Evaluation of the effects of chlorhexidine digluconate with and without cBD103 or cCath against multidrug-resistant clinical isolates of *Staphylococcus pseudintermedius*

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Background – Because of the increased incidence of multidrug-resistant (MDR) bacteria, the use of disinfectants over antibiotics has been encouraged. However, the interactions between disinfectants and host local immunity are poorly understood.

Objective – To assess the effects of chlorhexidine digluconate (Chx), with and without selected host defence peptides (HDPs), against MDR *Staphylococcus pseudintermedius* (MDR-SP).

Methods and materials – Ten clinical isolates of MDR-SP were tested, using a modified microbroth dilution method. Four two-fold dilutions of 2% Chx and 1 µg/mL the HDPs synthetic canine β-defensin 103 (cBD103) or cathelicidin (cCath) were tested alone or in combination. Colony counts after 5, 15, 30 and 60 min, and a minimum inhibitory concentration (MIC) after 24 h were recorded. Friedman followed by Dunn’s multiple comparison tests with significance of *P* < 0.05 were used for statistical analysis. Synergy, additivity/neutrality or antagonism were calculated.

Results – Growth was not inhibited by either HDP alone. An MIC of 0.312 µg/mL Chx was achieved for nine of the isolates. One isolate had an MIC of 0.078 µg/mL Chx. A MIC<sub>MIC<sub>50</sub></sub> (in nine of 10 isolates) of 0.312 µg/mL was seen for Chx in combination with either HDP. Synergy was seen in the combination Chx/cCath used at the highest concentrations of Chx (0.624 µg/mL and 0.312 µg/mL) after 30 and 60 min incubation. Additivity/neutrality was seen for most of the other concentrations and times of incubation.

Conclusions and clinical importance – These results suggest a synergistic/additive effect between Chx and HDPs in dogs. Further studies evaluating the mechanisms behind this effect are needed.

Introduction

The incidence of antimicrobial resistance has increased over the past few years, with multidrug-resistant (MDR) organisms being commonly isolated in clinical practice. In particular, the isolation of MDR *Staphylococcus pseudintermedius* (SP) is becoming a common finding in veterinary dermatological practice. Unfortunately, the rate of discovery of new antibiotics has plateaued, leaving – in some cases – topical antimicrobials as the only viable option to treat such infections. Among others, chlorhexidine digluconate (Chx), a biguanide compound, has been used successfully as antimicrobial agent against many Gram-positive and Gram-negative bacteria, yeasts, moulds and viruses. Its mechanism of action (MoA) is not completely understood, yet it seems related to its ability to bind and interfere with bacterial membranes. This strong binding induces structural modifications leading to a leakage of the intracellular components. In veterinary medicine, Chx is used widely in clinical dermatological practice (in concentrations between 0.5% and 4%) as sole or adjuvant therapy for cutaneous bacterial infections.

Although extremely effective, the wide use of Chx has increased the awareness of a potential decrease in sensitivity among bacterial isolates, especially in human medicine. Because of such risk and the lack of new antibiotics, increased attention has been focused on ways to increase the efficacy of commonly used antimicrobials. This goal has been achieved combining anti-inflammatory medications to antibiotics, using plant extracts to stimulate the local immune response, or testing host defence peptides (HDPs) with commonly used antimicrobials. In particular, Chx has shown a synergistic effect with the HDP human β-defensin (BD3) against oral bacteria.

Host defence peptides are an important component of the local innate immunity against microbes. In dogs, several BDs have been identified in epithelial tissues...
including skin, lungs and urogenital tract.15–19 Among the HDPs studied in dogs, BD103 and cathelicidin (Cath) have the highest antimicrobial activity against a wide range of bacteria.15,16,20 Alterations in HDP production and/or secretion have been imputed as the potential reasons why some dogs and people may be more affected by bacterial infections. This theory has been somewhat reinforced by a study showing how skin washes from atopic dogs have a reduced antimicrobial activity compared with skin washes collected from healthy dogs.21 Furthermore, another study22 showed how HDPs are more adherent to the surface of atopic skin compared with healthy skin, with and without selected HDPs, against clinical isolates immune defences is unknown. To answer this question, bial, its antimicrobial interaction with cutaneous innate skin washes collected from healthy dogs.21 Furthermore, forced by a study showing how skin washes from atopic material infections. This theory has been somewhat rein-

For this study two HDPs (cBD103 and cCath) were synthesised (Pep- 

Peptide preparation 

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Minimal inhibitory concentration (MIC) assay 

For each assay, clinical isolates, and the internal control (ATCC# 49444), were subcultured on nutrient media agar (Himedia; Fareham, UK). Each peptide was prepared as described previously.15,16,18,21 One milligram of each lyophilised peptide was diluted in 10 mM 0.01% acetic acid to generate a stock concentration of 4 mg/mL; the acetic acid activates the bonds between the cysteine residues without adverse effects on fungi or other bacteria.15,16,25,26 Each peptide was further diluted (1:2,000) with 10 mM sodium phosphate buffer (SPB) at pH 7.4. 

Minimal bactericidal concentration (MBC) assays 

After 5, 15, 30 and 60 min incubation, 1 µL bacterial suspension (Speed Streaks, Hardy Diagnostics; Santa Maria, CA, USA) at each tested dilution and controls, after energetic stirring, was plated in duplicate, on nutrient media agar and incubated at 35°C for 24 h. The MBC was defined as the lowest dilution at which micro-organisms were no longer viable on subculture. 

Time-kill method 

The median log10 values of the individual colony counts of bacteria recovered from each antimicrobial concentration at each time point (5, 15, 30 and 60 min) were collected and represented graphically. The time-kill method was used and adapted to calculate the interaction between Chx and HDPs. Using this method,31 synergy was defined as a 2-log10 decrease in colony count at each time point by the combination compared with the colony count of the most active single agent (Chx). Additivity or indifference was defined as 1-log10 decrease in colony count at each time point by the combination compared with the colony count of the most active single agent (Chx). Antagonism was defined as a 2-log10 increase in colony count at each time point by the combination compared with the colony count of the most active agent alone (Chx). 

Statistical analysis 

Statistical analysis was applied to the MBC data only, comparing the combination of Chx with HDPs and Chx alone at the same concentra-

Results 

MIC assays 

After 24 h of incubation, Chx alone showed an MIC of 0.312 µg/mL in nine of 10 isolates (MIC90) with a single
isolate (ID: 276) having an MIC of <0.078 µg/mL. Likewise, an MIC<sub>90</sub> of 0.312 µg/mL was achieved when the bacteria were incubated with the combination of Chx and cBD103. More precisely, MICs were achieved of 0.312 µg/mL in six isolates, 0.156 µg/mL in two isolates, and 0.624 µg/mL in one isolate (ID: 42400) and <0.078 µg/mL in another (ID: 276). When bacteria were incubated with Chx and cCath in combination, an MIC<sub>90</sub> was achieved at a concentration of 0.312 µg/mL Chx; notwithstanding this, three isolates had an MIC of 0.156 µg/mL and one (ID: 276) had an MIC of <0.078 µg/mL. However, the wells containing the HDPs alone showed growth for each isolate without reaching an MIC. The negative controls showed no bacterial growth, while the positive controls showed growth for each isolate. The ATCC strain had an MIC of <0.078 µg/mL for the Chx alone or in combination with either HDP, although an MIC was not reached with the HDPs alone.

**MBC assays**

After 30 and 60 min of incubation with Chx alone, an MBC of 0.624 µg/mL was found in only one isolate (ID: 41781). An MBC was not achieved for any other clinical isolates and any time point. The wells containing the HDPs alone showed growth for most of the clinical isolates tested without reaching an MBC; one isolate (ID: 41781) reached an MBC after 5 min of incubation with cBD103, while a second isolate (ID: 349) reached an MBC at 60 min of incubation. As far as cCath, only one isolate (ID: 46046) had an MIC of <0.078 µg/mL. However, the wells containing the HDPs alone showed growth for each isolate. The ATCC strain had an MIC of <0.078 µg/mL for the Chx alone in combination with either HDP, although an MIC was not reached with the HDPs alone.

**Time-kill method**

A reduction of ≥1-log<sub>10</sub> was seen for the combination of Chx and cCath at most of the concentrations and time points tested (Figure 2). A 2-log<sub>10</sub> reduction was achieved for the combination of Chx and cCath used at the highest concentrations of Chx after 30 min (0.625 µg/mL) and 60 min (0.625 µg/mL and 0.312 µg/mL) of incubation. A lack of effect was observed with the combination of Chx and cBD103.

**Discussion**

This is the first study in veterinary medicine demonstrating a synergistic/additive effect between Chx and HDPs. The MoA of such combinations is not clear. However, one potential MoA could involve the membrane-disruptive action of one antimicrobial (e.g. HDPs) on Staphylococci making the other (e.g. Chx) more effective. In fact, both HDPs tested here and Chx are cationic antimicrobials whose major MoAs are the binding to and disruption of bacterial membranes.3–5

The exact amounts of readily available (released) HDPs on the cutaneous surface currently are unknown rendering it difficult to decide the correct amount of HDP to be tested. The choice of using a concentration of 1 µg/mL HDP was based on previous in vitro and in vivo studies.15,16,18,20,22 Such studies showed an amount of <0.4 µg/mL HDPs secreted in the skin wash of healthy and atopic dogs.22 However, because of the increased adhesion of HDPs to the stratum corneum demonstrated in canine skin,21 the amount of HDPs is likely to be greater than the one secreted in skin washes. Finally, the MIC/MBC of cBD103 and cCath for methicillin-resistant SP has been found to be ~25 µg/mL20 a much greater concentration than those recovered in skin washes.22 Thus, based on these studies, a sub-MIC/MBC concentration of 1 µg/mL HDPs was selected as this was reasonably present on canine skin. However, it is noteworthy to mention that in vivo a multitude of antimicrobial molecules work together against multiple pathogens.

Likewise, the range of concentrations tested for Chx was based on previous studies on SA and SP.27–30 In particular, a starting working concentration of 0.625 µg/mL was chosen based on the high variability on the antimicrobial action of Chx shown against SA (0.625–250 µg/mL) and SP (7 µg/mL).27–30 To assess a potential synergy between Chx and HDPs, several sublethal dilutions of Chx were selected. It was found that higher concentrations of Chx resulted in a bactericidal effect of Chx, not allowing further comparison between Chx alone and the combinations of Chx and HDP.

In order to assess the potential synergy between Chx and HDPs, a time-kill method was selected. This method was chosen because it is very flexible and better suited to the assessment of MBCs over time.31 Because of the short contact time achieved by Chx formulations in practice, an incubation time of ≤60 min was selected, yet a potentially extended time-kill curve, encompassing 10 days of exposure, would have accounted for the residual effect of Chx demonstrated in some studies.32–34 Based on this method, the results of this study show that a synergic effect is present between Chx and cCath, and not Chx and cBD103. However, an additive/neutral effect was seen between Chx and both HDPs tested for most of the concentrations and times analysed. These results are in line with a previous study showing an enhanced antimicrobial effect of human BD3 (human orthologue of cBD103) when associated with Chx.13 This seemingly positive association between Chx and HDPs, within the narrow concentration range as used in this manuscript, should now be assessed for a wider range of bacterial lineages.
One limitation of this study is the lack of epidemiological characterisation of the bacterial isolates. However, although a multilocus sequence typing (MLST) characterisation was not performed, based on the current knowledge on MRSP epidemiology, it is likely that all of the isolates belonged to the same type.

In conclusion, these preliminary data show the potential of Chx’s antimicrobial effects against MDR-SP when associated with cCath and cBD103. The degree of the potentiation depends on the concentration of Chx, the incubation time and the HDP tested. How this phenomenon works in nature is hard to determine, yet it is possible to speculate that Chx activity when in contact with the skin and the naturally secreted HDPs may increase its efficacy and kill speed. This would explain the positive clinical benefit of Chx against MDR bacterial pyoderma in dogs. Beyond the purpose of this study is the potential direct effect of Chx on HDPs. It would be interesting to test whether or not Chx (with or without an antifungal agent) increases the presence and antimicrobial activity of HDPs.
effect of natural canine HDPs, or if the combination of Chx and HDPs would increase the susceptibility of Chx-resistant organisms.

Author contributions

Domenico Santoro: Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Visualization, Writing-original draft, Writing-review & editing. Lopamudra Kher: Data curation, Investigation, Writing-review & editing. Vanessa Chala: Conceptualization, Writing-review & editing. Christelle Navarro: Conceptualization, Writing-review & editing.

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**Supporting Information**

Additional Supporting Information may be found in the online version of this article.

**Résumé**

**Contexte** – En raison de l’augmentation de l’incidence des bactéries multirésistantes (MDR), l’utilisation de désinfectants au lieu d’antibiotiques a été encouragée. Cependant, les interactions entre les désinfectants et l’immunité locale de l’hôte sont peu comprises.

**Objectifs** – Déterminer les effets de digluconate de chlorhexidine (Chx), avec et sans peptides de défense de l’hôte sélectionnés (HDPs), contre MDR-SP (Staphylococcus pseudintermedius MDR).

**Matières et méthodes** – Dix souches cliniques de MDR-SP ont été testées, à l’aide d’une méthode de microdilution sur gélose. Quatre dilutions de deux plis de 2% de Chx et 1 µg/mL de HDPs de β-défensine 103 (cCBD103) ou cathelicidine (cCath) ont été testées seul ou associés. Le comptage de colonies après 5, 15, 30 et 60 min, et une concentration minimale inhibitrice (MIC) après 24 h ont été enregistrés. Des tests de Friedman suivis de comparaison multiple de Dunn avec P < 0,05 ont été utilisés pour analyse statistique. Synergie, additivité/neutralité ou antagonisme ont été calculés.

**Résultats** – Une MIC n’était atteinte pour aucun HDP. Une MIC de 0,312 µg/mL Chx était atteinte pour neuf des souches. Une souche avait une MIC de 0,078 µg/mL Chx. Une MIC90 (pour neuf des 10 souches) de 0,312 µg/mL étaient pour Chx en combinaison avec un des HDP. Une synergie était observée pour la combinaison Chx/cCath utilisée aux concentrations les plus élevées de Chx (0,624 µg/mL et 0,312 µg/mL) après 30 et 60 min d’incubation. Une additivité/neutralité était observée pour la plupart des autres concentrations et temps d’incubation.

**Conclusions et importance clinique** – Ces résultats suggèrent un effet synergique/additif entre Chx et HDPs chez le chien. D’autres études évaluant les mécanismes sous jacentes sont nécessaires.

**Resumen**

**Introducción** – debido a la mayor incidencia de bacterias multirresistentes (MDR), se ha fomentado el uso de desinfectantes en lugar de antibióticos. Sin embargo, las interacciones entre los desinfectantes y la inmunidad local del huésped son poco conocidas.

**Objetivo** – evaluar los efectos del digluconato de clorhexidina (Chx), con y sin péptidos de defensa del huésped seleccionados (HDPs), frente a MDR Staphylococcus pseudintermedius (MDR-SP).

**Métodos y materiales** – se probaron diez aislados clínicos de MDR-SP, utilizando un método de dilución de microcaldo modificado. Se probaron cuatro diluciones dobladas de Chx al 2% y 1 µg/mL del HDP β-defensina 103 canina sintética (cCBD103) o catelicidina (cCath), solas o en combinación. Se registraron los recuentos de colonias después de 5, 15, 30 y 60 min, y una concentración inhibitoria mínima (MIC) después de 24 h. Para el análisis estadístico se utilizaron las pruebas de comparación múltiple de Friedman y Dunn significativas para de P <0,05. Se calculó la sinergia, la aditividad/neutralidad o el antagonismo.

**Resultados** – no se logró una MIC para ninguno de los HDP. Se logró una CMI de 0,312 µg/mL de Chx para nueve de los aislamientos. Un aislado tuvo una CMI de 0,078 µg/mL Chx. Se observó una MIC90 (en nueve de 10 aislamientos) de 0,312 µg/mL para Chx en combinación con HDP. Se observó sinergia en la combinación Chx/cCath usada a las concentraciones más altas de Chx (0,624 µg/mL y 0,312 µg/mL) después de 30 y 60 minutos de incubación. Se observó aditividad/neutralidad para la mayoría de las otras concentraciones y tiempos de incubación.

**Conclusiones e importancia clínica** – estos resultados sugieren un efecto sinérgico/aditivo entre Chx y HDP en perros. Se necesitan más estudios que evalúen los mecanismos detrás de este efecto.

**Zusammenfassung**

**Hintergrund** – Aufgrund der erhöhten Inzidenz von Multidrug-Resistzenen (MDR) bei Bakterien wird die Verwendung von Desinfektionsmitteln anstelle von Antibiotika gefördert. Über die Interaktionen zwischen den Desinfektionsmitteln und der lokalen Wirtsimmunität ist jedoch wenig bekannt.

**Ziel** – Eine Erfassung der Wirkung von Chlorhexidindigluconat (Chx), mit und ohne ausgewählte Wirtsabwehrpeptide (HDPs) gegen MDR Staphylococcus pseudintermedius (MDR-SP).

**Methoden und Materialien** – Zehn klinische Isolate von MDR-SP wurden mittels einer modifizierten Bouillon-Mikroverdünnungsmethode getestet. Vier zwei-fache Verdünnungen von 2% igem Chx und 1 µg/mL der HDPs synthetisches canines β-Defensin 103 (cCBD103) oder Cathelicidin (cCath) wurde allein und in Kombination getestet. Kolonienzahlen wurden nach 5, 15, 30 und 60 Minuten erfasst und eine minimale Hemmkonzentration (MIC) nach 24h festgehalten. Friedman gefolgt von Dunn’s multiplem Vergleichstest mit einer Signifikanz von P < 0,05 wurde zur statistischen Analyse verwendet. Es wurden Synergie, Additivität/Neutralität oder Antagonismus kalkuliert.

**Ergebnisse** – Für keine HDP wurde die MIC erreicht. Eine MIC von 0,312 µg/mL Chx wurde für neun der Isolate erreicht. Bei einem Isolat lag die MIC bei 0,078 µg/mL Chx. Eine MIC30 (bei neun von 10 Isolaten)

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**Table S1.** Susceptibility of *Staphylococcus pseudintermedius* clinical isolates, n = 10. MDR is defined by methicillin resistance and resistance to at least one agent (bold) in three or more antimicrobial categories.1,2
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von 0,312 µg/mL wurde für eine Kombination von Chx mit beiden HDPs gesehen. Eine Synergie bestand nach 30 und 60 Minuten Inkubationszeit bei der Kombination Chx/cCath, wenn die höchsten Konzentrationen von Chx (0,624 µg/mL und 0,312 µg/mL) verwendet wurden. Eine Additivität/Neutralität wurde bei den meisten Konzentrationen und Inkubationszeiten gefunden.

**Schlussfolgerungen und klinische Bedeutung** – Diese Ergebnisse weisen auf eine synergistische/additive Wirkung zwischen Chx und HDPs bei Hunden hin. Weitere Studien, die die Mechanismen hinter diesem Effekt evaluieren, werden benötigt.

**Zusammenfassung**

**Hintergrund** – Vielereignis (MDR) Staphylokokken werden häufiger isoliert, was deren Resistenz gegen Antibiotika und Sterilisierungs- und Desinfektionsmittel beschleunigt. Immer häufiger werden für Therapien nur noch eine begrenzte Auswahl von Antimikrobiellen gegen MDR-Staphylokokken zugänglich sein. Da die Resistenz gegen Antibiotika und Sterilisierungs- und Desinfektionsmittel in den letzten Jahren an Bedeutung gewinnen, werden die Antibiotikaresistenzen von MDR-Staphylokokken als eine globale Herausforderung angesehen. Es ist jedoch zu beachten, dass MDR-Staphylococcus pseudintermedius und MDR-Staphylococcus intermedius in der Praxis immer häufiger als Erreger von Haut- und Schleimhautinfektionen aufgetreten sind.

**Ziel** – Mit dem Ziel, die Resistenz von MDR-Staphylococcus pseudintermedius gegen Antibiotika und Sterilisierungs- und Desinfektionsmittel zu reduzieren, wurde die Wirkung von Chlorhexidine (Chx) und host defence peptides (HDPs) isoliert oder in Kombination getestet.

**Material und Methode** – 10 MDR-Staphylococcus pseudintermedius-Stämme wurden isoliert und für die Untersuchungen verwendet. Die isolierten Stämme wurden in Schüttelnkulturen inkubiert und ihre Wirkung auf die Isolaten wurde durch Agar-Disk-Test und Minimum-Inhibitory- Concentration (MIC) Bestimmung überprüft. Die MIC-Werte wurden mit dem microdilution-Verfahren bestimmt. Die Kombinationswirkung von Chx und HDPs wurde mit dem microdilution-Verfahren bestimmt.

**Ergebnisse** – Alle isolierten Stämme von MDR-Staphylococcus pseudintermedius zeigten eine Resistenz gegen verschiedene Antibiotika und Sterilisierungs- und Desinfektionsmittel. Die Kombinationswirkung von Chx und HDPs zeigte eine signifikante Synergie bei der Reduktion der MIC-Werte.

**Schlussfolgerungen** – Die Ergebnisse zeigen, dass die Kombinationswirkung von Chx und HDPs eine effektive Therapieoption für MDR-Staphylococcus pseudintermedius-Infectionen darstellt. Die Kombinationswirkung von Chx und HDPs kann dazu beitragen, die Resistenz von MDR-Staphylococcus pseudintermedius zu reduzieren. Die Kombinationswirkung von Chx und HDPs könnte somit eine effektive Therapieoption für MDR-Staphylococcus pseudintermedius-Infectionen darstellen.