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Original article

Inhibition of *in vitro Histophilus somni* biofilm production by recombinant Hsp60 antibodies

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Abstract

Histophilus somni is an opportunistic pathogen causing respiratory, genitourinary and generalized infections in cattle. An important virulence factor is its ability to produce a biofilm. The aim of this work was to confirm that *H. somni* Hsp60 (Gro-EL) is a constituent of the biofilm produced by this bacterium *in vitro* and to check whether or not the presence of a specific antibody within the culture medium can inhibit biofilm production. Biofilm production by *H. somni* cultured *in vitro* was confirmed by crystalline violet staining. The presence of Hsp60 in the biofilm was confirmed by using specific antibodies produced in a mouse and goat hyperimmunized with *H. somni* recombinant Hsp60 (rHsp60). Large complexes of biofilm stained with Hsp60 antibodies were microscopically detected. This indicates that the Hsp60 protein is a common constituent of the biofilm produced by *H. somni in vitro*. In a second experiment, mouse serum containing anti- *H. somni* rHsp60 antibodies was added to an *H. somni* culture. It was found that the presence of anti-rHsp60 antibodies in the culture medium inhibited biofilm production *in vitro*. Only small biofilm particles were seen in the presence of the specific antibody, whereas in control cultures (without specific antiserum) large biofilm complexes were produced. The results indicate that antibodies specific to Hsp60 may be useful for preventing *H. somni* biofilm formation *in vitro*. If this also occurs *in vivo*, it may be helpful for eradicating *H. somni* infection in cattle through the elimination of carriers. Further *in vivo* studies are needed to confirm this idea.

Key words: biofilm, *Histophilus somni*, Hsp60, *H. somni* rHsp60 antibody

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Introduction

Histophilus somni is known as a facultative pathogen that is commonly isolated from the respiratory and genito-urinary tract of cattle (Inzana et al. 1997). This Gram-negative coccobacillus is involved in the bovine respiratory disease complex (BRDC), which causes significant losses and mortality in feedlot cattle in North America (Sandal et al. 2009). It causes thrombo-embolic meningoencephalomyelitis (ITEME) and many organ pathologies, including myocarditis, polyarthritis and systemic disease (Sandal et al. 2007). Respiratory tract and central nervous system infections are frequently associated with sepsis, fever and a fatal outcome. Sudden death during histophilosis is associated with myocarditis and after degenerative and necrotic processes, with abscess formation mainly occurring in the papillar muscles of the left ventricle (Harris and Janzen 1989). *Histophilus somni* possesses many virulence factors utilized in relation to the host (Corbeil 2008). Biofim formation increases the stability of bacteria on mucosal membranes and prevents eradication of the infection in the herd (Corbeil 2008).

Bacterial biofilms consist of aggregations of bacteria that can survive in highly complex and organized groups (Greiner et al. 2004). Factors important in biofilm formation are adhesive interactions between bacterial cells, adhesion to the base, and the secretion of extracellular polysaccharides and intercellular adhesins (Jefferson et al. 2006). The extracellular matrix contains many organic substances, including nucleic acids, saccharides and proteins (Moxon et al. 2008). The biofilm allows the bacteria to survive under conditions that would be lethal to individual bacterial cells. The biofilm is resistant to phagocytosis and to antibiotic concentrations 10-1000 times higher than those that cause the inhibition and death of scattered bacteria (Davey and O'Toole 2000). Based on these features, infections caused by biofilm-producing bacteria are frequently chronic, recurrent and resistant to antibacterial chemotherapy (Jefferson et al. 2006). Sandal et al. showed that all *H. somni* strains tested (commensal as well as pathogenic) were able to produce a biofilm (Sandal et al. 2007). The structure of the biofilm significantly differs according to the pathogenicity of the strain. The biofilm produced by commensal strains was shown to be thinner and more sensitive and consists of a lower amount of extracellular matrix compared to pathogenic strains (Sandal et al. 2007).

The heat shock proteins (HSP) are highly evolutionarily conserved molecules that occur in the majority of organisms from *Archea* to *Eucaryota.* According to their molecular weight and sequence homology they are divided into different families, including HSP110, HSP90, HSP70, HSP60, HSP40 and low molecular HSP (sHSP). HSP are molecular chaperones (Smith and al. 1998) and play many other roles (Bolhassani and Rafati 2008). In the development of *E.coli* biofilm an important role is played by IbpA and IbpB proteins, which belong to the sHSP family (Kuczyńska-Wiśnik et al. 2010). In *Haemophilus influenzae* it was found that the biofilm contains heat shock protein 60 kDa (Hsp60). The presence of this protein within bacterial cells and in the extracellular matrix could indicate their important role in biofilm production (Gallaher et al. 2006).

Among other functions, Hsp60 binds partly folded intermediate products of translation, preventing their aggregation, facilitating their folding and achieving the final structure. It prevents denaturation of the proteins exposed to stressors such as high temperature (Tsan et al. 2009). Bacterial Hsp60 is highly immunogenic and induces antibody production and T lymphocyte activation (Tsan et al. 2004). Its activity is regulated by 10 kDa chaperonin (cpn 10 or GroES in *Escherichia coli*) (Fink 1999). Antibodies raised against bacterial Hsp60 react cross with Hsp60 of evolutionarily different organisms, such as mammals.

The aim of this study was to confirm the presence of Hsp60 in the biofilm produced by *Histophilus somni.* The influence of recombinant Hsp60-antibodies on biofilm production as well on Hsp60 expression in the biofilm was also evaluated.

Materials and Methods

Bacterial culture

Histophilus somni strain CAMP 6280 (Czech Collection of Microorganisms in Brno, Brno, Czech Republic) was grown on chocolate agar plates and incubated at 37°C in microaerophilic conditions (air with 5% CO_2) (Shell Lab, Sheldon Manufacturing INC., Cornelius, OR, USA), for 36 h. Harvested colonies were transferred to BHI broth supplemented with 0.1% thiamine and 0.01% Tris (BHI-TT) and incubated for 24 h under microaerophilic conditions, and then for 1 hour at 43°C. The *H. somni* culture was then diluted to 1:100 in BHI-broth (Sandal et al. 2007). 100 µl samples of the culture were then transferred, in triplicate, to a 96-well microplate and to 8-well plates (LabTek 8-well chambered Coverglass 1.0, *NUNC*, Roskilder, Denmark). The plates were incubated at 37°C under microaerophilic conditions for 48 h. The experiments were performed twice, with 10 repetitions.

Biofilm detection

The culture supernatant was removed and the microplates were gently washed using PBS containing 0.02% Tween 20 (PBS-T). 25 µl of 1% crystal violet was then added per well and incubated for 15 min. The wells were then washed three times with sterile distilled water and filled with 200 µl of 96% ethanol. The presence of a biofilm was spectrophotometrically confirmed at λ=630 nm (µQuant, Bio-Tek Instruments, Winooski, VT, USA) (Sandal et al. 2007). Wells filled with growth medium (BHI-TT) were used as the controls.

Immune sera

The animals were kept in a vivarium at the Institute of Immunology and Experimental Therapy Polish Academy of Science Wroclaw and the Veterinary Faculty Vivarium of Wroclaw University of Environmental and Life Sciences. The experimental procedure was accepted by the II Local Ethical Committee in Wroclaw (decision No. 38/2006, 27.02.2006 and No. 6/2007, 22.01.2007).

Mouse *H. somni* rHsp60 antibodies were produced in specific pathogen free (SPF) animals to minimize the presence of cross reacting antibodies induced by contact with bacteria. Six male individuals of BALB/CJ-H2, DBA/2J-H2, FVB/NJ-H2 and B6 SPF mice strains respectively were subcutaneously immunized with 25 µg of *H. somni* recombinant Hsp60 (rHsp60), and 0.1 ml of 0.9% NaCl containing rHsp 60 was emulsified with 0.1 ml of Freund's incomplete adjuvant (Sigma), immediately before the injection. The antigen was given four times at 2 week intervals. Twelve days after the last immunization the animals were anaesthetized with halothane and blood was obtained by extirpation of the eyeball. Blood was collected in tubes containing EDTA. The blood collected was then centrifuged (3000 × *g*/5 min at room temperature). After blood collection the mice were killed by cervical spine interruption. Goat Hsp60 IgG antibodies were affinity isolated from the hyperimmune serum of a goat immunized with *H. somni* rHsp60, using a 4B Sepharose column coupled with *H. somni* rHsp60. The SPF plasma of normal mice was obtained from four individuals of BALB/CJ-H2, DBA/2J-H2, FVB/NJ-H2 and B6 SPF mice respectively. The blood plasma was pooled and stored at -22°C, and goat rHsp60 antibodies were stored at -80°C until use.

Immunochemical detection of *Histophilus somni* Hsp60 in the biofilm

The culture supernatant in 8-well plates (LabTek 8-well chambered Coverglass 1.0, *NUNC*, Roskilder, Denmark) was removed and the plates were washed three times with PBS-T. Hyperimmune goat serum containing anti-*Histophilus somni* rHsp60 antibodies or pooled hyperimmune SPF mouse serum containing *H. somni* rHsp60-antibodies diluted at 1:10 and 1:100 in PBS-T, respectively, was then added and incubated for 1 h. The wells were then washed with PBS-T and rabbit anti-goat IgG HRP (Sigma 1:1000) or goat anti-mouse IgG HRP (Sigma 1:1000) conjugates were added, respectively, and incubated for 1 h at room temperature. After washing with PBS-T, the substrate reaction (0.1% diaminobenzidine in PBS) was developed for 20 min in the dark. The reaction was stopped by rinsing with water. The reaction was evaluated using a Nikon Eclipse 80i microscope (Nikon, Melville, NY, USA) and archived in the NIS-Elements AR 2.30 program.

The influence of rHsp60 antibodies on biofilm formation by *Histophilus somni*

The colonies of *H. somni* that were cultured on chocolate agar for 36 h were transferred to sterile glass tubes containing 10 ml of BHI-TT supplemented with 100 µl of pooled SPF mouse hyperimmune anti-*H. somni* rHsp60 plasma or 100 µl of PBS (control). The tubes were incubated under microaerophilic conditions for 24 h at 37°C and then 1 h at 43°C. The cultures were then diluted to 1:100 with BHI broth (Sandal et al. 2007), mixed, and 100 µl of each culture was transferred to a 96-well microplate and 8-well plates (LabTek 8-well chambered Coverglass 1.0, *NUNC*, Roskilder, Denmark) in triplicate. The plates were incubated for 48 h under the same conditions. The presence of a biofilm and *H. somni* Hsp60 within the biofilm were detected using the methods described. An *H. somni* culture with non-immunized SPF mouse pooled serum and culture in growth medium only were used for the control.

Statistical analysis

The morphometric data were subjected to statistical analysis using Statistica 10.0 software (StatSoft Polska Sp. z o.o., Kraków, Poland). The significance of the differences between the results was appraised using the Kruskal-Wallis test.

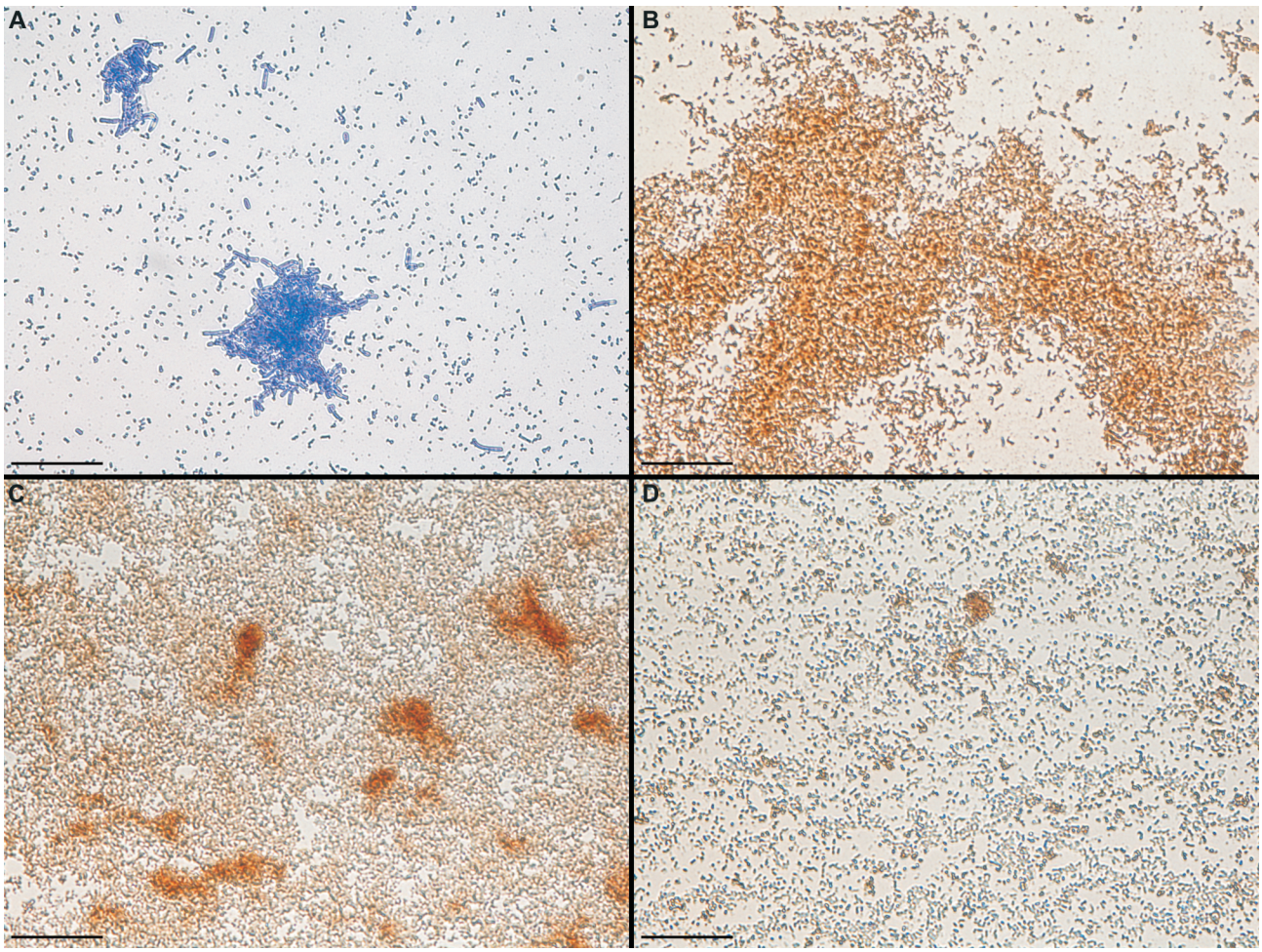

Fig. 1. A – *H. somni* biofilm (crystal violet staining); B – *H. somni* biofilm, immunochemical staining anti-Hsp60; C – *H. somni* biofilm, growth medium (BHI-TT) with addition of mouse SPF normal serum, immunochemical staining anti-Hsp60; D – *H. somni* biofilm, growth medium with mouse SPF hyperimmune *H.somni* rHsp60-antiserum, immunochemical staining anti-Hsp60; scale bar = 30 μm.

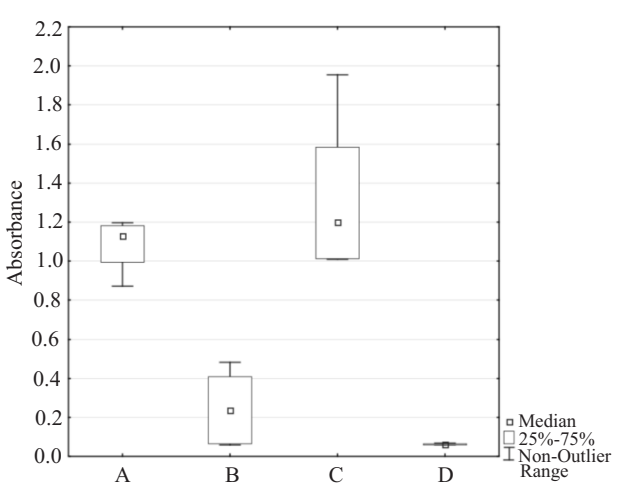

Fig. 2. Biofilm production by *H.somni* strain CAMP 6280 stained with cristal violet (absorbance at λ=630 nm): A – *H. somni* cultured with growth medium (BHI-TT) containing mouse normal SPF serum; B – *H. somni* cultured with growth medium (BHI-TT) containing mouse SPF hyperimmune *H.somni* rHsp60-antiserum; C – *H. somni* cultured in BHI-TT medium (control); D – growth medium (BHI-TT).

Results

Detection of *H. somni* biofilm

In vitro biofilm production by *H. somni* was confirmed using a spectrophotometer (λ=630 nm) and light microscope. The microplate wells containing the *H. somni* culture showed a significantly higher absorbance level (mean ± SD; 1.327 ± 0.374) than the wells containing growth medium (0.062 ± 0.004) ($p < 0.001$) (Fig. 2). The *H. somni* colonies that produced the extracellular matrix were seen under the light microscope (Fig. 1A).

Presence of Hsp60 in *Histophilus somni* biofilm

Using goat *H. somni* rHsp60-antibodies and mouse anti-*H. somni* rHsp60 hyperimmune plasma, the presence of Hsp60 in the *H. somni* biofilm was

confirmed (Fig. 1B). The continued presence of this protein within the *H. somni* biofilm structure was found under light microscopy. The staining was more intense in areas of intensive bacterial growth, which produced large amounts of extracellular matrix.

Influence of presence of rHsp60-antibodies in culture medium on growth and biofilm production by H. somni

Biofilm production by *H. somni* was evaluated by measuring the absorbance of *H. somni* cultures stained with crystal violet. The absorbance (λ=630 nm) of *H. somni* cultured with hyperimmune SPF mouse anti-rHsp60 plasma (0.249 ± 0.202) was significantly lower ($p < 0.01$) than that of the same strain cultured with the addition of normal SPF mouse plasma (1.084 ± 0.126), as well as that cultured in pure BHI-TT medium (1.327 ± 0.374). This indicates an inhibitory effect of rHsp60 antibodies in biofilm production (Fig. 2). Simultaneously, no differences were found between the absorbance of *H. somni* cultured in pure growth medium and *H. somni* grown in the same medium supplemented with normal SPF mouse plasma. This indicates that other factors present in the blood plasma had no inhibitory effect on *H. somni* growth and biofilm production. The absorbance of *H. somni* cultured with Hsp60 antibodies did not significantly differ from that in pure growth medium, which indicates strong inhibition of *H. somni* growth by these antibodies.

The cultures of *H. somni* grown with Hsp60 antibodies observed under light microscopy (Fig. 1D) presented numerous individual bacteria that did not form a compact biofilm structure. Rare, small clusters of bacteria formed a biofilm that was markedly weaker than that in the control cultures or in cultures grown with normal SPF mouse plasma (Fig. 1C). Large areas covered by biofilm and marked Hsp60 expression were observed in the cultures containing normal SPF mouse plasma.

Discussion

The biofilm produced by the bacteria allows them to survive conditions that would be lethal for individual or colony-forming organisms (Sandal et al. 2009). The ability to produce a biofilm has been indicated in many representatives of the *Pasteurellaceae* family, among them *H. somni*. This last one differs markedly from the others because it is able to produce a biofilm without being under specific conditions (Sandal et al. 2007). Biofilm production by *H. somni* has been indicated *in vivo*, and its presence could contribute to the high level of virulence of this bacterium (Sandal et al. 2009); eradicating this bacterium from carrier animals in the herd is particularly difficult, and infected animals have a high mortality rate (Stefaniak et al. 2008).

The biofilm produced *in vitro* by *H. somni* in the present study was similar to that described by Sandal et al. (2007). Using goat and mouse rHsp60-antibodies, the presence of Hsp60 was immunochemically confirmed within the structure of the biofilm. One significant aspect of the *H. somni* biofilm is the fact that the Hsp60 protein can be seen over its entire surface. The presence of Hsp60 was also detected in the biofilm produced *in vitro* by *Haemophilus influenzae* (Gallaher et al. 2006).

An inhibitory effect of rHsp60 antibodies on *in vitro H. somni* biofilm production was found. The binding of rHsp60 antibodies to *H. somni* Hsp60 probably caused the inhibitory effect on biofilm production by this bacterium. The inhibition of biofilm production was confirmed by the lower absorbance of cultures containing mouse anti-rHsp60 antibodies compared to cultures containing plasma-free medium and medium containing normal SPF mouse plasma. Further studies are needed to explain whether or not this inhibitory effect occurs *in vivo*. It could cause easier exposure of bacterial cells to antibiotics, as well as to the host immune system. This finding is interesting in terms of the prevention of *H. somni* infection in cattle herds. The early immunization of calves with Hsp60 and the active production of specific antibodies could disturb biofilm formation by *H. somni* and other organisms. The broad cross-reactivity of Hsp60 from one bacterial strain to another might enable the easier elimination of different pathogens from mucosal membranes, decreasing the rate of infection dissemination within a herd and lowering the number of carriers, amongst other beneficial effects; for example, decreasing resistance to antibiotics.

It is concluded that the biofilm produced by *Histophilus somni in vitro* contained Hsp60. Furthermore, the addition of *H. somni* rHsp60 antibodies to culture medium inhibited biofilm production by *H. somni*.

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