Research on the biofilm formation of *Staphylococcus pseudintermedius* clinical isolates from dogs and cats

Summary of Doctoral Thesis

Sayuri Arima

Graduate School of Veterinary Medicine and Life Science

Nippon Veterinary and Life Science University

Bacterial infection is the most common disease in the medical field, and it has been considered that biofilm may be involved as one of the causative agents. Biofilm is the state of forming colony in extracellular glycoprotein produced by bacteria that attached to the surface of tissue or medical equipment. It was suggested that biofilm is important for prevention of infection progression so that biofilm formation was associated with the chronicity and refractory infections. Staphylococci is listed as representative biofilm-forming bacteria. *Staphylococcus pseudintermedius* is also known as a major bacterium isolated as causative organism of various infections in small animal. Biofilm of *S. pseudintermedius* is not only few reports but also low recognition in veterinary medicine. Therefore, we investigated the following studies.

In Chapter 1, we identified 250 isolates of *S. pseudintermedius* obtained from clinical cases of dogs and cats at the Veterinary Medical Teaching Hospital of Nippon Veterinary and Life Science University (NVLU-VMTH) and general animal hospitals from January in 2012 to December in 2015 using polymerase chain reaction (PCR). After measured the ability of biofilm formation of the isolates in OD_{590nm} and classifying them, they were 62 strong biofilm producers (24.8 %), 130 moderate biofilm producers (52.0 %) and 58 weak biofilm producers (23.2 %). We compared the isolation rate of *S. pseudintermedius* in host species, and it was shown that the isolates derived from dogs were more than the isolates derived from cats conspicuously. However, comparing the ability of biofilm formation, no differences were found between the hosts. This suggested that biofilm infection caused by cat-derived *S. pseudintermedius* may have the same risk as dogs. On the other hand, when the ability of biofilm formation was compared with each lesion

sites, specific difference was not shown, so the association between lesion sites and forming biofilm could not be clarified. The isolation rate of *S. pseudintermedius* in general animal hospitals was higher than in NVLU-VMTH. But after classifying isolates according to the ability of biofilm formation, the remarkable difference was not found between these facilities. Therefore, the risk of the biofilm infection caused by *S. pseudintermedius* was proved to be not different regard of animal species, infection sites and medical facilities.

In Chapter 2, we investigated the effect of *S. pseudintermedius* biofilm formation ability isolated in Chapter 1 on antibiotic resistance. We compared the susceptibilities of ampicillin (ABPC), amoxicillin (AMPC), oxacillin (MPIPC), enrofloxacin (ERFX), orbifloxacin (OBFX), gentamicin (GM), erythromycin (EM), chloramphenicol (CP), vancomycin (VCM), cephalexin (CEX), cefovezin (CFV), and minocycline (MINO). The minimum inhibitory concentration (MIC) of each antibiotics was measured by the agar plate dilution method according to Clinical Laboratory Standard Institute (CLSI) guidelines, and the correlation between MIC and OD_{590nm} of each isolates were examined. As a result, positive correlations were significantly observed in ABPC, AMPC, MPIPC, CEX, CFV, ERFX, OBFX, EM, and GM, indicating that the strong biofilm producers were high resistant to these antibiotics. In addition, methicillin-resistant *S. pseudintermedius* (MRSP) and methicillin-sensitive *S. pseudintermedius* (MSSP) were identified by detection of *mecA* gene using PCR and antibiotic susceptibility test of MPIPC. As a result, MRSP and MSSP were isolated 145 (58.0 %) and 75 (30.0 %), respectively. Comparing MRSP to animal species, the cat-derived MRSP (81.8 %) surpassed the dog-derived MRSP (55.7 %). It was suggested that cats have a

high significance as MRSP reservoirs. Meanwhile, the ABPC resistant rate of MSSP was high (57.3 %). It was shown that ABPC resistance of *S. pseudintermedius* was increased independent of *mecA* gene. Moreover, antibiotic resistance tended to increase in proportion to the ability of biofilm formation. It was suggested that biofilm formation is related to antibiotic resistance of MSSP.

In Chapter 3, we examined pathogenicity of *S. pseudintermedius* biofilm. Biofilm has been suggested association with not only antibiotic resistance but also inflammatory response as a cause of chronic and refractory infections. To investigate the effect that *S. pseudintermedius* provide to mammalian cells, we focused on the metabolites produced in biofilm forming process and investigated inflammatory response and its attractants. The culture medium which formed biofilm was sterilized by filtration, and made biofilm-conditioned medium (BCM) for the experiments. BCMs prepared from weak and strong biofilm producers were cocultivated with RAW264.7 cells which is the cell line of mouse macrophage for 24 hours and measured expression of inflammatory cytokines using real-time RT-PCR. As a result, the expression levels of interleukin-1β (IL-1β) and tumor necrosis factor α (TNF-α) in BCM of strong biofilm producer were significantly increased than in that of weak biofilm producer ($P < 0.01$). These results suggest that strong biofilm-producing isolates of *S. pseudintermedius* enhance the inflammatory response compared with weak biofilm producers.

To identify the components secreted by *S. pseudintermedius* biofilms and analyse the induction of inflammatory reaction in RAW264.7 cells, BCM was treated with proteinase K, DNase, and RNase at 37 °C. When RAW264.7 cells were cultured with proteinase K-treated BCM, the expression of IL-1β and

IL-6 significantly decreased compared with that of RAW264.7 cells cultured in non-treated BCM ($P < 0.05$). No specific change in inflammatory cytokine expression level was observed for BCM treated with heat (95 °C for 10 min). As the results, it was suggested that the inflammatory attractants were heat-resistant extracellularly secreted proteins.

To elucidate a mechanism of the inflammatory reaction, we focused on the toll like receptor (TLR) of the macrophage and conducted confirmation of the expression of TLR signals including myeloid differentiation primary response 88 (MyD88), phospho interleukin-1 receptor associated kinase 4 (p-IRAK4), total IRAK4, phospho-nuclear factor kappa B (NFκB) p65 (p-p65), and total NFκB p65 by western blot analysis. The expression of both MyD88 and p-IRAK4 increased at 15 min after treatment. The expression of p-p65 significantly increased at 15 and 30 min after treatment. These results indicate that BCM from *S. pseudintermedius* biofilms induced the inflammatory response via stimulation of the TLR signaling pathway in RAW 264.7 cells. Moreover, in order to investigate which TLRs are remarkably expressed, the expression levels of TLR 1 to 9 were measured by real-time RT-PCR. Comparison of mRNA expression levels of TLRs for each reaction time with BCM showed a tendency to increase after 15 minutes in TLR 1, 2, 3, 4, 6, 7, 8 and 9. This suggested that the inflammatory attractant contained in BCM are involved in various TLRs.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI TOF-MS) were used to examine the difference in the extracellularly secreted proteins between weak and strong biofilm producers. In SDS-PAGE, specific bands were particularly detected at 35 kDa and 50

kDa in strong biofilm producers. In addition, three other bands at 39, 42, and 60 ~ 120 kDa were increased in strong biofilm producers. Whereas, by MALDI TOF-MS analysis, the peaks that was specific for strong biofilm producers were observed in 2,715 Da and 2,789 Da. From the above, it was revealed that there are differences in extracellularly secreted proteins due to the ability of biofilm formation. Future studies may be needed to reveal the protein and investigate the TLR-specific mechanism of action to elucidate defense mechanisms against biofilm-related disease.

The results obtained in this study are useful findings in the treatment of *S. pseudintermedius* infections in dogs and cats, and are considered to contribute to the prevention of biofilm-related infections in the veterinary medicine.