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Original article

In vitro antibacterial and antibiofilm effects of mupirocin spray against *Staphylococcus pseudintermedius*

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Abstract

Mupirocin is an effective antibiotic for infectious skin diseases. However, mupirocin is formulated as an ointment and is difficult to apply in canine systemic pyoderma. Therefore, many clinicians reformulate mupirocin off-label ointment into a spray. This study aimed to evaluate the antibacterial and antibiofilm effects of different concentrations of mupirocin spray (2%, 1%, and 0.5%) on *Staphylococcus pseudintermedius* over 21 days. Mupirocin spray was prepared by mixing mupirocin ointment and distilled water. The antibacterial effects were evaluated by measuring the optical density using broth microdilution assay and by live/dead staining. The antibiofilm activity of mupirocin spray was measured using a crystal violet staining method. All concentrations of mupirocin spray inhibited the growth of *S. pseudintermedius*. Mupirocin spray also inhibited biofilm formation of each isolate, although the degree of inhibition was influenced by the mupirocin concentration. The antibacterial and antibiofilm effects of mupirocin spray were maintained for 21 days. The 2% and 1% mupirocin sprays exhibited significantly better antibacterial and antibiofilm efficacy than the 0.5% mupirocin spray. Thus, 1-2% mupirocin spray may be effective for clinical use. Mupirocin spray is convenient and effective for the treatment of canine systemic pyoderma caused by *S. pseudintermedius* infection.

Keywords: antibacterial, antibiofilm, canine, mupirocin, spray, *Staphylococcus pseudintermedius*

Introduction

Canine pyoderma is one of the most common bacterial skin infections diagnosed in dogs (Lynch and Helbig 2021). It is a pyogenic cutaneous bacterial infection characterized by papules, pustules, and epidermal collarettes (Baeumer et al. 2017, Azzarity et al. 2022). The infection is also accompanied by pruritus, which can affect the quality of life of both patients and their owners. Canine pyoderma can vary from a moderate to severe infection and is triggered by underlying factors such as allergic skin disease, ectoparasites and endocrinopathies (Lynch and Helbig 2021). The disease may become chronic or recurrent if the primary underlying disease is not controlled (Bajwa 2016). The predominant pathogen that causes canine pyoderma is *Staphylococcus pseudintermedius* (Silva et al. 2021).

S. pseudintermedius is a gram-positive opportunistic pathogen frequently isolated from canine skin infections (Bannoehr and Guardabassi 2012). Almost 85% of canine skin, ear, and urinary tract infections test positive for *S. pseudintermedius* (Ruscher et al. 2009). *S. pseudintermedius* secretes immunomodulating virulence factors, expresses many adhesion factors and can produce biofilms (Singh at el. 2013, Stefanetti et al. 2017). Biofilms are formed by a complex community of microorganisms that attach to biological or nonbiological surfaces using adhesion factors and an extracellular polymeric matrix (Meroni et al. 2019, Rosman et al 2021). Biofilm formation is an important virulence factor that protects bacteria from the host immune system (Sritharadol et al 2018, Andrade et al 2022) and prevents antibiotics from penetrating the bacteria, resulting in antibiotic resistance (Stewart and Costerton 2001, Stewart 2002, Jamal et al. 2018). Consequently, biofilms make infections difficult to treat, leading to severe and persistent infections.

Canine pyoderma is treated with systemic antibiotics and topical therapy to rapidly resolve lesions and decrease the frequency and duration of antibiotic use. In addition, the use of topical treatments may reduce antibiotic resistance (Hillier et al. 2014). Mupirocin is an effective topical antibiotic for treating infectious skin diseases in dogs (Valentine 2019, Ganwar et al. 2021) with a high level of antibacterial activity against *Staphylococcus* spp., *Pseudomonas*, and *Streptococci* (Sanju et al 2015). However, due to its ointment formulation, mupirocin is challenging to apply effectively in canine pyoderma because of the spread of the infection across large areas of fur-covered skin. Therefore, many clinicians reformulate mupirocin ointment into a spray. Several studies have focused on the application of mupirocin spray in human infection (Allen 2019, Uren et al. 2009); however, the application of mupirocin spray in canine pyoderma has not been studied.

This study evaluated the antibacterial and antibiofilm effects of several concentrations of mupirocin spray (2%, 1%, and 0.5%) against *S. pseudintermedius* over 21 days and compares the effects of the spray across the studied concentrations.

Materials and Methods

Institutional animal care and use approval was not required for this *in vitro* study.

Mupirocin preparation

Mupirocin spray (2%, 1% and 0.5% concentration) was prepared by blending the appropriate amount of mupirocin ointment (20 mg/g) and distilled water (DW). The mixture was double boiled at 85°C to completely dissolve the mupirocin. DW was included as the control. The test solutions were stored at room temperature (21°C-23°C) without light protection for 21 days to simulate the conditions of clinical use.

Bacterial isolation

Six strains of *S. pseudintermedius* isolated from dogs with superficial pyoderma treated at Kyungpook National University animal clinics were used in this study. Clinical samples were cultivated on blood agar and incubated aerobically at 35°C-37°C for 24 h. *S. pseudintermedius* was identified using 16S rRNA gene sequencing and using 27F and 1492R primers. Sequences obtained from the isolates were compared with DNA sequences in the National Center for Biotechnology Information database.

Study design

Tests were conducted on days 0, 7, 14, and 21 after preparation of the mupirocin solution to evaluate the antibacterial and antibiofilm effects of mupirocin spray against *S. pseudintermedius*. A one-day difference in testing may have occurred depending on the time required for the tests. The antibacterial effects were determined using two methods: first, six strains of *S. pseudintermedius* were tested in triplicate using a broth microdilution assay and, second, three randomly selected strains were tested once using a live/dead assay. For antibiofilm testing, three strains were evaluated in triplicate using a crystal violet staining method.

Antibacterial effects 1: Broth microdilution assay

Each strain of *S. pseudintermedius* was incubated in tryptic soy broth (TSB; Kisanbio, Korea) at 35°C-37°C

for 24 hours. A total of 100 µL of bacterial suspension (0.5 McFarland) was inoculated by 5-fold dilution in a 96-well microtiter plate. The same volumes of the test solutions (2%, 1%, 0.5%, and DW) were added to each well. Medium without an antimicrobial agent was inoculated as a control. The plates were sealed and incubated at 35°C-37°C, and bacterial growth was determined at 0, 2, 4, 6, 8, and 10 h after inoculation by measuring the optical density (OD) at 595 nm.

Antibacterial effects 2: Live/dead cell staining

The live/dead bacterial viability of three randomly chosen isolates was measured using the LIVE/DEAD BacLight Bacterial Viability kit (L-7007, Invitrogen; Carlsbad, CA, USA). Bacteria were incubated in tryptic soy agar (BD Difco, ThermoFisher Scientific; Waltham, MA USA) at 35°C-37°C for 24 hours. The bacteria were collected from the plate using a platinum loop, and DW was added to make a suspension. After mixing and centrifuging the solution, the supernatants were removed. After resuspending the pellets, test solutions (2%, 1%, and 0.5%) or DW were added to the bacterial suspension followed by a dye mixture of SYTO 9 and propidium iodide (PI). The samples were incubated at room temperature in the dark for 15 minutes and observed with fluorescence microscopy.

Live bacterial cells with intact cell membranes were stained with SYTO 9, which appears as green fluorescence. Dead cells with compromised cell membranes were stained with PI, which infiltrates into the damaged cells, exhibiting red fluorescence. The excitation/emission maxima were approximately 450/490 nm for live and dead cells and 510/560 nm for only dead cells. Images were obtained using fluorescence microscopy of separate channels within the same field of view.

The numbers of live or dead cells in each image were quantified using ImageJ software (NIH freeware). The bactericidal percentage was calculated as the ratio of red fluorescence to the total fluorescence values (red + green fluorescence) (Zhou et al. 2011).

Antibiofilm effects

The crystal violet staining method was used to confirm the antibiofilm effects of mupirocin spray. *S. pseudintermedius* isolates were diluted with TSB to obtain a turbidity equivalent to that of a 0.5 McFarland standard. A bacterial suspension (100 µL) was added to each well of a 96-well microtiter plate. The same volumes of 2%, 1%, or 0.5% were added to the wells. The plate was incubated at 35°C-37°C for 24 hours. Positive (200 µL bacterial suspension) and negative (TSB) controls were also included. After incubation, the wells were washed three times with 250 µL of phosphate-buffered saline (Gibco, ThermoFisher Scientific) to remove nonadherent cells. Adherent biofilms were fixed with 200 µL of 99% methanol (Duksan Science, South Korea) for 15 minutes and dyed with 200 µL of 0.1% crystal violet (Sigma-Aldrich; St Louis, MO, USA) for 15 minutes at room temperature. The excess dye was rinsed with DW, and the microtiter plate was dried at room temperature. The dye bound to the biofilm was resolubilized with 160 µL of 33% acetic acid (Sigma-Aldrich) per well. Following resolubilization, the OD of each well was assessed at a wavelength of 570 nm (OD570). Each isolate was run in triplicate, and the average value was obtained.

Statistical analysis

The results of the broth microdilution and crystal violet staining assays are presented as mean ± standard deviations. Differences in bacterial growth and biofilm formation at different concentrations of mupirocin spray were evaluated using the Kruskal-Wallis test and using GraphPad Prism for Windows (GraphPad Software, La Jolla, CA, USA). Bonferroni corrections were used for post-hoc analyses. A p-value of ≤ 0.01 was considered statistically significant.

Results

Antibacterial effects 1: Broth microdilution assay

Staphylococcus pseudintermedius growth was inhibited on days 0, 7, 14, and 21 after production of mupirocin spray for all test solutions compared with the control group. Fig. 1 shows the antibacterial effects of mupirocin spray 10 hours after inoculation on the specified day. Both 2% and 1% mupirocin solutions showed significantly greater inhibition of bacterial growth than 0.5% ($p \leq 0.01$). However, the 2% and 1% solutions did not differ significantly from each other in terms of their inhibitory effects. The degree of antibacterial activity of each test solution is shown in Fig. 2.

Antibacterial effects 2: Live/dead cell staining

Three *S. pseudintermedius* isolates were evaluated using live/dead assay to confirm the antibacterial effects of mupirocin spray. Fluorescence microscopy images of *S. pseudintermedius* treated with the test solutions are shown in Fig. 3. Most cells were alive in the control group (DW). Table 1 shows the percentages of dead and live cells. Each concentration of mupirocin spray inhibited bacterial growth over 21 days, and 2% and 1% mupirocin solution inhibited bacterial growth to a greater extent than the 0.5% solution.

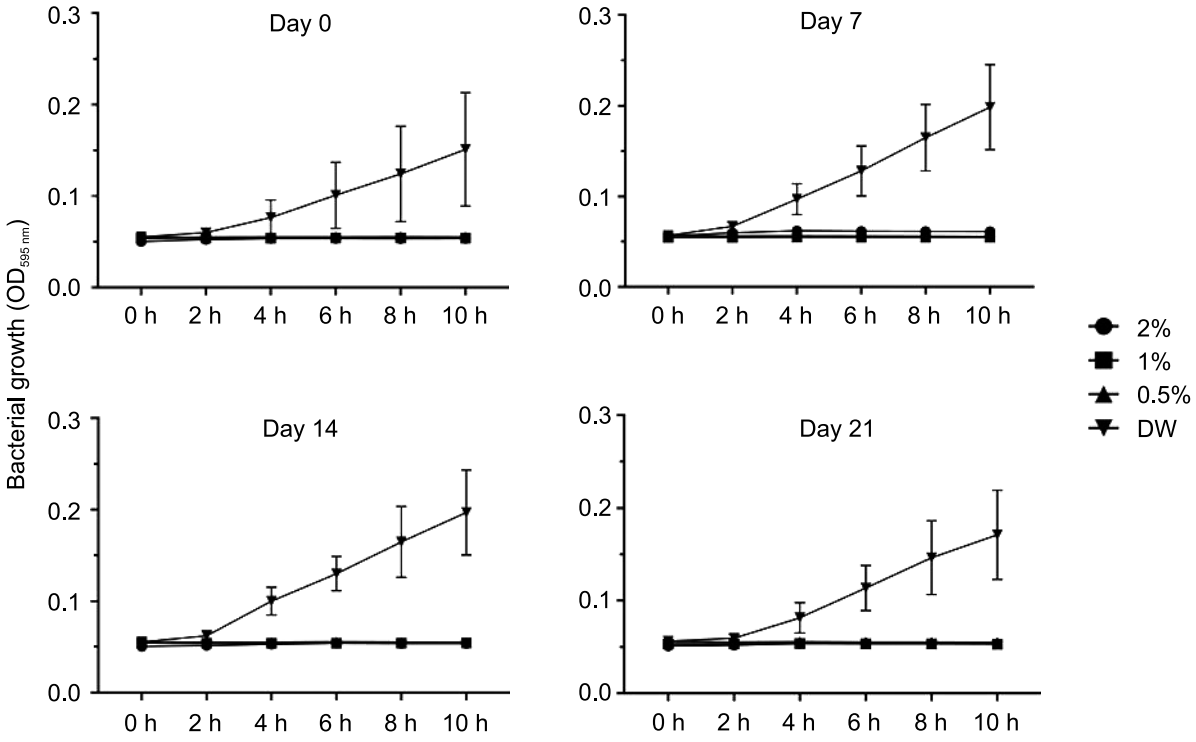

Fig. 1. Antibacterial effects of mupirocin spray against *Staphylococcus pseudintermedius* applied for 10 hours on day 0, 7, 14, 21.

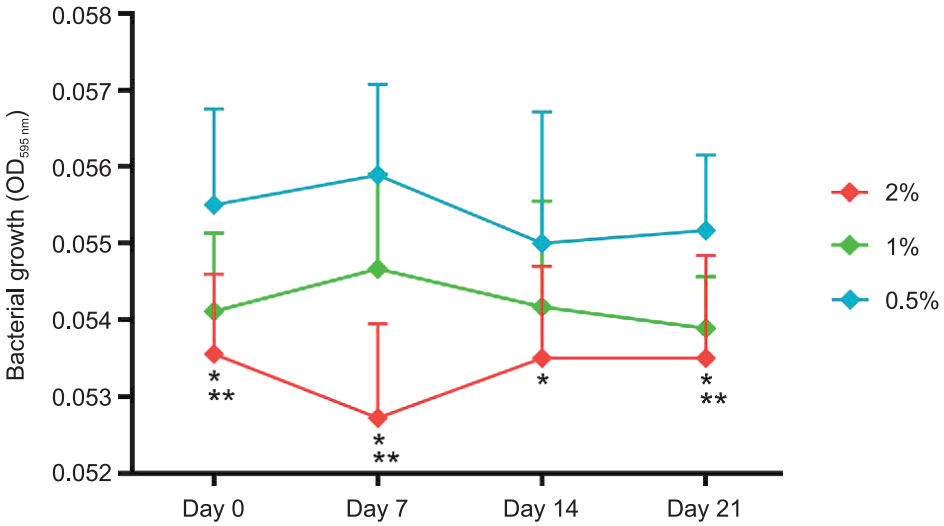

Fig. 2. Degree of antibacterial effects against *S. pseudintermedius* for each test solution on 10 hours.
* p≤0.01, indicating a significant difference of 2% mupirocin spray compared with 0.5%, **: p≤ 0.01, indicating a significant difference of 1% mupirocin spray compared with 0.5%.

Antibiofilm effects

Figure 4 shows the antibiofilm effects of mupirocin spray on days 0, 7, 14, and 21. The antibiofilm effects were maintained for 21 days. 2% and 1% mupirocin solutions exerted significantly stronger antibiofilm effects than the 0.5% solution (p≤0.01).

Discussion

Canine pyoderma is one of the most prevalent skin diseases in small animal clinics (Lynch and Helbig 2021). *S. pseudintermedius*, the primary pathogen responsible for canine pyoderma, can produce a biofilm that protects the bacteria from the host immune system, conferring antibiotic resistance (Sritharadol et al. 2018, Jamal et al. 2018). Previous studies have reported that 51%–96% of *S. pseudintermedius* strains isolated from dogs with canine pyoderma produced biofilm (Singh

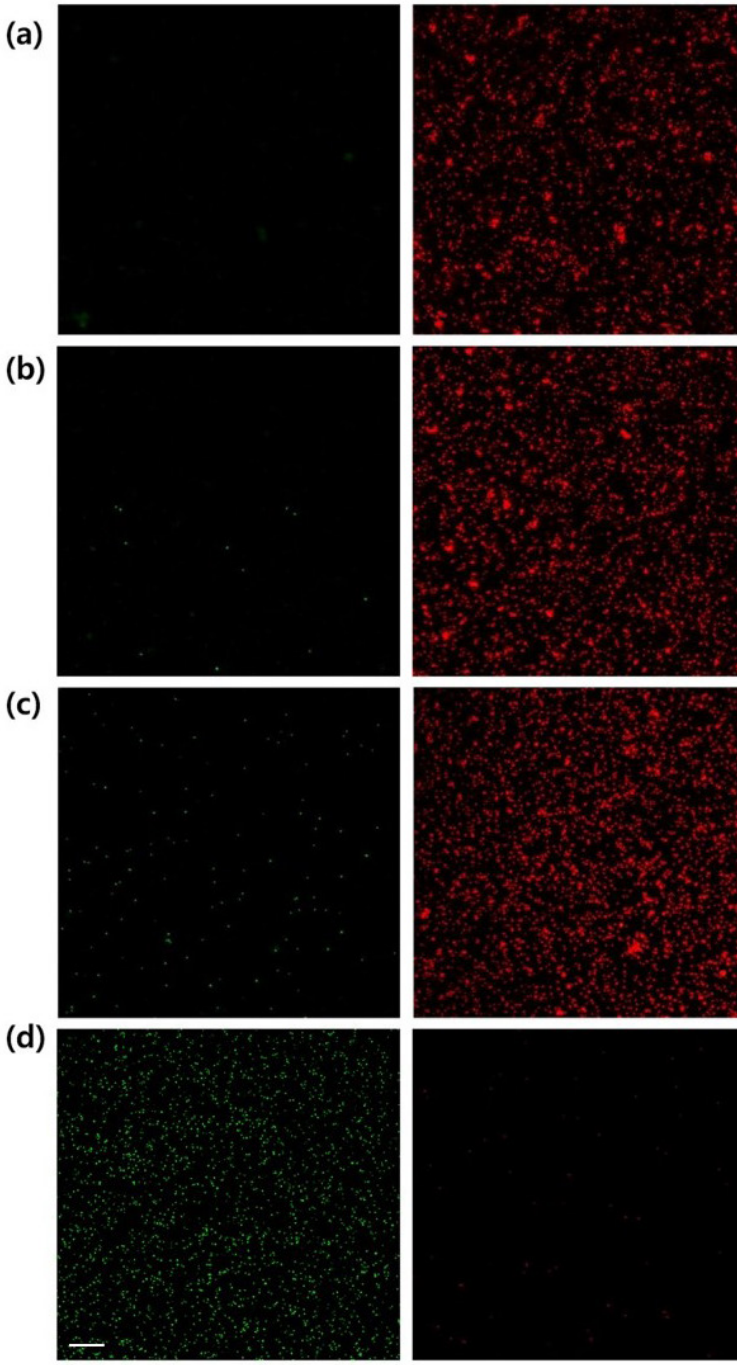

Fig. 3. Fluorescence microscopy images of *S. pseudintermedius* using Live/dead Bacterial Viability kit on day 21. The live cells are stained green (on the left side of each images) and the dead cells are stained red (on the right side of each images). (a): treated with 2%; (b): treated with 1%; (c): treated with 0.5%; (d): treated with DW (scale bar = 50 μm)

et al. 2013, Meroni et al. 2019, Andrade et al. 2022). Systemic antibiotics and topical therapy are generally prescribed to treat canine pyoderma (Summers et al. 2012). Topical treatments can rapidly resolve lesions through direct contact, decreasing the frequency and duration of antibiotic use and thereby minimizing systemic effects (Hillier et al. 2014). Mupirocin is an effective topical antibiotic for canine pyoderma (Valentine 2019). Mupirocin is typically formulated as an ointment, which causes discomfort when applied to lesions covered with fur. Therefore, most clinicians convert mupirocin to a spray for convenience. However, the effectiveness of mupirocin spray in canine pyoderma has not been established. Therefore, this study aimed to evaluate the antibacterial and antibiofilm effects of mupirocin spray (2%, 1%, and 0.5%) against *S. pseudintermedius* over 21 days and to evaluate the effective concentration of mupirocin spray.

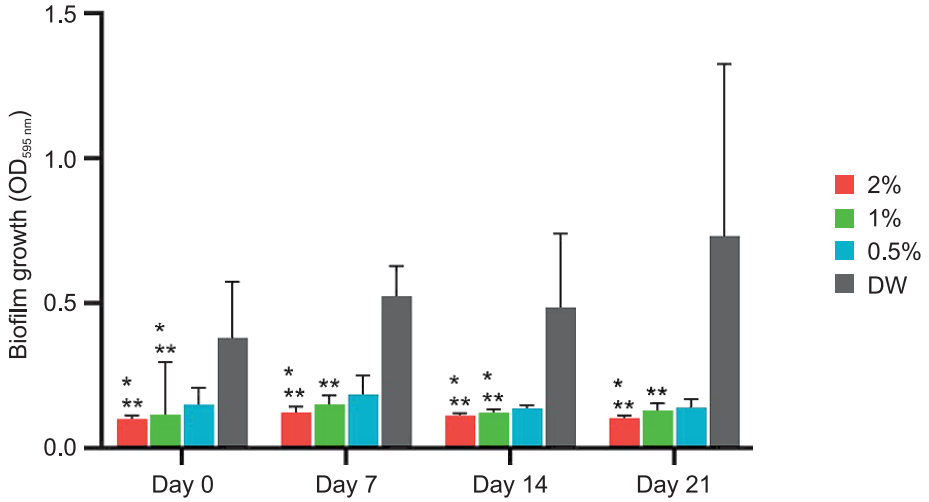

Fig. 4. Antibiofilm effects of mupirocin spray on day 0, 7, 14, 21.
* p≤0.01, indicating a significant difference compared with 0.5% mupirocin spray, **: p≤0.01, indicating a significant difference compared with the nontreatment group.

Table 1. Percentages of live and dead cells of *Staphylococcus pseudintermedius* applied with each test solution in fluorescence microscopy images (DW – Distilled Water).

	Day 0		Day 7		Day 14		Day 21	
	Live	Dead	Live	Dead	Live	Dead	Live	Dead
2%	0.086%	99.914%	0.192%	99.808%	0.043%	99.957%	0.039%	99.961%
1%	0.683%	99.317%	1.656%	98.344%	0.393%	99.607%	0.289%	99.711%
0.5%	10.27%	89.73%	12.658%	87.342%	7.114%	92.886%	8.535%	91.465%
DW	98.222%	1.778%	99.044%	0.956%	98.752%	1.248%	97.354%	2.646%

In a study by Khoshnood et al., topical mupirocin reduced *Staphylococcus aureus* biofilm mass by more than 90% (Ha et al. 2008, Sritharadol et al. 2018, Khoshnood et al. 2019). Furthermore, topical mupirocin reduced biofilm formation *in vitro* in *Pseudomonas aeruginosa* isolates (Ishikawa and Horii 2005). In this study, mupirocin exhibited antibacterial and antibiofilm effects against *S. pseudintermedius*. Thus, mupirocin is an effective treatment option for concurrent infection with various bacteria.

According to Bakkiyaraj et al., mupirocin spray exhibited similar antibacterial and antibiofilm activities as mupirocin ointment (Bakkiyaraj et al. 2017). A topical formulation of mupirocin spray was successfully developed, which could be used instead of the ointment formulation. Similarly, the present study demonstrated the antibacterial and antibiofilm effects of the spray formulation against *S. pseudintermedius*; the effects were maintained for at least 21 days.

The spray form of mupirocin has several advantages. In mupirocin spray, a humectant, such as glycerol, facilitates wound healing by preserving wound moisture and preventing drying of the mupirocin (Bakkiyaraj et al. 2017, Sritharadol et al. 2017). Polyethylene glycol in mupirocin ointment also acts as a humectant and keeps the wound moist. In addition, the spray formulation is more convenient compared with the ointment when the lesion is widespread, since it can be easily applied without the need for swabs or dressings (Bakkiyaraj et al. 2017). The spray can be applied to a wide area of the wound, acting as a film on the wound surface, thereby facilitating effective wound management. Thus, mupirocin spray may have good therapeutic effects on wounds and burns as well as canine pyoderma.

Mupirocin resistance was initially confirmed in human S. aureus and is currently observed in canine Staphylococcus spp. (Kizerwetter-Świda et al. 2019). For small animals, topical mupirocin is an excellent therapeutic option for treating infections caused by various bacteria; thus, mupirocin use in veterinary clinical practice is currently increasing (Valentine et al. 2012). As the frequency of mupirocin use increases, topical treatment should be prescribed only when necessary, and monitoring mupirocin resistance in various bacteria in companion animals is strongly recommended.

There are several limitations to this study. First, we used only a small number of samples without a reference strain to evaluate the antibacterial and antibiofilm effects of mupirocin. In addition, antibacterial and antibiofilm effects were assessed over 21 days only. However, we conducted our research using micro-

organisms collected directly from dogs with otitis externa who visited our veterinary teaching hospital. Therefore, further evaluation of mupirocin antibacterial and antibiofilm activity using a larger number of strains and a longer treatment period is needed to assess clinical significance in more detail.

To the best of our knowledge, this is the first study to describe the antibacterial and antibiofilm effects of mupirocin spray. This off-label formulation maintained efficacy for 21 days. In clinical practice, mupirocin spray at a concentration higher than 1% is recommended. Based on these findings, off-label mupirocin spray may be conveniently and effectively used to treat canine pyoderma caused by *S. pseudintermedius* infection.

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