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Combined antimicrobial effect of two peptide nucleic acids against *Staphylococcus aureus* and *S. pseudintermedius* veterinary isolates

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ABSTRACT

Background: *Staphylococcus aureus* and *S. pseudintermedius* are the major etiological agents of staphylococcal infections in humans, livestock, and companion animals. The misuse of antimicrobial drugs has led to the emergence of antimicrobial-resistant *Staphylococcus* spp., including methicillin-resistant *S. aureus* (MRSA) and methicillin-resistant *S. pseudintermedius* (MRSP). One novel therapeutic approach against MRSA and MRSP is a peptide nucleic acid (PNA) that can bind to the target nucleotide strands and block expression. Previously, two PNAs conjugated with cell-penetrating peptides (P-PNAs), antisense PNA (ASP)-cmk and ASP-deoD, targeting two essential genes in *S. aureus*, were constructed, and their antibacterial activities were analyzed.
Objectives: This study analyzed the combined antibacterial effects of P-PNAs on *S. aureus* and *S. pseudintermedius* clinical isolates.
Methods: *S. aureus* ATCC 29740 cells were treated simultaneously with serially diluted ASP-cmk and ASP-deoD, and the minimal inhibitory concentrations (MICs) were measured. The combined P-PNA mixture was then treated with *S. aureus* and *S. pseudintermedius* veterinary isolates at the determined MIC, and the antibacterial effect was examined.
Results: The combined treatment of two P-PNAs showed higher antibacterial activity than the individual treatments. The MICs of two individual P-PNAs were 20 and 25 μM, whereas that of the combined treatment was 10 μM. The application of a combined treatment to clinical *Staphylococcus* spp. revealed *S. aureus* isolates to be resistant to P-PNAs and *S. pseudintermedius* isolates to be susceptible.
Conclusions: These observations highlight the complexity of designing ASPs with high efficacy for potential applications in treating staphylococcal infections in humans and animals.

Keywords: *Staphylococcus aureus*; *Staphylococcus pseudintermedius*; peptide nucleic acid

INTRODUCTION

Staphylococcal infections are some of the common zoonotic diseases found in human and companion animal populations. Many human, livestock, and pet diseases are caused by *Staphylococcus aureus* and *S. pseudintermedius*. Both species are frequently isolated from

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livestock and are found in human communities and hospitals, spreading via direct contact with contracted hosts or contaminated objects [1,2]. The prevalence, adaptability, and elicitation of antimicrobial resistance threaten public and animal healthcare. In particular, the emergence of methicillin-resistant *S. aureus* (MRSA) and methicillin-resistant *S. pseudintermedius* (MRSP) has become a challenge in the medical field because these "superbugs" restrict the spectrum of available antimicrobial drugs [3]. Furthermore, the uncontrolled prescription and misuse of antimicrobial drugs exacerbate the emergence of multidrug-resistant (MDR) MRSA and MRSP [4]. Such incidences may expedite the spread of antibiotic resistance genes via horizontal gene transfer (HGT) [5], and there are cases where HGTs have occurred between *Staphylococcus* spp., including MRSA and MRSP [6,7]. This is alarming from a veterinary perspective because the number of companion animal owners has increased over the years, and the increased interaction between companion animals and their owners may increase the exposure risk to pathogenic *Staphylococcus* spp. and the rate of HGT events between pathogens.

Combined efforts to develop novel antimicrobials have been made in response to the health threats of antimicrobial-resistant *Staphylococcus* spp. One of the novel agents is the peptide nucleic acid (PNA), a synthetic DNA mimic that can be designed to form a triplex with mRNA or DNA strands. Antisense binding of PNA to the target nucleic acids silences their transcription or translation, preventing their expression and impeding their biological functions [8,9]. PNAs have been assessed as alternatives to antimicrobials designed to target essential genes [10,11]. PNAs are often conjugated with a cell-penetrating peptide (CPP), such as the KFF motif peptide and its variants, for efficient delivery across the bacterial membrane [12,13]. Several studies have developed PNAs to inhibit the expression of the essential genes in *S. aureus* and *S. pseudintermedius* [14,15]. Previously, two antisense CPP-conjugated PNAs (P-PNAs) were designed, antisense PNA (ASP)-cmk and ASP-deoD, targeting two essential genes in *S. aureus*: a cytidylate kinase *cmk* and a purine nucleoside phosphorylase *deoD* [16]. Both genes are involved in nucleotide metabolism, which is linked directly to bacterial homeostasis. Disrupting their expression led to cell death [17]. Both P-PNAs showed *in vitro* and *in vivo* antibacterial activity towards *S. aureus* Rosenbach ATCC 29740 (also known as Newbould 305) originally isolated from a cow with bovine mastitis [18]. Based on these observations, it was speculated that combining both P-PNAs would enhance the overall antibacterial activity and exert similar effects on *Staphylococcus* clinical isolates. This study examined the synergistic antibacterial effects of two P-PNAs when treated simultaneously and assessed their antibacterial activity towards *S. aureus* and *S. pseudintermedius* clinical isolates collected from veterinary origins: pigs, chickens, and dogs.

MATERIALS AND METHODS

Bacterial strains and culture conditions
S. aureus ATCC 29740 was used as the reference strain [18]. **Table 1** lists the *S. aureus* and *S. pseudintermedius* veterinary isolates. Twenty *S. aureus* isolates and 10 *S. pseudintermedius* isolates were collected from various animal sources: *S. aureus* from either chickens or pigs and *S. pseudintermedius* from dogs. All isolates were streaked on tryptic soy agar (TSA) plates (Difco) and grown at 37°C for 19 h before preparing the McFarland standards.

Table 1. Bacterial strains used in this study

Strains	Description (Source)	References
ATCC 29740	*S. aureus* reference strain (bovine mastitis)	ATCC
PG01	*S. aureus* isolate (pig feces)	This study
PG02	*S. aureus* isolate (pig feces)	This study
PG03	*S. aureus* isolate (pig feces)	This study
PG04	*S. aureus* isolate (pig feces)	This study
PG05	*S. aureus* isolate (pig feces)	This study
PG06	*S. aureus* isolate (pig feces)	This study
PG07	*S. aureus* isolate (pig feces)	This study
PG08	*S. aureus* isolate (pig feces)	This study
PG09	*S. aureus* isolate (pig feces)	This study
PG10	*S. aureus* isolate (pig feces)	This study
CK01	*S. aureus* isolate (chicken feces)	This study
CK02	*S. aureus* isolate (chicken feces)	This study
CK03	*S. aureus* isolate (chicken feces)	This study
CK04	*S. aureus* isolate (chicken feces)	This study
CK05	*S. aureus* isolate (chicken feces)	This study
CK06	*S. aureus* isolate (chicken feces)	This study
CK07	*S. aureus* isolate (chicken feces)	This study
CK08	*S. aureus* isolate (chicken feces)	This study
CK09	*S. aureus* isolate (chicken feces)	This study
CK10	*S. aureus* isolate (chicken feces)	This study
DG01	*S. pseudintermedius* isolate (canine wound swab)	This study
DG02	*S. pseudintermedius* isolate (canine wound swab)	This study
DG03	*S. pseudintermedius* isolate (canine wound swab)	This study
DG04	*S. pseudintermedius* isolate (canine wound swab)	This study
DG05	*S. pseudintermedius* isolate (canine wound swab)	This study
DG06	*S. pseudintermedius* isolate (canine wound swab)	This study
DG07	*S. pseudintermedius* isolate (canine wound swab)	This study
DG08	*S. pseudintermedius* isolate (canine wound swab)	This study
DG09	*S. pseudintermedius* isolate (canine wound swab)	This study
DG10	*S. pseudintermedius* isolate (canine wound swab)	This study

PNA design and synthesis

The PNA oligomers were designed to bind the translation initiation regions (TIRs) of *cmk* and *deoD* of *S. aureus* [16]. All P-PNAs were synthesized, purified, and conjugated with the (KFF)$_3$K-L bacterial penetration peptide at PANAGENE, Inc. (Korea). The synthesized P-PNAs were dissolved in distilled water to a final concentration of 1 mM. The combined P-PNA mixture was prepared by mixing both P-PNAs 1:1 to a final concentration of 0.5 mM.

Determination of minimal inhibitory concentration (MIC) of PNAs against *S. aureus* ATCC 29740

The bacterial inoculum was prepared by adjusting the turbidity of the isolates in 0.9% (w/v) saline to a 0.5 McFarland standard. The bacterial colonies from a streaked TSA plate were resuspended in 0.9% (w/v) saline, and the turbidity was measured and adjusted using a nephelometer. The adjusted suspension (10 µL) was then transferred to 10 ml Mueller Hinton (MH) broth.

Microdilution assays were performed on a 96-well plate to determine the MICs of P-PNAs used in this work. The bacterial inoculum was aliquoted to wells with the following volumes: the initial two wells, 100 µL; the other wells, 50 µL. The initial wells were treated with the P-PNAs to a final concentration of 100 or 80 µM. The P-PNA-treated suspensions were then diluted to 1:2 until 3.125 or 2.5 µM. The plate was then sealed and incubated at 37°C. After 4- and 19-h incubation, 5 µL of the bacterial suspensions were spotted on MH agar plates and further incubated at 37°C for 19 h to determine the cell viability.

Combined antimicrobial effect of two P-PNAs against *S. aureus* and *S. pseudintermedius* clinical isolates

The *S. aureus* and *S. pseudintermedius* veterinary isolates were precultured in MH broth at 37°C for 19 h and diluted with fresh MH broth to obtain a bacterial concentration of 5.0×10^4 colony-forming units/mL. The P-PNA mixture (40 µM) was then added to the bacterial diluents, incubated at 37°C for 4 h, and placed in ice to stop bacterial growth. For the spot assay, five microliters of each grown culture were dropped onto MH agar plates and incubated at 37°C for 19 h.

RESULTS

Synergistic antibacterial effect of the combined P-PNA treatment on S. aureus ATCC 29740

The antibacterial effects of ASP-cmk, ASP-deoD, and the combined P-PNA mixture against *S. aureus* ATCC 29740 were examined by treating 40 µM of P-PNAs, as determined by the previous study [16], and ATCC 29740 cells were incubated for 4 and 8 h. As shown in **Fig. 1A**, the growth of ATCC 29740 treated with P-PNAs was inhibited. The samples treated with either ASP-cmk or ASP-deoD were less susceptible to growth inhibition after 8-h incubation than those treated with the combined P-PNA mixture. The total number of viable cells was also reduced by the combined treatment, followed by the ASP-cmk and ASP-deoD (**Fig. 1B**). These results validated the antibacterial effects of P-PNAs designed in previous research and showed that the combined treatment with P-PNAs exerts a stronger bactericidal effect.

The MICs of the tested P-PNAs were also determined by microdilution assays. The survival of ATCC 29740 cells after the PNA treatment was observed by spot assays. As a result, the individual PNA treatments revealed ASP-cmk and ASP-deoD to have MIC values of 20 and 25 µM, respectively. By contrast, the combined treatment had a MIC value of 10 µM (**Fig. 2**). Morphological observations also showed that ATCC 29740 is more susceptible to the combined treatment than to the individual P-PNA treatments, suggesting that combining two P-PNAs enhanced their antibacterial activities toward the target pathogen synergistically.
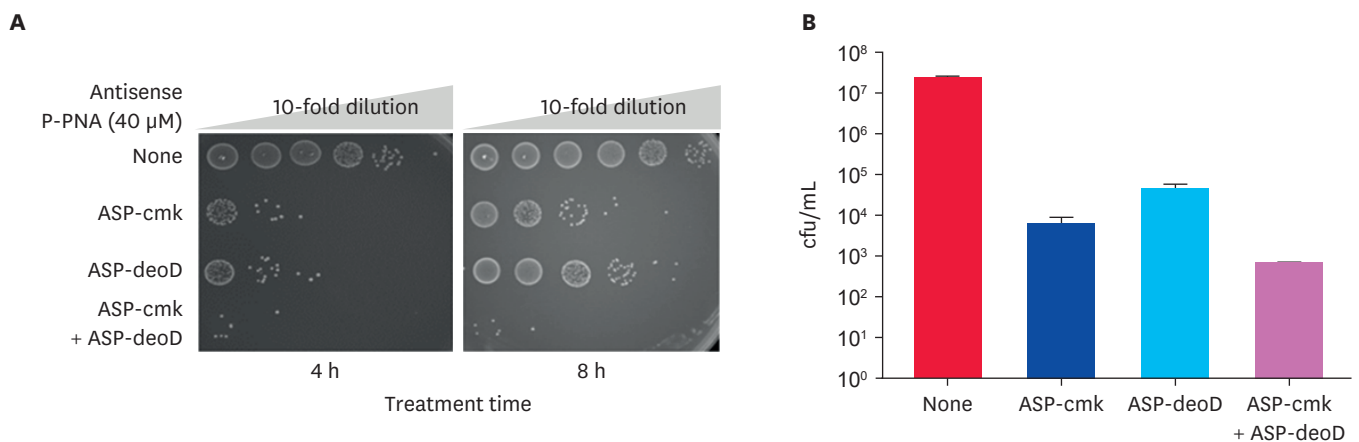
Fig. 1. Antibacterial effects of the individual and combined P-PNAs on *S. aureus* ATCC 29740. Bacterial cells (McF = 0.5, 11 mL MH broth) were treated with 40 µM of P-PNAs individually or combined (ASP-cmk, ASP-deoD, or ASP-cmk + ASP-deoD). (A) Cells were spotted (5 µL) on MH agar plates after 4- or 8-h treatment and incubated at 37°C for 19 h. (B) Cells were serially diluted, spread on Luria-Bertani agar plates after 8 h treatment, and incubated at 37°C for 19 h. After incubation, the CFUs were counted manually. All experiments were conducted in triplicate.
P-PNA, peptide nucleic acids conjugated with cell-penetrating peptide; MH, Mueller Hinton; ASP, antisense peptide nucleic acid; CFU, colony-forming unit.
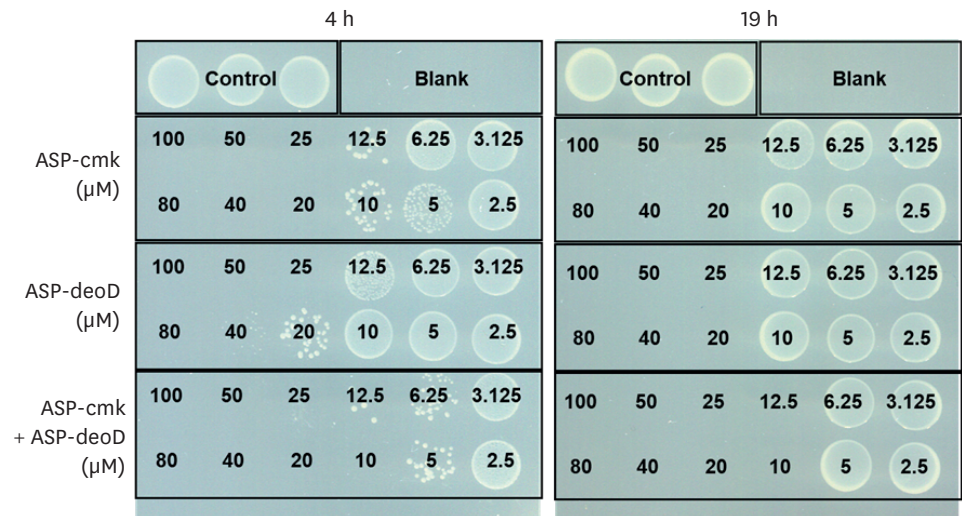
Fig. 2. Minimal inhibitory concentration determination of the individual and combined P-PNAs against *S. aureus* ATCC 29740. Bacterial cells (McF = 0.5, 11 mL MH broth) were treated with 100 or 80 µM of the antisense P-PNAs individually or combined (ASP-cmk, ASP-deoD, or ASP-cmk + ASP-deoD). The initial mixtures were then diluted serially to a final concentration of 3.125 or 2.5 µM. The cells were spotted (5 µL) on MH agar plates after 4- or 19-h treatment and incubated at 37°C for 19 h. For the controls, ATCC 29740 cells were treated with distilled water for 19 h and spotted on MH agar. For the blanks, sterilized MH broth was spotted. All experiments were conducted in triplicate, and a representative result is shown.
P-PNA, peptide nucleic acids conjugated with cell-penetrating peptide; ASP, antisense peptide nucleic acid; MH, Mueller Hinton.

Antibacterial activity of the P-PNA against *S. aureus* and *S. pseudintermedius* veterinary isolates

Previous findings and current data suggest that antisense P-PNAs designed in this study exert antibacterial activity toward *S. aureus* ATCC 29740 [16]. Next, the potential bactericidal effects of the combined P-PNA treatment on *Staphylococcus* veterinary isolates were investigated. Twenty *S. aureus* (pig and chicken) and ten *S. pseudintermedius* isolates (dogs) were treated with 40 µM P-PNA mixture for up to 19 h, and the viable cells were counted by spotting on MH agar plates. At 4 h post-treatment, 25 isolates were susceptible, and five *S. aureus* isolates displayed intermediate resistance to the combined treatment. At 19-h post-treatment, 18 *S. aureus* isolates were either resistant or intermediate to the treatment, unlike the reference strain ATCC 29740, and two isolates were susceptible (**Table 2**). On the other hand, all *S. pseudintermedius* isolates were susceptible to the combined P-PNA treatment (**Fig. 3**). These observations suggest that ASP-cmk and ASP-deoD can inhibit *S. pseudintermedius*, whereas *S. aureus* inhibition may be dependent on the strain types.

DISCUSSION

The emergence of MDR bacterial pathogens, such as MRSA and MRSP, has threatened human and animal healthcare, and global efforts have been made. Attempts to develop novel antimicrobials have also been made, but many have failed, and the potential emergence of drug resistance remains [19]. Scientists have searched for alternatives to overcome the shortcomings of conventional antimicrobials, and one of the approaches is to design ASPs conjugated to a CPP that can complementarily bind to RNA expressed by their targets, which in turn affect translation or post-transcriptional processing [8-10,12]. Previously, two antisense P-PNAs targeting the essential genes in *S. aureus* were developed: ASP-cmk and

ASP-deoD. Their molecular mechanisms and *in vivo* efficiency were analyzed [16]. This study focused on the synergistic effects of the combined treatment with the P-PNAs.
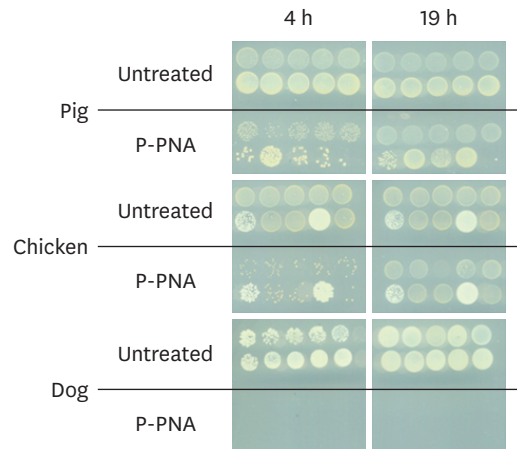

Fig. 3. Susceptibility of *S. aureus* and *S. pseudintermedius* veterinary isolates to the combined P-PNA treatment. Precultured cells (5.0×10^4 colony-forming units/mL) were treated with distilled water (untreated) or 40 μM of ASP-cmk + ASP-deoD (P-PNA) for 4 and 19 h, spotted (5 μL) on Mueller Hinton agar plates, and incubated at 37°C for 19 h. All experiments were conducted in triplicate, and a representative result is shown.
P-PNA, peptide nucleic acids conjugated with cell-penetrating peptide.

Table 2. Susceptibility of *S. aureus* and *S. pseudintermedius* veterinary isolates to the combined peptide nucleic acids conjugated with cell-penetrating peptide mixture

Isolates	Susceptibility to the combined treatment[a]	
	4 h	19 h
PG01	S	R
PG02	S	R
PG03	S	I
PG04	I	R
PG05	I	R
PG06	S	I
PG07	I	R
PG08	S	R
PG09	S	R
PG10	S	S
CK01	S	I
CK02	S	I
CK03	S	S
CK04	S	R
CK05	S	R
CK06	I	R
CK07	S	R
CK08	S	R
CK09	I	R
CK10	S	I
DG01	S	S
DG02	S	S
DG03	S	S
DG04	S	S
DG05	S	S
DG06	S	S
DG07	S	S
DG08	S	S
DG09	S	S
DG10	S	S

[a]Susceptibility relative to the untreated control: R, resistant; I, intermediate; S, susceptible.

The combined treatment with two P-PNAs inhibited *S. aureus* ATCC 29740 at a lower concentration than the treatment with the individual P-PNAs. Targeting essential genes for bacterial growth and survival effectively controls potential pathogens. The *cmk* and *deoD* enzymes are involved in the nucleotide metabolism and were confirmed as essential genes using allelic replacement mutagenesis [17,20]. A previous study reported that two antisense P-PNAs targeting *cmk* or *deoD* mRNA had high antibacterial activities to 29740 with minor differences in the MICs [16], which is consistent with the present observation. The combined treatment lowered the MIC, suggesting that two P-PNAs may have improved the effectiveness. A few examples of combined treatments with two PNAs [21] or a PNA and another therapeutical agent [22,23] have been published. Combined treatment with P-PNAs targeting two essential genes may be an effective alternative to treating potential pathogens.

The combined treatment of *S. aureus* clinical isolates with two P-PNAs revealed an interesting result. Unlike the control strain, *S. aureus* veterinary isolates resisted the combined treatment. A previous report showed that the MICs of ASPs on human clinical isolates were not different, regardless of the strain types [15]. On the other hand, there are genetic and phenotypic differences in human and animal isolates [24,25]. In this study, the color and morphology of *S. aureus* veterinary isolates varied when grown on MH agar plates. These isolates displayed resistance to the P-PNA treatment to different degrees (**Fig. 3**). Wall teichoic acids (WTAs) and staphyloxanthin play essential roles in the physiology and pigmentation of bacteria. Different *S. aureus* isolates have different WTAs and staphyloxanthin compositions, conferring differences in the stress response, fluidity, virulence, and antimicrobial resistance [26-28]. This study speculates that these veterinary isolates may harbor different membrane components or have acquired mutations in the genes associated with the bacterial membrane that would confer resistance to PNAs. Antimicrobial resistance phenotypes associated with the membrane have been observed in *S. aureus* [29]. Interestingly, a positive cell-surface charge acquired by constant exposure to pediocin conferred *Enterococcus faecalis* resistance to cationic antimicrobial peptides [30]. As (KFF)$_3$K is cationic, similar changes in *S. aureus* may confer resistance to P-PNAs because it would be difficult for P-PNAs to pass through a positively charged membrane efficiently. In addition, the uptake of antisense P-PNA was affected by the overall chemical composition of the outer lipopolysaccharide core in *E. coli* [31]. Whether the veterinary isolates in this study acquired such mutations that would render different WTA compositions or cell-surface charges is unclear, but these findings suggest that designing P-PNAs may need to overcome the additional complexity in the bacterial membrane physiology.

S. pseudintermedius veterinary isolates tested in this study exhibited susceptibility to combined P-PNA treatment, possibly because of the low MIC required for P-PNAs in *S. pseudintermedius* compared to *S. aureus* conferred by differences in the cell wall structures. Two previous studies showed that the MIC of the (KFF)$_3$K peptide against MRSP isolates was low, ranging from 2 to 8 μM [14,32]. Passage across the cell wall is one of the limiting factors for effective PNA treatments. Moreover, although structural differences between *S. aureus* and *S. pseudintermedius* have not been assessed experimentally, the collective results suggest that *S. pseudintermedius* isolates are potentially more susceptible to a PNA treatment. Furthermore, the P-PNAs used in this study may have targeted other genes with enhanced inhibitory effects. *In silico* analysis showed that the *cmk* and *deoD* TIR sequences in *S. pseudintermedius* differ from *S. aureus*. In addition, there are other potential sites to which P-PNAs may bind in the *S. pseudintermedius* genome (data not shown). Whether these newly identified genes are essential to bacterial survival or their transcription levels are lowered by the treated P-PNAs is unclear. Nevertheless, the current results address the potential of applying the antisense

P-PNAs to *S. pseudintermedius*. The increase in the number of pet owners has contributed to the increase in contracting this potential zoonotic pathogen, and the number of dog-to-human cases of *S. pseudintermedius* infections has increased in the 21st century [33,34]. Moreover, studies of potential HGT between MRSA and MRSP suggest that *S. pseudintermedius* is a potential human pathogen [35]. The findings show that designing P-PNAs to inhibit two different *Staphylococcus* spp. by targeting the bacterial essential genes is possible.

The application of ASPs to antimicrobial development may provide an alternative against the ever-growing number of MDR bacteria. On the other hand, there is a level of complexity in designing antisense P-PNAs to inhibit target pathogens effectively, and applications to clinical isolates require additional validation of the PNA efficacy. This work addresses the potential of treating *Staphylococcus* spp. with two P-PNAs simultaneously to determine the antibacterial effects on veterinary clinical isolates. Optimization of both CPPs and ASP sequences will be necessary to inhibit the target pathogens with high efficacy.

ACKNOWLEDGMENTS

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