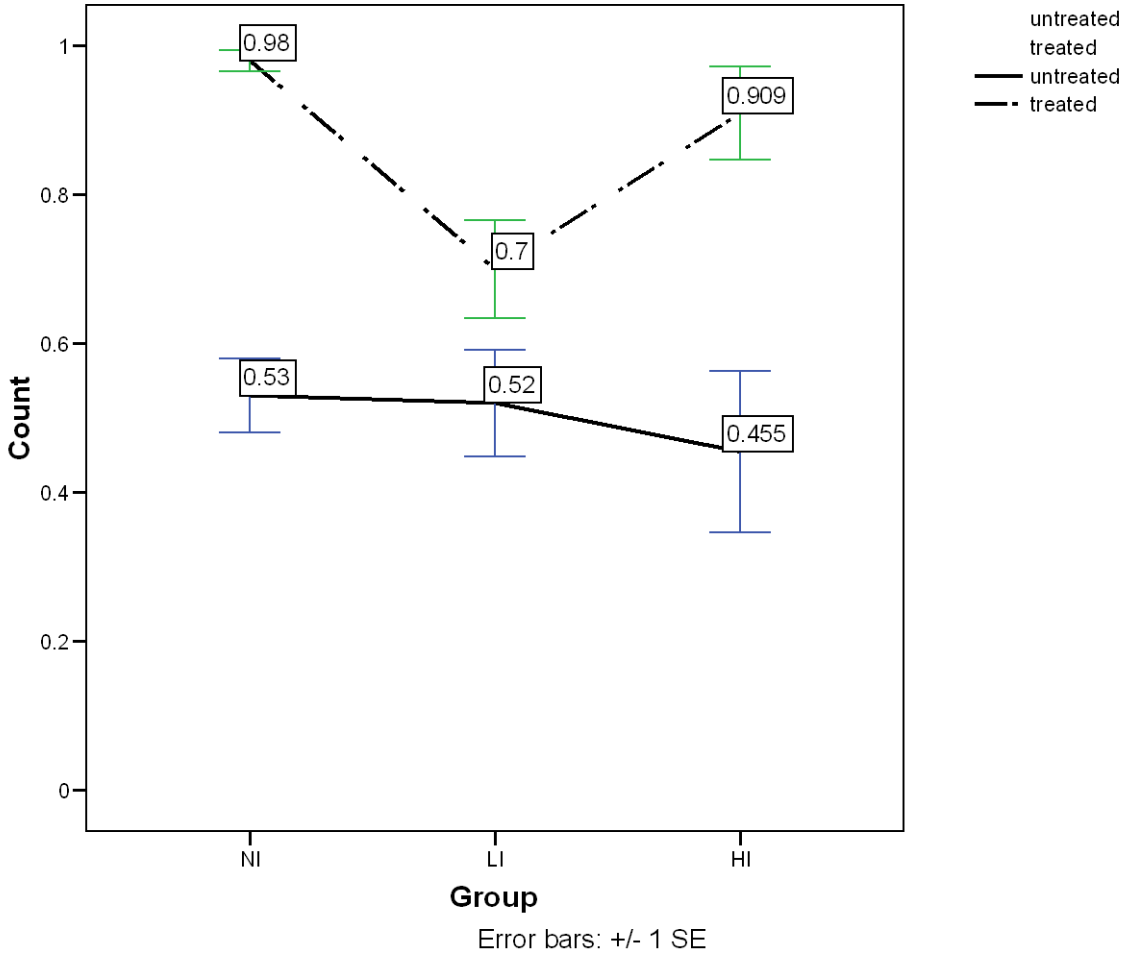

Fig. 1. Proportion of adherent isolates of NI, LI and HI group before and after hyaluronidase treatment.

Growth conditions

Briefly, 1-2 colonies were picked from CBA, inoculated in 2 ml Todd-Hewitt broth supplemented with 1% yeast extract (THY) and incubated for 18-24 h at 37°C in air. All isolates were tested for adherence, biofilm formation and SpeB production before and after hyaluronidase treatment, e.g. capsule removal.

Hyaluronidase treatment

Bovine testicular hyaluronidase, type VI-S (Sigma-Aldrich), was diluted in enzyme diluent (20 mM sodium phosphate, 77 mM sodium chloride, 0.01% bovine albumin, pH 7.0 at 37°C) according to the manufacturer's instructions. After overnight incubation in THY, the broth culture was centrifuged at 18000 rpm for 15 min, the pellet was washed with PBS (0.2 mM, pH 7), resuspended in PBS (0.2 mM, pH 6) and treated with bovine testicular hyaluronidase, at a concentration of 5 U/ml broth culture for 45 min at 37°C, as previously described (Ofek et al., 1983). The absence of a capsule was confirmed by India ink preparation.

Biofilm formation and adherence in uncoated polystyrene microtiter plates

Biofilm production was determined by the method described by Stepanović et al. (2000) with minor

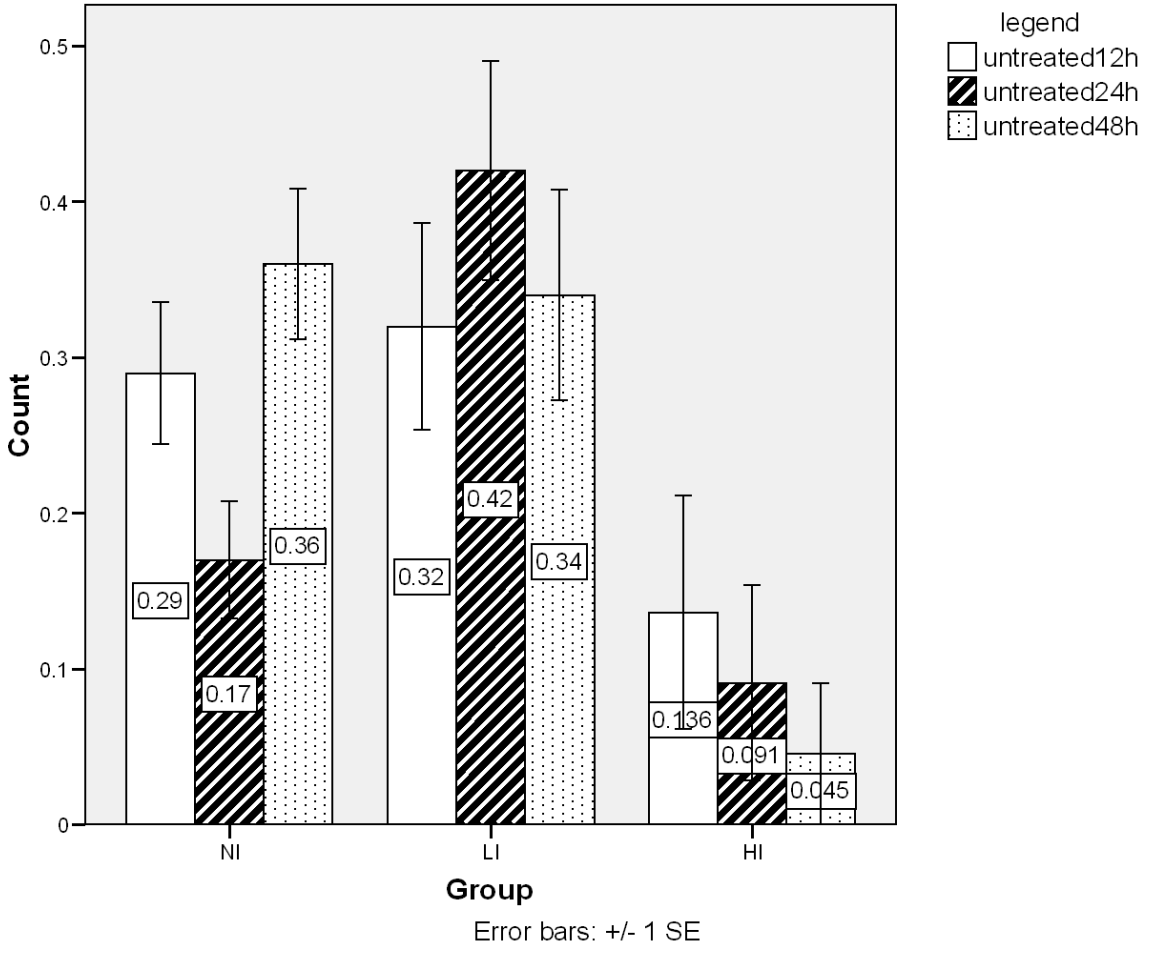

Figure 2. Proportion of untreated isolates that produce biofilm after 12, 24 and 48 h of incubation.

modification in inoculum concentration. Briefly, 1-2 colonies from CBA were inoculated in 2 ml Todd-Hewitt broth supplemented with 1% yeast extract (THY). After overnight incubation at 37°C in air, 20 µl of the broth culture were pipetted in 2 ml of fresh THY broth and from this prepared culture 100 µl were transferred to microtiter plates (Kartell, Italy) and incubated for 12, 24 and 48 h at 37°C. The negative control contained broth only. The positive control was *Staphylococcus epidermidis* ATCC 14990. The plates were incubated at 37°C without shaking. Based on the OD produced by bacterial films and cut-off OD (ODc), strains were classified into the following categories: no biofilm producers, weak, moderate or strong biofilm producers. The cut-off OD (ODc) was defined as three standard deviations above the mean OD of the negative control.

In order to test adherence to polystyrene, we incubated microtiter plates with inoculum for 30 min at 37°C in air. Strains with OD above the ODc were considered adherent to the microtiter plates.

SpeB production

SpeB production was tested according to the method described by Hynes and Tagg (1985). The GAS colony was stab inoculated into azocasein agar (Co-

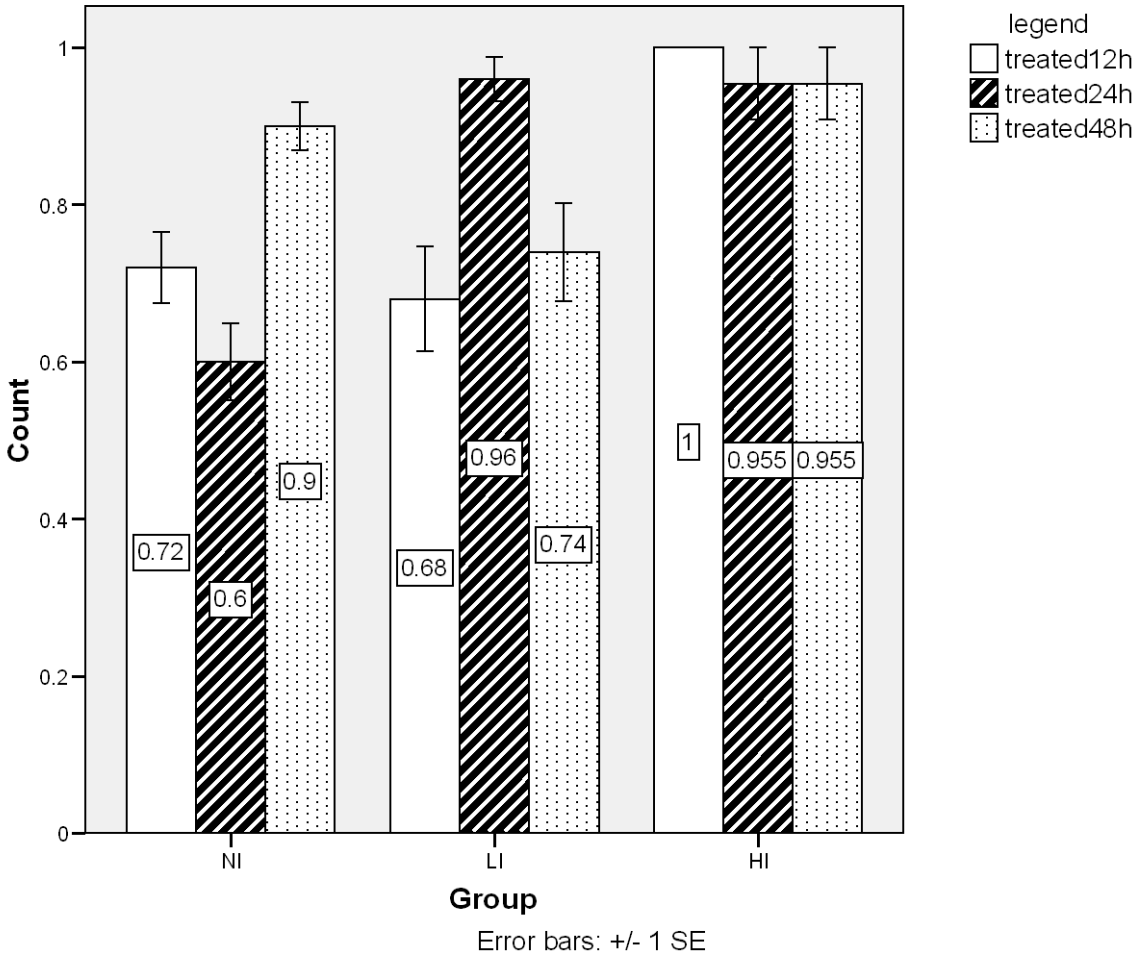

Fig. 3. Proportion of treated isolates that produce biofilm after 12, 24 and 48 h of incubation.

lumbia agar supplemented with 1.5% skimmed milk) and incubated overnight at 37°C in air. Proteinase-positive strains formed a translucent halo around the site of the stab. SpeB production was measured in millimeters as the radius of translucent zone around the GAS colony in azocasein agar.

Statistical analysis

Data analyses were done with SPSS version 20. ANOVA was applied to the results of adherence, SpeB and biofilm production between the three groups. Student's t-test was applied to the results when comparing the data within the same group after and before the treatment. Both tests were considered significant if $p<0.05$, and very significant if $p<0.01$.

RESULTS

As shown in Figure 1, there was no difference in adherence to the polystyrene plate between untreated GAS strains ($p=0.816$). However, after capsule removal, NI and HI isolates adhered significantly better than the LI group ($p \leq 0.001$).

All tested *S. pyogenes* isolates were weak biofilm producers (OD values ODc <OD< 2xODc). There was no difference in biofilm production among all

the GAS strains after 12 h of incubation (p=0.262). After 24 h, the low invasive strains produced biofilm significantly better than the other two groups (p=0.01), while after 48 h, LI and NI isolates produced considerably more biofilm than the HI group (p=0.014). However, after 24 and 48 h, the highly invasive strains were the worst biofilm producers.

After hyaluronidase treatment, all tested GAS strains were still weak biofilm producers, but were better than before treatment. However, after 12 h of incubation, the highly invasive strains produced biofilm significantly better than the other two groups of isolates (p=0.011). In the course of incubation (after 24 h), the low invasive strains increased biofilm production ($p \leq 0.001$), while after 48 h, both HI and NI isolates produced biofilm in a similar amount (p=0.011) (Figure 3.).

Regardless of the hyaluronidase treatment, there was a similar pattern of biofilm growth among all three groups of strains. For the NI, LI and HI groups, SpeB production before treatment was 11.7, 10.22 and 11.09 millimeters, and 11.67, 10.56 and 11.36 millimeters, respectively, after treatment There was no difference in SpeB production among GAS isolates, regardless of the hyaluronidase treatment (p= 0.253, p= 0.417).

DISCUSSION

This work revealed that the adherence and biofilm production of both invasive and noninvasive GAS isolates increased after capsule removal. The capsule is a cell surface structure involved in virulence and colonization by virtue of its ability to create a hydrated protective layer around the bacteria and increase their resistance to phagocytosis and to the bactericidal effects of human serum. The virulence of S. pyogenes correlates closely with the expression of the hyaluronic acid capsule. Heavily capsulated strains spread more readily, while capsule production is repressed during carriage. On the other hand, the attachment of group A streptococci to pharyngeal or dermal epithelial cells is the most important initial step in the colonization of the host. Without strong adherence mechanisms, group A streptococci could not attach to host tissues and would be removed by mucous and salivary fluid flow mechanisms and exfoliation of the epithelium. Adhesins enable interactions with host cells and extracellular matrix components. The adhesion process involves multiple group A streptococcal adhesins (lipoteichoic acid, M protein, fibrinogen, fibronectin and laminin binding protein, pili and many other proteins and carbohydrates) (Nobbs et al., 2009). The presence of multiple adhesins in the strains could give them the advantage of more avid adherence and potentially enhanced virulence. It could be assumed that the capsule hinders the important adhesins or hydrophobic molecules that mediate biofilm formation and absence of a capsule uncovers the components and allows better adherence and biofilm formation (Schembri et al., 2004).

Although there are no data in literature that could explain the weaker adherence of the LI group, our presumption was that the isolates from patients with pharyngeal carriage (NI group) and invasive isolates (HI group) possess more adhesins or adhesins with higher affinity for cell receptors that allow isolates to strongly adhere to epithelial cells during carriage into deeper tissues in invasive infections. Our next goal will be to test the adherence of the same isolates to fibrinogen and laminin-coated microtiter plates to verify our conclusions.

Originally identified as a secreted product, SpeB was found to be associated with the cell surface, possibly via its pro-peptide moiety (Hytonen et al., 2001). In this form, SpeB mediates adhesion to glycoproteins and laminin, functions that may promote the colonization capabilities of GAS. When SpeB is associated with the cell surface, the capsule hinders SpeB, like other adhesins. SpeB is an extracellular cysteine protease that has been shown to cleave fibronectin and vitronectin (Kapur et al., 1993a), extracellular matrix proteins and human interleukin-1b into the active form of the molecule (Kapur et al, 1993b). Therefore, the protease may be important in inflammation, shock and tissue destruction. Humans with a diverse range of invasive disease (erysipelas,

cellulitis, pneumonia, bacteremia, septic arthritis, toxic shock syndrome and necrotizing fasciitis) all produced elevated levels of antibodies against streptococcal pyrogenic exotoxin B following infection (Gubba et al., 1998). Hence, we expected invasive isolates to produce more efficiently SpeB. However, we did not observe differences between the groups in SpeB production either before or after hyaluronidase treatment. We could this explain in two ways. Firstly, our method was not sensitive and precise enough to detect such fine differences between the isolates in SpeB production. Secondly, after capsule removal the isolates were grown for 24 h in azocasein agar containing polysaccharides and, hence, they had enough time and substrate to synthesize a new capsule. Our goal in the future will be to measure SpeB production by other methods that are more sensitive and exact.

The different capabilities of biofilm formation of *S. pyogenes* isolates were observed depending on their clinical origin. These differences were observed after 24 h and 48 h of incubation. It was noticed that initially, invasive non-treated isolates were the worst biofilm producers, while pharyngeal strains were the best. These results were expected, since we presumed that biofilm formation is not an essential virulence factor for highly invasive isolates. Baldassari et al. (2006) have found that the isolates that were able to invade cells with high efficiency were those producing the lowest amount of biofilm; the strong biofilm formers entered cells only with low efficiency. Similar results were found in experiments with *S. pneumoniae* (Moscoso et al., 2006, Garcia et al., 2007), although the role of pneumococcal adhesins in pathogenesis and their contribution to biofilm production have not been investigated as well as GAS adhesins. However, we expected that among all strains the noninvasive isolates would be the most potent biofilm producers, because it was assumed that pharyngeal carriage might be due to biofilm formation (Baldassari et al., 2006). Another possible explanation for streptococcal carriage is internalization into epithelial cells (Marouni and Sela, 2004), although GAS were considered to be extracellular bacteria. Our results support intracellular internalization as a possible mechanism of carriage in the throat. There was no difference in biofilm stability among the strains. These results were expected, because there is no correlation in literature between GAS and diseases associated with biofilm and indwelling medical devices.

After hyaluronidase treatment, biofilms production was improved in all isolates. Surprisingly, the highly invasive isolates were the best biofilm producers of all the strains. The pattern of biofilm growth in all the GAS isolates was comparable before and after capsule removal.

It is important to note that although the capsule was removed during hyaluronidase treatment, the strains synthesized a new capsule during incubation in the broth. It is well known that a capsule is essential for the three-dimensional architecture of biofilm (Danese et al., 2000). We believe that at the beginning of incubation capsule removal enabled the good adherence of the highly invasive isolates. However, during the incubation, these isolates produced thicker capsules, which probably contributed to subsequent better biofilm formation among all the isolates. In an experiment with *S. intermedius*, Pecharki et al. (2008) obtained similar results. Nevertheless, Sugareva et al. (2010) did not find a correlation between capsule and biofilm formation. Cho and Caparon (2005) found that the GAS hyaluronic acid capsule was not required for biofilm formation in a static system, but that following initial attachment, no bacterial aggregation or biofilm production under flow conditions occurred in mutants lacking the capsule. In addition, none of the authors could not estimate if serotype specificity affected biofilm formation. Another important question is why the GAS capacity to form biofilm appears not to be universal but rather strain-dependent.

To the best of our knowledge, this is the first work in which biofilm production was examined after enzymatic removal of the GAS capsule. We do not exactly know the mechanism whereby the removal of the capsule affects biofilm formation, and plan to investigate this in the future. At present we still don't know enough about biofilm formation in *S. pyogenes*.

Further study of GAS biofilm formation will provide new insights into the pathogenesis of GAS.

REFERENCES

Bjarnsholt, T., Kirketerp-Muller, K., Østrup Jensen, P., Madsen, K.G., Phipps, R., Krogfelt K., Hoiby N. and *M. Givskov* (2008). Why chronic wounds will not heal: a novel hypothesis. *Tech. Wound Rep. Reg.* **16,** 2-10.

Busscher, H.J., Bos, R. **and** *H.C. van der Mei* (1995). Initial microbial adhesion is a determinant for the strength of biofilm adhesion. *FEMS Microbiol. Lett.* **128,** 229-234.

Chiang-Ni C. and *J.J. Wu* (2008) Effects of streptococcal pyrogenic exotoxin B on pathogenesis of Streptococcus pyogenes. *J. Formos. Med. Assoc.* **107**: 677-685.

Cho, K.H. and *M.G. Caparon* (2005). Patterns of virulence gene expression differ between biofilm and tissue communities of *Streptococcus pyogenes. Mol. Microbiol.* **57**, 1545-1556.

Connolly, K.L., Roberts, A.L., Holder, R.C. and *S.D. Reid* (2011). Dispersal of Group A streptococcal biofilms by the cysteine protease SpeB leads to increased disease severity in a murine model. *PLoS One.* **6**(4)

Costerton, J.W., Stewart, P.S. and *E.P. Greenberg* (1999). Bacterial biofilms: a common cause of persistent infections. *Science* **284**, 318-322.

Cunningham, M.W. (2000) Pathogenesis of group A streptococcal infections. *Clin Microbiol Rev*; 13, 470-511.

Cywes, C. and *M.R. Wessels* (2001). Group A *Streptococcus* tissue invasion by CD44- mediated cell signaling. *Nature* **414**, 648-52.

Danese, P. N., Pratt, L.A. and *R. Kolter* (2000). Exopolysaccharide Production Is Required for Development of *Escherichia coli* K-12 Biofilm Architecture. *J. Bacteriol.* **182** (12), 3593-3596**.**

Doern, C.D., Roberts, A.L., Hong, W., Nelson, J., Lukomski, S., Swords, W.E. and *S.D. Reid* (2009). Biofilm formation by group A Streptococcus: a role for the streptococcal regulator of virulence (Srv) and streptococcal cysteine protease (SpeB). *Microbiology* **155**, 46-52.

Donlan, R.M. and *J.W. Costerton* (2002). Biofilms: Survival Mechanisms of Clinically Relevant Microorganisms. *Clin. Microbiol. Rev.* **15**(2), 167-193.

Ellen, R. P., Lepine, G. and *P. M. Nghiem***.** (1997). In vitro models that support adhesion specificity in biofilms of oral bacteria. *Adv. Dent. Res***. 11,** 33-42.

García-Castillo, M., Morosini, M.I., Valverde, A., Almaraz, F., Baquero, F., Cantón, R. and *R. del Campo (*2007) Differences in biofilm development and antibiotic susceptibility among Streptococcus pneumoniae isolates from cystic fibrosis samples and blood cultures. *J Antimicrob Chemother.* **59**(2), 301-4.

Girish, K. S. and *K. Kemparaju* (2007). The magic glue hyaluronan and its eraser hyaluronidase: a biological overview. *Life Sci.* **80**, 1921-1943.

Gryllos, I., H. J. Tran-Winkler, M. F. Cheng, H. Chung, R. Bolcome III, W. Lu, R. I. Lehrer **and** *M. R. Wessels***.** 2008. Induction of group A *Streptococcus* virulence by a human antimicrobial peptide. *Proc. Natl. Acad. Sci. USA* **105,**16755-16760.

Gubba, S., Low, D.E. and *J.M. Musser* (1998). Expression and characterization of group A streptococcus extracellular cysteine protease recombinant mutant proteins and documentation of seroconversion during human invasive disease episodes. *Infect. Immun.* **66**, 765–770.

Hidalgo-Grass, C., Dan-Goor, M., Maly, A., Eran, Y., Kwinn L.A., Nizet, V., Ravins, M., Jaffe, J., Peyser, A., Moses, A. E. and *E. Hanski.* (2004). Effect of a bacterial pheromone peptide on host chemokine degradation in group A streptococcal necrotizing soft-tissue infections. *Lancet* **363:**696-703.

Hynes, W. L. and *J.R. Tagg* (1985). A simple plate assay for detection of group A streptococcus proteinase. *J. Microbiol. Methods.* **4**, 25-31.

Hytonen, J., S. Haataja, D. Gerlach, A. Podbielski and *J. Finne* (2001). The SpeB virulence factor of Streptococcus pyogenes, a multifunctional secreted and cell surface molecule with strepadhesin, laminin-binding and cysteine protease activity. *Mol. Microbiol.* **39**, 512-519.

Jefferson, K. K. (2004). What drives bacteria to produce biofilm? *FEMS Microbiol. Lett.* **236,** 163-173.

Kapur, V., Majesky, M.W., Li, L.-L., Black, R.A. and *J.M. Musser.* (1993 b). Cleavage of interleukin 1b precursor to produce active IL-1b by a conserved extracellular cysteine protease from *Streptococcus pyogenes*. *Proc. Natl.Acad. Sci. USA* **90**, 7676-7680.

Kapur, V., Topouzis, S., Majesky, M.W., Li, L.-L., Hamrick, M.R., Hamill, R.J., Patti, J. M. and *J.M. Musser.* (1993a). A conserved Streptococcus pyogenes extracellular cysteine protease cleaves human fibronectin and degrades vitronectin. *Microb. Pathog.* **15**, 327-346.

Lukomski, S., Burns, E.H., Wyde, P.R., Podbielski, A., Rurangirwa, J. et al. (1998) Genetic inactivation of an extracellular cysteine protease (SpeB) expressed by Streptococcus pyogenes decreases resistance to phagocytosis and dissemination to organs. *Infect Immun* **66**, 771-776.

Moscoso, M., Garcia, E. and *R. Lopez* (2006). Biofilm formation by *Streptococcus pneumoniae*: role of choline, extracellular DNA and capsular polysaccharide in microbial accretion. *J. Bacteriol.* **188**(22), 7785-7795.),

Neely, M. N., Pfeifer, J. D. and *M. Caparon* **(**2002). Streptococcus-zebra fish model of bacterial pathogenesis. *Infect. Immun.* **70,** 3904-3914.

Nobbs, A.H., Lamont, R.J. and *F. Jenkinson* (2009) Streptococcus adherence and colonization. *Microbiol. Mol. Biol. Rev.* **73** (3), 407-450.

Offek, I., Whitnack, E. and *E. Beachey* (1983). Hydrophobic interaction of Group A streptococci with hexadecane droplets. *J. Bacteriol.* **154**(1), 139-145.

Ogawa, T., Terao, Y., Okuni, H., Ninomiya, K., Sakata, H., Ikebe, K., Maeda, Y. and *S. Kawabata* (2011). Biofilm formation or internalization into epithelial cells enable *Streptococcus pyogenes* to evade antibiotic eradication in patients with pharyngitis. *Microb Pathog.* **51**(1-2), 58-68

Pecharki, D., Petersen, F.C. and *A.A. Sheie* (2008) Role of hyaluronidase in *Streptococcus intermedius* biofilm. *Microbiology* **154**, 932-938.

Schembri, M. A., Dalsgaard D. and *Klemm P.* 2004. Capsule shields the function of short bacterial adhesins. *J. Bacteriol.* **186**(5), 1249-1257.

Schrager, H.M., Alberti, S., Cywes, C., Dougherty, G.J. and *M.R. Wessels* (1998). Hyaluronic acid capsule modulates M protein-mediated adherence and acts as a ligand for attachment of group A streptococcus to CD44 on human keratinocytes. *J. Clin. Invest.* **101**, 1708-16.

Stepanović, S., Vuković, D., Dakić, I., Savić, B. and *M. Švabić-Vlahović* (2000). A modified microtiter-plate test for quantification of staphylococcal biofilm formation. *J. Microbiol. Methods.* **40**(2), 175-9.

Sugareva, V., Arlt, R., Fiedler, T., Riani, C., Podbielski, A. and *B. Kreikemeyer* (2010) Serotype- and strain- dependent contribution of the sensor kinase CovS of the CovRS two-component system to *Streptococcus pyogenes* pathogenesis. *BMC Microbiology* **10**, 34-39