

# Antibiofilm activity of silver nanoparticles against biofilm forming *Staphylococcus pseudintermedius* isolated from dogs with otitis externa

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**Background:** Silver nanoparticles (AgNPs) are known to possess antimicrobial properties. Although the antibiofilm activity of AgNPs has been demonstrated in humans, this activity has not yet been elucidated in veterinary medicine.

**Objectives:** The purpose of this study was to evaluate the antibiofilm activity of silver nanoparticles against *Staphylococcus pseudintermedius*.

**Methods:** Ten isolates of *S. pseudintermedius* obtained from dogs with otitis externa were treated with AgNPs, and the antibiofilm activity was measured using a modified microtiter plate and Congo red agar (CRA) method and scanning electron microscopy.

**Results:** AgNPs displayed a significant dose-dependent antibiofilm activity and reduced biofilm formation at concentrations of 20 and 10 µg/ml ( $p < 0.05$ ). *S. pseudintermedius* exposed to 20 µg/ml of AgNPs formed less bacterial slime compared to the controls on CRA plates. Scanning electron micrographs showed that the biofilm had few individually scattered cells along its surface when treated with AgNP concentrations of 20 and 10 µg/ml. Untreated surfaces showed an aggregated biofilm.

**Conclusions:** Our results suggested that AgNP may be a valuable alternative antibiofilm agent for canine otitis externa.

## KEYWORDS

antibiofilm, dog, otitis externa, silver nanoparticles, *Staphylococcus pseudintermedius*

## 1 | INTRODUCTION

Bacterial biofilms are bacterial communities embedded in a self-produced polymeric matrix (extracellular polymeric substance [EPS]) that adheres to surfaces and their interface (Costerton, 1999). This biofilm protects bacteria from immunological reactions of the hosts and from antimicrobial agents. Therefore, they are an important factor in the pathophysiology of diseases and resistance to treatment (Clutterbuck et al., 2007; Costerton, 1999). Biofilm formation by bacteria is studied *in vitro* by testing either in microtiter plates or on Congo red agar (CRA) (Freeman et al., 1989; Stepanović et al., 2000). Microtiter plates assess the ability to adhere to surfaces and

to grow in a biofilm layer. CRA detects the production of slime, a basic extracellular component of staphylococcal biofilms (Chaudhari et al., 2012). Scanning electron microscopy (SEM) can be used to observe the microstructure of biofilms.

The recent evolution of nanotechnology has accelerated the development of metal nanoparticles with antimicrobial properties. The most frequently used are silver nanoparticles (AgNPs), which are effective bactericidal agents and less toxic than silver ions (Bilberg et al., 2012; Greulich et al., 2012; Seil & Webster, 2012).

The efficacy of AgNPs, including antibiofilm activity, has been demonstrated against various bacteria in human medicine (Gurunathan et al., 2014; Martinez-Gutierrez et al., 2013). Many

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products containing AgNPs are now used in human medical care. Some products intended for dogs that contain AgNPs are also available, but little is known about their microbial activity against veterinary relevant pathogens (Bae et al., 2017). A single preliminary study of the antibiofilm activity of AgNPs on *S. pseudintermedius* isolated from dogs currently exists (Meroni et al., 2020).

*Staphylococcus pseudintermedius* is a member of the normal cutaneous microflora, colonizing up to 90% of dogs, and is also a major opportunistic pathogen responsible for a wide range of infections including otitis, and pyoderma in dogs (Ruscher et al., 2009). The majority of *S. pseudintermedius* were able to produce biofilm, and this is considered an important virulence factor in veterinary medicine (Meroni et al., 2019; Turk et al., 2013).

The objectives of this study were to evaluate AgNP inhibition of biofilm formation by clinical isolates of *S. pseudintermedius* obtained from dogs and to generate evidence for the use of AgNPs for dogs.

## 2 | MATERIALS AND METHODS

### 2.1 | Silver nanoparticles

Spherical AgNPs were obtained from NanoComposix (San Diego, USA). The concentration of AgNP was 1.08 mg/ml, and diameters was 10 nm. These AgNPs are sold as standard particles commissioned by the Organization for Economic Cooperation and Development (OECD) and will be referred to as OECD standard particles. Characterization data for these particles are available on the manufacturer's website (Nanocomposix Biopure). The AgNPs were diluted as required with a tryptic soy broth (TSB, Difco, Detroit, MI, USA) culture medium.

### 2.2 | Bacterial strains

Ten different strains of *S. pseudintermedius* were isolated from dogs that visited our animal hospital due to OE. Clinical samples were cultivated on blood agar (Kisanbio, Seoul, South Korea) and incubated aerobically at 37°C for 18–24 h. Colony morphology was used to identify suspected *S. pseudintermedius* that was then sub-cultured on tryptic soy agar (Difco, Detroit, MI, USA) for genus identification. A conventional Vitek system using the bioMerieux card was adjusted to identify bacteria. Fresh colonies were used to prepare an inoculum in 0.5% sterile saline, equivalent to a specified turbidity standard. Test cards were inoculated according to the protocol and transferred to the Reader/Incubator. All procedures were performed in accordance with the manufacturers' recommendations. *S. pseudintermedius* was confirmed using 16S rRNA gene sequencing with 27F and 1492R primers. The National Center for Biotechnology Information (NCBI) basic local alignment search tool was used to search the NCBI database for bacterial DNA nucleotide sequences matching the sequences of the microorganisms isolated. The antibiotic susceptibility testing for each strain was not conducted.

### 2.3 | Determination of biofilm formation

#### 2.3.1 | Microtiter plate method

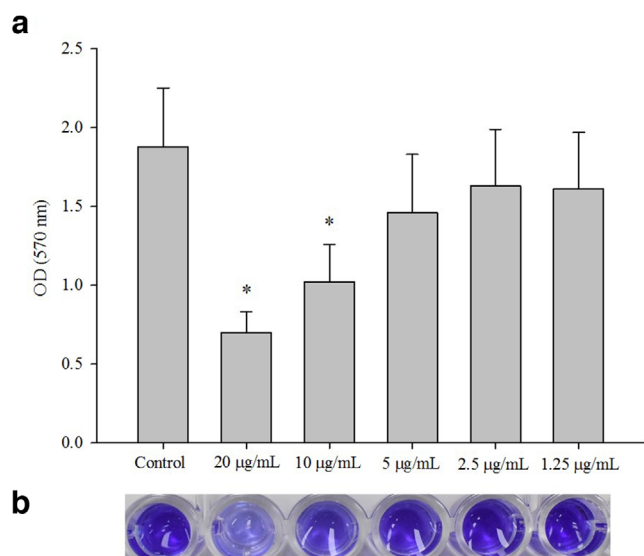
*S. pseudintermedius* isolates, grown for 18–24 h, were inoculated into 0.5 McFarland standard suspensions. AgNPs were added to obtain final concentrations of 20 µg/ml, 10 µg/ml, 5 µg/ml, 2.5 µg/ml, 1.25 µg/ml or 0 µg/ml. Bacterial cell concentrations were adjusted to 10<sup>5</sup> colony forming units per millilitre using the TSB. Aliquots of 200 µl bacteria were inoculated into flat-bottom, clear plastic cell culture plates with lids (SPL life science, Gyeonggi-do, South Korea) and incubated aerobically for 24 h at 37°C. Negative control wells contained TSB only. Following incubation, optical density (OD) at 540 nm was determined for each plate, and OD value was greater than 1 for all wells except negative controls. Liquid contents were discarded, and each well was washed three times with 250 µl sterile phosphate-buffered saline. Residual adherent bacteria were fixed with 200 µl of 99% methanol and stained using 150 µl of 1% crystal violet for 15 min at room temperature. Excess stain was rinsed away under running tap water. Plates were air-dried, and stained biofilms were resolubilised in 160 µl of 33% (v/v) glacial acetic acid. OD was measured with a micro ELISA automatic plate reader (Spectramax 250 Microplate reader, Molecular Devices, Sunnyvale, CA, USA) at 570 nm to quantify biofilm formation. Each experiment was conducted three times.

#### 2.3.2 | CRA method

Biofilm formation was screened using the CRA method, as previously described (Freeman et al., 1989). Depending on the degree of biofilm formation, black colonies with a dry crystalline consistency indicate biofilm production; weaker producers usually remained pink, although occasional darkening at the centre of colonies was observed. CRA was prepared using brain heart infusion agar (BHIA) supplemented with 5% sucrose and Congo red solution. Autoclaved Congo red solution was added to agar previously cooled to 55°C to final concentrations of 47 g/L BHIA, 50 g/L sucrose and 0.8 g/L Congo red stain. After 24 h incubation of strains with AgNPs at concentrations of 20 µg/ml, 10 µg/ml, 5 µg/ml, 2.5 µg/ml, 1.25 µg/ml or 0 µg/ml, strains were inoculated into AgNP-free CRA media and incubated aerobically for 24 h at 37°C. The colour of individual colonies was inspected after 24 h.

#### 2.3.3 | SEM

Two *S. pseudintermedius* strains used in the microtiter plates test were selected. Biofilms were grown on 18 × 18 mm glass coverslips for 24 h in the presence of 20 µg/ml, 10 µg/ml, 5 µg/ml, 2.5 µg/ml, 1.25 µg/ml or 0 µg/ml AgNPs. Each coverslip was stored in one well of a 6-well plate, and plates were sterilised by irradiation with ultraviolet light at least 48 h before incubation. Biofilms were washed twice with pre-warmed media to remove planktonic bacteria. Subsequent fixation, dehydration and drying methods were followed using previously



**FIGURE 1** Microtiter plates demonstrating the antibiofilm activity of silver nanoparticles (AgNPs). (a) Microtiter plate optical density (OD)<sub>570</sub> reading (mean  $\pm$  standard error) of biofilm formation with AgNPs added at concentrations of 0 µg/ml, 20 µg/ml, 10 µg/ml, 5 µg/ml, 2.5 µg/ml and 1.25 µg/ml. (b) Photographs of respective wells prior to OD measurement. (\* $p < 0.05$  compared with control group)

published protocols with minor modifications (Hazrin-Chong & Mane-field, 2012). Biofilms were fixed using 2.5% glutaraldehyde for 2.5 h in the phosphate-buffered saline and dehydrated with a graded series of ethanol concentrations (10 min each with 35%, 50%, 70%, 80%, 90%, 95%, 100%, 100% and 100%). Coverslips were dried using hexamethyl-disilane evaporation (50% and 100% for 10 min each) and left to air dry in a fume hood at 22°C for at least 48 h. The samples were metal coated by Pt and observed with a 5 keV electron beam at room temperature.

## 2.4 | Statistical analysis

Data from triplicate experiments are presented as mean  $\pm$  standard error. To verify the normality of the distributions, the Shapiro–Wilk

normality test was performed. Statistical analysis was performed using one-way analysis of variance (one-way ANOVA), multiple comparison versus control using SigmaPlot for Windows version 12.0 (Systat Software, San Jose, CA, USA). The value of statistical significance was set at  $p < 0.05$ .

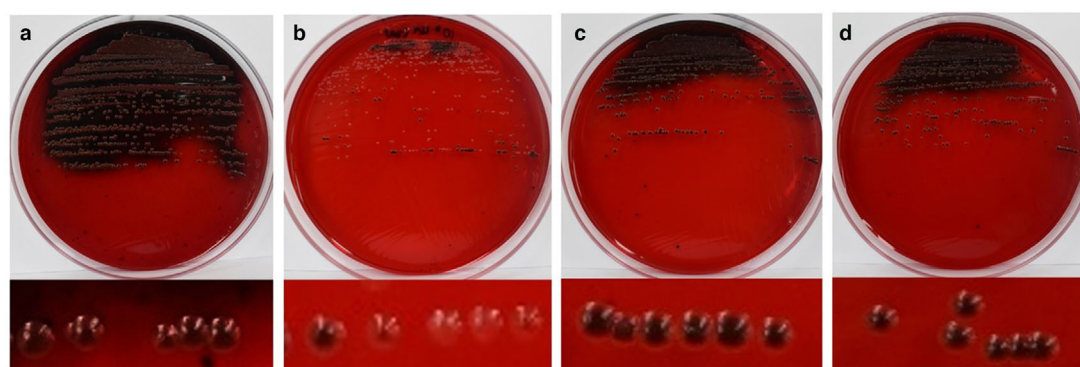
## 3 | RESULTS

### 3.1 | Antibiofilm activity of silver nanoparticles using a microtiter plate method

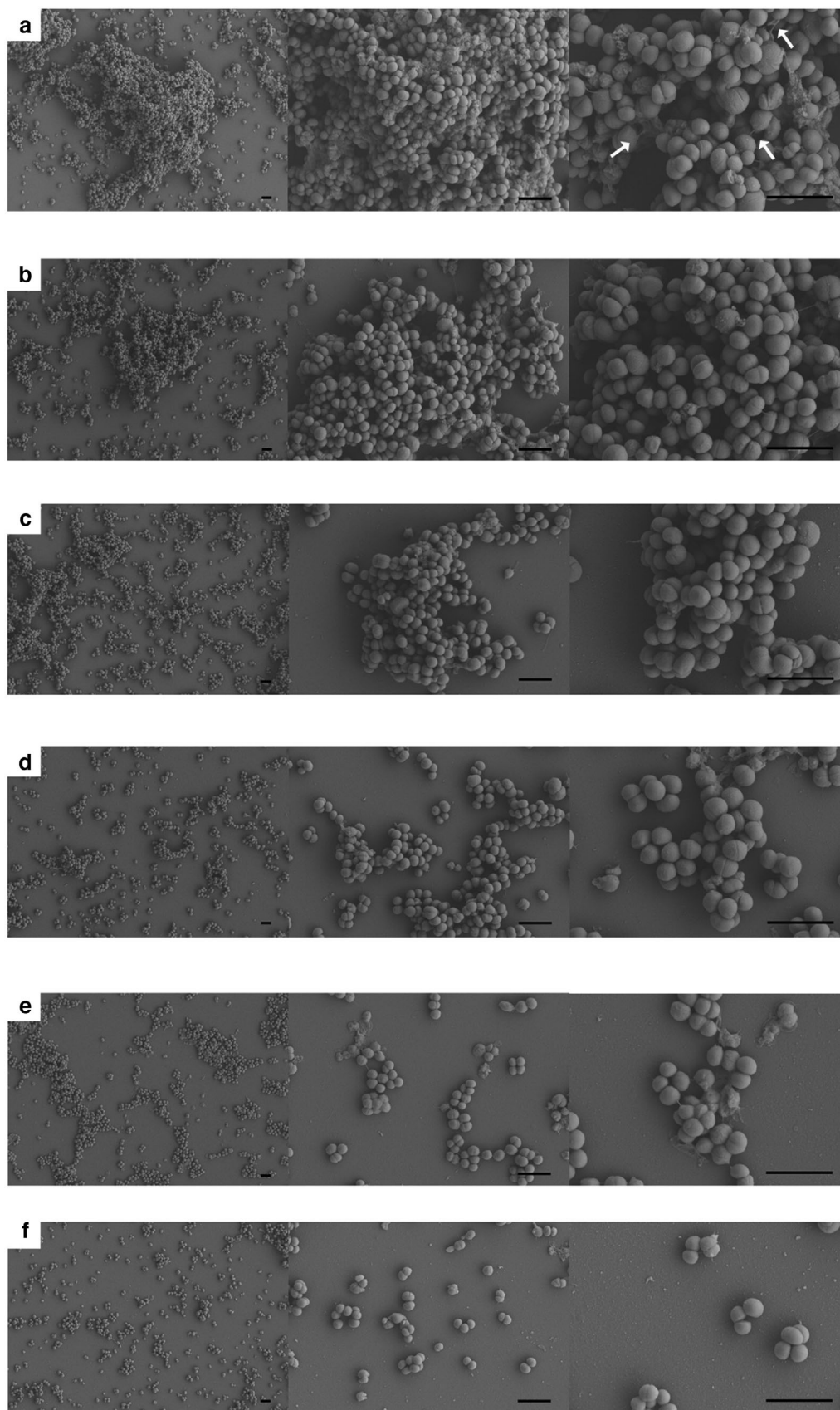
Antibiofilm activity of AgNPs was evaluated by measuring biofilm growth with crystal violet in the presence of varying concentrations of AgNPs. AgNPs successfully reduced biofilm formation at concentrations of 20 µg/ml and 10 µg/ml ( $p = 0.004$  and  $0.045$ ). AgNPs showed significant dose-dependent antibiofilm activity against *S. pseudintermedius* (Figure 1a). The positive control without AgNPs exhibited growth at an OD of 1.88. Notable inhibition of colony growth would decrease OD. Bacterial growth was mildly inhibited by the addition of 1.25 µg/ml AgNPs (OD of 1.61) and was decreased further with the addition of increasing AgNP concentrations (OD of 0.7 at 20 µg/ml).

### 3.2 | Silver nanoparticles impede biofilm formation on CRA plates

Congo red staining was used to further assess the antibiofilm activity of AgNPs. The Congo red assay measures biofilm formation in BHIA by staining biofilm exopolysaccharides; black colonies with a dry crystalline appearance indicate that bacteria have produced exopolysaccharides and formed biofilms. Bacteria cultured without AgNPs formed black colonies (Figure 2a), as did bacteria grown on plates treated with 1.25 µg/ml, 2.5 µg/ml, 5 µg/ml and 10 µg/ml of AgNPs (Figure 2c). Bacteria treated with 20 µg/ml of AgNPs continued to grow for 24 h and formed both red and small black colonies. Colonies red in colour indicate that AgNPs have impeded biofilm formation, although bacterial viability is not affected (Figure 2b).



**FIGURE 2** Congo red agar plates (CRA) demonstrating the antibiofilm activity of silver nanoparticles (AgNPs). (a–d) Photographs of CRA plates without AgNPs colonized by planktonic bacteria previously treated with AgNPs for 36 h, with close-ups of representative colonies in the lower panel. (a) Positive control without AgNPs, (b) 20 µg/ml AgNPs, (c) 10 µg/ml AgNPs and (d) 5 µg/ml AgNPs



**FIGURE 3** Antibiofilm activity of silver nanoparticles (AgNPs) as observed by scanning electron microscopy (SEM). SEM micrographs of *S. pseudintermedius* biofilm grown on glass coverslips after 24 h of incubation (magnification of panels from the left: 1500 $\times$ , 5000 $\times$ , 10000 $\times$ ). White arrows indicate likely extracellular polymeric substance (EPS) strands. (a) Positive control without AgNPs, (b) 1.25  $\mu\text{g/ml}$  AgNPs, (c) 2.5  $\mu\text{g/ml}$  AgNPs, (d) 5  $\mu\text{g/ml}$  AgNPs, (e) 10  $\mu\text{g/ml}$  AgNPs and (f) 20  $\mu\text{g/ml}$  AgNPs



### 3.3 | Observation of the antibiofilm activity of silver nanoparticles by SEM

SEM was used to characterize the morphology of biofilms treated with AgNPs. *S. pseudintermedius* biofilms grown on glass coverslips for 24 h consisted of aggregated and clumped bacterial cells, as well as EPS strands (Figure 3a). Addition of AgNPs reduced the size of biofilm clusters in a dose-dependent manner (Figures 3b–3f). At the highest concentration of 20 µg/ml, AgNPs almost completely inhibited bacterial clustering and assembly of EPS matrices (Figure 3f). Clustered biofilm was visible when bacteria were exposed to low AgNP concentrations (1.25 µg/ml and 2.5 µg/ml), although the biofilm remained smaller and had lesser EPS than the untreated control (Figures 3b and 3c).

## 4 | DISCUSSION

Many reports on antibiofilm activity of AgNPs against human bacterial pathogens are available, including *Pseudomonas aeruginosa*, *Escherichia coli* and *S. epidermis* (Ansari et al., 2013; Kalishwaralal et al., 2010), highlighting their potential as alternative antibiofilm treatments (Markowska et al., 2013). The size and shape of the AgNPs influence the activity of the nanoparticles, because smaller sizes increase the surface contact area of AgNPs with microorganisms. This experiment used a spherical AgNP with a diameter of 10 nm. Similar previous studies have been conducted. Meroni et al analyzed the antibiofilm abilities of biological-derived AgNPs with a size of 11 nm. The abilities against *S. pseudintermedius* were not identified. In this study, we verified the antibiofilm abilities of AgNPs at various concentration compared with a study reported previously (Meroni et al., 2020).

AgNPs act by increasing bacterial membrane permeability; by inducing oxidative damage via reactive oxygen species; by interacting with phosphate residues in DNA, and membrane and intracellular proteins (Kim et al., 2007; Morones et al., 2005; Sondi & Salopek-Sondi, 2004); and by quenching bacterial quorum sensing by an unknown mechanism, thus blocking signals for biofilm formation (Chaudhari et al., 2012). *S. pseudintermedius* exposed to 20 µg/ml of AgNPs for 24 h was inoculated in CRA plates without AgNP. Bacteria formed red colonies compared to controls. This difference in colour is due to the amount of slime stain, indicating that exposure to AgNPs causes a decrease in EPS produced by *S. pseudintermedius*. This finding is consistent with recent work by Ansari et al. using 20 µg/ml AgNPs against gram-negative bacterial biofilm formation. This can be assumed that during the time exposed to AgNP, the particles damaged the bacterial EPS production ability. Previous studies have shown that AgNP inhibits the EPS production of other bacteria (Siddique et al., 2020).

Otitis externa is an acute or chronic inflammation of the external ear. Bacterial infection, as secondary cause of otitis, is a common complication in otitis externa with primary causes (e.g., adverse food reaction, atopic dermatitis) that mostly require lifelong treatment (Nuttall, 2016). Recurrent infection and inflammation can lead to selection for

antimicrobial resistance which increases the difficulty of treatment. Biofilms inhibit cleaning, prevent antimicrobial penetration and form a protective environment surrounded by EPS (Devriese et al., 2005). The use of AgNPs may circumvent these problems and avoid resistance even during long-term use a critical consideration given the emergence of multidrug-resistant bacteria.

The toxicity of AgNPs is known to depend on its concentration, size, shape and surface (Dos Santos et al., 2014). In dermatology, AgNPs induce cytotoxicity in keratinocytes *in vitro*, and AgNPs are deposited in or on top of the stratum corneum *in vivo* (Samberg et al., 2010). In contrast, at high concentrations, no acute dermal toxicity was observed (Kim et al., 2013). Compared to previous studies that evaluated the toxicity of 10 nm nanosphere, the concentration used in this experiment was relatively very low. Like other topical agents with potential ototoxicity (e.g., polymyxin, chlorhexidine, acetic acid etc.), dogs with intact tympanum will be candidates to evaluate the toxicity of AgNP as a topical agent for otitis externa.

However, our study had several limitations. First, 10 strains are not sufficient to represent the *S. pseudintermedius* bacterial species. Second, these surface conditions are quite different from the *in vivo* conditions of OE in a clinical setting, and more work is needed to clarify the clinical effectiveness. In addition, this experiment confirmed the inhibition of the formation of early biofilm for 24 h, but did not evaluate the eradication and suppression of the mature biofilm.

AgNPs may be ideal supplements for contemporary treatment for OE. Currently, the most effective treatment of canine OE is the topical application of a range of antimicrobials and anti-inflammatory agents. AgNPs potentially display both properties (Jain & Agarwal, 2009; Tian et al., 2007), and a combination of AgNPs with a suitable antibiotic may effectively combat biofilms. AgNPs have previously been found to be synergistic with conventional antibiotics including polymyxin B, ampicillin and gentamycin (Fayaz et al., 2010; Hwang et al., 2012; Ruden et al., 2009).

## 5 | CONCLUSION

AgNPs showed significant dose-dependent antibiofilm activity against cultured *S. pseudintermedius*. Since these conditions are quite different from the *in vitro* conditions of OE in a clinical case, more work is needed to clarify the clinical effectiveness, stability in solution and toxicity of AgNPs before clinical use. Nevertheless, our results indicate that silver nanoparticles may become valuable antibiofilm agents for canine OE.

### CONFLICT OF INTEREST

The authors declare that they have no competing interests.

### AUTHOR CONTRIBUTIONS

Conceptualization, formal analysis, investigation, methodology, resources, validation, visualization and writing - original draft preparation: Mimi Seo. Funding acquisition, resources and project administration: Taeho Oh.

Conceptualization, data curation, methodology, project administration, supervision and writing - review and editing: Seulgi Bae.

## ETHICS STATEMENT

The protocol of collecting *S. pseudintermedius* isolates from canine patients was carried out with the consent of the owner. The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. No ethical approval was required as this is an original article with *in vitro* experiments, and no experimental animal models were used in the study.

## PEER REVIEW

The peer review history for this article is available at <https://publons.com/publon/10.1002/vms3.554>.

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**How to cite this article:** Seo, M., Oh, T., & Bae, S. (2021). Antibiofilm activity of silver nanoparticles against biofilm forming *Staphylococcus pseudintermedius* isolated from dogs with otitis externa. *Veterinary Medicine and Science*, *7*, 1551–1557. <https://doi.org/10.1002/vms3.554>