In Vitro Efficacy of Ionophores and Antimicrobial Adjuvants Against Planktonic and Biofilm Producing Pathogens Associated with Canine Otitis Externa

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A thesis submitted in fulfilment of the requirements of the degree of Doctor of Philosophy (PhD)

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September 2019

Table of Contents

Abstract 4
Thesis declaration 6
Acknowledgements 7
List of publications 9
Contribution to other publications 10
List of abbreviations 12

Chapter 1: Introduction and Literature Review 13
1.1 Canine otitis externa 13
1.2 Clinical signs and aetiologies of canine otitis externa 13
1.3 Pathogens associated with canine otitis externa 15
1.4 Antimicrobial susceptibility and resistance profiles 20
1.4.1 Antimicrobial susceptibility and resistance profiles of *Staphylococcus pseudintermedius* . 20
1.4.2 Antimicrobial susceptibility and resistance profiles of *Pseudomonas aeruginosa* 23
1.4.3 Antimicrobial susceptibility and resistance profiles of *Malassezia pachydermatis* 26
1.5 Treatment of canine otitis externa 27
1.5.1 Topical otic treatment 27
1.5.2 Topical ear cleaners 32
1.6 Antimicrobial resistance 33
1.6.1 Development of antimicrobial resistance 34
1.6.2 Antimicrobial resistance in canine otitis externa pathogens 35
1.6.3 Biofilm-associated canine otitis externa 38
1.6.4 Zoonotic transmission 39
1.7 Strategies to combat antimicrobial resistance 41
1.7.1 Development of new drugs 41
1.7.2 Antimicrobial stewardship 41
1.7.3 Alternative therapies 43
1.7.4 Repurposing of old drugs for new purposes 45
1.8 Carboxylic polyether ionophores (ionophores) 46
1.8.1 Monensin 47
1.8.2 Narasin 49
1.9 Antimicrobial adjuvants 51
1.9.1. N-acetylcysteine 52
1.9.2 Ethylenediaminetetraacetic acid (EDTA) and ethylenediaminetetraacetic acid – tromethamine (Tris-EDTA) 56
1.9.3 Monoglycerides 60
1.9.4 Polymyxin B nonapeptide 61
1.10 Research aims 62
1.11 Thesis outline – a summary of experimental chapters 63

Chapter 2: *In vitro* antimicrobial activity of narasin against common clinical isolates associated with canine otitis externa ... 65
Statement of Authorship ... 66
Original article ... 68

Chapter 3: *In vitro* antimicrobial activity of monensin against common clinical isolates associated with canine otitis externa ... 75
Statement of Authorship ... 76
Original article ... 78

Chapter 4: *In vitro* antimicrobial activity of seven adjuvants against common pathogens associated with canine otitis externa ... 83
Statement of Authorship ... 84
Original article ... 86

Chapter 5: *In vitro* antimicrobial activity of narasin and monensin in combination with adjuvants against pathogens associated with canine otitis externa ... 95
Statement of Authorship ... 96
Original article ... 98

Chapter 6: Biofilm production by pathogens associated with canine otitis externa, and the antibiofilm activity of ionophores and antimicrobial adjuvants ... 108
Statement of Authorship ... 109
Original article ... 111

Chapter 7: General discussion and Conclusion ... 122
7.1 Major findings ... 122
7.2 Future directions ... 127
7.3 Conclusions ... 128

Appendices ... 130
Appendix 1 ... 130
Appendix 2 ... 131
Appendix 3 ... 133
Appendix 4 ... 134
Appendix 5 ... 136
Appendix 6 ... 137

Collated references ... 206

Abstract

Antimicrobial resistance is an emerging global concern in human and veterinary medicine. Antibiotics that are critical for human health are also used in veterinary medicine, including for treatment of non-life threatening conditions such as otitis externa. Otitis externa is one of the most frequently diagnosed dermatological conditions in dogs with a prevalence of up to 20%, and it is traditionally treated with products containing antibiotics and antifungal agents. Though the use of antimicrobials can potentially contribute to the development of antimicrobial resistance, antimicrobials are an essential component to treat, manage and improve the welfare of dogs suffering from otitis externa. However, this may not represent good antimicrobial stewarship. One approach to minimising the use of critical or new antibiotics is to repurpose old drugs for new purposes. This approach has not yet been explored in the field of veterinary otology.

The experiments in this thesis explored the efficacy of polyether ionophores and antimicrobial adjuvants against pathogens associated with canine otitis externa. *Staphylococcus pseudintermedius*, multidrug-resistant *S. pseudintermedius*, β-haemolytic *Streptococcus* spp., *Pseudomonas aeruginosa*, *Proteus mirabilis* and *Malassezia pachydermatis* clinical otic isolates, as well as Gram-positive and Gram-negative American Type Culture Collection (ATCC) reference strains, were used in minimal inhibitory concentration (MIC) testing, checkerboard assays and biofilm assays *in vitro*. Narasin and monensin are polyether ionophores conventionally used as rumen modifiers in ruminants and as anticoccidial agents in avian and mammalian production animals. Both narasin and monensin were found to be effective against Gram-positive otic and ATCC strains, including the multidrug-resistant staphylococci. Gram-positive pathogens in the planktonic state were more susceptible to narasin than monensin, whereby the MIC_{50} and MIC_{90} of narasin were 32 times lower than those of monensin. Also, narasin had anti-yeast activity against *M. pachydermatis* otic isolates at higher concentration. However, both ionophores lacked activity

against Gram-negative pathogens which would preclude their use as sole antimicrobial agents in cases of otitis involving rods or mixed infections.

The non-antibiotic mucolytic agent, N-acetylcysteine (NAC), and two metal chelating agents, Tris-EDTA and disodium EDTA, were found to be promising adjuvants that had both intrinsic antimicrobial activity and the ability to enhance the efficacy of ionophores against Gram-negative bacterial infections. The combination of narasin with either Tris-EDTA or disodium EDTA produced additive effects against *P. aeruginosa* ATCC strains. An additive effect was also found against the *Staphylococcus aureus* ATCC strain when narasin or monensin was combined with NAC.

The ability of otic pathogens to produce j is an increasing concern in chronic and relapsing cases of otitis externa in dogs. In our experiments, all *S. pseudintermedius* otic isolates (n=20) formed biofilms with an 80:20 ratio between weak and moderate biofilm production. 95% of *P. aeruginosa* otic isolates (n=20) were biofilm producers, evenly distributed amongst weak (30%), moderate (35%) and strong (30%) production. The presence of biofilms may play an essential role in the resistance of otic pathogens to antimicrobial agents. *S. pseudintermedius* otic isolates which were inherently susceptible to narasin and monensin in the planktonic state became resistant to them in the biofilm state. NAC was demonstrated to be an effective antibiofilm agent that eradicated preformed biofilms of *S. pseudintermedius* and *P. aeruginosa* otic isolates. The presence of Tris enhances the ability of disodium EDTA (Tris-EDTA) to eradicate preformed *P. aeruginosa* biofilms whereby disodium EDTA only reduced biofilms when used alone.

In conclusion, the use of ionophores could represent a future strategy for the treatment of Gram-positive bacterial and *Malassezia* infections in canine otitis externa, and when combined with adjuvants, could be effective in Gram-negative infections. In addition, NAC and Tris-EDTA are potential antibiofilm agents for the treatment of biofilm-associated otitis externa. The study results have provided the basis for further development of these repurposed drugs which would represent an example of good antimicrobial stewardship.

Thesis declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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Wei Yee, Chan

September 2019

Acknowledgements

First and foremost, I would like to thank God Almighty for giving me the strength, wisdom, ability and opportunity to undertake this research study and to persevere and complete it satisfactorily. Without his blessings, this achievement would not have been possible.

A huge thank you and appreciation must go to my supervisors, Professor Peter B. Hill, Professor Darren J. Trott and Dr Stephen W. Page. Without your expertise, patience and persistence, I would not be where I am today. You have all made my studies an incredible learning experience, more rewarding and less stressful.

My sincerest thanks to all of my exceptional co-authors and mentors, Ms Elizabeth Hickey, Dr Manouchehr Khazandi and Dr David Ogunniyi for your mentoring and assistance in the project, and drafting and editing manuscripts. I truly could not have achieved as much as I have in the past three and a half years without you all. Special thanks to my research group teammates, Alex Pi and Hang Nguyen for assisting and supporting help in some of my experimental works. I would also like to express my gratitude to Ken Lee, Bhumi Savaliya, and Tania Veltman for their laboratory and technical assistance in microbiological culture and identification, and MALDI-TOF microbial identification. My sincere thanks to the otic formulation team, Dr Sanjay Garg, Ms Amanda Bergamin, Dr Ankitkumar Parikh, and Dr May Song, for their technical assistance and continuous support. I am also thankful to Dr Andrea McWhorter and Dr Farhid Hemmatzadeh for their helpful advice and being accommodating while I was working in the PC2 laboratory, and even to Janelle Trott for her kindness and encouragement.

I would like to express my appreciation to all PhD mates, Sugiyono Saputra, Farida Mazumder, Putri Pandarangga, Esther Yap, Mohammad Rabiei, Phuong Doan, Sangay Tenzin, Sri Dwi Hastuti, Yan Hui Wang, Ness Tamlin, Jo Aldersey, Tamsyn Stephenson, Alejandra Arbe,

Nitish Joat, Hue Thi Do, Hanh Nguyen, Mohammad Farouq, Saad Gilani, Janet Pandi, Entesar Shuaib, Pardeep Sharma and Vivek Pande for their friendship and moral support. I'm also grateful to my brothers and sisters in Christ, Irinda Toh, Rachel Liew, Tony Cheah, Jacqueline Thong, Melissa Phoon, Elaine Chin, Allan Foo, Ling Tan, Danwin Chan, Aaron Wong, Esther Chew, Eunis Fong, Wendy Loo and Pastor Wendy Rayford for their prayers, emotional support and motivation in my PhD journey.

Last, but not least, thank you to my beloved husband, Chok Ngee Keet, my parents and family who have supported and encouraged me throughout the past three and a half years of my PhD study.

List of publications

1. **Chan, W. Y.,** Hickey, E. E., Khazandi, M., Page, S. W., Trott, D. J., & Hill, P. B. (2018). *In vitro* antimicrobial activity of narasin against common clinical isolates associated with canine otitis externa. *Veterinary Dermatology*, *29*(2), 149-e157. doi:10.1111/vde.12516

2. **Chan, W. Y.,** Hickey, E. E., Khazandi, M., Page, S. W., Trott, D. J., & Hill, P. B. (2018). *In vitro* antimicrobial activity of monensin against common clinical isolates associated with canine otitis externa. *Comparative Immunology, Microbiology and Infectious Diseases*, *57*, 34-38. doi:10.1016/j.cimid.2018.05.001

3. **Chan, W. Y.,** Khazandi, M., Hickey, E. E., Page, S. W., Trott, D. J., & Hill, P. B. (2019). *In vitro* antimicrobial activity of seven adjuvants against common pathogens associated with canine otitis externa. *Veterinary Dermatology*, *30*(2), 133-e138. doi:10.1111/vde.12712

4. **Chan, W. Y.,** Hickey, E. E., Khazandi, M., Page, S. W., Trott, D. J., & Hill, P. B. (2019). *In vitro* antimicrobial activity of narasin and monensin in combination with adjuvants against pathogens associated with canine otitis externa. *Veterinary Dermatology*. doi:10.1111/vde.12803

5. **Chan, W. Y.,** Hickey, E. E., Page, S. W., Trott, D. J., & Hill, P. B. (2019). Biofilm production by pathogens associated with canine otitis externa, and the antibiofilm activity of ionophores and antimicrobial adjuvants. *Veterinary Pharmacology and Therapeutics, 42*, 682-692. doi:10.1111/jvp.12811

Contribution to other publications not specifically related to this PhD thesis

1. Misan, A., **Chan, W. Y.,** Trott, D., & Hill, P. B. (2017). Survival of *Staphylococcus pseudintermedius* in modified Romanowsky staining solutions. *Veterinary Dermatology, 28*(4), 333-e371. doi:10.1111/vde.12435

2. Ogunniyi, A. D., Kopecki, Z., Hickey, E. E., Khazandi, M., Peel, E., Belov, K., Boileau, A., Garg, S., Venter, H., **Chan, W. Y.,** Hill, P. B., Page, S. W., Cowin, A. J., & Trott, D. J. (2018). Bioluminescent murine models of bacterial sepsis and scald wound infections for antimicrobial efficacy testing. *PLoS ONE, 13*(7), e0200195. doi:10.1371/journal.pone.0200195

3. Pansara, C., **Chan, W. Y.,** Parikh, A., Trott, D. J., Mehta, T., Mishra, R., & Garg, S. (2018). Formulation Optimization of Chitosan Stabilised Silver Nanoparticles using In-vitro Antimicrobial Assay. *Journal of Pharmaceutical Sciences, 108*(2), 1007-1016. doi:10.1016/j.xphs.2018.09.011

4. Khazandi, M., Pi, H., **Chan, W. Y.,** Ogunniyi, A. D., Sim, J. X. F., Venter, H., . . . Trott, D. J. (2019). *In vitro* Antimicrobial Activity of Robenidine, Ethylenediaminetetraacetic Acid and Polymyxin B Nonapeptide Against Important Human and Veterinary Pathogens. *Frontiers in Microbiology, 10*, 837. doi:10.3389/fmicb.2019.00837

5. Graham, M., **Chan, W.Y.,** & Hill, P. (2019). Lesion distribution in cases of canine atopic dermatitis in South Australia. *Australian Veterinary Journal*, *97*(8), 262-267. doi:10.1111/avj.12828

6. Sim, J., Khazandi, M., **Chan, W.Y.,** Trott, D., & Deo, P. (2019). Antimicrobial activity of thyme oil, oregano oil, thymol and carvacrol against sensitive and resistant pathogens associated with canine otitis externa. *Veterinary Dermatology, 30*, 524-e159. doi:10.1111/vde.12794.

7. Han, C., **Chan, W.Y.,** & Hill, P. (2019). Prevalence of positive reactions in intradermal and IgE serological allergy tests in dogs from South Australia, and the subsequent outcome of allergen specific immunotherapy. *Australian Veterinary Journal*. doi:10.1111/avj.12892

List of abbreviations

AMR = Antimicrobial resistance

ATCC = American type culture collection

CFU = Colony forming unit

DRI = Dose reduction index

EDTA = Ethylenediaminetetraacetic acid

EPI = Efflux pump inhibitor

EPS = Extracellular polymeric substance

ESKAPE = *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa,* and *Enterobacter* spp.

FICI = Fractional inhibitory concentration index

LD_{50} = Median lethal dose

MBC = Minimum bactericidal concentration

MBEC = Minimum biofilm eradication concentration

MDR = Multidrug-resistant

MDRSP = Multidrug-resistant *Staphylococcus pseudintermedius*

MDRPA = Multidrug-resistant *Pseudomonas aeruginosa*

MFC= Minimum fungicidal concentration

MIC = Minimum inhibitory concentration

MRSA = Methicillin-resistant Staphylococcus aureus

MRSP = Methicillin-resistant *Staphylococcus pseudintermedius*

NAC = N-acetylcysteine

NOEL = No observed effect level

OE = Otitis externa

PGFE = Pulsed-field gel electrophoresis

VRE = Vancomycin-resistant enterococci

Chapter 1: Introduction and Literature Review

1.1 Canine otitis externa

Otitis externa (OE) is one of the most common skin disorders in dogs attended by small animal practitioners, with a prevalence up to 20% reported over the past five decades (Angus, 2004; August, 1988; Grono, 1969; Miller, Griffin, & Campbell, 2013). The earliest literature reported an incidence of OE in 4.8% (409/8585) of dogs presented to an Australian veterinary school clinic for over 5 years (Grono, 1969). A comprehensive survey between 1972 and 1973 found that on average, a small animal veterinarian in the UK was presented with skin/ear conditions in 19.6% of dogs (Evans, Lane, & Hendy, 1974). In 1995, a prevalence study of common diseases diagnosed by American private practitioners who examined 31,484 dogs, revealed OE being the third most common disorder with a prevalence of 13% (Lund, Armstrong, Kirk, Kolar, & Klausnor, 1999). A survey of types of small animal consultation, diagnosis and treatment of skin conditions in general practices in England over 4 years (1998-2001) found that 104 of 2322 dogs (4.5%) were diagnosed with otitis (Hill et al., 2006). In a recent survey in 89 primary care veterinary practices in the UK between 2009 and 2013, OE was recorded as the most frequent disorders in dogs with a prevalence of 10.2% (396 out of 3884 dogs) (O′Neill, Church, McGreevy, Thomson, & Brodbelt, 2014).

1.2 Clinical signs and aetiologies of canine otitis externa

Canine OE is a multifactorial inflammatory skin disease affecting the external ear canal of dogs with presenting signs of pain, erythema, swelling, pruritus, head shaking, head shyness, ceruminous or suppurative ear discharges and malodour (August, 1988; Miller et al., 2013; Rosser, 2004). Aetiologies of canine OE are classified by primary and secondary causes and predisposing and perpetuating factors. Primary and secondary causes are responsible for the onset and development of ear inflammation and disease. Predisposing and perpetuating factors do not cause ear infection in their own, but contribute to the ear disease and may prevent

resolution and lead to the recurrence of the ear disease if they are not managed appropriately and sufficiently (Miller et al., 2013; Paterson, 2016a). In a clinical scenario, chronologically, primary causes and predisposing factors of canine OE commonly lead to secondary bacterial and fungal infections, followed by perpetuating factors that further aggravate and prevent the resolution of the disease (Miller et al., 2013).

Primary causes of OE include allergies (atopic dermatitis and adverse food reactions), keratinisation disorders, ectoparasites, foreign bodies, autoimmune diseases and neoplastic diseases (August, 1988; Rosser, 2004; Saridomichelakis, Farmaki, Leontides, & Koutinas, 2007). The primary causes may not be obvious to the owner until secondary causes such as bacteria, yeast or fungal infections develop (Miller et al., 2013). Secondary causes are those that cause pathological changes or disease in an existing abnormal ear condition, which in general is considered more straightforward and requires a shorter term of therapy to resolve compared to primary causes and perpetuating factors (Miller et al., 2013; Paterson, 2016a). Secondary causes are usually discussed extensively as bacterial and yeast or fungal infections. Other secondary causes include overcleaning and inappropriate use of cotton-tipped applicators that could lead to excessive moisture and trauma in the ear canal (Miller et al., 2013). Adverse reactions and irritation to some of the ingredients in topical treatment such as propylene glycol, alcohol and acidic solutions is another secondary cause (Miller et al., 2013).

Predisposing causes of OE include conformational abnormalities, heat and high humidity in the environment that lead to excessive moisture, obstructive ear diseases, systemic diseases and treatment effects (August, 1988; Rosser, 2004). Pendulous pinnae and hairy ear canals in various breeds were the most common predisposing causes reported in a Greek study (Saridomichelakis et al., 2007). In contrast, another study did not find any significant difference in the temperature and humidity of external ear canals among various dog breeds with either erected or pendulous ear types that predisposed to OE (Yoshida, Naito, & Fukata, 2002). Perpetuating factors are advanced pathologic changes in the auditory meatus, tympanum and middle ear as a result of predisposing, primary and secondary causes of canine OE. Some

breeds of dogs are over-represented for canine OE in relation to the perpetuating factors. Cocker spaniels with chronic and severe OE were found to have predominant ceruminous tissue response pattern (ceruminous gland hyperplasia and ceruminous gland ectasia) in comparison to other dog breeds with prominent fibrous tissue response (Angus, Lichtensteiger, Campbell, & Schaeffer, 2002). A retrospective study of 100 cases of canine OE found that ear canal stenosis and tympanic membrane perforation-otitis media were the two most important perpetuating causes (Saridomichelakis et al., 2007). Furthermore, this study also suggested that bacterial (cocci and rods) and yeast infections in OE were perpetuating factors, in which OE complicated by an overgrowth of cocci was significantly related to stenotic ear canals and OE complicated by rods was strongly associated with otitis media. On the other hand, Zur, Lifshitz, and Bdolah-Abram (2011) identified a strong association of rods with endocrine diseases and *Malassezia* yeast infection with allergies.

1.3 Pathogens associated with canine otitis externa

Numerous studies from various worldwide geographical locations have investigated microbial isolation and identification along with antimicrobial sensitivity profiles in canine OE. *Staphylococcus pseudintermedius* (formerly *Staphylococcus intermedius*), *Streptococcus spp*. *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Escherichia coli* and *Malassezia pachydermatis* are the commonly reported bacterial and yeast pathogens associated with canine OE (see appendix 1) as shown in Table 1. It is important to note that the total percentages of isolates in each study do not equate to 100% due to overlapping results from several bacteria and yeast isolated concurrently from each ear swab as well as insignificant bacterial strains not listed in Table 1. The most common bacteria isolated from cases of otitis externa are Gram-positive *S. pseudintermedius* (23 – 91.9%) followed by Gram-negative *P. aeruginosa* (8.6 – 27.6%). However, an opposite scenario was observed in several studies where *P. aeruginosa* (18.4 – 49.3 %) was isolated most commonly followed by *S. pseudintermedius* (1.9 – 24.3 %) (Bugden, 2013; Demırbılek & Yılmaz, 2019; Fernández et al., 2006; Subapriya et al., 2015). In contrast

to the usual trend, β-haemolytic *Streptococcus* spp. was a more frequently isolated Gram-positive bacteria in one study (Demırbılek & Yılmaz, 2019) and another two studies found a higher frequency of *P. mirabilis* or *E. coli* compared to *P. aeruginosa* in the Gram-negative bacteria category (Lyskova, Vydrzalova, & Mazurova, 2007; Oliveira, Leite, Brilhante, & Carvalho, 2008). Generally, these studies revealed that *P. mirabilis* (4.1 – 18.9 %) and *E. coli* (1 – 17%) infections are interchangeable as the second or third most cultured Gram-negative bacteria.

In several studies, the percentages of *M. pachydermatis* (69.9 – 76.3 %) appeared to be higher or equal to the proportion of *S. pseudintermedius* and *P. aeruginosa* combined (Fernández et al., 2006; Kiss, Radvanyi, Szigeti, Lukats, & Nagy, 1997; Oliveira et al., 2008). Concurrent yeast cultured with bacterial pathogens is not uncommon (Kiss, Radvanyi, & Szigeti, 1997; Rigaut, Sanquer, Maynard, & Rème, 2011). Graham-Mize and Rosser Jr. (2004) demonstrated that more than two to six bacteria and yeast could be cultured concurrently. A 5-year retrospective study on 143 dogs with canine OE diagnosed in a veterinary teaching hospital found that mixed infection with Gram-positive cocci, Gram-negative rods and *Malassezia* yeast was more frequently observed (26.3%) than the combination of cocci and *Malassezia* yeast (21.1%) or cocci and rods (19.5%) (Zur et al., 2011).

However, these studies may not be a meaningful representation of the microbiological profiles in canine OE by describing the order from the most to the least frequently isolated pathogens. Prevalence figures in this table from the studies varied dramatically. For instance, an Iranian study isolated 91.9% *S. pseudintermedius* (n=74) while Venezuelan and Turkish studies only had 1.9% (n=53) and 5.4% (n=277), respectively (Demırbılek & Yılmaz, 2019; Fernández et al., 2006; Malayeri, Jamshidi, & Salehi, 2010). A true geographical variation or diversity in laboratory diagnosis is possible. Furthermore, several studies did not identify *Staphylococcus*, *Pseudomonas* and *Proteus* at the species level (Bugden, 2013; Lyskova et al., 2007; Moraes, Pereira, Silva, Moreira, & Casseb, 2014; Petrov et al., 2013; Subapriya et al., 2015; Türkyilmaz, 2008). Moreover, the frequency and types of microorganism isolated from

canine OE reported in these studies can differ for many reasons, depending on the type of canine OE cases, stages of infection and diagnostic approach. A first time consultation with an acute canine OE will most probably detect Gram-positive cocci with or without yeast if cytology and ear swab are routinely performed for culture and identification (Miller et al., 2013; Paterson, 2016a). On the other hand, a referral case or a chronic and relapsing case of canine OE with treatment failure, underlying conditions or severe external ear pathological changes will most likely find Gram-negative rods by cytology and microbiological diagnoses (Paterson & Matyskiewicz, 2018; Rosser, 2004; Zur et al., 2011).

Recent studies identified and compared aural bacterial and fungal microbiota in dogs with and without OE by molecular methods comprising DNA extraction, PCR amplification and sequencing (Korbelik, Singh, Rousseau, & Weese, 2018, 2019). Korbelik et al. (2019) analysed approximately 1.7 million sequences from dogs with bacterial otitis and identified four predominant genera: *Staphylococcus*, *Pseudomonas*, *Parvimonas* and *Fusobacterium*. It is not unexpected to find *Staphylococcus* spp. and *Pseudomonas* spp. associated with canine OE as discussed earlier in this review, but the findings of both anaerobic Gram-positive *Parvimonas* spp. and Gram-negative *Fusobacterium* spp. in canine OE might have been under-reported in the culture-based studies. Additionally, Korbelik et al. (2019) found that the detection of the genus *Escherichia* increased in healthy ears compared to infected ears. *Escherichia* may play a protective role in healthy ears as well as being an indicator of bacterial dysbiosis in disease conditions (Ngo, Taminiau, Fall, Daube, & Fontaine, 2018). In another study analysing approximately 1.4 million sequences from dogs with fungal otitis, three main genera *Malassezia*, *Filobasidium* and *Holtermanniella* were identified, and *M. pachydermatis* (55.7 – 98.4%) was the most common species (Korbelik et al., 2018). Molecular methods in microbiology are sensitive and specific to identify a more diverse range of species that may not be detected and limited by the conventional culture-based methodology (Rhoads, Wolcott, Sun, & Dowd, 2012). Previous studies using molecular methods demonstrated that the skin

microbiota of dogs was, in reality, more abundant and diverse compared to those identified by culture-based methods (Bradley et al., 2016; Hoffmann et al., 2014; Weese, 2013).

Table 1: Comparison of the microbiological profiles of canine otitis externa in various countries

References: Author (Year)	**Country of origin**	**Number of dogs/ swabs**	**Gram-positive bacteria**		**Gram-negative bacteria**			**Yeast**
			Staphylococcus pseudintermedius	β-haemolytic *Streptococcus* spp. or *Strep. canis*	*Pseudomonas aeruginosa*	*Proteus mirabilis*	*Escherichia coli*	*Malassezia pachydermatis*
Kiss, Radvanyi, and Szigeti (1997)	Hungary	515	39.2%	[c] 1.2%	12.6%	-	-	76.3%
Hariharan, Coles, Poole, Lund, and Page (2006)	Canada	1819	36.3%	1.9%	17.5%	9.6%	9.8%	-
Fernández et al. (2006)	Venezuela	53	1.9%	-	30.2%	18.9%	7.5%	69.9%
Lyskova et al. (2007)	Czech Republic	97	58.8%	29.9%	7.2%	[b] 14.4%	10.3%	30.9%
Oliveira et al. (2008)	Brazil	100	70%	[c] 2%	3%	7%	1%	73%
Türkyilmaz (2008)	Turkey	88	[a] 25%	[c] 9.1%	18.2%	[b] 9.1%	11.4%	-
Penna et al. (2010)	Brazil	151	23.2%	-	-	-	-	-
Malayeri et al. (2010)	Iran	74	91.9%	-	13.5%	4.1%	1.4%	-
Engelen, De Bock, Hare, and Goossens (2010)	US and Canada	176	52.8%	3.4%	10.8%	6.3%	-	44.3%
Rigaut et al. (2011)	France, German, and Spain	176	46.6%	-	8.6%	5.2%	5.2%	32.9%
Petrov et al. (2013)	Bulgaria	241	[a] 70%	10.4%	17.4%	11.2%	11.6%	40.2%
Bugden (2013)	Australia	3541	24.3%	6.2%	35.5%	[b] 6.8%	4.2%	-
Moraes et al. (2014)	Brazil	57	[c] 37.8%	[c] 12.2%	17.7%	[b] 11.0%	3.7%	15.4%
Subapriya et al. (2015)	India	240	[c] 21.8%	[c] 10.0%	[c]49.3%	[c] 6.6%	17.0%	-
De Martino et al. (2016)	Italy	196	23.0%	0.51%	11.7%	4.6%	3.1%	10.7%
Bourély et al. (2019)	France	7623	33.0%	5.9%	27.6%	13.6%	-	-
Demırbılek and Yılmaz (2019)	Turkey	277	5.4%	7.6%	18.4%	9.7%	4.3%	9.7%

Notes:

[a] CoPS= Coagulase-positive *Staphylococcus* spp., speciation not done

[b] *Proteus* spp., speciation not done

[c] speciation not done

1.4 Antimicrobial susceptibility and resistance profiles

The antimicrobial susceptibility and resistance profiles of *S. pseudintermedius*, *P. aeruginosa* and *M. pachydermatis* in the following sections were reviewed using clinical breakpoints of systemic therapy typically described in the majority of literature. However, clinical breakpoints are not defined for topical antimicrobial agents which are the most common treatment modality used to treat canine OE. Besides, topical antimicrobial therapy usually delivers a high concentration of antimicrobial in the mg/mL concentration range that can overcome apparent *in vitro* resistance to concentrations in the μg/mL range (Nuttall, 2013). Therefore, the results of *in vitro* antimicrobial susceptibility tests are less applicable and poorly predictive of the outcome to topical therapy.

1.4.1 Antimicrobial susceptibility and resistance profiles of *Staphylococcus pseudintermedius*

Table 2 shows the resistance profiles of *S. pseudintermedius* to a range of antimicrobials commonly available in commercial otic products. Overall, *S. pseudintermedius* showed a trend of reduction in susceptibility to enrofloxacin and gentamicin over two decades. Studies between 1997 and 2014 demonstrated high susceptibility of *S. pseudintermedius* to enrofloxacin from 82% to 100% and gentamicin from 96% to 100% (Bugden, 2013; Hariharan et al., 2006; Kiss, Radvanyi, & Szigeti, 1997; Lyskova et al., 2007; Malayeri et al., 2010). In comparison to more recent studies between 2013 and 2019, *S. pseudintermedius* showed resistant to enrofloxacin up to 38.6% and gentamicin up to 33.3% (De Martino et al., 2016; Dégi et al., 2013; Scherer et al., 2018). Polymyxin B resistance was reported in the earliest study with only 31% resistant, but 100% resistant were demonstrated in the Australian and Romanian studies (Bugden, 2013; Dégi et al., 2013; Kiss, Radvanyi, & Szigeti, 1997). *S. pseudintermedius* was highly susceptible to neomycin in the Australian study, but resistance was found in other studies from 31.1 to 91.4% (Bugden, 2013; De Martino et al., 2016; Kiss, Radvanyi, & Szigeti, 1997; Penna et al., 2010). A more recent retrospective survey in dogs

with OE from 2012 to 2016 by Bourély et al. (2019) revealed resistance of *S. pseudintermedius* to enrofloxacin, gentamicin and fusidic acid in 13.5%, 13% and 6.1%, respectively.

However, there were more susceptibility and resistance data reported in the listed studies in relation to systemic antimicrobials. The majority of these studies demonstrated high resistance to penicillin G from 62% to 69% (Bourély et al., 2019; Hariharan et al., 2006; Kiss, Radvanyi, & Szigeti, 1997; Lyskova et al., 2007; Malayeri et al., 2010). A survey conducted throughout Europe between 2002 and 2009 analysed that *S. pseudintermedius* (n=199) isolated from cats and dogs with otitis were highly susceptible to enrofloxacin (96.5%), marbofloxacin (97%) and amoxicillin-clavulanic acid (100%), but highly resistant to ampicillin (66.3%) and penicillin (73%) (Kroemer et al., 2014). On the other hand, a Brazilian study in 2010 reported significant resistance in *S. pseudintermedius* (35 isolates), to enrofloxacin (48.6%), gentamicin (54.3%) and neomycin (91.4%) but susceptibility to amoxicillin-clavulanic acid (85.7%), oxacillin (85.7%) and cephalexin (74.3%) (Penna et al., 2010). Additionally, Penna et al. (2010) highlighted that 89% of *Staphylococcus* spp. exhibited multiple drugs resistance as well as 100% resistance to at least one drug. Recent studies have reported that no single drug tested was 100% effective for *Staphylococcus* spp. (Moraes et al., 2014; Petrov et al., 2013).

Table 2: Comparison of resistance (%) of *Staphylococcus pseudintermedius* isolated from canine otitis externa to a range of antimicrobials commonly available in commercial otic products

References: Author (Year)	Country of origin	Number of isolates	Enrofloxacin[a]	Marbofloxacin[b]	Polymyxin B[c]	Gentamicin[d]	Neomycin[e]	Fusidic acid[f]
Kiss, Radvanyi, and Szigeti (1997)	Hungary	515	2%	-	31%	4%	35%	-
Hariharan et al. (2006)	Canada	660	1%	-	-	2%	-	3%
Lyskova et al. (2007)	Czech Republic	100	0%	-	-	0%	-	-
Penna et al. (2010)	Brazil	35	48.6 %	-	-	54.3%	91.4%	-
Malayeri et al. (2010)	Iran	68	0%	-	-	3.03%	-	-
Bugden (2013)	Australia	861	2%	-	100%	1%	4%	-
Dégi et al. (2013)	Romania	24	-	-	100%	33.3%	-	-
Kroemer et al. (2014)	France/ Europe	199	3.5%	3.0%	-	-	-	-
De Martino et al. (2016)	Italy	45	15.5%	-	-	11.1%	31.1%	-
Scherer et al. (2018)	Brazil	44	38.6%	-	-	27.3%	-	-
Bourély et al. (2019)	France	Varies	13% (n=2115)	-	-	13.5% (n=2491)	-	6.1% (n=1757)

Notes: a = Baytril® otic; b = Aurizon®; c = Surolan™ or Dermotic or PMP; d = Oribiotic®; e = Easotic® or Otomax® or Momentamax®; f = Canaural®; refer to table 4 for additional details of commercial otic products.

1.4.2 Antimicrobial susceptibility and resistance profiles of *Pseudomonas aeruginosa*

Table 3 shows the resistance profiles of *P. aeruginosa* isolated from canine OE to a range of antimicrobials commonly available in commercial otic products. This table lists the studies in chronological order in which the prevalence of resistance might be expected to increase over time. However, the table does not show such a trend but identifies geographical differences. A Brazilian study revealed the most comprehensive resistance profiles in 167 *P. aeruginosa* strains and reported 63.6% strains resistant to enrofloxacin, 54.4% strains resistant to polymyxin B, 71.4% strains resistant to gentamicin, and 94.7% strains resistant to neomycin (Penna, Thomé, Martins, Martins, & Lilenbaum, 2011). In a French surveillance network for antimicrobial resistance, data were analysed on *P. aeruginosa* isolated from dogs with otitis. Resistance to enrofloxacin and gentamicin were detected in 64.3% of 1996 strains and 17.8 % of 2096 strains, respectively (Bourély et al., 2019). Another study revealed that *P. aeruginosa* canine OE isolates were highly resistant to enrofloxacin and gentamicin in 51.9% and 43.3% of the strains, respectively (Mekić, Matanović, & Šeol, 2011). Overall, enrofloxacin and gentamicin are more frequently included in antimicrobial susceptibility testing against *P. aeruginosa* and detected for resistance.

Interestingly, a 100% resistance as well as a 100% susceptibility of *P. aeruginosa* to enrofloxacin were reported by Graham-Mize and Rosser Jr. (2004) and Malayeri et al. (2010), respectively whereby both studies examined less than 25 isolates. On the other hand, Bugden (2013) examined 1,256 isolates and Bourély et al. (2019) surveyed 1996 isolates. These studies demonstrated that 36% and 64.3% of *P. aeruginosa* strains showed enrofloxacin resistance, respectively. A larger number of samples in a study may prevent an overestimation of enrofloxacin–susceptible or –resistant strains of *P. aeruginosa.* The majority of the studies listed in table 3 show susceptibility of *P. aeruginosa* strains to gentamicin ranging from 81 to 100%. Resistance to marbofloxacin was not found in the previous study by Martin Barrasa, Lupiola Gomez, Gonzalez Lama, and Tejedor Junco (2000), but 26.4 % to 33.3% of *P. aeruginosa* strains were resistant to marbofloxacin in the more recent studies (Kiss, Radvanyi,

Szigeti, et al., 1997; Vingopoulou et al., 2018; Wildermuth, Griffin, Rosenkrantz, & Boord, 2007).

As Gram-negative *P. aeruginosa* is inherently resistant to penicillins and first-generation cephalosporins, the susceptibility or resistance to those antibiotics is not included in Table 3. As expected, multiple studies reported *P.* aeruginosa being totally (100%) resistant to amoxicillin/clavulanic acid, ampicillin, penicillin G, cephalexin and cephalothin (Hariharan et al., 2006; Lyskova et al., 2007; Malayeri et al., 2010; Petrov et al., 2013). *P. aeruginosa* was also highly resistant (≥ 87 to 100%) to other antimicrobial classes including tetracycline (e.g. doxycycline), chloramphenicol, macrolides (e.g. erythromycin) and lincosamides (e.g. clindamycin and lincomycin) (Hariharan et al., 2006; Lyskova et al., 2007; Malayeri et al., 2010; Petrov et al., 2013; Türkyilmaz, 2008).

Table 3: Comparison of resistance (%) of *Pseudomonas aeruginosa* isolated from canine otitis externa to a range of antimicrobials commonly available in commercial otic products.

References: Author (Year)	Country of origin	Number of isolates	Enrofloxacin[a]	Marbofloxacin[b]	Polymyxin B[c]	Gentamicin[d]	Neomycin[e]	Fusidic acid[f]
Kiss, Radvanyi, and Szigeti (1997)	Hungary	515	-	-	21%	8%	43%	-
Martin Barrasa et al. (2000)	Spain	19	26.3%	0%	-	15.8%	-	-
Graham-Mize and Rosser Jr. (2004)	Michigan	24	100%	-	-	50%	-	-
Hariharan et al. (2006)	Canada	319	38%	-	0%	15%	-	100%
Lyskova et al. (2007)	Czech Republic	9	71.4%	-	-	0%	-	-
Wildermuth et al. (2007)	California	32	53.1%	33.3%	-	-	-	-
Türkyilmaz (2008)	Turkey	16	-	-	-	19%	-	-
Malayeri et al. (2010)	Iran	10	0%	-	-	10%	-	-
Penna et al. (2011)	Brazil	167	63.6%	-	54.4%	71.4%	94.7%	-
Mekić et al. (2011)	Croatia	104	51.9%	-	-	43.3%	-	-
Petrov et al. (2013)	Bulgaria	42	38%	-	0%	2%	-	-
Bugden (2013)	Australia	1256	36%	-	7%	5%	-	-
Kroemer et al. (2014)	France	329	83%	26.4%	-	-	-	-
De Martino et al. (2016)	Italy	23	43.5%	-	-	8.7%	65.2%	-
Vingopoulou et al. (2018)	Greece	75	44%	32%	-	-	-	-
Demırbılek and Yılmaz (2019)	Turkey	51	35%	-	1%	1%	-	-
Bourély et al. (2019)	France	Varies	64.3% (n=1996)		-	17.8% (n=2096)	-	-

Notes: a = Baytril® otic; b = Aurizon®; c = Surolan™ or Dermotic or PMP; d = Oribiotic®; e = Easotic® or Otomax® or Momentamax®; f = Canaural®; refer to table 4 for additional details of commercial otic products.

1.4.3 Antimicrobial susceptibility and resistance profiles of *Malassezia pachydermatis*

There is increased concern about treatment failure and recurrence of *Malassezia* yeast otitis due to antifungal resistance along with poor management of primary causes and predisposing factors (Chiavassa, Tizzani, & Peano, 2014). In a study evaluating the potency of antifungal agents used in otic formulations, *M. pachydermatis* was most susceptible to miconazole with the lowest *in vitro* minimum inhibitory concentration (MIC) ranges compared to clotrimazole and thiabendazole (Peano, Beccati, Chiavassa, & Pasquetti, 2012). In a recent study, 54.3% of *M. pachydermatis* was found to be susceptible to gentamicin, an aminoglycoside antibiotic used in some topical otic formulations (F. A. Silva et al., 2017).

However, due to previously non-standardised methodology in antifungal susceptibility testing of *M. pachydermatis*, comparison of results with different studies becomes challenging (Cafarchia et al., 2012; Eichenberg et al., 2003; Peano et al., 2017). Thus, a susceptible strain in one study could be interpreted as resistance in other testing conditions and vice versa (Peano et al., 2017). Eichenberg et al. (2003) demonstrated that *M. pachydermatis* isolated from canine OE (n= 82) was susceptible to antifungal agents such as ketoconazole (56%), fluconazole (54%), itraconazole (69%) using modified broth microdilution methodology. However, an earlier study using disk diffusion methodology was only comparing the average diameter of inhibition zones to determine the susceptibility of *M. pachydermatis* otic isolates (n=80) to five antifungal agents (Kiss, Radvanyi, & Szigeti, 1997). Kiss, Radvanyi, and Szigeti (1997) revealed that *M. pachydermatis* was most susceptible to ketoconazole and least sensitive to nystatin with econazole and miconazole showing intermediate efficacy. In a later study using disk diffusion methodology, clinical breakpoints were available to determine the susceptibility of *M. pachydermatis* otic isolates (Lyskova et al., 2007). *M. pachydermatis* otic isolates were 100% susceptible to amphotericin B, bifonazole, ciclopiroxolamin, clotrimazole, miconazole, econazole, itraconazole, ketoconazole, nystatin and pimaricin except for fluconazole where 4.4% resistant strains were detected (Lyskova et al., 2007).

1.5 Treatment of canine otitis externa

1.5.1 Topical otic treatment

Topical antimicrobial and anti-inflammatory therapy is the first-line treatment to manage bacterial and yeast overgrowth and to relieve signs of pain and inflammation in cases of canine OE cases (Dowling, 1996). However, the choice of topical antimicrobial will depend on the initial findings in the cytological examination, or be based on microbiological culture and sensitivity testing in cases of chronic OE complicated by rods overgrowth or following unsuccessful treatment (Dowling, 1996). Topical ear treatments can also be beneficial in the management of primary, predisposing and perpetuating causes of otitis (Paterson, 2016b). For instance, a topical product containing glucocorticoids will be chosen according to its potency to manage the inflammation caused by allergies or immune-mediated diseases (Paterson, 2016b). Table 4 shows a list of commercially available topical otic preparations. The active ingredients in each otic product are categorised by antibiotic, antifungal agent and anti-inflammatory agent. Efficacy studies on the antibiotic and antifungal agent combination relevant to each otic formulation are recorded in the last column of the table.

Several clinical studies compared two or more combinations of antimicrobials and anti-inflammatory in the otic preparations for their efficacy and tolerability in canine OE. These *in vivo* studies utilised controlled, randomised and double-blinded designs according to the good clinical practice (GCP) guidelines (Blake, Keil, Kwochka, Palma, & Schofield, 2017; Engelen et al., 2010; Kiss, Radvanyi, Szigeti, et al., 1997). Efficacy assessment often includes clinical scoring of pain, erythema, swelling, ulceration and exudates pre- and post-treatment at weekly intervals and other evaluations based on cytology, microbiology, treatment response and cure rate (Engelen et al., 2010; Rigaut et al., 2011). Safety evaluation includes haematology and biochemistry profiles, urinalysis, hearing assessment and observation of the frequency and severity of adverse events (Blake et al., 2017; Rigaut et al., 2011). Rougier, Borell, Pheulpin, Woehrlé, and Boisramé (2005) demonstrated that both marbofloxacin-clotrimazole-dexamethasone (Aurizon®) and miconazole-polymyxin B-prednisolone (Surolan®) otic

formulations did not show a significant difference in efficacy and tolerability, but the former was superior in terms of glucocorticoid potency to reduce pain and once daily dosing that encouraged higher client compliance. Studdert and Hughes (1991) found that miconazole-polymyxin B-prednisolone (Surolan®) had the fastest cure and lowest recurrence rate when compared to two other combination therapies containing neomycin-thiostrepton-nystatin-triamcinolone (Panalog®) and neomycin-monosulfiram-betamethasone (Oterna®, Jurox). Engelen et al. (2010) recommended miconazole-polymyxin B-prednisolone (Surolan®) as a first-line combination topical therapy for canine OE that does not require a potent glucocorticoid. Furthermore, miconazole-polymyxin B-prednisolone (Surolan®) had a clinical efficacy of 97% in 91 cases compared to gentamicin-clotrimazole-betamethasone (Otomax®) with 95% clinical efficacy in 85 cases, hence demonstrating equivalent efficacy to eliminate infections and improve overall clinical response (Engelen et al., 2010). On the other hand, Rigaut et al. (2011) claimed that the otic product (Easotic®) containing gentamicin, miconazole and hydrocortisone aceponate administered once daily for 5 days, was an efficient and safe treatment for canine OE and it was non-inferior to gentamicin-clotrimazole-betametasone (Otomax®) that requires twice daily application for 7 days.

Newer topical otic formulations have been developed to improve treatment success and client compliance by reducing frequency and difficulty to deliver ear treatment and handling of dogs. Blake et al. (2017) demonstrated that a single treatment of florfenicol-terbinafine-mometasone (Claro®) achieved 72.5% clinical efficacy for up to 30 days in cases of otitis associated with *S. pseudintermedius* and *M. pachydermatis.* Similarly, florfenicol-terbinafine-betamethasone (Osurnia®) only requires two doses a week apart to effectively treat *S. pseudintermedius* and *M. pachydermatis* infection with resolution for up to 35 days (Nuttall & Forster, 2015). Furthermore, Noli, Sartori, and Cena (2017) demonstrated that the administration of single-dose or once a week ear treatment in dogs with OE by a veterinarian instead of their owners, significantly improved the quality of life of dogs and owners.

Other studies demonstrated the efficacy of antimicrobials in otic formulations *in vitro*. Combination antimicrobial therapy is crucial to prevent or delay the development of antimicrobial resistance (Domalaon, Idowu, Zhanel, & Schweizer, 2018). Pietschmann et al. (2013) demonstrated that the combination of polymyxin B and miconazole produced a synergistic effect against *E. coli and P. aeruginosa* but not with *S. pseudintermedius.* Similarly, Boyen et al. (2012) did not find synergistic activity with polymyxin B and miconazole combinations *in vitro* against methicillin-resistant *Staphylococcus aureus* (MRSA) and meticillin-resistant *S. pseudintermedius* (MRSP) isolates but effectiveness against these isolates was still possible with the high concentration of miconazole in commercial topical products (e.g. Surolan®). Another study found synergism in the combination of fusidic acid and framycetin (Canaural®) against *S. pseudintermedius* and β-haemolytic *Streptococcus* spp. whereas the combination of polymyxin B and miconazole had a synergistic effect only against β-haemolytic *Streptococcus* spp., but not *S. pseudintermedius* (Nuttall, Allison, Hale, & Williams, 2011).

Table 4: Various types of commercial otic products categorised by active ingredients (antibiotic, antifungal and anti-inflammatory) with the recommended dosages for canine otitis externa and the relevant efficacy studies.

Commercial otic products	**Company, Country**	**Antibiotic**	**Antifungal agent**	**Anti-inflammatory agent**	**Dosage and administration in dogs**	**Efficacy studies: Author (Year)**
Aurizon®	Vetoquinol, UK	Marbofloxacin (3 mg/ml)	Clotrimazole (10 mg/ml)	Dexamethasone (0.9 mg/ml)	10 drops per ear once daily for 7-14 days	Rougier et al. (2005)
Baytril® Otic	Bayer, USA	Enrofloxacin (5 mg/ml)	Silver Sulfadiazine (10 mg/ml)	Not available	5-10 drops(<16kg), 10-15 drops (>16kg) twice daily for 14 days	Bae, Choi, Kim, Lee, and Oh (2013)
Claro®	Bayer, USA	Florfenicol (16.6 mg/ml)	Terbinafine (14.8 mg/ml)	Mometasone (2.2 mg/ml)	1 tube (1ml) per ear once	Blake et al. (2017)
Osurnia®	Elanco, USA	Florfenicol (10 mg/ml)	Terbinafine (10 mg/ml)	Betamethasone (1 mg/ml)	1 tube (1ml) per ear every 7 days	(Nuttall & Forster, 2015), Forster, Real, Doucette, and King (2018), Noli et al. (2017)
Surolan™	Elanco, USA	Polymyxin B (0.5293 mg/ml)	Miconazole (23 mg/ml)	Prednisolone (5 mg/ml)	5 drops twice daily for 7 days	Studdert and Hughes (1991), Rougier et al. (2005), Engelen et al. (2010), Boyen et al. (2012), Pietschmann et al. (2013)
Dermotic	Ilium, AU	Polymyxin B (0.696 mg/ml)	Miconazole (23 mg/ml)	Prednisolone (5 mg/ml)	3-5 drops twice daily for 14-21 days	
PMP	Apex Lab., AU or Dechra	Polymyxin B (0.696 mg/ml)	Miconazole (23 mg/ml)	Prednisolone (5 mg/ml)	3-5 drops twice daily	
Easotic®	Virbac, France	Gentamicin (1505 IU/ml)	Miconazole (15.1 mg/ml)	Hydrocortisone aceponate (1.11mg/ml)	1 pump (1ml) per day for 5 days	Rigaut et al. (2011)
Otomax®	Merck, USA	Gentamicin (3 mg/ml)	Clotrimazole (10 mg/ml)	Betamethasone (1 mg/ml)	4 drops (<13.6kg), 8 drops (≥13.6kg) twice daily for 7 days	Engelen et al. (2010), Rigaut et al. (2011)
Mometamax®	Merck, USA	Gentamicin (3 mg/ml)	Clotrimazole (10 mg/ml)	Mometasone (1mg/ml)	4 drops (<13.6kg), 8 drops (≥13.6kg) once daily for 7 days	

Table 4 (continued)

Commercial otic products	**Company, Country**	**Antibiotic**	**Antifungal agent**	**Anti-inflammatory agent**	**Dosage and administration in dogs**	**Efficacy studies: Author (Year)**
Oribiotic®	Vetoquinol, France	Neomycin (3 mg/ml), Bacitracin (430 IU/ml)	Nystatin (86,000 IU/ml)	Triamcinolone (0.86 mg/ml)	A pea of ointment per ear 2-3 times a day for 7-14 days.	-
Panalog®	Fort Dodge, USA	Neomycin (2.5 mg/ml), Thiostrepton (2,500 units/ml)	Nystatin (100,000 IU/ml)	Triamcinolone (1 mg/ml)	3-5 drops twice daily	Rycroft and Saben (1977)
Dermalone™	Vedco, USA	Neomycin (2.5 mg/ml), Thiostrepton (2,500 units/ml)	Nystatin (100,000 units/ml)	Triamcinolone (1 mg/ml)	3-5 drops twice daily	
Tresaderm®	Merial, USA	Neomycin (3.2 mg/ml)	Thiabendazole (40 mg/ml)	Dexamethasone (1 mg/ml)	5-15 drops twice daily	Rycroft and Saben (1977)
Canaural® ear drop	Dechra, UK	Fusidic acid (5 mg/ml), Framycetin (5 mg/ml)	Nystatin (100,000 IU/ml)	Prednisolone (2.5 mg/ml)	5-10 drops twice daily for 7-14 days	Nuttall et al. (2011)
Posatex™	Merck, USA	Orbifloxacin (10 mg/ml)	Posaconazole (1 mg/ml)	Mometasone (1 mg/ml)	4 drops (<13.6kg), 8 drops (≥13.6kg) once daily for 7 days	Grecu et al. (2018)

1.5.2 Topical ear cleaners

Various studies have demonstrated the potential of chelating agents or ear cleaning solutions to increase the *in vitro* antimicrobial susceptibility of pathogens associated with canine OE to a primary antimicrobial agent. The earliest study described successful treatment of canine OE associated with resistant Gram-negative and Gram-positive pathogens within 7 to 15 days by application of topical EDTA-tromethamine/EDTA-Tris for 10 mins before the use of previously ineffective antibiotics (cephaloridine or kanendomycin or enrofloxacin) that were applied alone (Farca, Piromalli, Maffei, & Re, 1997). Likewise, an investigation by Metry et al. (2012) showed that *P. aeruginosa* was more susceptible to enrofloxacin compounded in four types of ear cleansers (Tris-EDTA, Tris-EDTA and 0.15% chlorhexidine, Epi-Otic® (Virbac) and Epi-Otic Advanced® (Virbac) containing 0.5% EDTA) and hypothesised that previously resistant bacteria can become susceptible to enrofloxacin when compounded with ear cleaner solutions. Furthermore, Pye, Singh, and Weese (2014) reported that Triz EDTA® (Dechra) aqueous flush containing tromethamine-EDTA and deionised water used in combination with either neomycin or gentamicin, significantly increased antimicrobial susceptibility of *Pseudomonas* otitis associated with biofilm formation.

Hensel, Austel, Wooley, Keys, and Ritchie (2009) demonstrated that a third-generation chelating agent (Tris/EDTA or Tricide®, Molecular Therapeutic LCC) potentiated the antifungal activity of miconazole against *M. pachydermatis* ear infections in dogs, and this combined therapy was recommended for cases with predominantly yeast otitis. Another study revealed that the use of ear cleaner containing tromethamine-EDTA alone was ineffective against *M. pachydermatis* unless 0.1% ketoconazole was added (Cole, Luu, Rajala-Schultz, Meadows, & Torres, 2007). In agreement with the previous study, no antimicrobial activity was found against *S. pseudintermedius* (*S. intermedius)*, *P. aeruginosa* or *M. pachydermatis* with the used of Tris-EDTA alone (Swinney, Fazakerley, McEwan, & Nuttall, 2008). Mason, Steen, Paterson, and Cripps (2013) demonstrated that five out of nine topical ear cleaners showed *in vitro* efficacy against *M. pachydermatis* otic isolates. Two of the five effective ear

cleaners were Epi-Otic® (Virbac) and Triz-Ultra+Keto™ (Dechra), and each similarly contains a chelating agent EDTA or Tris-EDTA and an antifungal agent such as salicylic acid and ketoconazole, respectively (Mason et al., 2013).

Additionally, ear cleaners containing lactic acid and salicylic acid (Sancerum®, MSD) and boric acid and citric acid (MalAcetic Aural®, Dechra), both had anti-*Malassezia* and antipseudomonal activities (Mason et al., 2013; Steen & Paterson, 2012). On the other hand, an ear cleaner containing isopropanol, propylene glycol, tromethamine and citric acid (Cleanaural® Dog, Dechra) was concluded the most effective ear cleaner against *S. pseudintermedius* (*S. intermedius)*, *P. aeruginosa* and *M. pachydermatis* (Swinney et al., 2008). The aforementioned organic acids in the ear cleaners have a mild acidifying effect to lower pH in the ear canal and inhibiting microbial growth (Swinney et al., 2008). Otodine® (Vetruus) containing propylene glycol, chlorhexidine, and Tris-EDTA, a ceruminolytic agent was used to clean ears daily (up to 7 days) prior to administration of ear formulations containing orbifloxacin-posaconazole-mometasone (Posatex®, Merck) to increase the clinical efficacy and treatment success in canine OE (Grecu et al., 2018).

1.6 Antimicrobial resistance

Globally, in recent decades, the emergence of antimicrobial resistance (AMR) associated with superbugs such as methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci (VRE), multidrug-resistant *P. aeruginosa* and other ESKAPE pathogens have led to threatening infectious diseases with an estimated mortality by 2050 of up to10 million people annually and global economic losses of up to 100 trillion USD if no action is taken(O'Neill, 2016). Other ESKAPE pathogens (*Klebsiella pneumoniae* and *Acinetobacter baumannii)* exhibit multi-drug resistance, including resistance to carbapenems; and *Enterobacter* spp., which contains extended-spectrum β-lactamases (ESBL)s and carbapenemases (Pendleton, Gorman, & Gilmore, 2013; Santajit & Indrawattana, 2016). Unfortunately, there is no escape from the development of AMR in production and companion animals and the increasing reports

of AMR in human populations involving zoonotic pathogens, poses further risk to public health (Pomba et al., 2017; Rendle & Page, 2018).

Existing effective antibiotics are becoming increasingly inadequate to meet the current clinical requirement. Moreover, only a few new antimicrobial classes (e.g. lipopeptides and oxazolidinones) have been approved for use since the 1980s, and the current research and development of new antimicrobials and antimicrobial classes is unable to keep up with the dissemination of AMR (Blaskovich, 2019). Antimicrobial misuse and overuse both in human and veterinary medicine are the other contributing factors to the development and spread of resistance (Weese, Page, & Prescott, 2013). Awareness in prudent use of antimicrobials and global enforcement on good practices of antimicrobial stewardship are urgently needed to reduce resistance selection and to preserve the efficacy of existing antimicrobials (Weese et al., 2013).

1.6.1 Development of antimicrobial resistance

In general, there are three types of antimicrobial resistance mechanisms in bacteria: acquired, intrinsic and adaptive (Alekshun & Levy, 2007). Acquired resistance develops through the process of chromosomal gene mutation or horizontal transfer of genes incorporated into new genetic material such as plasmids, integrons or naked DNA. This provides a selective advantage when the bacteria are in the presence of antimicrobial compounds that can be passed on to their progeny causing emergence of antimicrobial resistance strains (Kostyanev & Can, 2017; Tenover, 2006). Intrinsic resistance is commonly found in Gram-negative bacteria due to the presence of an extra outer membrane consisting of a double layer of phospholipids and lipopolysaccharides which restricts the penetration of hydrophobic and high molecular weight antibiotics (Cox & Wright, 2013; Domalaon et al., 2018). Furthermore, most Gram-negative bacteria possess efflux pump systems that remove drug compounds targeting intracellular pathways. (Cox & Wright, 2013; Kostyanev & Can, 2017). Adaptive resistance involves antimicrobial selective pressure that increases the ability of bacteria to survive in an

antimicrobial rich environment by alterations in gene or protein expression triggered by environmental conditions (Bernal, Molina-Santiago, Daddaoua, & Llamas, 2013). Subinhibitory levels of antibiotics, stress, nutrient condition and growth state are some of the contributing factors to adaptive resistance in bacteria (Poole, 2012). Biofilm or exopolysaccharide matrix formation is another bacterial resistance mechanism that produces an extra barrier to prevent diffusion of drugs into the bacterial cell (Costerton, Stewart, & Greenberg, 1999). Biofilm resistance mechanisms mainly involve physiological changes in biofilm-grown bacteria such as slow growth, nutrient limitation, induction of general stress response, increasing expression of multidrug-resistant (MDR) pumps, activating quorum-sensing systems and changing profiles of outer membrane proteins (Bjarnsholt, Ciofu, Molin, Givskov, & Hoiby, 2013; Mah & O'toole, 2001; Stewart, 2002). Biofilm formation can make bacteria ten to a thousand times more resistant to antibiotics compared to the planktonic state of the same bacterial strain (Mah & O'toole, 2001; Olson, Ceri, Morck, Buret, & Read, 2002).

1.6.2 Antimicrobial resistance in canine otitis externa pathogens

1.6.2.1 Methicillin-resistant and multidrug-resistant Staphylococcus pseudintermedius

There is an increasing amount of literature reporting methicillin-resistant *Staphylococcus pseudintermedius* (MRSP) and multidrug-resistant *Staphylococcus pseudintermedius* (MDRSP) associated with otitis and other skin diseases in companion animals (Morris, Rook, Shofer, & Rankin, 2006; Saputra et al., 2017; Weese & van Duijkeren, 2010). Note that in the older literature, *S. pseudintermedius* from canine isolates was previously identified as *S. intermedius* (Bannoehr et al., 2007; Sasaki et al., 2007). A retrospective survey from 2001 to 2005 in a US veterinary teaching hospital reported an increasing prevalence of oxacillin-resistance which corresponded with multidrug-resistance in *S. pseudintermedius* isolated from dogs (Jones, Kania, Rohrbach, Frank, & Bemis, 2007). In 2005, another study revealed that 94% (31 out 33) of *S. pseudintermedius* isolates were MDRSP, resistant to ≥ 4 antimicrobial classes, oxacillin-resistant and mecA-positive (Jones et al., 2007). In an European study, twelve

S. pseudintermedius isolates from 11 dogs and a cat with skin and ear infections were shown to be positive for the mecA gene indicating methicillin resistance (MRSP) and multidrug resistance to at least five antimicrobial classes including penicillins, cephalosporins, fluoroquinolones, macrolides, tetracyclines and aminoglycosides (Loeffler et al., 2007). A prospective study in Brazil revealed that 85.7% (33/35) of *S. pseudintermedius* in canine OE were MDRSP, highly resistant to amoxicillin-clavulanic acid (85.7%) and least resistant to neomycin (8.6%) (Penna et al., 2010). In a two-year Korean study, 78.4% of *S. pseudintermedius* isolated from the skin or ears in dogs were MDRSP, resistant to $\geq$ 4 antimicrobial classes excluding β-lactams, and 17.6% were simultaneously mecA-positive MRSP (Yoon et al., 2010). Another Korean study found that 52% of mecA-positive MRSP (n=25) from canine OE or pyoderma were highly resistant to at least one fluoroquinolone – enrofloxacin, marbofloxacin, ciprofloxacin, levofloxacin or moxifloxacin (J.-H. Yoo, Yoon, Lee, & Park, 2010). Dégi et al. (2013) demonstrated that 24 *S. pseudintermedius* isolated from canine OE before any antimicrobial treatment, were resistant to at least two drugs and were all resistant to polymyxin B. In a recent Brazilian study, 86% (38/44) of *S. pseudintermedius* isolated from canine OE were resistant to at least one antibiotic and 40.9% (18/44) were classified as MRSP, based on resistance to oxacillin by disk diffusion and mecA gene detection by PCR (Scherer et al., 2018). In the latest 4-year AMR survey on canine OE, 56% of *S. pseudintermedius* (n=1066) were resistant to one or two antibiotic classes, 20.7% were multidrug-resistant and 2 isolates were pan-resistant to eight antimicrobial classes: aminoglycosides, cephalosporins, fluoroquinolones, macrolides, penicillins, chloramphenicol, fusidic acid and trimethoprim-sulfamethoxazole (Bourély et al., 2019).

1.6.2.2 Multidrug-resistant Pseudomonas aeruginosa

Recent studies have documented the emergence of multidrug-resistant *P. aeruginosa* (MDRPA) with resistance to at least one antimicrobial agent in three or more antimicrobial classes commonly reported (Ciocan et al., 2015). This has been coupled with an increasing frequency of fluoroquinolone resistance (Mekić et al., 2011; Penna et al., 2011). In a study from 2000 (Martin Barrasa et al., 2000), *P. aeruginosa* was associated with chronic and recurrent OE in dogs with the highest fluoroquinolone resistance seen to enrofloxacin (57.9%), followed by ciprofloxacin (21.1%) and marbofloxacin (10.1%). However, this study lacked data on MDR strains. Likewise, Wildermuth et al. (2007) found that *P. aeruginosa* isolated from dogs with OE showed resistance (descending order) to enrofloxacin (53.1%), marbofloxacin (33.3%) and ciprofloxacin (25%). This study also found that 50% of the isolates (n=30) were resistant to at least one of the three fluoroquinolones and 20% were classified multidrug-resistant to all three fluoroquinolones (Wildermuth et al., 2007). In a recent study, an almost similar trend was reported wherein 44% and 32% of *P. aeruginosa* canine otic isolates (n=75) were resistant to enrofloxacin and marbofloxacin respectively, whereas 48% of the isolates were resistant to pradofloxacin, a third-generation fluoroquinolone (Vingopoulou et al., 2018). More worryingly, Arais, Barbosa, Carvalho, and Cerqueira (2016) demonstrated that MDRPA was present in canine OE and pyoderma that was resistant to at least four antimicrobials from different classes, not only fluoroquinolones, but also carbapenems, monobactams, and extended-spectrum antipseudomonal penicillins that are critically important for human medicine. Another study revealed that MDRPA otic isolates were highly resistant to cefepime, a fourth-generation cephalosporin followed by levofloxacin, a third-generation fluoroquinolone which are both listed as highest priority critically important antimicrobials for human medicine (Ciocan et al., 2015; WHO, 2019).

Pseudomonas otitis in dogs is commonly associated with MDR strains due to its intrinsic and acquired resistance mechanisms (Arais et al., 2016; Vingopoulou et al., 2018). Fluoroquinolone resistance in *P. aeruginosa* can be associated with mutation in DNA gyrase

(gyrA and gyrB) genes in a quinolone resistance determinant region (QRDR) whereas *P. aeruginosa* strains carrying class 1 integron were associated with multidrug resistance (Arais et al., 2016). However, Vingopoulou et al. (2018) demonstrated that the majority of *P. aeruginosa* canine OE isolates with fluoroquinolone resistance were associated with topoisomerase substitution pattern in GyrA, and only 6.7% was a carrier of plasmid-mediated quinolone resistance (PMQR) genes. Intrinsic resistance in MDRPA has been associated with its outer membrane permeability and MDR efflux pumps such as MexAB-OrPM and MexXY (Cox & Wright, 2013; Llanes et al., 2004).

1.6.3 Biofilm-associated canine otitis externa

Biofilm formation by *S. pseudintermedius* and *P. aeruginosa* canine otic pathogens may play an important role in bacterial persistence and resistance. However, few studies have reported biofilm-forming ability in these otic pathogens. One study found that 39.3% of *S. intermedius* otic isolates from dogs were biofilm producers, but no further classification of biofilm formation was undertaken (Moreira et al., 2012). Similarly, 40% (33/83) of *P. aeruginosa* canine otic isolates were shown to produce biofilms, and were categorised as weak (8.4%), moderate (18.1%) or strong (13.3%) biofilm producers (Pye, Yu, & Weese, 2013). Two studies investigated the association of biofilm-forming ability, biofilm-associated genes (*ica*A and *ica*D) and methicillin resistance in *S. pseudintermedius* isolated from healthy dogs or dogs with skin infections (Han, Yang, & Park, 2015; Singh, Walker, Rousseau, & Weese, 2013). Both studies reported that *S. pseudintermedius* isolates expressed *ica*A and *ica*D genes regardless of resistance, and there was no difference in the ability to produce biofilm between methicillin-susceptible *S. pseudintermedius* and MRSP. On the other hand, in a study investigating the sequence types (ST)s of MRSP isolated from European dogs, MRSP isolates belonging to ST71 demonstrated significantly higher biofilm production in comparison with MRSP ST106 and other MRSP isolates (Osland, Vestby, Fanuelsen, Slettemeås, & Sunde, 2012).

Biofilms can play an important role in persistent infection in canine OE (Costerton et al., 1999). Biofilms form a secure reservoir for planktonic bacteria that protect them from the host immune system (Clutterbuck et al., 2007; Nuttall, 2016). Moreover, the development of the exopolysaccharide matrix forms a barrier that reduces the penetration of antimicrobial agents that are normally effective against the planktonic state of bacteria (Mah & O'Toole, 2001). For instance, Pye et al. (2013) demonstrated that the MIC90 of gentamicin increased by 16-fold in the biofilm-embedded *P. aeruginosa* canine OE isolates in comparison to the planktonic state. The presence of persister cells in the slow-growing and stationary growth phases of both planktonic and biofilm cultures has been proposed to increase AMR (Jacques, Aragon, & Tremblay, 2010; Spoering & Lewis, 2001). Treatment failure and recurrence of OE can also occur when some antimicrobials manage to diffuse into biofilms causing bacteria to be exposed to an intermediate concentration of antimicrobials in which leads to selective pressure and mutational resistance (Nuttall, 2016).

1.6.4 Zoonotic transmission

Zoonotic transmission of AMR from companion animals to humans is a public health concern (Pomba et al., 2017). Humans and animals sharing the same environment or living in close contact were shown to harbour identical strains of methicillin-resistant *Staphylococcus* spp. (Morris, Boston, O’Shea, & Rankin, 2010). From an investigation of 171 veterinary dermatology staff and their respective pets (dogs and cats), three households were found to be sharing genetically identical MRSP strains using pulsed-field gel electrophoresis (PGFE) (Morris et al., 2010). In a companion animal clinic, MRSP strains isolated concurrently from healthy animals, animals with wounds, veterinary staff and environmental samples revealed indistinguishable PGFE profiles with similar resistance patterns (Van Duijkeren et al., 2008). Furthermore, veterinary professionals are at higher risk of cross-colonisation with MRSP strains due to their daily contact with companion animals (Rodrigues et al., 2018; Sasaki et al., 2007). Indirect evidence of zoonotic transmission was shown from a survey involving 128

small animal dermatologists, where 3.9% of them were carriers of MRSP (Paul, Moodley, Ghibaudo, & Guardabassi, 2011).

There are also increasing reports of owners diagnosed with *S. pseudintermedius* skin and soft tissue infections, transmitted from their pet dogs. *S. pseudintermedius* is a normal skin and nasal flora inhabitant in healthy dogs but can become an opportunistic pathogen in dogs and susceptible individuals, including humans (Bannoehr et al., 2007). In a Canadian survey, twenty-four human cases of skin and soft tissue infections were associated with *S. pseudintermedius* of canine origin with three cases of MRSP identified (Somayaji, Priyantha, Rubin, & Church, 2016). Tanner, Everett, and Youvan (2000) confirmed that *S. pseudintermedius* (previously known as *S. intermedius*) isolated both from a healthy dog and its owner who suffered from otitis externa was identical by molecular phylogenetic techniques, whereas Lozano et al. (2017) demonstrated identical strains of *S. pseudintermedius* in two patients diagnosed with skin diseases and their respective healthy dogs by PGFE. In another case, *S. pseudintermedius* was isolated both from a man suffering from a skin infection and his three healthy dogs indicating possible zoonotic transmission (Robb, Wright, Foster, Walker, & Malone, 2017). The majority of reports mentioned earlier involved detection of identical *S. pseudintermedius* strains from sick owners and their healthy dogs, but there were also cases involving dogs with dermatological conditions. *S. pseudintermedius* was isolated from patients suffering from chronic rhinosinusitis with underlying immune disorders as well as from their respective dogs with otitis and soft tissue infections (Kuan, Yoon, Vijayan, Humphries, & Suh, 2016).

1.7 Strategies to combat antimicrobial resistance

1.7.1 Development of new drugs

There is an urgency to develop new antibiotics to cope with the emerging of AMR. However, there are multiple challenges in the process of antibiotic discovery from preclinical stages to post-approval clinical trials. The first five to six years of preclinical trials has a low (17.3%) and unpredictable probability of success (O'Neill, 2015). Antibiotic discovery can take up 13 years and be very expensive, with research, post-approval and marketing costs of up 2.8 billion USD (DiMasi, Grabowski, & Hansen, 2016; O'Neill, 2015). Since 1960, only four new antibiotic classes have emerged – the quinolones, lipopeptides, streptogramins and diarylquinolines with a majority of analogues of existing antibiotics being approved (Chaudhary, 2016; Lewis, 2013). Drug approval has been problematic due to stringent regulatory requirements to demonstrate the safety and efficacy of a new drug (Projan, 2003). Daptomycin, a lipopeptide class antibiotic was discovered in 1986 but was only approved for use against Gram-positive bacteria after 17 years in 2003 (Lewis, 2013). Furthermore, newly introduced antibiotics can rapidly become ineffective as soon as resistance develops. For instance, ciprofloxacin was discovered in 1961 but resistance was documented in 1968, the same year the antibiotic was introduced to clinics (Lewis, 2013). Consequently, major pharmaceutical companies have exited the field of antibiotic research and development due to a poor return on investment and lack of incentives (Blaskovich, 2019; Lewis, 2013).

1.7.2 Antimicrobial stewardship

Responsible use of antimicrobials is necessary to cope with the emergence of antimicrobial resistance and exhaustion of effective antibiotics. Effective antimicrobial stewardship requires multidimensional approaches and involvement of everyone associated with the use of antimicrobials to preserve the efficacy of existing antibiotics and minimise the development of resistance (Prescott, 2014; Scott Weese et al., 2013). Veterinary practice guidelines, collection

of clinical microbiology data, consideration of drug pharmacokinetics and pharmacodynamics, antibiotic resistance and use surveillance, national and international drug regulations, infection control practices, and client compliance are part of the multifaceted approaches of antimicrobial stewardship in veterinary medicine (Guardabassi & Prescott, 2015; Weese et al., 2013).

There are five R's in the general principle of antimicrobial stewardship including responsibility, reduction, refinement, replacement and review (Page, Prescott, & Weese, 2014). For example, a prescriber uses an antimicrobial responsibly by making an accurate diagnosis, following antimicrobial guidelines for the right drug, frequency, dosage and duration, reducing and replacing the use of antimicrobials preferably with alternatives and finally, reviewing the need for the therapy (Dyar, Huttner, Schouten, & Pulcini, 2017; Scott Weese et al., 2013). Whenever possible, a narrow-spectrum antimicrobial should be chosen over a broad-spectrum drug based on culture and susceptibility testing, a topical rather than systemic therapy should be used in superficial skin diseases, and the use of critically important antimicrobials for human medicine should be avoided in veterinary medicine (Guardabassi & Prescott, 2015).

Establishing categorisation of antimicrobials used in veterinary practice is an excellent way to implement antimicrobial stewardship (Guardabassi & Prescott, 2015; Prescott, 2014; Weese et al., 2013). The categorisation should take into consideration the World Health Organisation (WHO) critically important antimicrobial (CIA) list for human medicine as well as World Organisation for Animal Health (OIE) CIA list for veterinary medicine (OIE, 2019; WHO, 2019). The first-, second-, and third- line of antimicrobial classifications are commonly adopted based on the severity of infections (minor to life-threatening), culture and sensitivity testing, the level of importance to human medicine and the likelihood of resistance development (Weese et al., 2013). Another type of antimicrobial categorisation involves communication between prescribers, an antimicrobial stewardship coordinator and a panel of experts as follows: first-choice drugs (prescribed without restriction), restricted drugs (only

prescribed for specific indications) and reserve drugs (prescribed only with permission) (Guardabassi & Prescott, 2015).

1.7.3 Alternative therapies

The remaining effective antimicrobials are limited (Blaskovich, 2019). Until recently, major pharmaceutical companies have abandoned antibiotic discovery, which will lead to no new antibiotics to combat AMR in the near future (Blaskovich, 2019; Projan, 2003). Many studies have now focused on alternative therapies to combat methicillin- and multidrug-resistance in resistant pathogens such as vaccines, bacteriophage therapy and monoclonal antibodies (Rello, Parisella, & Perez, 2019).

The first investigation into the use of bacteriophage mixtures as an ototopical therapy for *P. aeruginosa* canine OE demonstrated promising results of increase bacteriophage count that corresponded with clinical improvement and reduction in *Pseudomonas* bacterial count (46 – 96.8%) without apparent adverse effects (Hawkins, Harper, Burch, Änggård, & Soothill, 2010). A more recent study found that bacteriophages effectively lysed a majority (71.8%) of the *P. aeruginosa* strains (n=39) isolated from canine skin diseases including OE as well as 66.7% of the fluoroquinolone resistant *P. aeruginosa* strains (n=6) (Furusawa et al., 2016).

Natural antimicrobial therapies, such as honey bee products have been explored as alternative OE treatments. Argentine propolis extract formulation was used as a topical antibacterial and anti-inflammatory therapy in 38 dogs, with 71% showing satisfactory improvement, clinically and microbiologically (Lozina et al., 2010). The propolis extract was also found to be effective against *M. pachydermatis* (100%) and *Staphylococcus aureus* (94%) at two weeks post-treatment (Lozina et al., 2010). Another study tested 12 Argentine propolis ethanolic extracts, and they were found to be bacteriostatic against *S. aureus*, *S. intermedius* and *S. haemolyticus* isolated from canine otitis (Salas et al., 2014). Brazilian propolis extract demonstrated bactericidal and fungicidal activities against *S. intermedius* and *M.*

pachydermatis canine otic isolates with minimum bactericidal concentration $(MBC)_{90}$ at 21 mg/ml and minimum fungicidal concentration (MFC) at 5.3 mg/ml, respectively (Cardoso, Maboni, Machado, Alves, & de Vargas, 2010). The use of medical grade honey (MGH) gel in 15 dogs with OE achieved 70% clinical cure within 1-2 weeks and overall improvement in otitis clinical signs of erythema, swelling, edema and exudates (Maruhashi et al., 2016). Additionally, MGH was found to possess biocidal activity against various OE isolates, including MRSP (Maruhashi et al., 2016).

Other alternative therapies for canine OE found in the literature include herbal medicines and essential oils. An ethyl acetate leaf extract of *Harugana madagascariensis* exhibited antimicrobial activities against *S. intermedius*, *P. aeruginosa*, and *M. pachydermatis* otic isolates from dogs and cats with MICs ranging from 50 to 250 µg/ml and MBCs ranging from 125 to 500 µg/ml (Moulari, Pellequer, Chaumont, Guillaume, & Millet, 2007). A comprehensive study of the efficacy of nine commercial essential oils revealed that canine/feline otic MRSP strains were susceptible to oregano (*Origanum vulgare*), basil (*Ocimum basilicum*) and clary sage (*Salvia sclarea*) whereas MDRPA strains were sensitive to rosemary (*Rosmarinus officinalis*), basil, and clary sage, with highest MICs reported up to 18.34 µg/µl (18,340 mg/ml) (Ebani et al., 2017). Another study demonstrated that cinnamon essential oil and cinnamaldehyde were excellent antimicrobials against MDRSP, MDRPA and *M. pachydermatis* associated with canine OE (Sim et al., 2019). Additionally, a combination of either cinnamon oil or cinnamaldehyde with a chelating agent, EDTA produced synergistic activity against MDRPA with 15- to 30- fold reduction in the MICs without EDTA (Sim et al., 2019).

1.7.4 Repurposing of old drugs for new purposes

Repurposing the use of existing antimicrobials for new purposes is an alternative way to overcome the challenges in AMR and unavailability of new antibiotics, whilst adhering to good principles of antimicrobial stewardship. Repurposing old drugs to fight pathogens that are resistant to conventional antimicrobials has become more common (Chong & Sullivan, 2007). To discover, develop and obtain marketing approval for new drugs in the U.S. market can cost over 2.8 billion dollars and take over 10 years (DiMasi et al., 2016). There is a high risk of failure (82.7%) in the preclinical stage alone that may take up to 6 years (Hong, Ipema, Gabay, & Lodolce, 2011; O'Neill, 2015). Using old drugs with known toxicological and pharmacokinetic profiles with an acceptable level of safety and tolerability is a much cheaper and efficient option than developing entirely new antibiotics (Oprea & Mestres, 2012; Strittmatter, 2014). (Ashburn & Thor, 2004).

As a classic example of drug repurposing, two types of salicylanilide used exclusively as anthelmintic drugs, namely niclosamide and oxyclozanide, have potential efficacy in treating antimicrobial-resistant staphylococcal infections (Rajamuthiah et al., 2015). Similarly, auranofin, an antirheumatic drug, was repurposed as a topical antimicrobial agent for skin and wound infections caused by methicillin-resistant *S. aureus*, vancomycin-resistant *S. aureus* and vancomycin-intermediate *S. aureus* (Thangamani, Mohammad, Abushahba, Sobreira, & Seleem, 2016). An antifungal agent, ciclopirox has shown potential antimicrobial effectiveness against MDR infections caused by Gram-negative pathogens such as *Acinetobacter baumannii, Escherichia coli* and *Klebsiella pneumoniae* (Carlson-Banning et al., 2013). In another study, ciclopirox was used as an adjuvant to enhance the antimicrobial activity of polymyxin B, which successfully produced *in vitro* synergistic effects against Gram-negative bacteria including MDR *E. coli* and MDR *A. baumannii* strains (Kim, Kim, & Pan, 2015).

1.8 Carboxylic polyether ionophores (ionophores)

“Ionophore” means carrier of ions in the translation of the original Greek (Pressman & Fahim, 1982). Carboxylic polyether ionophores such as narasin and monensin are important secondary metabolites produced by *Streptomyces* spp., and they are commonly used as coccidiostats in poultry and as rumen modulators or growth promoters in ruminants (Callaway et al., 2003; Dutton, Banks, & Cooper, 1995; Russell & Strobel, 1989). Ionophores are only licenced for use in animals and have demonstrated little evidence of bacterial resistance or co-selection for resistance to other antimicrobial classes (Butaye, Devriese, & Haesebrouck, 2003; Subbiah, Mitchell, & Call, 2016). The mechanism of action of ionophores involves the formation of lipid-soluble complexes that allow monovalent (and in some cases divalent) cations to be transported across cellular membranes by passive diffusion processes (Cybulski, Radko, & Rzeski, 2015). Early literature describes carboxylic polyether ionophores as being highly lipophilic mobile ion carriers of approximately 520 – 950 MW that could rapidly dissolve into bacterial cell membranes resulting in the following effects: ion binding, shielding of ionic changes, translocation of ions across the bacterial membrane and disruption of ion gradients (Pressman, 1976). Other studies describe ionophores as compounds that interfere with the natural ion transport system, lowering the energy barrier necessary for the transmembrane transport of ions, and catalysing an electroneutral cation-proton exchange across the barrier which abolishes the gradient of Ca^{2+}, Mg^{2+}, K^+ and Na^+ across bacterial cell membranes (Butaye et al., 2003). Gram-positive bacteria are highly susceptibility to ionophores due to their porous peptidoglycan layer that allows small molecules such as the lipophilic ionophores to pass through the bacterial cell wall, permeate and dissolve into the cytoplasmic membrane (Callaway et al., 2003). In general, ionophores are not effective against Gram-negative bacteria. The Gram-negative bacterial cell wall contains a lipopolysaccharide layer, outer membrane and periplasmic space that does not permit penetration of hydrophobic molecules with a MW > 600 (Butaye et al., 2003).

Ionophores are safe and effective in the target animals at the prescribed and recommended dosage levels for oral consumption. However, ionophore toxicity has been reported due to mixing errors or ingestion of a ration, premix or concentrated product accidentally or intentionally in non-target animals such as horses, goats, pigs, dogs and cats as well as overdosing in target animals (i.e. chickens, cattle and sheep) (Novilla, 1992; Roder, 2011). Ionophore toxicity is typically associated with clinical signs of anorexia, diarrhoea, dyspnoea, ataxia, depression, recumbency and death, and pathological features of degenerative cardiomyopathy, musculoskeletal necrosis and congestive heart failure (Novilla, 1992). Horses were found to be the most sensitive non-target species with the lowest estimated oral median lethal dosage (LD_{50}) of monensin at 2 mg/kg which was comparatively 100x, 13x, 6.5x, > 5x, and 5x lower than the estimated LD_{50} for chicken, cattle, swine, dog, and sheep respectively (Todd, Novilla, & Howard, 1984). Ionophore toxicity is generally observed after oral consumption, but limited literature describes toxicity after topical exposure.

1.8.1 Monensin

Monensin, previously known as monensic acid, is a monovalent carboxylic polyether ionophore produced by *Streptomyces cinnamonensis* (Butaye et al., 2003). Monensin is a lipophilic ion carrier with a MW of 670 that transports Na^+ more efficiently than K^+ (Butaye et al., 2003; Pressman, 1976). Monensin disrupts the balance of intracellular and extracellular cations of the target bacterial cell by exchanging proton H^+ with either K^+ or Na^+ which causes accumulation of intracellular protons and disruption of ionic and pH gradients (Dutton et al., 1995; Russell, 1987). Then, multiple ATP linked efflux pumps are activated to re-establish ion and pH gradients until intracellular ATP pools are exhausted, leading to cell death (Callaway et al., 2003; Russell & Strobel, 1989). Monensin sodium (Coban®, Elancoban®, Rumensin®) is commercially available as an anticoccidial agent, feed additive and growth promoter in chickens, turkeys and cattle (EFSA, 2004b; EMEA, 2007). In ruminants, monensin improves feed efficiency by changing ruminal fermentation resulting in decreased production of

methane, ammonia and lactic acid, increased propionate production, nitrogen retention and reduction in ruminal acidosis leading to efficient energy and protein utilisation (Callaway et al., 2003; Russell & Strobel, 1989). The antimicrobial activity of monensin has been widely investigated in isolates associated with commercial poultry and ruminants. *Clostridium perfringens* isolated from broiler chickens (n=26) and turkeys (n=22) were both susceptible to monensin with MIC_{50} and MIC_{90} at 1 µg/ml (Watkins, Shryock, Dearth, & Saif, 1997). A Belgian antimicrobial resistance survey on *C. perfringens* (n=44) isolated from 31 broiler chickens farm revealed 100% susceptibility to monensin with both MIC_{50} and MIC_{90} at four-fold lower concentrations (0.25 µg/ml) than findings by Watkins et al. (1997). R. O. S. Silva et al. (2009) determined effectiveness of monensin against *C. perfringens* (n=55) isolated from broiler chickens by agar dilution method revealing MIC_{50} at 0.25 µg/ml and MIC_{90} at 0.5 µg/ml. However, monensin has also been evaluated for its efficacy against other pathogens. In an Australian bovine mastitis study (n=141), monensin was effective against *S. aureus*, coagulase-negative staphylococci, methicillin-resistant staphylococci and *Streptococcus* spp. with MIC_{50} and MIC_{90} at 2 and 8 µg/ml, respectively (Hickey et al., 2018). Other studies have looked into the potential of monensin in treating human infections (Rajendran, Ilamathi, Dutt, Lakshminarayana, & Ghoshi, 2018). *S. epidermidis*, a normal inhabitant of human skin and mucous membranes, but a primary cause of chronic nosocomial infections, was sensitive to monensin (n=12) with MIC ranging from 0.5 to 2 µg/ml with MIC_{90} of 2 µg/ml and MBC_{90} at 16 µg/ml (Stefańska, Stępień, Huczyński, & Tyski, 2015). Monensin was also effective against MRSA (n=5) at 4 µg/ml and VRE (n=3) at 16 µg/ml (J. C. Yoo et al., 2007).

The toxicology profile of monensin has been studied extensively in laboratory animals and livestock. However, toxicity in dogs has also been reported. Monensin poisoning in dogs was reported due to accidental contamination of dog food with monensin in feed milling factories or farm dogs exposed to the ration prepared for cattle and chicken. Clinical signs included neurological disorders, cardiac and skeletal myopathy, gastrointestinal disorders and death. (Warnock, 2016; Wilson, 1980). In a 3-month and 1-year chronic oral monensin toxicity

study in Beagle dogs the lowest no observed effect levels (NOEL) were seen at doses of ≤ 5 mg/kg (~200ppm) and ≤ 2.5 mg/kg, respectively (Todd et al., 1984). Dogs given doses above the NOEL (15 mg/kg and 50 mg/kg) showed signs of toxicity including anorexia, weakness, ataxia and elevated serum muscle enzyme while pathologic lesions of skeletal muscle and cardiac degeneration and necrosis were found in dead animals (Todd et al., 1984). In terms of topical toxicity, a single application of mycelial monensin (Elancoban®) at 500 mg/kg on clipped and abraded skin of rabbits for 24 hours and observation for 2 weeks revealed no dermal toxicity or irritation except slight skin redness in one animal (EFSA, 2004b). However, the application of crystalline monensin on mice ear pinnae caused a delayed contact hypersensitivity using a local lymph node assay, indicating monensin is a weak contact sensitiser (EMEA, 2007).

1.8.2 Narasin

Narasin is a monovalent carboxylic polyether ionophore (MW 700-800) produced by the fermentation process of *Streptococcus aureofaciens* followed by extraction and purification with organic solvents (Berg & Hamill, 1978; Dutton et al., 1995). Narasin (Monteban® G100, Maxiban®, Elanco) is commercially available as a feed additive to improve feed efficiency and growth as well as control and prevention of coccidiosis in poultry (Bampidis et al., 2018; EFSA, 2004a). However, the earliest study by Berg and Hamill (1978) also revealed the *in vitro* effectiveness of narasin against Gram-positive bacteria (*S. aureus* and *Streptococcus faecalis*, MIC=100 µg/ml; *Mycoplasma* spp., MIC=6.25 – 12.5 µg/ml), anaerobic bacteria (*Actinomyces bovis* and *Clostridium perfringens*, MIC < 0.5 µg/ml) and fungi (*Candida* spp., MIC= 100 µg/ml; *Trichophyton mentagrophytes*, MIC=12.5 µg/ml). Nagaraja and Taylor (1987) reported on the susceptibility of Gram-positive anaerobic ruminal bacterial strains, *Eucobacterium* spp., *Lactobacillus* spp., and *Streptococcus* spp. to narasin with MIC ranging from 0.19 to 3 µg/ml. Furthermore, Gram-negative staining ruminal bacterial strains such as *Butyrivibrio* spp., *Lachnospira* spp. and *Ruminococcus* spp. were identified as possessing a

Gram-positive-type cell wall structure during the period of study and being susceptible to narasin with an MIC range of 0.19 – 0.75 µg/ml (Nagaraja & Taylor, 1987). Antimicrobial activities of narasin were also frequently reported in *C. perfringens,* known to cause necrotic enteritis and cholangiohepatitis in chickens and turkeys, with MIC_{90} ranging from 0.03 to 0.5 µg/ml (Johansson, Greko, Engström, & Karlsson, 2004; Martel et al., 2004; R. O. S. Silva et al., 2009; Watkins et al., 1997). In a recent study, narasin and other ionophores were tested *in vitro* for the potential to be repurposed as intramammary therapies in bovine mastitis (Hickey et al., 2018). Narasin was found to effective against *S. aureus* (n=39; MIC_{50}=0.5 µg/ml; MIC_{90}=1 µg/ml), coagulase-negative staphylococci (n=22; MIC_{50}=0.25 µg/ml; MIC_{90}=0.5 µg/ml), methicillin-resistant staphylococci (n=7; MIC range=0.25-1 µg/ml) and *Streptococcus* spp. (n=72; MIC range= ≤0.06 – 0.25 µg/ml) isolated from bovine mastitis (Hickey et al., 2018).

Even though narasin has been widely used as a feed additive in livestock, low propensity for resistance was found, and there was no cross-resistance to other antimicrobials except salinomycin (Butaye, Devriese, & Haesebrouck, 2000; EFSA, 2004a; Subbiah et al., 2016). In a South Australian survey on antimicrobial resistance of *Enterococcus* spp. isolated from slaughtered pigs, 100% susceptibility was found to narasin at 1 µg/ml determined by the agar dilution method (Fard, Heuzenroeder, & Barton, 2011). However, in a study comparing the efficacy of narasin against *C. perfringens* isolated from piglets presented with and without diarrhoea, reduced susceptibility was found in *C. perfringens* causing diarrhoea where narasin MIC_{90} increased by 32-fold compared to *C. perfringens* isolated as normal flora (MIC_{90}=8 µg/ml) (Salvarani et al., 2012).

Toxicology profiles of narasin have been investigated extensively in laboratory animals including rats, mice, rabbits and dogs for acute and chronic oral toxicity, dermal toxicity and irritation, and skin sensitization (Novilla, Owen, & Todd, 1994). In an acute oral narasin toxicity study in dogs, LD_{50} was estimated at > 10 mg/kg body weight post oral administration of a single dose of a 5% mycelial narasin capsule and observation for 2 weeks (Novilla et al.,

1994). Clinical signs of acute oral narasin toxicity included anorexia, dullness and depression, diarrhoea, leg weakness, ataxia and death (Novilla et al., 1994). Similar observations were reported in 20 dogs poisoned by a batch of dog food contaminated with narasin premix intended for broilers in the factory packaging process (Karsai, Papp, Sályi, Bagó, & Kántás, 1990). Intravenous administration of narasin at concentrations of 0.153, 0.076 and 0.004 mg/kg increased heart rate, mean blood pressure and coronary artery blood flow, respectively, in both anaesthetised and conscious dogs (Novilla et al., 1994). The lowest NOEL in dogs was observed at concentrations of 1 mg/kg and 0.5 mg/kg body weight after 3 months and 1 year of oral daily dosing, respectively (EFSA, 2004a; Novilla et al., 1994). Narasin was determined to be a non-skin sensitiser in a skin sensitisation study on guinea pigs which were given ten intracutaneous injections of 0.25% crystalline narasin in safflower oil over 3 weeks (Novilla et al., 1994). However, application of 25% (v/v) of narasin (Monteban®) on the ear pinnae of mice resulted in increased ear thickness and a lymphoproliferative response indicating narasin to be a potential skin sensitiser (EFSA, 2004a). Narasin did not cause apparent dermal toxicity and irritation post 24h topical application on the shaved dorsum of rabbits with over a 2 week observation period (Novilla et al., 1994).

1.9 Antimicrobial adjuvants

The use of adjuvants to enhance the antimicrobial activity of an existing antimicrobial agent is an alternative strategy to the development of new antibiotics to treat resistant bacterial infection and sparing the use of critically important antibiotics for human medicine (Ejim et al., 2011; Wright, 2016). Antimicrobial adjuvants are active molecules, preferably with a weak or non-antibiotic activity, that augment the activity of an antibiotic or antimicrobial when present in combination against targeted resistant pathogens (Bernal et al., 2013). An antimicrobial adjuvant can function in different ways. It can inhibit bacterial cell physiological pathways such as inducing oxidative stress responses (e.g. tellurite), inhibiting synthesis and repair of bacterial cell wall (e.g. fosfomycin), and inhibiting folic acid biosynthetic pathways (e.g. the

synergistic combination of sulfamethoxazole and trimethoprim) (Bernal et al., 2013; Bushby, 1975; Kalan & Wright, 2011; Molina-Quiroz et al., 2012; Popovic, Steinort, Pillai, & Joukhadar, 2010). An adjuvant can also be a blocker of antibiotic resistance elements. For instance Augmentin® is a classic example of a commercially available antibiotic-adjuvant comprising amoxicillin and the β-lactamase inhibitor clavulanic acid to inhibit the cell wall synthesizing enzymes (penicillin-binding proteins) of pathogens resistant to β-lactam antibiotic (Domalaon et al., 2018; Gill, Franco, & Hancock, 2015; Worthington & Melander, 2013). There are also adjuvants that enhance the uptake of antibiotics through bacterial membranes by damaging the bacterial cell wall (e.g. bacitracin and vancomycin), increasing permeability of bacterial membranes (e.g. EDTA) and interfering with the integrity of the lipopolysaccharides of the outer membrane of Gram-negative bacteria (e.g. colistin or polymyxin E) (Bernal et al., 2013; Kalan & Wright, 2011). Efflux pump inhibition (EPI) is another mechanism of adjuvants to prevent antibiotics from being expelled from bacterial cells. Phenylalanine-arginyl β-naphthylamide (PaβN) is one of the identified EPIs against resistance nodulation division (RND) efflux pumps in Gram-negative bacteria including *P. aeruginosa* (Gill et al., 2015; Pieren & Tigges, 2012). Additionally, adjuvants can change resistant-cell physiology by dispersing biofilms to planktonic cells to increase the susceptibility of bacteria to antibiotics such as D-aminoacids, human peptide LL-37 and peptide IDR-1018 (Bernal et al., 2013; Pletzer & Hancock, 2016).

1.9.1. N-acetylcysteine

N-acetylcysteine (NAC) is commonly used as a mucolytic agent as well as a non-antibiotic agent with bactericidal and antibiofilm activities (Aslam & Darouiche, 2011; Samuni, Goldstein, Dean, & Berk, 2013). The antimicrobial effect of NAC is thought to be due to its ability to competitively inhibit the utilisation of amino acids such as cysteine by bacterial cells (Parry & Neu, 1977; Zhao & Liu, 2010). Also, NAC can efficiently reduce disulfide bonds in bacterial cell proteins, leading to alteration of cell structure, cell damage and death (Samuni et al., 2013). Several *in vivo* studies in laboratory animals demonstrated that NAC antioxidant properties are protective against chemotherapy-induced ototoxicity and noise-induced hearing loss (Dickey, Muldoon, Kraemer, & Neuwelt, 2004; Feghali, Liu, & Van De Water, 2001; Fetoni et al., 2009). However, there are conflicting reports on whether or not NAC reduces oxidative stress or is otoprotective in cases of aminoglycoside-induced ototoxicity (e.g. kanamycin and gentamicin) (Tepel, 2007). An early investigation found that NAC produced a synergistic effect with kanamycin to cause further hearing loss and cochlear damage in guinea pigs (Bock, Yates, Miller, & Moorjani, 1983), but in a subsequent study NAC reduced ototoxicity induced by gentamicin in a rat model by preventing cochlear cell apoptosis (Somdaş, Korkmaz, Gürgen, Sagit, & Akçadağ, 2015).

Multiple studies have demonstrated the *in vitro* antimicrobial activity of NAC against veterinary and human pathogens. NAC was effective against *S. pseudintermedius*, *P. aeruginosa*, *Corynebacterium* spp. and β-haemolytic *Streptococcus* spp. isolated from canine OE with MICs ranging from 5,000 to 20,000 μg/ml (May, Conklin, & Bemis, 2016). Similarly, *P. aeruginosa* strains associated with human chronic otitis media were susceptible to NAC at concentrations of ≥ 0.5% or 5,000 μg/ml (Lea et al., 2014). However, a previous study detected much lower inhibitory concentrations of NAC with MICs ranging from 2 to 20 μg/ml against *P. aeruginosa* strains isolated from the sputum of patients with lower respiratory infections (Parry & Neu, 1977). They found that the growth curve and inhibition of *P. aeruginosa* in the presence of NAC were both dose- and inoculum size-dependent (Parry & Neu, 1977). Another

study demonstrated that NAC had excellent bactericidal activity against various bacterial strains isolated from hospital device-associated infections (Aslam & Darouiche, 2011). Both MIC and MBC of NAC ranged from 5,000 to 10,000 µg/ml against *S. aureus*, *P. aeruginosa*, *Enterobacter cloacae*, *K. pneumoniae* and resistant pathogens such as MRSA and VRE (Aslam & Darouiche, 2011).

Despite its own antimicrobial activity, NAC is also an important modulator of the antimicrobial activity of other agents (Goswami & Jawali, 2010). Hence, although NAC can enhance the activity of a primary antimicrobial agent such as penicillin and polymyxin, it can also antagonise the antimicrobial activities of several types of antibiotics including aminoglycosides, fluoroquinolones, macrolides and imipenem against different bacterial strains (Goswami & Jawali, 2010; Parry & Neu, 1977; Rodríguez-Beltrán et al., 2015; Saggers & Lawson, 1966). NAC significantly potentiated the antimicrobial activity of carbenicillin against *P. aeruginosa* strains isolated from cystic fibrosis patients, whereby carbenicillin MICs reduced from 100 – 500 µg/ml to 0.4 – 62 µg/ml in the presence of 1% NAC (Roberts & Cole, 1981). Similar findings were reported by Parry and Neu (1977) in which additive or synergistic activity was found against *P. aeruginosa* when NAC and carbenicillin or ticarcillin were used in combination. On the other hand, NAC antagonised the antimicrobial activity of gentamicin against *P. aeruginosa* and *K. pneumoniae* strains with up to 16-fold increases in MIC values (Parry & Neu, 1977). Also, NAC inactivated the antimicrobial activity of neomycin but synergised with polymyxin B with a 4-fold reduction of MIC against *P. pyocyanea* (Saggers & Lawson, 1966). In the presence of NAC, the antimicrobial activity of two fluoroquinolones ciprofloxacin and ofloxacin reduced and their MIC values increased from 2- to 64-fold against *E. coli*, *P. aeruginosa* and *K. aerogenes* strains (Goswami & Jawali, 2010). In contrast, no antagonism was found in an NAC and ciprofloxacin combination against 20 *P. aeruginosa* strains, but a 50:50 split between synergism and no interaction was observed (Zhao & Liu, 2010). A recent study found that imipenem-susceptible *P. aeruginosa* strains became unresponsive to imipenem treatment in the presence of NAC (Rodríguez-Beltrán et al., 2015).

They demonstrated that NAC antagonised imipenem by competitively inhibiting the uptake of imipenem by OprD protein in the outer membrane of *P. aeruginosa* (Rodríguez-Beltrán et al., 2015).

The antibiofilm activity of NAC involves inhibition of bacterial adherence, reduction of extracellular polymeric substance (EPS) matrix formation, disruption to mature biofilm formation, and reduction of sessile cell viability (El-Feky et al., 2009; Marchese et al., 2003; Olofsson, Hermansson, & Elwing, 2003; Perez-Giraldo et al., 1997). NAC has also been shown to disrupt the disulfide bridges between the EPS matrix that maintains the integrity of biofilm (Schwandt et al., 2004). NAC is commonly used as a flushing solution to physically remove biofilm as part of the management of canine OE (Griffin & Aniya, 2017; Nuttall, 2016). However, the literature on NAC antibiofilm activity in canine otic and other veterinary pathogens is limited. The majority of studies are related to human respiratory and urinary tract diseases and medical device-associated infections (Dinicola, De Grazia, Carlomagno, & Pintucci, 2014). Multiple studies demonstrated that the antibiofilm activity of NAC is dose-dependent where the reduction in biofilm formation increases as the concentration of NAC increases simultaneously (El-Feky et al., 2009; Marchese et al., 2003; Perez-Giraldo et al., 1997). Perez-Giraldo et al. (1997) found that 8 mg/ml of NAC reduced biofilm growth by > 50% in the majority of *S. epidermidis* strains associated with prosthetic and intravascular devices. The combination of NAC with ciprofloxacin produced synergistic effects and increased the inhibitory effects on biofilm production and pre-formed biofilms of *S. aureus*, *S. epidermidis*, *E. coli*, *K. pneumoniae*, *P. aeruginosa* and *Proteus vulgaris* strains related to ureteral stent placement (El-Feky et al., 2009). Furthermore, they found that the combination of 2 µg/ml (2 MIC) of ciprofloxacin and 4,000 µg/ml of NAC entirely eradicated preformed *S. aureus* biofilm using scanning electron microscopy (El-Feky et al., 2009). Similarly, Zhao and Liu (2010) found that 500 µg/ml of NAC and ½ MIC of ciprofloxacin produced synergism against *P. aeruginosa* biofilms and EPS production significantly reduced by 44.6% with 1,000 µg/ml of NAC. Additionally, *P. aeruginosa* PAO1 biofilm was eradicated by NAC at 10,000

µg/ml, which was observed using fluorescence confocal laser scanning microscopy (Zhao & Liu, 2010). Another study revealed that 2 mg/ml of NAC potentiated the antimicrobial activity of fosfomycin (2000 mg/l) to disperse initial and mature *E. coli* biofilms up to 80% and 73%, respectively compared to 57% and 49%, respectively, if fosfomycin was used alone (Marchese et al., 2003). A recent study demonstrated that the growth and biofilm formation of Gram-positive skin pathogens, including *S. aureus* were completely inhibited at 25,000 µg/mL of NAC (Eroshenko, Polyudova, & Korobov, 2017).

1.9.2 Ethylenediaminetetraacetic acid (EDTA) and ethylenediaminetetraacetic acid – tromethamine (Tris-EDTA)

Ethylenediaminetetraacetic acid (EDTA) is a metal chelating agent with antimicrobial and antibiofilm properties (Finnegan & Percival, 2015). Tromethamine (Tris) buffer enhances the ability of EDTA to chelate and remove divalent cations such as Mg^{2+} and Ca^{2+} ions that are important in maintaining the integrity of the lipopolysaccharide cell wall of Gram-negative bacteria (Goldschmidt & Wyss, 1967; Gray & Wilkinson, 1965; Vaara, 1992). Disruption by EDTA or Tris-EDTA leads to solubilisation of the lipopolysaccharide molecules and increases the permeability of primary antimicrobial agents into the inner membrane of resistant Gram-negative organisms (Goldschmidt & Wyss, 1967; Gray & Wilkinson, 1965; Leive, 1965).

Tris-EDTA is a common ingredient found in ear cleaners or rinses to remove ear debris, reduce microbial load and increase the susceptibility of otic pathogens to topical antimicrobial therapy for canine OE (Nuttall, 2016; Paterson, 2016b). MDRPA canine otic isolates (n=11) were susceptible to Tris-EDTA with MICs ranging from 2,200/600 – 8,900/2,400 µg/ml (Buckley, McEwan, & Nuttall, 2013). In another study, a comprehensive range of canine otic isolates were susceptible to EDTA with MIC_{90} of 190 µg/ml (*S. pseudintermedius*), 750 µg/ml (*P. mirabilis* and β-haemolytic *Streptococcus* spp.) and 3,000 µg/ml (*P. aeruginosa*) (Khazandi et al., 2019).

Tris-EDTA and disodium EDTA were also assessed for their antimicrobial activities as ear cleaners as well as part of a formulation or poly-pharmaceutical commercial product (Guardabassi, Ghibaudo, & Damborg, 2010; Swinney et al., 2008). Among ear cleaners containing EDTA or Tris-EDTA, Epi-Otic® Advanced formula (Virbac, other ingredients: salicylic acid 0.1%, parachlorometaxylenol 0.1%, docusate sodium and propylene glycol) and TrizPlus® (Dermapet, other ingredients: chlorhexidine 0.15%) had equivalent antimicrobial activity against *S. intermedius* at ½ dilution and *M. pachydermatis* at 1/8 dilution, but the former was comparatively more effective against *P. aeruginosa* at 1/16 dilution (Swinney et al., 2008). However, TrizEDTA® (Dermapet), which only contains Tris-EDTA, was not effective at any dilution against *S. intermedius*, *P. aeruginosa* and *M. pachydermatis* canine otic isolates (Swinney et al., 2008). Another study also found that Epi-Otic® Advanced formula (Virbac) containing 0.5% EDTA effectively inhibited the growth of *M. pachydermatis* otic isolates with ≥ 40 mm zone inhibition compared to the other ear cleaners containing EDTA (Mason et al., 2013). However, the presence of salicylic acid and parachlorometaxylenol in Epi-Otic® (Virbac) may possess anti-yeast activity as well (Mason et al., 2013). Another study demonstrated that the ear cleaner, Otodine® (Vetruus) containing 48 µg/ml of Tris-EDTA and 1,500 µg/ml of chlorhexidine was bactericidal at ¼ dilution (12 /375µg/ml of Tris-EDTA/chlorhexidine) against a wide range of otic pathogens including *S. pseudintermedius*, *Streptococcus canis*, *Corynebacterium auriscanis*, *P.aeruginosa*, *P. mirabilis*, *E. coli* and *M. pachydermatis* (Guardabassi et al., 2010).

There is a considerable amount of literature describing the efficacies of EDTA or Tris-EDTA when combined with other antimicrobial agents, resulting in enhanced antimicrobial activities against Gram-negative and Gram-positive bacteria, yeasts and fungi (Brown & Richards, 1965; Farca et al., 1997; Wooley & Jones, 1983). An older study by Russell (1987) used EDTA to increase the uptake and susceptibility of ionophores such as monensin, against inherently resistant Gram-negative bacteria. Likewise, Tris-EDTA (50/250 mmol/l) was used as a rinse twice a day for a week before the administration of cephaloridine, kanendomycin or

enrofloxacin to potentiate the antimicrobial activity of these antibiotics against Gram-positive and Gram-negative otic pathogens (Farca et al., 1997). An ear rinse containing Tris-EDTA and benzyl alcohol was used to enhance the antifungal activity of 0.1% (1 mg/ml) ketoconazole against *M. pachydermatis* otic isolates from dogs (Cole et al., 2007). Another study found that Tris-EDTA increased *in vitro* efficacy of gentamicin and marbofloxacin against MDRPA canine otic isolates (Buckley et al., 2013). They found that the median MBC for gentamicin significantly reduced by 16-fold from 625 to 39.1 µg/ml whereas the median MBC for marbofloxacin reduced slightly from 625 to 468.8 µg/ml in the presence of Tris-EDTA (Buckley et al., 2013). In a recent study, Tris-EDTA (2250/600 µg/ml) potentiated the antibacterial activity of gentamicin and orbifloxacin against MDRSP (n=20) and also, enrofloxacin, marbofloxacin, orbifloxacin and gentamicin against *P. aeruginosa* (n=20) skin isolates from dogs (Boyd, Santoro, & Gram, 2019). However, the potentiating activity of Tris-EDTA with these antibiotics became pointless as their concentrations found in otic formulations (e.g. 3,000 µg/ml of gentamicin in Otomax®) were 27 x and 279 x higher than the combined antibiotic/Tris-EDTA MICs against *S. pseudintermedius* (123.46 µg/ml) and *P. aeruginosa* (4.12 µg/ml), respectively (Boyd et al., 2019).

The antibiofilm activity of EDTA or Tris-EDTA is associated with its ability to chelate divalent cations Ca^{2+}, Mg^{2+}, and Fe^{2+} that causes destabilisation and dispersion of the EPS matrix leading to detachment and killing of biofilms (Banin, Brady, & Greenberg, 2006; Z. Liu et al., 2017). Z. Liu et al. (2017) demonstrated that 30,000 µg/ml (30 mg/ml) EDTA significantly reduced EPS formation and viable bacterial cells in biofilms as well as decreasing the thickness, average dispersal distance and textural entropy of *Pseudomonas* biofilms. According to Banin et al. (2006), 18,600 µg/ml (50 mM) of EDTA reduced *P. aeruginosa* PAO1 biofilms from approximately 10^8 to 10^6 colony forming units (CFU) and a further reduction to $\approx 10^2$ CFU in the presence of 2,420 µg/ml (20 mM) of Tris (Tris-EDTA). Furthermore, Tris-EDTA potentiated the antibiofilm activity of gentamicin (50 µg/ml) to completely eradicate *P. aeruginosa* PAO1 biofilms (CFU=0), which was far more effective

than the combination of gentamicin and EDTA alone (CFU≈10^4) or gentamicin alone (CFU≈10^7) (Banin et al., 2006). In an observation using confocal laser scanning microscopy, a meagre concentration of EDTA at 5 µg/ml was able to synergistically reduce *P. aeruginosa* PAO1 biofilms when combined with 50 µg/ml of phenyl-arginine-β-napthylamide (PaβN), an efflux pump inhibitor (Y. Liu, Yang, & Molin, 2010). Conversely, the PAO1 biofilm formation increased in 5 µg/ml of EDTA alone, which was likely due to the capability of EDTA to release lipopolysaccharide and the latter serving as a matrix for biofilm cell attachment (Leive, 1965; Y. Liu et al., 2010). In a recent investigation, 7,400 µg/ml (20 mM) of EDTA was found to enhance the antibiofilm activities of commercial antiseptics such as Octenillin® (Schülke & Mayr GmbH), Prontosan® (B. Braun) and Betadine® (Meda Pharma) by 20,000-fold, 4000-fold and 200-fold, respectively against *S. aureus* and *P. aeruginosa* biofilms associated with chronic wound infections (Lefebvre, Vighetto, Di Martino, Garde, & Seyer, 2016).

Few studies have described the antibiofilm efficacy of EDTA or Tris-EDTA on biofilm-associated OE in dogs. A previous investigation demonstrated that Triz-EDTA® (Dechra), an ear rinse containing 530 µg/ml of Tris and 140 µg/ml of EDTA increased susceptibility of canine OE *P. aeruginosa* biofilms (n=31) to neomycin and gentamicin with reduction of MIC_{90} by 2- and 16-fold, respectively (Pye et al., 2014). However, an antagonistic effect was observed in 64.5% of the biofilm-embedded *P. aeruginosa* isolates when Triz-EDTA® (Dechra) was used in combination with enrofloxacin (Pye et al., 2014). A recent study found that 380 µg/mL (1 mM) of EDTA disrupted and reduced the growth of 24h pre-formed biofilms in *S. pseudintermedius* (n=2) and *P. aeruginosa* (n=2) canine OE isolates (Khazandi et al., 2019).

1.9.3 Monoglycerides

Monolaurin and monocaprin are bactericidal monoglycerides esterified from lauric acid and capric acid respectively with glycerol (Da Silva, Medeiros, Langone, & Freire, 2003; Pereira, da Silva, & Langone, 2004; Rarokar, Menghani, Kerzare, & Khedekar, 2017). They have increasing applications in the food, cosmetic and pharmaceutical industries as emulsifiers, preservatives and food additives, but they have also been used as antimicrobial agents for mucosal and skin infections (John J Kabara, 1984; Thormar & Hilmarsson, 2007). Monocaprin has been used as a model to identify the antimicrobial mode of action of monoglycerides against both Gram-positive and Gram-negative bacteria using transmission electron microscopy, in which disintegration of bacterial cell plasma membranes and cytoplasm granules is seen but little effect observed on the cell wall (Gudmundur Bergsson, Arnfinnsson, Karlsson, Steingrímsson, & Thormar, 1998; Gudmundur Bergsson, Arnfinnsson, Steingrimsson, & Thormar, 2001). The combination of these two monogylcerides enhances antimicrobial activity against Gram-positive organisms (Batovska, Todorova, Tsvetkova, & Najdenski, 2009) while the combination of monolaurin with other natural antimicrobials such as nisin and lactoperoxidase has shown additive to synergistic antibacterial activity against Gram-negative organisms (Dufour, Simmonds, & Bremer, 2003). In general, monolaurin has greater inhibitory activity against Gram-positive bacteria than monocaprin, but the latter has been described as having a broader spectrum of activity against some Gram-negative and yeast organisms (Jon J Kabara, Swieczkowski, Conley, & Truant, 1972; Růžička, Velclová, Janiš, & Krejčí, 2003). Buňková et al. (2011) found that monolaurin and monocaprin had antimicrobial activity against *S. aureus* CCM 3953 at 250 µg/ml and 500 µg/ml, respectively and against *P. aeruginosa* CCM 3955 at 1500 µg/ml. In another study, monolaurin and monocaprin displayed much lower MICs against three *S. aureus* strains; 7.8 – 31.25 µg/ml and 62.5 - 125 µg/ml, respectively (Batovska et al., 2009). The discrepancy in these results could be attributed to differences in the chemical composition of the monoglycerides, experimental methods and variation in the susceptibility characteristics of the tested strains.

1.9.4 Polymyxin B nonapeptide

Polymyxin B nonapeptide (PMBN) is a derivative of polymyxin B with reported negligible bactericidal activity but a higher affinity to bind to, disrupt and permeate the lipopolysaccharide outer membrane of Gram-negative bacteria (Vaara, 1992; Martti Vaara & T Vaara, 1983; Martti Vaara & Timo Vaara, 1983; Vaara & Viljanen, 1985). One study found that *P. aeruginosa* ATCC 27853 was sensitive to PMBN at 4 µg/ml, but *S. aureus* ATCC 29213 was less susceptible to PMBN with a much higher MIC at 64 µg/ml (Duwe, Rupar, Horsman, & Vas, 1986). Similarly, *P. aeruginosa* IFO 3080 strain was highly susceptible to PMBN at 128 nmol/ml or 0.12 µg/ml (Sato, Shindo, Sakura, Uchida, & Kato, 2011).

Furthermore, PMBN was used as an antibiotic adjuvant to increase the susceptibility of resistant Gram-negative bacteria to hydrophobic antimicrobials such as valinomycin, novobiocin, fusidic acid, erythromycin, clindamycin and carbenicillin by at least 10-fold (Alatossava, Vaara, & Baschong, 1984; Ofek et al., 1994; Viljanen & Vaara, 1984). For example, PMBN at 5 µg/ml enhanced the antibacterial activity of valinomycin against *E. coli* B strain with a 50-fold reduction in MIC (Alatossava et al., 1984). In the presence of 30 µg/ml of PMBN, *P. aeruginosa* strains became susceptible to novobiocin with an MIC range of 0.1 – 8 µg/ml, having previously been resistant (MIC > 1000 µg/ml) (Ofek et al., 1994). On the other hand, 30 µg/ml of PMBN reduced the MIC of fusidic acid by 300-fold against *E. coli* strains (Viljanen & Vaara, 1984). In a recent study, PMBN at the sub-inhibitory concentration of 1 µg/ml was used to synergise the antimicrobial activity of an antibiotic-EPI combination, e.g. azithromycin-PAβN against MexAB-OprM overexpressing *P. aeruginosa* and MDRPA strains (Ferrer-Espada et al., 2019). They found that both PMBN and PaβN potentiated antibacterial and antibiofilm activities of azithromycin with up to a 2,133-fold decrease in MIC and a 10 million times reduction in biofilm growth of *P. aeruginosa* strains (Ferrer-Espada et al., 2019).

1.10 Research aims

The aims of this PhD project were as follows:

A. To investigate the antimicrobial activity of the ionophores narasin and monensin against pathogens associated with canine otitis externa *in vitro*.

B. To investigate the antimicrobial activity of seven adjuvants – N-acetylcysteine, Tris-EDTA, disodium EDTA, Tris-HCL, monolaurin, monocaprin, and polymyxin B nonapeptide against pathogens associated with canine otitis externa *in vitro*.

C. To determine the interaction between ionophores and adjuvants against pathogens associated with canine otitis externa *in vitro*.

D. To determine the ability of pathogens associated with canine otitis externa to produce biofilms.

E. To investigate the antibiofilm activity of ionophores (narasin and monensin) and adjuvants (N-acetylcysteine, Tris-EDTA and disodium EDTA) against *Staphylococcus pseudintermedius* biofilms and *Pseudomonas aeruginosa* biofilms.

1.11 Thesis outline – a summary of experimental chapters

This thesis is presented as a series of five published or accepted journal articles. Each chapter forms a separate scientific paper. Accordingly, some repetition between chapters exists in their introduction and or methods sections.

Chapter 2 (Paper 1): *In vitro antimicrobial activity of narasin against common clinical isolates associated with canine otitis externa*. In this paper, narasin, a polyether ionophore conventionally used as a rumen modifier and anticoccidial agent in production animals, was investigated for its efficacy against pathogens associated with canine otitis externa. Gram-positive otic pathogens comprising methicillin-susceptible *Staphylococcus pseudintermedius*, multidrug-resistant *S. pseudintermedius* and β-haemolytic *Streptococcus* spp. were highly susceptible to narasin. Narasin was also found to have a weak anti-yeast activity against *Malassezia pachydermatis* isolates. However, no antimicrobial activity was found against the Gram-negative otic pathogens *Pseudomonas aeruginosa* and *Proteus mirabilis*.

Chapter 3 (Paper 2): *In vitro antimicrobial activity of monensin against common clinical isolates associated with canine otitis externa*. Monensin was another polyether ionophore that was investigated for its efficacy against pathogens associated with canine otitis externa. Monensin was effective against all Gram-positive bacteria including the multidrug-resistant staphylococci but lacked antimicrobial activity against Gram-negative bacteria and yeast isolates.

Chapter 4 (Paper 3): *In vitro antimicrobial activity of seven adjuvants against common pathogens associated with canine otitis externa*. Following the finding that both ionophores lacked Gram-negative activity, seven potential adjuvants which might enhance antimicrobial activity were investigated. Adjuvants with limited spectrum or no antimicrobial activity were omitted after initial screening against *Staphylococcus* and *Pseudomonas* reference strains. N-

acetylcysteine (NAC), Tris-EDTA and disodium EDTA were found to be the most promising adjuvants with intrinsic antimicrobial activity that could be used to enhance the efficacy of ionophores against Gram-negative and multi-drug-resistant bacterial infections.

Chapter 5 (Paper 4): *In vitro antimicrobial activity of narasin and monensin in combination with adjuvants against pathogens associated with canine otitis externa.* Further investigations were performed to evaluate the interaction between ionophores and adjuvants *in vitro* using a checkerboard assay. The antimicrobial activity of narasin or monensin in the presence of an adjuvant (NAC, Tris-EDTA or disodium EDTA) against bacterial strains representing pathogens associated with canine otitis externa was investigated. The combination of narasin with either Tris-EDTA or disodium EDTA produced additive effects against *P. aeruginosa* strains. Also, an additive effect was found against *Staphylococcus aureus* when narasin or monensin was combined with NAC.

Chapter 6 (Paper 5): *Biofilm production by pathogens associated with canine otitis externa, and the antibiofilm activity of ionophores and antimicrobial adjuvants.* The phenotypic characterisation of biofilm production in *S. pseudintermedius* and *P. aeruginosa* canine otic isolates were described and evaluated. The majority of the otic isolates in this study were biofilm producers. Further studies investigated the *in vitro* antibiofilm activity of ionophores (narasin and monensin) and adjuvants (NAC, Tris-EDTA and disodium EDTA) against moderate to strong biofilm producers of both strains. NAC eradicated biofilms produced by *S. pseudintermedius* and *P. aeruginosa* strains while Tris-EDTA had antibiofilm activity against *P. aeruginosa* biofilms only.

Chapter 2: *In vitro* antimicrobial activity of narasin against common clinical isolates associated with canine otitis externa

Statement of Authorship

Title of Paper	*In vitro* antimicrobial activity of narasin against common clinical isolates associated with canine otitis externa.
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Chan, W. Y., Hickey, E. E., Khazandi, M., Page, S. W., Trott, D. J., & Hill, P. B. (2018). *In vitro* antimicrobial activity of narasin against common clinical isolates associated with canine otitis externa. *Veterinary Dermatology, 29*(2), 149-e157. doi:10.1111/vde.12516

Principal Author

Name of Principal Author (Candidate)	Wei Yee, Chan		
Contribution to the Paper	Performed literature search, planned and conducted the experiments, analysis on all samples, interpreted data, wrote manuscript and acted as corresponding author.		
Overall percentage (%)	75%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

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Veterinary Dermatology

Vet Dermatol 2018 DOI: 10.1111/vde.12516

In vitro antimicrobial activity of narasin against common clinical isolates associated with canine otitis externa

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Background – Antimicrobial resistance and antimicrobial stewardship are of ever-increasing importance in veterinary medicine. Re-purposing of old drugs that are not used in human medicine is one approach that addresses the emergence of multidrug resistance in canine skin and ear infections, and can reduce the use of critically important human antibiotic classes.

Hypothesis/Objectives – To determine the antimicrobial activity of narasin, a polyether ionophore conventionally used as a rumen modifier and anticoccidial agent in production animals, against common clinical isolates of canine otitis externa (OE).

Animals/Isolates – Clinical isolates (n = 110) from canine OE were tested, including 17 meticillin-susceptible *Staphylococcus pseudintermedius* (MSSP), 13 multidrug-resistant *Staphylococcus pseudintermedius* (MDRSP), and 20 each of β-haemolytic *Streptococcus* spp., *Pseudomonas aeruginosa*, *Proteus mirabilis* and *Malassezia pachydermatis*.

Methods – Bacterial and yeast isolates were subcultured, suspended in broth and inoculated into 96-well plates. Organisms were tested against concentrations of narasin ranging from 0.03 to 128 μg/mL. Minimal inhibitory concentrations (MICs) were determined after overnight incubation.

Results – Narasin MICs for staphylococcal and streptococcal isolates ranged from 0.06 to 0.25 μg/mL; MIC_{50} and MIC_{90} values for both organisms were 0.125 μg/mL. No MICs were achieved for *Pseudomonas* or *Proteus* isolates. There was a weak antifungal effect against *M. pachydermatis* isolates (MIC 32 to >128 μg/mL).

Conclusions and Clinical Relevance – Narasin was effective against Gram-positive bacteria and had antifungal activity at higher concentrations against *M. pachydermatis*. However, the lack of Gram-negative activity would prevent its use as a sole antimicrobial agent in cases of canine OE.

Introduction

Otitis externa (OE) is one of the most common disorders to affect dogs. A 1995 prevalence study involving over 31,000 dogs from 52 general veterinary practices in the United States concluded that canine OE was the third most common disorder with a prevalence of 13%.[1] A subsequent UK survey from 1998 to 2001 found that otitis was the most common dermatological diagnosis, accounting for 4.5% of all consultations in dogs.[2] A further study in the UK, using data derived from the VetCompass (Veterinary Companion Animal Surveillance) database of 3,884 dogs from 89 practices, listed canine OE as the most frequently recorded disorder, with a prevalence of 10.2%.[3]

Micro-organisms typically associated with OE are *Staphylococcus pseudintermedius* (formerly *S. intermedius*), *Pseudomonas aeruginosa*, β-haemolytic *Streptococcus* spp., *Proteus mirabilis* and *Malassezia pachydermatis*.[4–6] Emergence of antimicrobial resistance has been described in a number of these bacterial species with increasing frequency, especially meticillin-resistant *Staphylococcus pseudintermedius* (MRSP) which are often multidrug-resistant to other antimicrobial classes,[7–9] and multidrug-resistant *P. aeruginosa* which often show resistance to at least three to four classes of antimicrobials.[10–13]

One approach to the problem of antimicrobial resistance in the past is to incorporate more potent classes of antimicrobial agents into topical otic treatments,

Accepted 19 November 2017

Abbreviations: BHS, b-haemolytic *Streptococcus* spp.; CAMHB, Cation-adjusted Mueller Hinton Broth; CLSI, Clinical and Laboratory Standards Institute; MDRSP, Multiple drug resistant *Staphylococcus pseudintermedius*; MIC, Minimum inhibitory concentration; MP, *Malassezia pachydermatis*; MRSA, meticillin-resistant *Staphylococcus aureus*; MRSP, meticillin-resistant *Staphylococcus pseudintermedius*; MSSP, meticillin-sensitive *Staphylococcus pseudintermedius*; OD, Optical density; OE, Otitis externa; PA, *Pseudomonas aeruginosa*; PM, *Proteus mirabilis*; VRE, vancomycin-resistant enterococci.

Sources of Funding: Supported with funding from ARC Linkage grant LP130100736 with Luoda Pharma Pty. Ltd as a partner organization.

Conflict of Interest: Stephen W. Page is a director of Luoda Pharma Pty. Ltd. Darren J. Trott has received research funding from Luoda Pharma, Neoculi, Zoetis, Bayer, Merial, Virbac and Elanco.

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particularly the newer generation fluoroquinolones such as enrofloxacin and marbofloxacin. However, fluoroquinolone resistance is emerging, with *Pseudomonas* otitis isolates showing resistance rates ranging from 47.9 to 53.1% for enrofloxacin and from 8.7 to 33.3% for marbofloxacin.[10,11,14] The development of resistance to these and other antibiotics among veterinary pathogens also poses a potential threat to human health, particularly among zoonotic multidrug-resistant strains with potential to cause severe, life-threatening infections.[15–17] Restricting the use of critically important antibiotics to safeguard their future effectiveness, a fundamental element of antimicrobial stewardship, is essential and is driving a search for alternative antimicrobial agents to treat infections such as canine OE. However, discovering, developing and obtaining marketing approval of new drugs for humans can cost over US$ 1.3 billion, can take an average of over 10 years, and comes with a high risk of failure (up to 84% in the preclinical stage alone).[18,19]

An alternative approach to address both the issue of antibiotic resistance and antimicrobial stewardship is the concept of repurposing old drugs for new purposes.[20–22] For example, the salicylanilide anthelmintic drugs niclosamide and oxyclozanide have been studied as potential agents for the treatment of resistant bacterial infections, with *in vitro* activity being demonstrated against meticillin-resistant *Staphylococcus aureus* (MRSA) and *Enterococcus faecium*.[23] Polyether ionophores are highly lipophilic molecules which transport monovalent or divalent cations across the cell membrane of susceptible bacteria. These drugs are only licensed for animal use in which they are commonly used as rumen modulators and anticoccidial agents in production animals.[24–26] There is little evidence of bacterial resistance or co-selection for resistance to other classes of antimicrobials by the ionophores.[27–30] These drugs are not suitable for development as systemic antibiotics due to a low margin of safety and potential toxicity in nontarget species. Poisoning or fatalities have been observed in horses, sheep, goats, pigs, cats and wild birds that accidentally ingested poultry or cattle feed containing ionophores.[31]

Narasin, a monovalent polyether ionophore discovered in the late 1970s by the fermentation process of *Streptomyces aureofaciens*, has been shown to be effective against Gram-positive bacteria, anaerobic bacteria and fungi.[32] However, it has a very low therapeutic index and has resulted in neurotoxicity, cardiac toxicity and toxic myopathy in dogs following consumption of narasin-contaminated commercial dog food,[33] and following systemic administration.[34] Despite this, narasin may have value as a topical agent for the treatment of surface infections such as those found on the skin or in the ear.

The aim of this study was to investigate the *in vitro* efficacy of the polyether ionophore narasin as an antimicrobial agent against common bacterial and yeast OE isolates from dogs.

Materials and methods

Bacteria and yeast isolates

One hundred and ten clinical isolates from cases of canine OE were collected from 22 government, private and university diagnostic laboratories throughout Australia. These organisms were speciated by standard methods employed in the accredited laboratories using Clinical and Laboratory Standards Institute (CLSI) guidelines. Seventeen meticillin-susceptible *Staphylococcus pseudintermedius* (MSSP) and 13 multidrug-resistant *Staphylococcus pseudintermedius* (MDRSP) were obtained from the first national survey of antimicrobial resistance in animal pathogens conducted in Australia.[35] Other organisms comprised 20 each of β-haemolytic *Streptococcus* spp. (BHS), *Pseudomonas aeruginosa* (PA), *Proteus mirabilis* (PM) *and Malassezia pachydermatis* (MP). Type strains *S. aureus* ATCC 29213, *P. aeruginosa* ATCC 27853, *Streptococcus pneumoniae* ATCC 49619 and *Candida albicans* ATCC 90028 were used to determine appropriate narasin testing concentrations as well as for internal quality control to monitor the reproducibility of minimal inhibitory concentrations (MIC) during testing of clinical isolates. ATCC strains were purchased from Abacus ALS, Queensland 4131, Australia.

Antimicrobial agents

Technical grade narasin, >98% purity (BioAustralis; Smithfield, NSW, Australia) was used for determining the MIC for each isolate. Testing concentrations were determined based on CLSI guidelines[36] and preliminary experiments (data not shown). Stock solutions of each antimicrobial [1.6 mg/mL for MSSP, MRSP and *Streptococcus* spp. (BHS); and 12.8 mg/mL for *P. aeruginosa*, *P. mirabilis* and *M. pachydermatis*] were prepared in dimethyl sulfoxide, ≥99.9% (DMSO, Sigma-Aldrich; St. Louis, MO, USA) and stored at −80°C until used. Preliminary testing found that the ≤1% concentration of DMSO used in final experiments did not affect the MIC results.

Antimicrobial susceptibility testing

The antimicrobial susceptibility for each isolate was determined using MIC microdilution methodology as recommended by CLSI,[36] with minor modifications. Briefly, bacterial isolates were subcultured on 5% Sheep Blood Columbia Agar (Thermo Fisher Scientific; Scoresby, Victoria, Australia) and incubated overnight at 37°C. A bacterial suspension for each isolate was prepared in phosphate buffered saline (Oxoid™; Basingstoke, Hampshire, UK) and adjusted to 0.5 McFarland standard followed by 1 in 20 dilutions. Two-fold serial dilutions of narasin stocks were performed in DMSO and diluted 1:100 in cation-adjusted Mueller Hinton Broth (CAMHB) (Becton Dickinson Pty Ltd, Sparks, MA, USA) in 96-well microtitre plates (Nunclon™ Delta Surface, Thermo Fisher Scientific; Hvidovre, Denmark). Finally, 20 μL of the bacterial inoculum was added to 180 μL narasin solution to achieve a final bacterial concentration of 5×10^5 colony forming units (cfu)/mL and narasin concentrations of 0.03–16 μg/mL (MSSP, MDRSP and BHS) and 0.25–128 μg/mL (*P. aeruginosa*, *P. mirabilis*). Antimicrobial challenge plates for streptococcal isolates were prepared using CAMHB supplemented with 5% lysed sheep blood. All isolates and control strains were tested in duplicate. Negative growth controls contained only CAMHB, and positive growth controls contained CAMHB and bacterial suspension. Growth was assessed visually after overnight incubation at 37°C, with streptococcal isolates being incubated in 5% CO_2; MIC was determined as the lowest concentration of narasin that completely inhibited visible growth of the organism. The MIC range (minimum and maximum), MIC_{50} and MIC_{90} were recorded and calculated for each of the bacteria groups; MIC_{50} and MIC_{90} values were defined as the lowest concentrations of narasin at which 50 and 90% of the isolates were inhibited, respectively.

Antifungal susceptibility testing

The antifungal susceptibility of *M. pachydermatis* isolates was performed using a modified CLSI broth microdilution method.[37,38] Briefly, isolates were inoculated onto Sabouraud's dextrose agar (SDA) supplemented with 1% Tween 80 (Sigma-Aldrich) and incubated for 72 h at 32°C. Organisms were then suspended in Sabouraud's dextrose broth (SDB) (Oxoid™) supplemented with 1% Tween 80 to obtain a uniform yeast suspension with an optical density (OD)

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between 0.2 and 0.3 at 600 nm wavelength (Eppendorf BioPhotometer plus; Hamburg, Germany). A 1:100 dilution was performed and 50 μL of yeast suspension was added to 150 μL of narasin solution to create a final inoculum concentration of 4–5 × 10^3 cfu/mL and narasin concentrations of 0.25 to 128 μg/mL. The MIC end-point was determined using a combination of two methods: visual end-point reading of 50% growth inhibition compared with the positive growth control, and spectrophotometric end-point reading of OD_{570nm} less or equal to 50% of the positive growth control. The MICs were reported as described previously for bacteria antimicrobial susceptibility testing.

Results

Narasin had antimicrobial activity against *S. pseudintermedius* (MSSP and MDRSP) and *Streptococcus* spp. (BHS) with MICs ranging from 0.06 to 0.25 μg/mL (Table 1). The narasin MIC_{50} and MIC_{90} values for these isolates were both 0.125 μg/mL. Furthermore, the 13 isolates of *S. pseudintermedius* that were multidrug-resistant were all susceptible to narasin, with MICs ranging from 0.06 to 0.25 μg/mL. At the concentrations tested there was no attainable narasin MIC for any *P. aeruginosa* or *P. mirabilis* isolates. Narasin MIC for *M. pachydermatis* ranged from 32 to >128 μg/mL, with MIC_{50} and MIC_{90} values of 128 μg/mL and >128 μg/mL, respectively (Table 1). These results were consistent with those obtained for the ATCC type strains. Consistent confluent growth was observed in all the positive growth control wells and there was no growth in any of the negative growth control wells.

Discussion

In this study, narasin was shown to have *in vitro* antimicrobial activity against the Gram-positive canine otic pathogens *S. pseudintermedius* and β-haemolytic *Streptococcus* spp., with MICs ranging from 0.06 to 0.25 μg/mL. To the best of the authors' knowledge, this is the first report of narasin activity against these common Gram-positive canine pathogens. Previous studies have focused on its efficacy against *Clostridium perfringens* in poultry, with MICs ranging from 0.03 to 0.25 μg/mL,[39–42] and multiple organisms isolated from the bovine rumen associated with bloat and lactic acidosis.[43]

The 13 isolates of multidrug-resistant *S. pseudintermedius* tested in this study were all susceptible to narasin. A previous study investigating the efficacy of four polyether ionophores against five MRSA and three vancomycin-resistant enterococci (VRE) found that narasin was the most effective *in vitro*, with MICs of 0.5 and 8 μg/mL, respectively.[44] These studies suggest that in addition to having excellent activity against Gram-positive organisms, narasin has potential as a topical treatment for bacteria that have become resistant to multiple antibiotic classes, including the critically important fluoroquinolones.

Narasin demonstrated antifungal activity against *M. pachydermatis* but only at higher concentrations. The narasin MICs ranged from 32 to >128 μg/mL. These results were similar to one of the earliest studies on the antimicrobial activity of narasin which reported MICs for each isolate of *Candida tropicalis*, *Trichophyton mentagrophytes* and *Ceratocystis ulmi* were 100, 12.5 and 50 μg/mL, respectively.[32] However, if narasin was to be used as a topical product, such as in an ear preparation, it is likely that the concentration in the formulation would exceed these higher MICs. Narasin may therefore also have potential as a treatment for canine OE caused by *M. pachydermatis.*

Narasin did not demonstrate any antibacterial activity against the Gram-negative otic pathogens *P. aeruginosa* and *P. mirabilis,* with MICs for both organisms exceeding the highest tested concentration of 128 μg/mL. Previous studies likewise reported that narasin and other ionophores are not active against Gram-negative bacteria.[28,43]

The mechanism of action of narasin relates to its highly lipophilic properties, allowing it to insert into bacterial cell membranes. It then chelates reversibly with Na^+ and K^+ conveying them rapidly across the cell membrane where they exchange for other monovalent cations such as hydrogen ions, disrupting the normal ionic and pH gradients.[45] In Gram-positive bacteria, it is likely that narasin can penetrate the porous peptidoglycan cell wall layer and cause an imbalance of intracellular concentration gradients, disrupting cellular division and potentially leading to cell death.[25,28,46] The inherent resistance of Gram-negative bacteria is most likely due to the two-layered cell wall structure of Gram-negative bacteria preventing narasin from permeating into the inner cytoplasmic membrane. This would stop the drug from interfering with ionic gradients and bacterial cell functions.[25,28]

Table 1. Minimum inhibitory concentration (MIC) range and lowest concentration at which 50% and 90% of the isolates were inhibited (MIC_{50} and MIC_{90}, respectively) for 110 clinical isolates of canine otitis externa and MIC for quality control strains with narasin

	Narasin concentration (μg/mL)		
Clinical isolates	MIC range	MIC_{50}	MIC_{90}
Staphylococcus pseudintermedius (*n* = 30) MSSP (*n* = 17), MDRSP (*n* = 13)	0.06–0.25	0.125	0.125
β-haemolytic *Streptococcus* spp. (*n* = 20)	0.06–0.25	0.125	0.125
Pseudomonas aeruginosa (*n* = 20)	>128	>128	>128
Proteus mirabilis (*n* = 20)	>128	>128	>128
Malassezia pachydermatis (*n* = 20)	32–>128	128	>128
Quality control strains			
Staphylococcus aureus ATCC 29213	0.125	–	–
Streptococcus pneumoniae ATCC 49619	0.25	–	–
Pseudomonas aeruginosa ATCC 27853	>128	–	–
Candida albicans ATCC 90028	>128	–	–

MSSP meticillin–sensitive *Staphylococcus pseudintermedius*, MDRSP Multiple drug resistant *Staphylococcus pseudintermedius*.

The results of this study have shown that narasin could be a suitable candidate to treat canine OE due to Gram-positive organisms and, at concentrations >128 μg/mL, *Malassezia* spp. However, further studies would be required to assess suitable formulations and topical safety in dogs. To date, there is only limited information about the potential of narasin to cause cutaneous irritation in laboratory animals. No evidence of skin irritation was seen when it was applied to the dorsum of rabbits,[34] but topical application to the pinnae of mice resulted in some swelling.[30]

Narasin would not be suitable as a sole antimicrobial agent in cases of otitis externa with mixed infections or those associated with Gram-negative organisms. Even with the high drug concentrations achieved in topical preparations, it is unlikely that this inherent resistance could be overcome. This problem could be solved by the addition of a second antimicrobial agent, but there is also a possibility that addition of an adjuvant that weakens the external cell wall of Gram-negative bacteria could remove this resistance mechanism. This could lead to the organisms becoming susceptible to narasin. Further studies are underway to test this hypothesis.

Acknowledgement

Wei Yee Chan was supported by the Ministry of Higher Education of Malaysia and Universiti Putra Malaysia.

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Résumé

Contexte – La résistance antimicrobienne et la gestion antimicrobienne sont d'importance croissante en médecine vétérinaire. L'utilisation d'anciens médicaments qui ne sont plus utilisés en médecine humaine est une approche qui pourrait être utile dans l'émergence des multi-résistances dans les infections cutanées et auriculaires canines et pourrait réduire l'utilisation d'antibiotiques de classe critique d'-portance humaine.

Hypothèses/Objectifs – Déterminer l'activité antimicrobienne de la narasine, un ionophore polyéther, conventionnellement utilisé comme agent anticoccidien et modificateur de la flore ruminale en production animale, dans le cadre des otites externes (OE) canines.

Sujets/Souches – Les isolats ($n = 110$) des OE externes ont été testés (17 meticillin-susceptible *Staphylococcus pseudintermedius* (MSSP), 13 multidrug-resistant *Staphylococcus pseudintermedius* (MDRSP), et 20 β-haemolytic *Streptococcus* spp., 20 *Pseudomonas aeruginosa*, 20 *Proteus mirabilis* et 20 *Malassezia pachydermatis*).

Méthodes – Les souches bactériennes et de levures ont été repiquées, mélangées et inoculées dans 96 puits. Les organismes ont été testés contre des concentrations de narasine allant de 0.03 à 128 µg/mL. Les concentrations minimales inhibitrices (MICs) ont été déterminées après une nuit d'incubation.

Résultats – Les MICs de narasine pour les souches de staphylocoques et de streptocoques allaient de 0.06 à 0.25 µg/mL; les valeurs de MIC_{50} et MIC_{90} pour les deux organismes étaient 0.125 µg/mL. Aucune MIC n'a été déterminée pour *Pseudomonas* ou *Proteus*. Il y avait un effet antifongique faible contre *M. pachydermatis* (MIC 32 à >128 µg/mL).

Conclusions et importance clinique – La narasine est efficace contre les bactéries Gram positives et a un effet antifongique à de hautes concentrations contre *M. pachydermatis*. Cependant, le manque d'-tivité contre les Gram positives pourrait empêcher son utilisation en tant que seul agent antimicrobien dans les cas d'OE canines.

Resumen

Introducción – la resistencia a los antimicrobianos y el uso responsable de los mismos tienen una importancia cada vez mayor en medicina veterinaria. Buscar un nuevo uso a los medicamentos antiguos que no se usan en medicina humana es un enfoque que afronta la aparición de resistencia a múltiples fármacos en infecciones de la piel y el oído de perros, y puede reducir el uso de clases de antibióticos importantes en medicina humana.

Hipótesis/Objetivos – Determinar la actividad antimicrobiana de narasina, un ionóforo de poliéter utilizado convencionalmente como modificador del rumen y agente anticoccidial en animales de producción, frente aaislados clínicos comunes de otitis externa canina (OE).

Animales/aislados – se analizaron aislados clínicos (n = 110) de casos de OE canina, incluidos 17 *Staphylococcus pseudintermedius* sensible a meticilina (MSSP), 13 *Staphylococcus pseudintermedius* resistente a múltiples fármacos (MDRSP) y 20 de cada uno de *Streptococcus spp.* beta-hemolíticos*, Pseudomonas aeruginosa, Proteus mirabilis y Malassezia pachydermatis.*

Métodos – Los aislados bacterianos y de levadura se subcultivaron, suspendidos en caldo y se inocularon en placas de 96 pocillos. Los organismos se expusieron a concentraciones de narasina que fueron desde 0,03 a 128 μg/mL. Las concentraciones inhibitorias mínimas (MICs) se determinaron después de la incubación durante la noche.
Resultados – Las MICs de narasina para aislados de estafilococos y estreptococos variaron de 0,06 a 0,25 μg/mL; los valores MIC_{50} y MIC_{90} para ambos organismos fueron 0,125 μg/ml. No se lograron MICs para aislamientos de *Pseudomonas* o *Proteus*. Hubo un efecto antifúngico débil contra los aislados de *M. pachydermatis* (MIC de 32 a >128 μg/ml).
Conclusiones y relevancia clínica – La narasina fue efectiva frente a bacterias Gram-positivas y tuvo actividad antifúngica en concentraciones más altas contra *M. pachydermatis*. Sin embargo, la falta de actividad Gram-negativa evitaría su uso como único agente antimicrobiano en casos de OE canino.

Zusammenfassung
Hintergrund – Antimikrobielle Resistenz und die mikrobielle Verantwortung sind von immer größerer Bedeutung in der Veterinärmedizin. Eine Zweckänderung alter Medikamente, die in der Humanmedizin nicht mehr verwendet werden ist eine Herangehensweise, die der zunehmenden Multi-Resistenzbildung bei Haut und Ohrinfektionen des Hundes entgegenwirken und die Verwendung der in der Humanmedizin kritischen Antibiotika Klassen reduzieren kann.
Hypothese/Ziele – Eine Bestimmung der antimikrobiellen Aktivität von Narasin, einem Polyether Ionophor, welches konventionell als Rumen-modifizierendes und anti-Coccidien- wirksames Produkt bei Farmtieren angewendet wird, gegenüber gewöhnlichen klinischen Isolaten von Otitis externa (OE) bei Hunden.
Tiere /Isolate – Es wurden klinische Isolate ($n = 110$) von Otitis externa von Hunden getestet. Es handelte sich um 17 Methicillin-empfängliche *Staphylococcus pseudintermedius* (MSSP), 13 multidrug-resistente *Staphylococcus pseudintermedius* (MDRSP), und jeweils 20 Isolate von β-hämolytischen *Streptococcus* spp., *Pseudomonas aeruginosa*, *Proteus mirabilis* und *Malassezia pachydermatis*.
Methoden – Die Bakterien- und Hefeisolate wurden subkultiviert, in Bouillon angereichert und auf 96-Loch Mikrotiterplatten inokuliert. Die Organismen wurden auf Konzentrationen von Narasin, die zwischen 0,03 und 128 μg/ml lagen, getestet. Die minimale Hemmkonzentration (MICs) wurden nach einer Inkubation über Nacht bestimmt.
Ergebnisse – Die MICs der Staphylokokken und Streptokokken Isolate gegenüber Narasin rangierten von 0,06 bis 0,25 μg/mL; MIC_{50} und MIC_{90} Werte betrugen für beide Organismen 0,125 μg/mL. Es konnten für *Pseudomonas* oder *Proteus* Isolate keine MICs erstellt werden. Es bestand eine schwache antimykotische Wirkung gegenüber *M. pachydermatis* Isolaten (MIC 32 bis > 128 μg/mL).
Schlussfolgerungen und klinische Relevanz – Narasin war wirksam gegenüber Gram-positiven Bakterien und zeigte eine antimykotische Wirkung in höherer Konzentration gegenüber *M. pachydermatis*. Aufgrund der fehlenden Wirkung gegenüber Gram-negativer Aktivität würde es jedoch als einziger Wirkstoff in Fällen von OE des Hundes nicht eingesetzt werden können.

要約
背景 – 薬剤耐性およよび抗菌剤のよび正使用は、獣医学領域においてますます重要になっている。人医療では現在使用されていない古いタイプの薬剤の再利用は、犬の皮膚や耳の感染症における多剤耐性菌の出現に対するアプローチの1つであり、また人医療において非常に重要な抗生物質クラスの使用を減らすことができる。
仮説/目的 – 家畜の反芻胃調整剤および抗コクシジウム剤として従来から使用されているポリエーテルイオノフォアであるナラシンの犬外耳炎(OE)の一般的な臨床分離株に対する抗菌活性を決定すること。
供与動物/分離株 – 17株のメチシリン感受性*Staphylococcus pseudintermedius*(MSSP)、13株の多剤耐性*Staphylococcus pseudintermedius*(MDRSP)、各20株ずつのβ溶血性レンサ球菌、緑膿菌、プロテウス・ミラビリス(*Proteus mirabilis)*およびマラセチアを含む犬OE由来の臨床分離株(n = 110)。
方法 – 細菌および酵母分離株を継代培養し、培養液中に懸濁させ、96ウェルプレートに接種した。各分離株を用いて、0.03〜128μg/mLのナラシンに対する検査を実施した。一晩培養後に、最小阻害濃度(MIC)を測定した。
結果 – ブドウ球菌および連鎖球菌分離株に対するナラシンのMICは、0.06〜0.25μg/mLであった。両分離株のMIC_{50}およびMIC_{90}値は0.125μg/ mLであった。緑膿菌またはプロテウス分離株ではMICは得られなかった。マラセチア分離株に対しては弱い抗真菌活性を認めた(MIC 32〜>128μg/mL)。
結論および臨床的な重要性 – ナラシンは、グラム陽性細菌に対して有効であり、高濃度ではマラセチアに対して抗真菌活性を示した。しかしながら、グラム陰性に対する活性を持たないことにより、犬OEに対する単剤使用は出来ないと考えられた。

摘要
背景 — 抗生素耐药性和抗生素管理在兽药领域的重要性日益增加。重新利用不再用于人医的旧药,是抑制犬皮肤和耳朵细菌多耐药性的方法,并且可以减少使用对人类极其重要的抗生素。

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假设/目的 — 确定甲基盐霉素的抗菌活性,聚醚离子载体通常用作生产动物的瘤胃调节剂和抗球虫剂,抗犬外耳炎(OE)的临床常见细菌。
动物/菌株 — 对来自犬OE的临床菌株(n = 110)进行测试,包括17个甲氧西林敏感的假中间型葡萄球菌(MSSP),13个多耐药假中间型葡萄球菌(MDRSP)和20个β-溶血性链球菌、绿脓假单胞菌、奇异变形杆菌和厚皮病马拉色菌。
方法 — 将细菌和酵母菌菌株传代培养,悬浮在肉汤中并接种到96孔板中。测试微生物对0.01至128μg/ mL范围内的甲基盐霉素浓度。过夜培养后测定最小抑制浓度(MIC)。
结果 — 葡萄球菌和链球菌菌株的甲基盐霉素 MIC范围为0.06至0.25μg/mL;两种微生物的MIC50和MIC90值均为0.125μg/mL。MIC值。对厚皮病马拉色菌有较弱的抗真菌作用(MIC 32至> 128μg/mL)。
结论和临床相关性 — 甲基盐霉素对革兰阳性菌有效,并且在较高浓度下,对厚皮马拉色菌具有抗真菌活性。然而,由于缺乏抗革兰氏阴性菌活性,在治疗犬OE时,甲基盐霉素不能做为单独的抗生素使用。

Resumo

Contexto – A resistência a antimicrobianos e o gerenciamento do uso racional dos mesmos tem ganhado importância crescente na Medicina Veterinária. A prescrição de drogas antigas que não são utilizadas em medicina humana é uma medida que aborda a emergência de multirresistência a antimicrobianos em infecções de pele e condutos auditivos de cães e pode reduzir o uso de classes de drogas humanas criticamente relevantes.
Hipótese/objetivos – Determinar a atividade antimicrobiana de narasin, um poliéter ionóforo convencionalmente utilizado como um modificador rumenal e agente anticoccidial em animais de produção, contra isolados clínicos comuns de otite externa (OE) canina.
Animais/Isolados – Os isolados clínicos ($n = 110$) de OE canina foram testados, incluindo 17 *Staphylococcus pseudintermedius* suscetíveisl a meticilina (MSSP), 13 *Staphylococcus pseudintermedius* mutltirresistentes (MDRSP), e 20 de cada β-haemolytic *Streptococcus* spp., *Pseudomonas aeruginosa*, *Proteus mirabilis* e *Malassezia pachydermatis*.
Métodos – Os isolados bacterianos e levedúricos foram subcultivados, suspensos em caldo e inoculados em placas de 96 poços. Os microrganismos foram testados para concentrações de narasin de 0,03 a 128 μg/mL. Concentrações inibitórias mínimas (MICs) foram determinados após incubação durante a noite.
Resultados – Os MICs de narasin para os isolados de *Staphylococcus e Streptococcus* variaram de 0.06 a 0.25 μg/mL; os valores de MIC_{50} e MIC_{90} para ambos os microrganismos foi 0.125 μg/mL. Nenhum MIC foi atingido para os isolados de *Pseudomonas* ou *Proteus.* Houve um efeito antifúngico fraco contra os isolados de *M. pachydermatis* (MIC 32 a >128 μg/mL).
Conclusões e relevância clínica – Narasin foi eficiente contra bactérias Gram-positivas e demonstrou atividade antifúngica em altas concentrações contra *M. pachydermatis*. Entretanto, a ausência de atividade contra Gram-negativos impediria o seu uso como agente antimicrobiano em casos de OE.

Chapter 3: *In vitro* antimicrobial activity of monensin against common clinical isolates associated with canine otitis externa

Statement of Authorship

Title of Paper	*In vitro* antimicrobial activity of monensin against common clinical isolates associated with canine otitis externa
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Chan, W. Y., Hickey, E. E., Khazandi, M., Page, S. W., Trott, D. J., & Hill, P. B. (2018). *In vitro* antimicrobial activity of monensin against common clinical isolates associated with canine otitis externa. *Comparative Immunology, Microbiology and Infectious Diseases, 57*, 34-38. doi:10.1016/j.cimid.2018.05.001

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Contribution to the Paper	Performed literature search, planned and conducted the experiments, analysis on all samples, interpreted data, wrote manuscript and acted as corresponding author.		
Overall percentage (%)	75%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Signature		Date	06/08/19

Comparative Immunology, Microbiology and Infectious Diseases 57 (2018) 34–38

Contents lists available at ScienceDirect

Comparative Immunology, Microbiology and Infectious Diseases

journal homepage: www.elsevier.com/locate/cimid

In vitro antimicrobial activity of monensin against common clinical isolates associated with canine otitis externa

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ARTICLE INFO

Keywords:
Monensin
Antimicrobial susceptibility
MIC
Canine
Otitis externa

ABSTRACT

Antimicrobial resistance and antimicrobial stewardship are of ever-increasing importance in veterinary medicine. Multidrug-resistant infections of the canine skin and ear continue to emerge, but the use of antibiotic classes of critical importance to human medicine may not represent good antimicrobial stewardship. Repurposing of old drugs that are not used in human medicine is one approach that addresses both these issues. In this study, the minimal inhibitory concentration (MIC) of monensin for 111 bacterial and yeast canine otitis isolates was determined using microdilution methodology according to Clinical Laboratory Standards Institute (CLSI) guidelines. Monensin was effective against all Gram-positive bacteria including the multidrug-resistant staphylococcal strains with MICs ranging from 1 to 4 μg/ml, but lacked antimicrobial activity against Gram-negative bacteria and yeast isolates. Monensin has potential to be incorporated as one of the main components in an otic formulation.

1. Introduction

The emergence of antimicrobial resistance (AMR) due to resistant superbugs is a worldwide problem both in human and animal medicine. It has been estimated that drug-resistant antimicrobial infections account for 700,000 human deaths each year and by 2050, the death toll may rise to 10 million people every year [1]. Adding to this global issue, there has not been a new class of antimicrobial agents approved for use since the 1980s. Discovering, developing and obtaining marketing approval for new human drugs can cost over US$2.6 billion, take an average of over 10 years, and comes with a high risk of failure of up to 84% in the preclinical stage alone [2,3].

Development of AMR in canine veterinary isolates from cases of otitis externa is frequently associated with methicillin-resistant *Staphylococcus pseudintermedius* (MRSP), multidrug-resistant *S. pseudintermedius* (MDRSP) [4–8], and multidrug-resistant *Pseudomonas aeruginosa,* which often show resistance to at least 3–4 classes of antimicrobials [9–12]. These pathogens represent a potential threat to human health, particularly among zoonotic multidrug-resistant strains with the potential to cause severe, life-threatening infections [13,14]. To prolong the life of existing antibiotics, good antimicrobial stewardship is essential [15,16] and should involve limiting the use of newer antimicrobial classes to safeguard their future effectiveness and avoidance of the use in animals of antimicrobial classes of critical importance to human medicine.

Repurposing existing drugs is an alternative approach to address both the issue of antimicrobial resistance and antimicrobial stewardship [1,17,18]. For example, the salicylanilide anthelmintic drugs niclosamide and oxyclozanide have been studied as potential agents for the treatment of resistant bacterial infections, with *in vitro* activity being demonstrated against methicillin-resistant *S. aureus* (MRSA) and *Enterococcus faecium* [19]. Our laboratory has been exploring the potential for repurposing polyether ionophores as antimicrobial agents in veterinary medicine. One example of such an ionophore, monensin, is the subject of this study. The highly lipophilic ionophores have compound specific affinity for transport of monovalent and divalent cations across the cell membrane of susceptible bacteria [20]. These drugs are only licensed for animal use where they are commonly used as rumen microbiota modulators and anticoccidial agents in production animals [21,22]. There is little evidence of bacterial resistance or co-selection for resistance to other classes of antimicrobials by ionophores [23–25]. These drugs are not suitable for development as systemic antibiotics

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https://doi.org/10.1016/j.cimid.2018.05.001
Received 12 December 2017; Received in revised form 26 March 2018; Accepted 5 May 2018
0147-9571/ © 2018 Published by Elsevier Ltd.

due to the low margin of safety and a high likelihood of systemic toxicity in non-target species [26]. Neurotoxicity and toxic myopathy have been reported in dogs following consumption of monensin contaminated commercial dog food [27,28]. However, the development of ionophores as topical antimicrobials can preclude systemic toxicity while retaining their potential benefit as a true class of "animal only" antimicrobials.

Canine otitis externa was identified as a condition that might be amenable to treatment with monensin. Otitis externa (OE) is one of the most frequently recorded disorders in dogs, with a prevalence of 10.2% in a large UK VetCompass (Veterinary Companion Animal Surveillance) database in 2014 [29]. An earlier UK study found that otitis was the most common dermatological diagnosis, accounting for 4.5% of all consultations in dogs [30]. A US prevalence study in 1995 involving over 31,000 dogs from 52 general veterinary practices concluded that canine OE was the third most common disorder with a prevalence of 13% [31].

Microorganisms typically diagnosed with OE are *S. pseudintermedius* (formerly *S. intermedius*), *P. aeruginosa*, β-haemolytic *Streptococcus* spp., *Proteus mirabilis* and *Malassezia pachydermatis* [32–37]. Resistance of these organisms to existing antimicrobials is an emerging problem. Staphylococcal otitis was commonly reported with resistance to polymyxin B ranging from 66% to 100% [32,33,38], and penicillin G ranging from 34.3% to 66% [35,38,39]. *Pseudomonas* otitis isolates had fluoroquinolone resistance rates ranging from 47.9% to 53.1% for enrofloxacin and 8.7% to 33.3% for marbofloxacin [9,10,40]. Recently, 52% (13/25) of MRSP strains isolated from canine OE or pyoderma showed resistance to two or more fluoroquinolones (enrofloxacin, ciprofloxacin, ofloxacin, levofloxacin, and moxifloxacin) [41]. There is a potential public health risk associated with emerging AMR in canine OE pathogens [42,43]. Thus, it is essential to reduce the use of critically important human drugs for animal treatment and seek alternative antimicrobial agents where appropriate.

The aim of this study was to investigate the *in vitro* efficacy of the polyether ionophore monensin as a topical antimicrobial agent against common bacterial and yeast otitis externa isolates from dogs.

2. Materials and methods

2.1. Test and control strains

A total of one hundred and eleven bacterial and yeast clinical canine otitis isolates were collected from government, private and university diagnostic laboratories throughout Australia. These organisms were speciated using biochemical testing and MALDI-TOF mass spectrometry (Bruker, Preston, VIC, Australia). Seventeen methicillin-susceptible *S. pseudintermedius* (MSSP) and thirteen multidrug-resistant *S. pseudintermedius* (MDRSP) were obtained from the first national survey of antimicrobial resistance in animal pathogens conducted in Australia [44]. Other organisms comprised twenty each of β-haemolytic *Streptococcus* spp. (BHS), *P. aeruginosa* and *Proteus mirabilis*, and twenty-one *Malassezia pachydermatis*. Type strains *S. aureus* ATCC 29213, *P. aeruginosa* ATCC 27853, *Streptococcus pneumoniae* ATCC 49619, *Candida albicans* ATCC 90028, and *Issatchenkia orientalis* (*Candida krusei)* ATCC 6258 were used to determine appropriate monensin testing concentrations as well as for internal quality control to monitor the reproducibility of minimal inhibitory concentrations (MIC) during testing of clinical isolates.

2.2. Antimicrobial agent

Monensin A sodium salt and/or monensin A crystalline acid, > 98% purity (BioAustralis, Smithfield, NSW, Australia) were used to determine the MIC for each isolate. The crystalline acid material is solubilised in the presence of sodium, it immediately complexes with sodium and becomes the sodium salt. Preliminary experiments were performed to compare the *in vitro* efficacy of both forms and there was no difference between the results obtained using sodium salt and acid form (data not shown). Stock solutions of monensin were prepared at 100 times maximum testing concentration (1.6 mg/ml for MSSP, MDRSP and *Streptococcus* spp. (BHS); and 6.4 mg/ml for *P. aeruginosa*, *P. mirabilis,* and *M. pachydermatis*) in dimethyl sulfoxide (DMSO ≥ 99.9%, Sigma-Aldrich, St. Louis, Mo, USA) and stored at −80 °C until used.

2.3. Control antimicrobial agents

Ampicillin, 99.4% purity (Sigma-Aldrich, Mo. USA) was used for both *S. aureus* and *S. pneumoniae* ATCC strains. Enrofloxacin, 99.8% purity (Sigma-Aldrich, Mo. USA) was used for *P. aeruginosa* ATCC strain. Amphotericin B, 250 μg/ml (Sigma-Aldrich, Mo. USA) was used to determine MIC for both *C. albicans* and *I. orientalis* ATCC strains. Both ampicillin and enrofloxacin stock solutions were prepared at 3.2 mg/ml and 12.8 mg/ml, respectively and stored at −80 °C until use. Amphotericin B was stored as its original solution at −20 °C and thawed for use on the day of antifungal susceptibility testing.

2.4. Antimicrobial susceptibility testing

The antimicrobial susceptibility of each isolate was determined using minimal inhibitory concentration (MIC) microdilution methodology as recommended by CLSI methods with some modifications [45]. Briefly, antimicrobial challenge plates were prepared by creating two-fold serial dilutions of monensin stock solution in DMSO and each dilution was then further diluted 1:100 in cation-adjusted Mueller Hinton Broth (CAMHB) (Becton Dickinson Pty Ltd, Maryland, USA) in 96-well microtiter plates (Nunclon™ Delta Surface; Thermo Fisher Scientific, Denmark). A bacterial suspension for each isolate was prepared in phosphate buffered saline (Oxoid™, Hampshire, UK) with a visual turbidity comparable to 0.5 McFarland turbidity standard. These suspensions were then adjusted using additional saline to give each well a final bacterial concentration of approximately 5×10^5 CFU/ml after inoculation. Monensin concentrations of 0.03–16 μg/ml (MSSP, MDRSP, and BHS) and 0.125–64 μg/ml (*P. aeruginosa* and *P. mirabilis*) were used for testing. These testing concentrations were determined based on CLSI guidelines[45] and preliminary experiments (data not shown). Antimicrobial challenge plates for streptococcal isolates were prepared using CAMHB supplemented with 5% lysed sheep blood. All isolates and control strains were tested in duplicate. Negative growth controls contained only CAMHB and positive growth controls contained CAMHB and bacterial suspension. Growth was assessed visually after overnight incubation at 37 °C, with streptococcal isolates being incubated in 5% CO_2, and MIC was determined as the lowest concentration of monensin that completely inhibited the growth of the organism. The MIC range (minimum and maximum), MIC_{50} and MIC_{90} were recorded and calculated for each of the bacterial groups. The MIC_{50} and MIC_{90} were the lowest concentrations of monensin at which 50% and 90% of the isolates were inhibited, respectively.

2.5. Antifungal susceptibility testing

The antifungal susceptibility of *M. pachydermatis* isolates was performed using a modified CLSI broth microdilution method [46,47]. Briefly, isolates were inoculated onto Sabouraud's dextrose agar (SDA) supplemented with 1% Tween 80 (Sigma-Aldrich, Mo., USA) [48,49] and incubated for 72 h at 32 °C. Antifungal challenge plates were prepared by creating two-fold serial dilutions of monensin stock solution in DMSO and each dilution was then further diluted 1:100 in Sabouraud's dextrose broth (SDB) (Oxoid™, Hampshire, UK) supplemented with 1% Tween 80 in 96-well microtiter plates (Nunclon™ Delta Surface; Thermo Fisher Scientific, Denmark). Yeast isolates were suspended in SDB supplemented with 1% Tween 80 to obtain a uniform yeast suspension

Table 1
MIC range, $MIC_{50,}$ and MIC_{90} for 111 clinical isolates of canine otitis externa and quality control strains against monensin.

		Monensin concentration (μg/ml)		
Clinical Isolates	**n**	**MIC range**	**MIC_{50}**	**MIC_{90}**
Staphylococcus pseudintermedius	30	2–4	4	4
i) MSSP	17	2–4	2	4
ii) MDRSP	13	2–4	4	4
β-haemolytic *Streptococcus* spp.	20	1–2	2	2
Pseudomonas aeruginosa	20	> 64	> 64	> 64
Proteus mirabilis	20	> 64	> 64	> 64
Malassezia pachydermatis	21	16– > 64	> 64	> 64
Quality Control Strains				
Staphylococcus aureus ATCC 29213		4	–	–
Streptococcus pneumoniae ATCC 49619		1–2	–	–
Pseudomonas aeruginosa ATCC 27853		> 64	–	–
Candida albicans ATCC 90028		> 64	–	–
Issatchenkia orientalis (*C. krusei*) ATCC 6258		> 64	–	–

with an optical density (OD) between 0.2–0.3 at 600 nm wavelength (Eppendorf BioPhotometer plus, Hamburg, Germany). A 1:100 dilution was then performed and 50 μl of yeast suspension was added to 150 μl of monensin solution to create a final inoculum concentration of 4–5 × 10^3 CFU/ml and monensin concentrations of 0.125–64 μg/ml. The MIC end-point was determined using a combination of two methods: visual end-point reading of 50% growth inhibition compared with the positive growth control and spectrophotometric end-point reading of OD_{570nm} less or equal to 50% of the positive growth control. The MICs were reported as described above for bacterial antimicrobial susceptibility testing.

3. Results

The MICs for all the otitis externa isolates are shown in Table 1. Both methicillin-susceptible *S. pseudintermedius* (MSSP) and multidrug-resistant *S. pseudintermedius* (MDRSP) had MICs ranging from 2 to 4 μg/ml. The monensin MIC_{50} and MIC_{90} values for all *S. pseudintermedius* isolates were both 4 μg/ml. *Streptococcus* spp. (BHS) isolates were more susceptible to monensin compared to *S. pseudintermedius* with MICs ranging from 1 to 2 μg/ml, and both MIC_{50} and MIC_{90} values were 2 μg/ml. At the highest concentrations tested, there was no attainable monensin MIC for any *P. aeruginosa* or *P. mirabilis* isolates. *M. pachydermatis* isolates had monensin MICs ranging from 16 to > 64 μg/ml, with both MIC_{50} and MIC_{90} values of > 64 μg/ml. These results were in alignment with those obtained for the ATCC type strains. Consistent confluent growth was observed in all the positive growth control wells, and there was no growth in any of the negative growth control well. MICs of ATCC type strains against selected control antimicrobials were within CLSI range (Table 2).

4. Discussion

To the best of our knowledge, this is the first report demonstrating monensin activity against Gram-positive pathogens isolated from ear infections in dogs. Monensin was shown to have *in vitro* antimicrobial activity against the Gram-positive canine OE pathogens *S. pseudintermedius* and β-haemolytic *Streptococcus* spp., with MICs ranging from 2 to 4 μg/ml. These results were comparable to those obtained using the type strains of these organisms. Previous clinical studies have focused on its efficacy against *Clostridium perfringens* in poultry, with MICs ranging from 0.03 to 2 μg/ml [52–55]. Other studies have revealed the activity of monensin against Gram-positive reference strains with MICs of 1–2 μg/ml for *Staphylococcus* spp., 1–16.5 μg/ml for *Bacillus* spp., 2–4 μg/ml for *Micrococcus* spp., and 12.5 μg/ml for *Enterococcus* spp. [56–58]. Though monensin is exclusively for veterinary use, there was an *in vitro* susceptibility study of clinical *Staphylococcus epidermidis* strains conducted in a search of an alternative antimicrobial therapy for chronic nosocomial infections in human. These twelve *S. epidermidis* strains were susceptible to monensin with MIC_{50} and MIC_{90} of 1 μg/ml and 2 μg/ml, respectively [59].

The thirteen isolates of multidrug-resistant *S. pseudintermedius* tested in this study were all susceptible to monensin. These resistant isolates were also characterised as methicillin-resistant strains based on the assessment of cefoxitin and/or oxacillin resistance and mecA PCR by Saputra and co-researcher [44]. This is in agreement with previous studies that have shown monensin to be effective against hospital-associated MRSA with MIC_{50} and MIC_{90} values of 2.9 μg/ml and 5.8 μg/ml, respectively [60], methicillin-resistant *S. epidermidis* (MIC range from 1 to 4 μg/ml) [61], and clinical MRSA (MIC of 4 μg/ml) and vancomycin-resistant enterococci (MIC of 16 μg/ml) [62]. These studies suggest that in addition to having excellent activity against Gram-positive organisms, monensin has potential as a topical treatment for bacteria that have become resistant to multiple classes of antimicrobial agents, including the critically important fluoroquinolones.

Monensin had poor antimicrobial activity against yeast isolates and it was not effective against Gram-negative pathogens. Antifungal activity of monensin against *M. pachydermatis* was only observed in 19% (4/21) of the isolates with MICs ranging from 16 to 32 μg/ml. Monensin was inactive against both *Candida* ATCC strains and this result was similarly observed in a Polish study [57]. Monensin did not demonstrate any antibacterial activity against the Gram-negative OE pathogens *P. aeruginosa* and *P. mirabilis,* with MICs for both organisms exceeding the highest tested concentration of 64 μg/ml. Previous studies similarly reported that monensin and other ionophores are not active against Gram-negative bacteria [24,56,57]. An alternative antimicrobial agent or adjuvant could be used in combination with monensin to target Gram-negative and yeast otitis pathogens.

The mechanism of action of monensin relates to its highly lipophilic properties, allowing it to insert into bacterial cell membranes. It then chelates reversibly with Na^+ and K^+ conveying them rapidly across the cell membrane where they exchange for other monovalent cations such as hydrogen ions, disrupting the normal ionic and pH gradients [63]. In Gram-positive bacteria, it is likely that it can penetrate the porous peptidoglycan cell wall layer and cause an imbalance of intracellular concentration gradients, disrupting cellular functions and potentially leading to cell death [21,24,64]. The inherent resistance of Gram-negative bacteria is most likely due to the two-layered cell wall structure

Table 2
MIC obtained and CLSI acceptable MIC range for quality control strains using control antimicrobials.

Quality control strains	Control antimicrobial agent	CLSI acceptable MIC range (μg/ml) [50,51]	MIC obtained (μg/ml)
Staphylococcus aureus ATCC 29213	Ampicillin	0.06–2	0.25
Streptococcus pneumoniae ATCC 49619	Ampicillin	0.06–0.25	0.125
Pseudomonas aeruginosa ATCC 27853	Enrofloxacin	1–4	4
Issatchenkia orientalis (*C. krusei*) ATCC 6258	Amphotericin B	0.5–2.0 (Post 24 h)	2.0
		1.0–4.0 (Post 48 h)	4.0
Candida albicans ATCC 90028	Amphotericin B	0.5–2.0 (Post 24 h)	1.0
		0.5–2.0 (Post 48 h)	1.0

of Gram-negative bacteria preventing monensin from permeating into the inner cytoplasmic membrane. This would stop the drug from interfering with ionic gradients and bacterial cell functions [21,24].

There have been multiple safety and toxicology studies of monensin in dogs. Dogs given oral monensin daily at a dose of ≤2.5 mg/kg for 1 year survived without toxicity, but suffered toxicity and death when given daily doses above 10 mg/kg for 3 months [65]. Over a 3 month study period in Beagle dogs, the No Observed Effect Level (NOEL) of monensin was determined to be ≤5 mg/kg [66]. However, further studies would be required to assess suitable formulations for topical use in dogs. To date, there is only limited information about the potential of monensin to cause cutaneous irritation in laboratory animals and humans. It was concluded that a weak contact sensitiser effect was present when delayed hypersensitivity was detected in the local lymph node assay following the application of 50 μg/ml of monensin to the ear pinnae of mice, and some cutaneous adverse effects such as erythema have been seen in the clipped and abraded skin of rabbits [66]. In the feed industry, workers exposed to monensin have been reported with allergic signs of contact dermatitis, urticaria, face or tongue swelling, nasal congestion, and local respiratory irritation [66,67]. Other reported adverse reactions following topical monensin exposure in humans include skin congestion, eye/lids discharges and swelling, pruritus and rash [68].

5. Conclusion

The results of this study suggest that monensin would not be suitable as a sole antimicrobial agent for the treatment of canine OE due to lack of activity against Gram-negative isolates and reduced activity against yeast isolates. However, an adjuvant could be added to disrupt the external cell wall of these pathogens to allow organisms to become susceptible to monensin. Further studies are underway to test this hypothesis.

Despite this limitation, monensin was shown to have *in vitro* efficacy against Gram-positive organisms, including multidrug-resistant strains. Therefore, it would be a suitable candidate for a single topical treatment for a dominantly or purely Gram-positive ear infection. It could also be included as one of the components of a commercial otic preparation for treatment of OE in dogs. This study provides a proof of concept of drug repurposing of an ionophore in the field of veterinary otology and would represent a good example of prudent antimicrobial stewardship if the compound was to be ultimately used clinically.

Conflicts of interest

Stephen W. Page is a director of Luoda Pharma Pty. Ltd. Darren J. Trott has received research funding from Luoda Pharma, Neoculi, Zoetis, Bayer, Merial, Virbac, and Elanco.

Sources of funding

This work was supported by ARC Linkage [grant numbers LP130100736] with Luoda Pharma Pty. Ltd as a partner organisation.

Acknowledgements

Wei Yee Chan is supported by the Ministry of Higher Education of Malaysia and Universiti Putra Malaysia.

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Chapter 4: *In vitro* antimicrobial activity of seven adjuvants against common pathogens associated with canine otitis externa

Statement of Authorship

Title of Paper	*In vitro* antimicrobial activity of seven adjuvants against common pathogens associated with canine otitis externa
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Chan, W. Y., Khazandi, M., Hickey, E. E., Page, S. W., Trott, D. J., & Hill, P. B. (2019). *In vitro* antimicrobial activity of seven adjuvants against common pathogens associated with canine otitis externa. *Veterinary Dermatology, 30*(2), 133-e138. doi:10.1111/vde.12712

Principal Author

Name of Principal Author (Candidate)	Wei Yee, Chan		
Contribution to the Paper	Performed literature search, planned and conducted the experiments, analysis on all samples, interpreted data, wrote manuscript and acted as corresponding author.		
Overall percentage (%)	75%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Prof. Gordon S. Howarth on behalf of Manouchehr Khazandi		
Contribution to the Paper	Provided input in analysing data, understanding of results and editing of manuscript.		
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Veterinary Dermatology

Vet Dermatol 2018 DOI: 10.1111/vde.12712

In vitro antimicrobial activity of seven adjuvants against common pathogens associated with canine otitis externa

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Background – An antibiotic adjuvant is a chemical substance used to modify or augment the effectiveness of primary antimicrobial agents against drug-resistant micro-organisms. Its use provides an alternative approach to address the global issue of antimicrobial resistance and enhance antimicrobial stewardship.

Hypothesis/Objectives – To determine the antimicrobial activity of a panel of potential antimicrobial adjuvants against common pathogens associated with canine otitis externa (OE).

Animals/Isolates – A number of type strains and clinical isolates (n = 110) from canine OE were tested including *Staphylococcus pseudintermedius,* β-haemolytic *Streptococcus* spp., *Pseudomonas aeruginosa*, *Proteus mirabilis* and *Malassezia pachydermatis.*

Methods and materials – Antimicrobial activities of monolaurin, monocaprin, *N*-acetylcysteine (NAC), polymyxin B nonapeptide, Tris-EDTA, Tris-HCL and disodium EDTA were tested using microdilution methodology according to CLSI guidelines.

Results – *N*-acetylcysteine, Tris-EDTA and disodium EDTA had antimicrobial activity against both type strains and otic pathogens. The other adjuvants tested had limited to no efficacy. NAC had a minimal inhibitory concentration (MIC) range of 2,500–10,000 μg/mL for the various organisms. *Pseudomonas aeruginosa* isolates were eight times more susceptible to disodium EDTA in the presence of Tris-HCL in comparison to disodium EDTA alone. *Malassezia pachydermatis* isolates were most susceptible to Tris-EDTA (MIC_{90} = 190/60 μg/mL) and disodium EDTA (MIC_{90} = 120 μg/mL).

Conclusions and clinical relevance – *N*-acetylcysteine, Tris-EDTA and disodium EDTA have intrinsic antimicrobial activity and represent promising adjuvants that could be used to enhance the efficacy of existing antibiotics against Gram-negative and multidrug-resistant bacterial infections. These agents could be combined with other antimicrobial agents in a multimodal approach for mixed ear infections in dogs.

Introduction

Otitis externa (OE) is one of the most commonly diagnosed dermatological diseases in dogs with a prevalence of 4.5–13%.[1–3] Bacterial and yeast pathogens typically associated with canine OE are *Staphylococcus pseudintermedius* (*S. pseudintermedius*, formerly *S. intermedius*), β-haemolytic *Streptococcus* spp., *Pseudomonas aeruginosa*, *Proteus mirabilis* and *Malassezia pachydermatis*.[4–9] Infections with these organisms are typically treated with antimicrobial drugs, but there are increasing concerns about bacterial resistance and the requirement for diligent antimicrobial stewardship.[10,11] Meticillin-resistant *S. pseudintermedius* (MRSP), which is often multidrug-resistant,[12–14] as well as multidrug-resistant *P. aeruginosa* strains[15–17] are frequently isolated from canine OE and/or skin infections. Alarmingly, studies have shown that antimicrobial resistance in the human population, especially in pet owners and veterinarians, can be associated with organisms of canine origin.[18–21] Therefore, public health risks mandate the prudent use of antimicrobials to safeguard important drugs for human medicine and the search for alternative approaches to treat animal infections.[22,23]

One way of broadening the spectrum of activity of antimicrobial agents is to combine them with an adjuvant.[24,25] Antimicrobial adjuvants can function in different ways including acting as inhibitors of enzymes that inactivate antibiotics, efflux pump inhibitors, bacterial membrane permeabilizers, biofilm dispersers, inhibitors of antibiotic resistance elements and inhibitors of bacterial cell physiological pathways.[26,27]

Accepted 8 November 2018

Conflicts of interest: Stephen W. Page is a director of Luoda Pharma Pty. Ltd. Darren J. Trott has received research funding from Luoda Pharma, Neoculi, Zoetis, Bayer, Merial, Virbac and Elanco.

Sources of funding: Supported by funding from ARC Linkage grant LP130100736 with Luoda Pharma Pty. Ltd as a partner organization

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This study aimed to determine the *in vitro* antimicrobial activity of seven nonantibiotic adjuvant agents against common clinical isolates associated with canine OE.

Methods and materials

Antibiotic adjuvants and antibiotics

The seven adjuvants used in this study were monolaurin, monocaprin, *N*-acetylcysteine (NAC), polymyxin B nonapeptide (PMBN), Tris-ethylenediaminetetraacetic acid (Tris-EDTA), tromethamine-hydrochloride (Tris-HCL) and disodium ethylenediaminetetraacetic acid (EDTA). Monolaurin (2-Lauroylglycerol), monocaprin (1-Decanoyl-rac-glycerol), PMBN and NAC (*N*-acetyl-L-cysteine) were obtained from Sigma-Aldrich (St Louis, MO, USA). Tris-HCL and disodium EDTA were purchased from Promega (Madison, WI, USA).

Monolaurin stock solution was prepared at 25× the highest concentration to be tested in 95% ethanol (VWR International S.A.S.; Fontenay-sous-Bois, France) as described previously.[28] In a preliminary experiment, no inhibitory effect was found on the control bacterial strains when exposed to ≤4% ethanol (highest tested concentration in antimicrobial susceptibility testing) (data not shown). Monocaprin stock solution was prepared at 50× the highest tested concentration in chloroform (Sigma-Aldrich). No inhibitory effect was found when ≤2% chloroform was tested against control bacterial strains in a preliminary experiment. PMBN stock solution was prepared at 10× the highest testing concentration in MilliQ water. These three adjuvants were stored at −80°C until used. On the day of testing, they were diluted to make working solutions of 2,000 μg/mL (monocaprin and monolaurin) and 256 μg/mL (PMBN) with the previously mentioned solvent or diluent. NAC was prepared at 160,000 μg/mL (double the highest concentration to be tested) in cation-adjusted Mueller Hinton Broth (CAMHB) (Becton Dickinson Pty Ltd; Cockeysville, MD, USA). Stock solutions of 1M Tris-HCL and 0.5 M disodium EDTA were prepared in MilliQ water. Tris HCL working solution was prepared in CAMHB at 100 mM (12,200 μg/mL), the highest concentration to be used for preliminary screening. Disodium EDTA working solution was prepared in CAMHB at 20 mM (7,500 μg/mL) for Gram-positive organisms and 80 mM (30,000 μg/mL) for Gram-negative organisms. Tris-EDTA was made by mixing and diluting 1 M Tris-HCL and 0.5M disodium EDTA in CAMHB and then buffered to pH 8.0 with sodium hydroxide (AnalaR®, Merks Pty. Ltd; Victoria, Australia). Tris-EDTA working solution was prepared at a concentration of 100 mM (12,200 μg/mL) Tris-HCL with 10 mM (3,800 μg/mL) disodium EDTA for preliminary screening of ATCC type strains and Gram-positive organisms, and 400 mM (48,500 μg/mL) Tris-HCL with 40 mM (15,000 μg/mL) disodium EDTA for testing of Gram-negative organisms. NAC, Tris-EDTA, Tris-HCL and disodium EDTA were stored at 2–8°C and were ready to be used as working solutions on the day of minimal inhibitory concentration (MIC) testing.

Ampicillin, 99.4% purity (Sigma-Aldrich) was used as a control for Gram-positive organisms. Enrofloxacin, 99.8% purity (Sigma-Aldrich) was used as a control for Gram-negative organisms. Amphotericin B, 250 μg/mL (Sigma-Aldrich) was used as the control for fungal organisms. Both ampicillin and enrofloxacin stock solutions were prepared at 3.2 and 12.8 mg/mL, respectively, and stored at −80°C until used. Amphotericin B was stored as its original solution at −20°C and thawed for use on the day of antifungal susceptibility testing.

Bacterial and yeast isolates

One hundred and ten clinical isolates from cases of canine OE were collected from diagnostic laboratories throughout Australia. These organisms were speciated using biochemical testing and matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF) mass spectrometry (Bruker; Preston, Victoria, Australia). These isolates comprised 17 meticillin-susceptible *Staphylococcus pseudintermedius* (MSSP) and 13 MRSP obtained from the first national survey of antimicrobial resistance in animal pathogens conducted in Australia,[29] and 20 each of β-haemolytic *Streptococcus* spp., *P. aeruginosa*, *P. mirabilis* and *M. pachydermatis*. American Type Culture Collection (ATCC) strains *S. aureus* (SA) 29213, *P. aeruginosa* (PA) 27853 and *P. aeruginosa* biofilm-producer PA-01 were used to preliminary screen the antimicrobial activities of the seven adjuvants. Based on these results, three adjuvants were selected to test against the full range of clinical isolates. ATCC SA 29213, ATCC PA 27853 and *Streptococcus pneumoniae* ATCC 49619 were used as bacterial internal quality controls to monitor the MIC reproducibility during testing of clinical isolates. For yeast internal quality controls, *Candida albicans* ATCC 90028 and *C. krusei* (*Issatchenkia orientalis*) ATCC 6258 were used as published previously,[30,31] rather than *Malassezia* spp. as there is no standard MIC testing for *Malassezia* spp. in the CLSI guidelines.

Antimicrobial susceptibility testing

The antimicrobial susceptibility of each isolate was determined using minimal inhibitory concentration (MIC) microdilution methodology as recommended by the Clinical and Laboratory Standards Institute (CLSI).[32] Briefly, bacterial isolates were subcultured on 5% Sheep Blood Columbia Agar (Thermo Fisher Scientific; Victoria, Australia) and incubated overnight at 37°C**,** with 5% CO_2 supplemented for streptococcal isolates. A bacterial suspension for each isolate was prepared in phosphate buffered saline Oxoid Limited; Basingstoke, Hampshire, UK. and adjusted to 0.5 McFarland standard. Two-fold serial dilution of each adjuvant working solution was performed with 90 μL CAMHB in 96-well microtitre antimicrobial challenge plates (Nunclon™ Delta Surface, Thermo Fisher Scientific, Roskilde, Denmark). Finally, 10 μL of the bacterial inoculum was added to the 90 μL adjuvant solution to achieve a final bacterial concentration of 5×10^5 colony forming units (cfu)/mL. The final tested concentration ranges of each adjuvant is shown in Table 1. Antimicrobial challenge plates for streptococcal isolates were prepared using CAMHB supplemented with 5% lysed sheep blood. All isolates and control strains were tested in duplicate. Negative growth controls contained only CAMHB and positive growth controls contained CAMHB and bacterial suspension. Bacterial growth was assessed visually after overnight incubation at 37°C and MIC was determined as the lowest concentration of adjuvant that completely inhibited the growth of the organism. The MIC range (minimum and maximum), MIC_{50} and MIC_{90} were recorded and calculated for each of the clinical bacterial groups. The MIC_{50} and MIC_{90} values were defined as the lowest concentrations of adjuvant at which 50% and 90% of the isolates were inhibited, respectively.

Antifungal susceptibility testing

The antifungal susceptibility of *M. pachydermatis* isolates was performed using a modified CLSI broth microdilution method.[30,31] Briefly, isolates were inoculated onto Sabouraud's dextrose agar (SDA) supplemented with 1% Tween 80 (Sigma-Aldrich)[33,34] and incubated for 72 h at 35°C. Yeast isolates were suspended in Sabouraud's dextrose broth (SDB) (Oxoid™) supplemented with 1% Tween 80 to obtain a uniform yeast suspension with an optical density (OD) between 0.2 and 0.3 at 600 nm wavelength (Eppendorf BioPhotometer plus; Hamburg, Germany) and further diluted 1:100 in the same medium. Antifungal challenge plates were prepared by creating two-fold serial dilutions of the adjuvant working solution in 150 μL SDB supplemented with 1% Tween 80 in 96-well microtiter plates (Nunclon™ Delta Surface; Thermo Fisher Scientific). Thereafter, 50 μL of yeast suspension was added to 150 μL of the adjuvant solution to create a final inoculum concentration of 2–2.5×10^3 cfu/mL and adjuvant (NAC, Tris-EDTA and disodium EDTA) concentrations as shown in Table 1. The MIC end-point was determined every 24 h (up to 72 h) using a combination of two methods: visual end-point reading of more than or equal to 50% growth inhibition (prominent decrease in turbidity) compared with the positive growth control and spectrophotometric end-point reading of $OD_{570\ nm}$ less or equal to 50% of the positive growth control.[30] The MICs were reported as described above for bacterial antimicrobial susceptibility testing.

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Results

Table 1 shows the preliminary screening of the seven adjuvants against one Gram-positive and two Gram-negative ATCC type strains. NAC, Tris-EDTA and disodium EDTA were effective against both Gram-positive (ATCC SA 29213) and Gram-negative bacteria (ATCC PA 27853 and ATCC PA-01) with MICs ranging from 2,500 to 5,000 μg/mL, 1,500/470 to 6,000/1,900 μg/mL and 470 to 3,800 μg/mL, respectively. Monolaurin was found to have antimicrobial activity only against Gram-positive ATCC SA 29123. PMBN was effective only against Gram-negative bacteria (ATCC PA 27853 and ATCC PA-01) with MICs ranging from 8 to 16 μg/mL. No MIC was attainable at the highest tested concentration of monocaprin and Tris-HCL against both Gram-positive and Gram-negative ATCC type strains. Precipitation also was observed at concentrations beyond 1,000 μg/mL during MIC testing of monocaprin.

Based on the antimicrobial activities found during the preliminary screening, three adjuvants (NAC, Tris-EDTA and disodium EDTA) were chosen for further antimicrobial susceptibility testing against the clinical bacterial and yeast isolates (Table 2) and quality control strains (Table S1). NAC was effective against all 110 bacterial and yeast isolates with MICs ranging from 2,500 to 10,000 μg/mL. Both *Staphylococcus* and *Streptococcus* clinical isolates showed similar values in MIC range, MIC_{50} and MIC_{90} for Tris-EDTA and disodium EDTA at 1,500/470 and 470 μg/mL, respectively. There was no difference in results between MSSP and MRSP for all three adjuvants. The Tris-EDTA MIC range was one to two dilutions lower for *Pseudomonas* clinical and control strains compared to the screening results in Table 1. *Proteus mirabilis* isolates were more susceptible to disodium EDTA (MIC_{90} of 930 μg/mL) than *P. aeruginosa* (MIC_{90} of 7,500 μg/mL). Compared to both Gram-positive and Gram-negative clinical isolates, *M. pachydermatis* yeast isolates were most susceptible to Tris-EDTA and disodium EDTA with a MIC range of 95/30–190/60 μg/mL and 30–120 μg/mL, respectively.

Consistent confluent growth was observed in all of the positive growth control wells and there was no growth in any of the negative growth control wells. MICs of ATCC type strains against selected control antimicrobials were within CLSI ranges (Table S1).

Discussion

This study has demonstrated the *in vitro* antimicrobial activity of NAC, Tris-EDTA and disodium EDTA against pathogenic Gram-positive (including MRSP), Gram-negative bacteria and *M. pachydermatis* isolated from cases of canine OE. The other adjuvants tested had either a limited spectrum or no antimicrobial activity.

N-acetylcysteine is a known mucolytic and nonantibiotic agent with bactericidal activity.[35] Its proposed mode of action is to competitively inhibit the uptake of amino acids such as cysteine by bacterial cells or to react with the bacterial cell proteins with its own sulfhydryl group.[36] It is used as a flushing solution to physically remove biofilm as part of the management of canine OE.[37] Studies related to human respiratory and urinary tract diseases have described the combination of NAC with other antibiotics such as carbenicillin, fosfomycin and ciprofloxacin to potentiate the antimicrobial activity of these antibiotics, to increase the inhibitory effect on biofilm formation and to eradicate mature biofilms.[38–40] In this study, NAC had a comprehensive range of antimicrobial activities against both Gram-positive and Gram-negative bacteria and yeast isolates associated with canine OE. In comparison to a previous study,[41] in which the NAC MIC ranged from 5,000 to 20,000 μg/mL, our MICs were typically one- to two-fold lower for all of the isolates except β-haemolytic *Streptococcus* (2,500–5,000 μg/mL) and 90% of each clinical isolate cohort was inhibited at 5,000 μg/mL. Conversely, an older study[42] observed that *P. aeruginosa* growth curve and inhibition in the presence of NAC were both dose- and inoculum size-dependent; consequently, they were able to detect a NAC MIC of *P. aeruginosa* at a lower concentration range (2–20 μg/mL). Another study used NAC as an adjunctive therapy against fluoroquinolone/ciprofloxacin-resistant *P. aeruginosa* strains associated with human chronic otitis media at concentrations ≥0.5% or 5,000 μg/mL.[43] Taken together, our results and those from previous studies suggest that NAC could potentially be a useful antimicrobial adjuvant that could be safely incorporated in the treatment and management of OE in dogs, especially in cases associated with antimicrobial resistance.

Tris-EDTA is a common ingredient found in ear cleaners or rinses to remove ear debris, reduce microbial load and

Table 1. Minimal inhibitory concentrations (MICs) of the seven adjuvants against the three ATCC type strains (*Staphylococcus aureus* ATCC 29213, *Pseudomonas aeruginosa* ATCC 27853 and *P. aeruginosa* biofilm-producer ATCC PA-01)

Adjuvant	*Staphylococcus aureus* ATCC 29213 MIC (μg/mL)	*Pseudomonas aeruginosa* ATCC 27853 MIC (μg/mL)	*Pseudomonas aeruginosa* biofilm-producer ATCC PA-01 MIC (μg/mL)	Tested concentration MIC range (μg/mL)
Monolaurin	125	>2,000	>2,000	8–2,000
Monocaprin	>2,000*	>2,000*	>2,000*	8–2,000
N-acetylcysteine	2,500	5,000	2,500	156–80,000
PMBN	>128	16	8	0.25–128
Tris-EDTA buffer pH 8.0	1,500 470	6,000 1,900	6,000 1,900	Tris: 24–12,200 EDTA: 7–3,800
Tris-HCL pH 8.0	>12,200	>12,200	>12,200	24–12,200
Disodium EDTA pH 8.0	470	3,800	3,800	15–30,000

EDTA ethylenediaminetetraacetic acid, PMBN Polymyxin B nonapeptide, Tris-HCL tromethamine-hydrochloride.
*Precipitation at concentration from 1,000–2,000 μg/mL.
The right-hand column indicates the range of concentrations tested.

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Table 2. MIC range, MIC_{50}, and MIC_{90} for *N*-acetylcysteine, Tris-EDTA and disodium EDTA against 110 clinical isolates of canine otitis externa

	N-acetylcysteine (μg/mL)			Tris-EDTA in combination (μg/mL)						Disodium EDTA (μg/mL)		
				MIC range		MIC_{50}		MIC_{90}				
Isolates	MIC range	MIC_{50}	MIC_{90}	Tris	EDTA	Tris	EDTA	Tris	EDTA	MIC range	MIC_{50}	MIC_{90}
Staphylococcus pseudintermedius (n=30)	2,500 –5,000	2,500	5,000	1,500	470	1,500	470	1,500	470	470	470	470
β-haemolytic *Streptococcus* spp. (n=20)	10,000	10,000	10,000	1,500	470	1,500	470	1,500	470	470	470	470
Pseudomonas aeruginosa (n=20)	2,500–5,000	5,000	5,000	1,500–3,000	470 –930	3,000	930	3,000	930	470 –7,500	1,900	7,500
Proteus mirabilis (n=20)	2,500 –10,000	5,000	5,000	1,500–6,000	470 –1,900	1,500	470	3,000	930	470 –1,900	470	930
Malassezia pachydermatis (n=20)	2,500–5,000	5,000	5,000	95 –190	30–60	190	60	190	60	30–120	60	120

	MIC range (μg/mL)			
		Tris-EDTA in combination		
Quality control strains	*N*-acetylcysteine	Tris	EDTA	Disodium EDTA
Staphylococcus aureus ATCC 29213	2,500–5,000	1,500	470	470
Streptococcus pneumoniae ATCC 49619	10,000	1,500	470	470
Pseudomonas aeruginosa ATCC 27853	2,500–5,000	3,000	930	3,800
Candida albicans ATCC 90028	80,000	380	120	120
Candida krusei ATCC 6258	80,000	380	120	120

Isolates included meticllin-susceptible *Staphylococcus pseudintermedius* (n = 17), meticillin-resistant *Staphylococcus pseudintermedius* (n = 13), β-haemolytic *Streptococcus* spp. (n = 20), *Pseudomonas aeruginosa* (n = 20), *Proteus mirabilis* (n = 20) and *Malassezia pachydermatis* (n = 20). Refer to Table 1 for the range of concentrations tested.

increase the susceptibility of otic pathogens to topical antimicrobial therapy for canine OE.[37,44] There is a significant literature on Tris-EDTA and disodium EDTA that describes these bacteriostatic agents as potentiating other antimicrobial agents[45–47] or assesses their antibacterial activity as a part of a formulation or poly-pharmaceutical commercial product.[48,49] Tris-buffer enhances EDTA's ability to chelate and remove divalent cations that are critically important in maintaining the integrity of the lipopolysaccharide cell wall. Disruption leads to solubilization of the cell wall and increases the permeability of the outer membrane of resistant Gram-negative organisms to primary antimicrobial agents.[50–52] We confirmed in the present study that the antibacterial activity of Tris-EDTA against the ATCC type strains was dependent on the presence of disodium EDTA as no activity was observed with Tris-HCL alone. Furthermore, for *P. aeruginosa*, there was a significant reduction in the MIC_{50} (two times lower) and MIC_{90} (eight times lower) for disodium EDTA in the presence of Tris-HCL compared to disodium EDTA used alone. However, this was not the case for *P. mirabilis* isolates, in which disodium EDTA was essentially as effective on its own. *Proteus mirabilis* clinical isolates also were four to eight times more susceptible to disodium EDTA than *P. aeruginosa* clinical isolates. In our study, the MIC range for Tris-EDTA against canine otic *Pseudomonas* isolates was comparatively lower (1,500/470–3,000/930) than those reported previously,[15] with the range of 2,200/600–8,900/2,400 μg/mL. The antimicrobial activity of disodium EDTA against both Gram-positive and Gram-negative ATCC strains in our study was quite close to the findings in a previous study.[53]

To the best of the authors' knowledge, this is the first study to demonstrate *in vitro* antifungal activity of disodium EDTA and Tris-EDTA against otic *Malassezia pachydermatis* from dogs using the MIC methodology. No previous report was identified which described the *in vitro* susceptibility of yeast to EDTA or Tris-EDTA alone. Previous studies only reported the anti-yeast activity of an otic cleaner which contained disodium EDTA or Tris-EDTA as one of a number of other ingredients, with inhibitory activity recorded in either the effect of growth reduction (cfu/mL)[54] or dilution ratio.[48,49] Our results also provide a clear comparison of the efficacy of disodium EDTA against yeast compared to Gram-positive and Gram-negative bacteria. With reference to MIC_{90}, *M. pachydermatis* was eight times more susceptible to *P. mirabilis* and 62.5× more susceptible than *P. aeruginosa,* but only four times more susceptible than *S. pseudintermedius* and *Streptococcus* spp.

The results of this study have shown that NAC, Tris-EDTA and disodium EDTA demonstrated extensive *in vitro* antimicrobial activities against Gram-positive organisms (including the 13 isolates of MRSP*)*, Gram-negative and yeast isolates associated with canine OE. If antimicrobial results are confirmed *in vivo*, these three agents could be used as antibiotic adjuvants or potentiating agents for other antibiotics to treat Gram-negative and multidrug-resistant bacterial infections. In alignment with

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the principles of antimicrobial stewardship, this might spare the use of important human medicines and prevent the ongoing development of antimicrobial resistance. There also is the potential for adjuvants to be incorporated with other antimicrobials in an otic formulation as a multimodal approach to treating mixed ear infections in dogs. Further studies are underway to test this hypothesis.

Acknowledgement

Wei Yee Chan was supported by the Ministry of Higher Education of Malaysia and Universiti Putra Malaysia.

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Supporting Information

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

Table S1. MIC obtained and CLSI acceptable MIC range for quality control strains using control antimicrobials.

Résumé

Contexte – Un adjuvant antibiotique est une substance chimique utilisée pour modifier ou augmenter l'efficacité d'agents antimicrobiens primaires contre les micro-organismes résistants; Leur utilisation fournit une approche alternative pour gérer les résistances antimicrobiennes et augmenter la gestion antimicrobienne.

Hypothèses/Objectifs – Déterminer l'activité antimicrobienne d'un panel d'adjuvants antimicrobien potentiels contre les pathogènes fréquents associés aux otites externes canines (OE).

Sujets/Isolats – Un nombre de souches types et de prélèvements cliniques (n = 110) d'OE canines ont été testés comprenant *Staphylococcus pseudintermedius*, β-haemolytic *Streptococcus* spp., *Pseudomonas aeruginosa*, *Proteus mirabilis* et *Malassezia pachydermatis*.

Méthodes – Les activités antimicrobiennes de monolaurine, monocaprine, N-acetylcystéine (NAC), polymyxin B nonapeptide, Tris-EDTA, Tris-HCL et disodium EDTA ont été testées par microdilution selon les recommandations du CLSI.

Résultats – Les *N*-acetylcystéine, Tris-EDTA et disodium EDTA avaient une activité antimicrobienne contre les deux types de souches et les pathogènes auriculaires. Les autres adjuvants testés avaient une efficacité limitée à pas d'efficacité. NAC avait une concentration minimale inhibitrice (MIC) qui variait de 2,500 à 10,000 μg/mL pour les différents organismes. Les souches de *Pseudomonas aeruginosa* étaient huit fois plus sensibles au disodium EDTA en présence de Tris-HCL en comparaison au disodium EDTA seul. Les souches de *Malasseiza pachydermatis* étaient plus sensibles au Tris-EDTA (MIC_{90} = 190/60 μg/mL) et disodium EDTA (MIC_{90} = 120 μg/mL).

Conclusions et importance clinique – *N*-acetylcystéine, Tris-EDTA et disodium EDTA avaient une activité antimicrobienne intrinsèque et représentaient des adjuvants prometteurs qui pourraient être utilisés pour augmenter l'efficacité des antibiotiques existants contre les infections bactériennes gram négatives et multi-résistantes. Ces agents pourraient être associés avec d'autres agents antimicrobiens dans une approche multimodale des infections de l'oreille du chien.

Resumen

Introducción – un antibiótico adyuvante es una sustancia química utilizada para modificar o aumentar la efectividad de los agentes antimicrobianos primarios contra microorganismos resistentes a los medicamentos. Su uso proporciona un enfoque alternativo para abordar el problema global de la resistencia a los antimicrobianos y mejorar la administración antimicrobiana.

Hipótesis/Objetivos – determinar la actividad antimicrobiana de un panel de potenciales adyuvantes antimicrobianos contra patógenos comunes asociados con la otitis externa canina (OE).

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Animales/aislamientos – se analizaron cepas tipo y aislamientos clínicos (n = 110) de OE canina, que incluían *Staphylococcus pseudintermedius*, *Streptococcus spp.* β-hemolítico, *Pseudomonas aeruginosa*, *Proteus mirabilis* y *Malassezia pachydermatis*.
Métodos – se analizaron las actividades antimicrobianas de monolaurina, monocaprina, N-acetilcisteína (NAC), polimixina B nonapéptido, Tris-EDTA, Tris-HCL y EDTA disódico utilizando la metodología de microdilución de acuerdo con las pautas CLSI.
Resultados – N-acetilcisteína, Tris-EDTA y EDTA disódico tuvieron actividad antimicrobiana contra cepas tipo y patógenos óticos aislados de campo. Los otros adyuvantes probados tuvieron eficacia limitada o no existente. La NAC tenía un rango de concentración inhibitoria mínima (CIM) de 2,500 a 10,000 μg/ml para los diversos organismos. Los aislamientos de *Pseudomonas aeruginosa* fueron ocho veces más susceptibles al EDTA disódico en presencia de Tris-HCL en comparación con el EDTA disódico solo. Los aislados de *Malassezia pachydermatis* fueron más susceptibles a Tris-EDTA (MIC90 = 190/60 μg/mL) y EDTA disódico (MIC90 = 120 μg/mL).
Conclusiones y relevancia clínica – la N-acetilcisteína, Tris-EDTA y EDTA disódico tienen actividad antimicrobiana intrínseca y representan adyuvantes prometedores que podrían usarse para mejorar la eficacia de los antibióticos existentes contra las infecciones bacterianas Gram-negativas y resistentes a múltiples fármacos. Estos agentes podrían combinarse con otros agentes antimicrobianos en un enfoque multimodal para infecciones de oído mixtas en perros.

Zusammenfassung

Hintergrund – Ein antibiotisches Adjuvans ist eine chemische Substanz, die eingesetzt wird, um die Wirksamkeit von primär antibiotischen Wirkstoffen gegenüber Medikamenten-resistenten Mikroorganismen zu modifizieren oder zu verbessern. Seine Verwendung bedeutet eine alternative Herangehensweise, um das globale Thema der antimikrobiellen Resistenz anzusprechen und Antimikrobielle Stewardship zu verbessern.
Hypothese/Ziele – Die Bestimmung der antimikrobiellen Aktivität eines Panels von potentiellen antimikrobiellen Adjuvantien gegen übliche Pathogene, die mit einer Otitis externa (OE) des Hundes in Zusammenhang gebracht werden.
Tiere/Isolate – Eine Anzahl von Erregerstämmen und klinische Isolate (n = 110) von OE von Hunden mit *Staphylococcus pseudintermedius*, β-haemolytic *Streptococcus* spp., *Pseudomonas aeruginosa*, *Proteus mirabilis* und *Malassezia pachydermatis*.
Ergebnisse – *N*-Acetylcystein, Tris-ETDA und Disodium EDTA zeigen eine antimikrobielle Aktivität gegenüber beiden Stämmen und Ohr Pathogenen. Die anderen getesteten Adjuvantien zeigten wenig bis keine Wirksamkeit. NAC hatte eine minimale Hemmkonzentration (MIC) im Bereich von 2,500-10,000 μg/mL für die verschiedenen Organismen. *Pseudomonas aeruginosa* Isolate waren achtmal empfänglicher auf Disodium EDTA wenn auch Tris-HCl verwendet wurde im Vergleich zu Disodium EDTA alleine. *Malassezia pachydermatis* Isolate waren am empfindlichsten auf Tris-EDTA (MIC_{90} = 190/60 μg/mL) und Disodium EDTA (MIC90 = 120 μg/mL).
Schlussfolgerungen und klinische Bedeutung – *N*-Acetylcystein, Tris-EDTA und Disodium EDTA haben eine intrinsische antimikrobielle Aktivität und repräsentieren vielversprechende Adjuvantien, die verwendet werden könnten, um die Wirksamkeit von bestehenden Antibiotika gegenüber Gram-negativen und Multidrug-resistenten bakteriellen Infektionen zu verbessern. Diese Wirkstoffe könnten mit anderen antimikrobiellen Wirkstoffen in einer multimodalen Herangehensweise für Mischinfektionen der Ohren von Hunden kombiniert werden.

要約

背景 – 抗菌アジュバントは、薬剤耐性菌に対する主要な抗菌薬の有効性を修飾または増強するために使用される化学物質である。抗菌アジュバントの使用は、世界的な抗生剤耐性問題に対処し、抗菌管理を強化するための代替アプローチとして提供される。
仮説/目的 – 本研究の目的は、犬の外耳炎(OE)に関連する一般的な病原体に対する潜在的な抗菌アジュバントパネルの抗菌活性を決定することである。
被験動物/分離株 – 犬のOE由来の多くの基準株および臨床分離株(n = 110)(Staphylococcus pseudintermedius、b-haemolytic Streptococcus spp.、Pseudomonas aeruginosa、Proteus mirabilisおよびMalassezia pachydermatis)を試験した。
方法 – monolaurin、monocaprin、N-acetylcysteine (NAC)、polymyxin B nonapeptide、Tris-EDTA、Tris-HCLおよびdisodium EDTAの抗菌活性を、CLSIガイドラインに従って微量希釈法を用いて試験した。
結果 – N-acetylcysteine、Tris-EDTAおよびdisodium EDTAは、基準株および耳病原体の両方に対して抗菌活性を有していた。試験した他のアジュバントの有効性は限定的であった。 NACは、様々な微生物に対して、2,500〜10,000lg/ mLの最小阻害濃度(MIC)範囲を有していた。 Pseudomonas aeruginosa分離株は、disodium EDTA単独と比較して、Tris-HCl存在下でdisodium EDTAに8倍の感受性を示した。 Malassezia pachydermatis分離株は、Tris-EDTA(MIC_{90} = 190 /60lg/ mL)およびdisodium EDTA(MIC_{90} =120lg/ mL)に対して最も感受性であった。

結論と臨床的関連性 – N-acetylcysteine、Tris-EDTAおよびdisodium EDTAは内因性の抗菌活性を有し、グラム陰性菌および多剤耐性細菌感染症に対する既存の抗菌薬の有効性を高めるために使用できる有望なアジュバントである。これらの薬剤は、犬の耳における混合細菌感染症に対する多様なアプローチの一つとして、他の抗菌剤と組み合わせることができた。

摘要

背景 — 抗生素佐剂是一种化学物质,用于改变或增强主要抗菌剂对抗耐药微生物的有效性。它的使用为解决全球抗菌素耐药性问题,以及加强抗菌药物管理提供了一种备选方法。

假设/目的 — 确定一组潜在抗菌佐剂对犬外耳炎(OE)常见病原体的抗菌活性。

动物/分离物 — 对多种来自犬OE的不同类型的正常和致病菌株进行测试(n = 110),包括假中间型葡萄球菌、b-溶血性链球菌、铜绿假单胞菌、奇异变形杆菌和厚皮马拉色菌。

方法 — 根据CLSI指南,采用微量稀释方法检测月桂酸甘油酯、单癸酸甘油脂、N-乙酰半胱氨酸(NAC),多粘菌素B非肽、Tris-EDTA、Tris-HCL和EDTA二钠的抗菌活性。

结果 — N-乙酰半胱氨酸,Tris-EDTA和EDTA二钠对两种类型菌株和耳病原体都具有抗菌活性;测试的其他佐剂功效有限。对于各种微生物,NAC最小抑制浓度(MIC)范围是2,500-10,000μg/ mL。与单独的EDTA二钠相比,在Tris-HCL存在下,铜绿假单胞菌株对EDTA二钠的敏感性高8倍。厚皮马拉色菌株对Tris-EDTA (MIC90 = 190 /60μg/ mL)和EDTA二钠(MIC90 =120μg/ mL)最敏感。

结论和临床相关性 — N-乙酰半胱氨酸、Tris-EDTA和EDTA二钠具有内在抗菌活性,是一种有前途的佐剂,可用于增强现有抗生素对抗革兰氏阴性和多药耐药性细菌感染的功效。这些药剂可以与其他抗微生物剂组合,以多种模式用于治疗犬耳的混合感染。

Resumo

Contexto – Um adjuvante de antibiótico é uma substância química utilizada para modificar ou aumentar a eficácia de agentes antimicrobianos primários contra microrganismos resistentes. O seu uso propicia uma alternativa para abordar o problema global da resistência a antimicrobianos e enfatizar as diretrizes para uso racional destes fármacos (*antimicrobial stewardship*).

Hipótese/Objetivos – Determinar a atividade antimicrobiana de um painel de potenciais adjuvantes de antimicrobianos contra patógenos comumente associados à otite externa canina (OE).

Animais/IsoladosTestou-se cepas padrão e isolados clínicos (n = 110) de OE canina, incluindo *Staphylococcus pseudintermedius*, *Streptococcus* spp. β-haemolítico, *Pseudomonas aeruginosa*, *Proteus mirabilis* e *Malassezia pachydermatis*.

Métodos – A atividade antimicrobiana de monolaurina, monocaprina, N-acetilcisteína (NAC), nanopeptídeo polimixina B, Tris-eDTA, Tris-HCL e EDTA dissódico foi testada utilizando o método de microdiluição de acordo com as diretrizes do CLSI.

Resultados – N-acetilcisteína, Tris-EDTA e EDTA dissódico apresentaram atividade antimicrobiana tanto contra as cepas padrão quanto contra os patógenos óticos. Os outros adjuvantes apresentaram pouca ou nenhuma eficácia. A NAC apresentou um intervalo de concentração inibitória mínima (MIC) de 2.500–10.000 μg/mL para os diversos microrganismos. Os isolados de *Pseudomonas aeruginosa* foram oito vezes mais suscetíveis ao EDTA dissódico na presença do Tris-HCL em comparação ao EDTA dissódico isoladamente. Os isolados de *Malassezia pachydermatis* foram mais suscetíveis ao Tris-EDTA (MIC_{90} = 190/60 μg/mL) e ao EDTA dissódico (MIC_{90} = 120 μg/mL).

Conclusões e relevância clínica – N-acetilcisteína, Tris-EDTA e EDTA dissódico apresentam atividade antimicrobiana intrínseca e são adjuvantes promissores que podem ser utilizados para potencializar a eficácia de antibióticos existentes no tratamento de infecções por bactérias Gram-negativas e multirresistentes. Estes agentes podem ser utilizados associados a outros agentes antimicrobianos em uma abordagem multimodal pra infecções mistas de ouvido de cães.

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Table S1: MIC obtained and CLSI acceptable MIC range[31,55] for quality control strains using control antimicrobials.

Quality control strains	Control antimicrobial agent	CLSI acceptable MIC range (µg/ml) [31,55]	MIC obtained (µg/ml)
Staphylococcus aureus ATCC 29213	Ampicillin	0.06 – 2	0.25
Streptococcus pneumoniae ATCC 49619	Ampicillin	0.06 – 0.25	0.125
Pseudomonas aeruginosa ATCC 27853	Enrofloxacin	1 – 4	4
Issatchenkia orientalis (*Candida krusei*) ATCC 6258	Amphotericin B	0.5 – 2.0 (Post 24 hours) 1.0 – 4.0 (Post 48 hours)	2.0 4.0
Candida albicans ATCC 90028	Amphotericin B	0.5 – 2.0 (Post 24 hours) 0.5 – 2.0 (Post 48 hours)	1.0 2.0

Chapter 5: *In vitro* antimicrobial activity of narasin and monensin in combination with adjuvants against pathogens associated with canine otitis externa

Statement of Authorship

Title of Paper	*In vitro* antimicrobial activity of narasin and monensin in combination with adjuvants against pathogens associated with canine otitis externa.
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Chan, W. Y., Hickey, E. E., Khazandi, M., Page, S. W., Trott, D. J., & Hill, P. B. (2019). *In vitro* antimicrobial activity of narasin and monensin in combination with adjuvants against pathogens associated with canine otitis externa. *Veterinary Dermatology.* doi:10.1111/vde.12803

Principal Author

Name of Principal Author (Candidate)	Wei Yee, Chan		
Contribution to the Paper	Performed literature search, planned and conducted the experiments, analysis on all samples, interpreted data, and wrote manuscript.		
Overall percentage (%)	75%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Contribution to the Paper	Principal supervisor, provided guidance on content, editing of manuscript and acted as corresponding author.		
Signature		Date	06/08/19

Veterinary Dermatology

Vet Dermatol 2019 DOI: 10.1111/vde.12803

In vitro antimicrobial activity of narasin and monensin in combination with adjuvants against pathogens associated with canine otitis externa

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Background – The emergence of antimicrobial resistance represents a serious human and animal health risk. Good antimicrobial stewardship is essential to prolong the lifespan of existing antibiotics, and new strategies are required to combat infections in man and animals.

Hypothesis/Objectives – To determine the *in vitro* interaction of ionophores (narasin or monensin) with antimicrobial adjuvants (*N*-acetylcysteine (NAC), Tris-EDTA or disodium EDTA) against bacterial strains representing pathogens associated with canine otitis externa (OE).

Animal/Isolates – American Type Culture Collection (ATCC) strains *Staphylococcus aureus* 29213, *Pseudomonas aeruginosa* 27853 and *P. aeruginosa* biofilm producer PAO1, and a clinical isolate of *Proteus mirabilis* from a case of canine OE were tested.

Methods and materials – A 2D microdilution checkerboard method was used, allowing calculation of fractional inhibitory concentration index (FICI), dose reduction index (DRI) and plotting of isobolograms.

Results – The combination of narasin with either Tris-EDTA or disodium EDTA produced additive effects (FICI = 0.75) against *P. aeruginosa* ATCC 27853 and *P. aeruginosa* biofilm producer ATCC PAO1. An additive effect (FICI = 0.53–0.75) was found against *S. aureus* ATCC 29213 when narasin or monensin were combined with NAC. The highest DRI (32-fold) was found with monensin/NAC where the MIC of monensin was reduced from 4 to 0.125 μg/mL.

Conclusions and clinical importance – The combination of narasin with Tris-EDTA or disodium EDTA is a promising strategy to inhibit the intrinsic resistance elements of Gram-negative bacteria. These novel combinations potentially could be useful as a multimodal approach to treat mixed infections in canine OE.

Introduction

Otitis externa (OE) is one of the most frequently reported skin disorders in dogs with a prevalence of ≤10.2% in the UK and 13% in the US.[1–3] Resistant pathogens often associated with canine OE are meticillin-resistant *Staphylococcus pseudintermedius* (MRSP), multidrug-resistant *S. pseudintermedius* (MDRSP) and MDR *Pseudomonas aeruginosa* (MDRPA).[4,5] *Pseudomonas* otitis has been reported with organisms demonstrating increasing fluoroquinolone resistance and multidrug resistance to at least three or four antimicrobial classes.[6,7]

The emergence of antimicrobial resistance (AMR) is a global threat to both human and animal populations.[8] A significant public health risk was identified with increasing reports of AMR transmission between companion animals and humans.[9,10] Zoonotic transmission of MRSP has been reported causing skin and soft tissue infections in patients with confirmed contact with dogs.[11] MRSP also has been isolated from human patients with chronic rhinosinusitis whose dogs are suffering from an ear infection.[12] There is, therefore, a significant responsibility within the veterinary profession to practice good antibiotic stewardship. The key principles of "reduce, refine and replace" are critically important in order to prolong the lifespan of existing antibiotics that are essential for human and animal health.[13]

A recent trend in the field of antimicrobial stewardship is the concept of drug repurposing (using old drugs for new purposes). This may allow existing antibiotics to be reserved for severe or life-threatening infections and less

Accepted 28 August 2019
[1]Joint senior authors
Sources of Funding: This work was supported by ARC Linkage (grant no. LP130100736) with Luoda Pharma Pty Ltd as a partner organization.
Conflicts of Interest: Stephen W. Page is a director of Luoda Pharma Pty Ltd. Darren J. Trott has received research funding from Luoda Pharma, Neoculi, Zoetis, Bayer, Merial, Virbac and Elanco.

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severe infections to be treated using less critical drugs. Furthermore, the concurrent use of nonantibiotic compounds (antimicrobial adjuvants) could help to broaden the spectrum of activity of these agents. In previous studies, we reported on the activity of the polyether ionophores narasin and monensin against pathogens involved in canine OE; these drugs are currently used as rumen modulators, growth promoters and coccidiostats in production animals.[14,15] There is little evidence for bacterial resistance or co-selection for resistance to other antimicrobial classes.[16,17] We showed that Gram-positive organisms including MRSP and MDRSP from cases of canine OE were highly susceptible to narasin and monensin although these agents lacked activity against Gram-negative bacteria and had weak anti-yeast activity.[18,19] In a study to identify potential adjuvants that might broaden the spectrum of activity of these agents, we reported that *N*-acetylcysteine (NAC), Tris-EDTA and disodium EDTA had intrinsic antimicrobial activity against otic pathogens, including Gram-negative organisms.[20]

The aim of this study was to determine the *in vitro* antimicrobial activity of narasin and monensin in the presence of an adjuvant (NAC, Tris-EDTA or disodium EDTA) against bacterial strains representing pathogens associated with canine OE.

Methods and materials

Test organisms

Due to the technical difficulties of performing multiple checkerboard assays, American Type Culture Collection (ATCC) strains of *Staphylococcus* aureus (ATCC SA 29213) and *Pseudomonas aeruginosa* (ATCC PA 27853 and *P. aeruginosa* biofilm producer ATCC PAO1) were used to assess minimum inhibitory concentrations (MICs). This was considered appropriate because previous studies demonstrated that susceptibility to the test compounds was consistent between the ATCC type strains and a range of clinical isolates from cases of canine OE (n = 110).[18–20] This was the case even with meticillin-susceptible *S. pseudintermedius,* MRSP and MDRSP. In this context, the ATCC type strains were being used as proxies for clinical isolates. ATCC SA 29213 and ATCC PA 27853 were purchased from Abacus ALS, Queensland, Australia and ATCC PAO1 was donated by the University of New South Wales, Sydney. A clinical isolate of *Proteus mirabilis* from a case of canine OE was obtained from the archive collection of Australian Centre for Antimicrobial Resistance Ecology (ACARE), South Australia. Before the day of antimicrobial susceptibility testing, bacterial isolates were subcultured on 5% sheep blood Columbia agar (Thermo Fisher Scientific; Melbourne, Victoria, Australia) and incubated at 36 $\pm$ 1°C for 24 h. Purity was confirmed by inspecting the overnight culture on an agar plate.

Ionophores and antimicrobial adjuvants

Technical grade narasin (>98% purity) and monensin A sodium salt (>98% purity) were purchased from BioAustralis (Smithfield, New South Wales, Australia). Stock solutions of narasin and monensin were prepared at 100 times the maximum testing concentration in dimethyl sulfoxide (DMSO $\geq$ 99.9%, Sigma-Aldrich; St Louis, MO, USA) and stored at −80°C until used. Stock concentrations for antimicrobial susceptibility testing were prepared according to the types of test organisms. For narasin, stock concentrations were made at 1.6 mg/mL for ATCC SA 29213; and 12.8 mg/mL for ATCC PA 27853, ATCC PAO1 and *P. mirabilis*. For monensin, 1.6 mg/mL stock concentration was prepared for all ATCC strains and clinical isolate. In a preliminary experiment with 1% DMSO, the maximum concentration used in susceptibility testing, did not show any antimicrobial activity.

The NAC (from Sigma-Aldrich) was prepared at 40,000 μg/mL (double the highest concentration to be tested) in cation-adjusted Mueller–Hinton broth (CAMHB) (Becton Dickinson and Company; Sparks, MD, USA) for the testing of ATCC strains and clinical isolate. Tromethamine-hydrochloride (Tris-HCL) and disodium ethylenediaminetetraacetic acid (EDTA) were purchased from Promega (Madison, WI, USA); 1 M Tris-HCL and 0.5 M disodium EDTA stock solutions were prepared in MilliQ water and stored at room temperature before preparation of Tris-EDTA. Tris-EDTA was then made by mixing and diluting 1 M Tris-HCL and 0.5 M disodium EDTA in CAMHB and then buffered to pH 8.0 with sodium hydroxide (AnalaR®, Merck Pty Ltd; Bayswater, Victoria, Australia). For the antimicrobial testing of ATCC SA 29213 and *P. mirabilis*, Tris-EDTA was prepared at a concentration of 100 mM (12,200 μg/mL) Tris-HCL with 10 mM (3,800 μg/mL) disodium EDTA. For testing of ATCC PA 27853 and ATCC PAO1, 400 mM (48,500 μg/mL) Tris-HCL with 40 mM (15,000 μg/mL) disodium EDTA working solution was prepared. When used alone, disodium EDTA was prepared in CAMHB at 20 mM (7,500 μg/mL) for ATCC SA 29213 and *P. mirabilis,* and 80 mM (30,000 μg/mL) for Gram-negative ATCC strains. NAC, Tris-EDTA and disodium EDTA working solutions were prepared fresh on the day of *in vitro* antimicrobial synergy testing and stored at 2–8°C.

In vitro antimicrobial synergy testing by microdilution checkerboard assays

A modified 2D microdilution checkerboard assay[21,22] was used to evaluate the potential synergistic activity between ionophores (narasin or monensin) and adjuvants (NAC, Tris-EDTA or disodium EDTA) (Figure 1). Briefly, 89 μL CAMHB was added to each well of a 96 well microtiter plate (Nunclon™ Delta Surface, Thermo Fisher Scientific; Roskilde, Denmark) which was used as the checkerboard challenge plate. Next, a two-fold serial dilution of an adjuvant working solution was performed along the ordinate from row H to C (e.g. from 20,000 to 625 μg/mL for NAC, from 400/40 to 12.5/1.25 mM for Tris-EDTA and 10–0.3125 mM for disodium EDTA). In another 96 well plate, each ionophore was two-fold serially diluted in DMSO from 12.8 to 0.025 mg/mL or 1.6 to 0.003 mg/mL for narasin and 1.6 to 0.003 mg/mL for monensin. Then, 1 μL of each concentration was dispensed along the abscissa (column 3–12 only) in the checkerboard challenge plate. This resulted in a final concentration range for narasin or monensin of 0.25–128 μg/mL or 0.03–16 μg/mL. Each plate was set up to test a single bacterial isolate. Ten μL of bacterial suspension (prepared at 1:20 dilution of 0.5 McFarland standard) was added to each well of the plate to achieve a final bacterial concentration of 5 $\times$ 10^5 cfu/mL. Following incubation at 36 $\pm$ 1°C for 24 h, MIC values for ionophore and adjuvant when tested alone and in combination were assessed both visually and spectrophotometrically ($OD_{600\ nm}$). Experiments were performed in duplicate and repeated twice.

Fractional inhibitory concentration index (FICI)

The fractional inhibitory concentration index (FICI) was used to describe the result of the checkerboard assay and it was calculated using the following formula (1).

$$\mathrm{FICI} = \frac{A}{\mathrm{MIC}_A} + \frac{B}{\mathrm{MIC}_B} \qquad (1)$$

A and B were the MICs of ionophore and adjuvant, respectively, when in combination. MIC_A and MIC_B were the MICs of ionophore and adjuvant, respectively, when tested alone. Based on the FICI, the interaction between two antimicrobial agents was interpreted as synergistic (FICI $\leq$ 0.5), additive or partially synergistic (1 $\geq$ FICI > 0.5), indifference (1 < FICI $\leq$ 4) or antagonism (FICI > 4).[22] Further analysis was performed by plotting an isobologram for each checkerboard assay. This creates a graphical interpretation of the results and is achieved by plotting the MIC for drug A (ionophore) on the *x*-axis and drug B (adjuvant) on the *y*-axis. A dotted line is then drawn between the intercepts of the x- and *y*-axis to indicate the line of indifference of activity between the two drug compounds. Curves or

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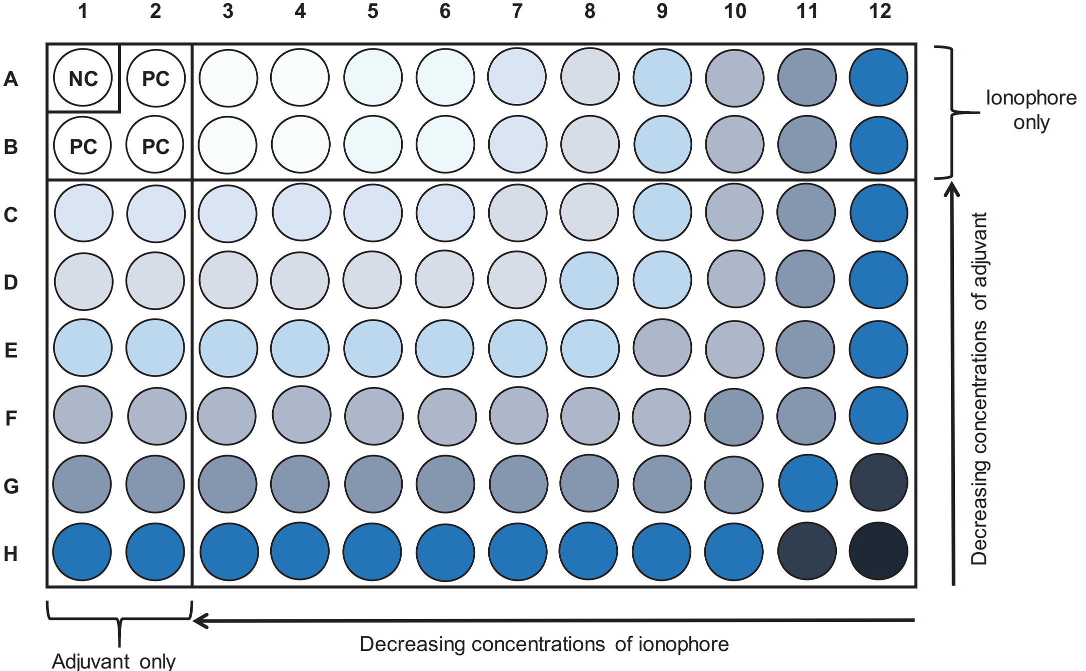

Figure 1. A modified 2D checkerboard assay of ionophore (narasin or monensin) and adjuvants *N*-acetylcysteine, Tris-EDTA and disodium EDTA. NC, negative control (broth only); PC, positive control (broth and inoculum only).

MIC values of the combination located below the indifference line indicate additive or synergistic interactions, whereas curves located above indicate no interaction or antagonistic interaction.[23,24] If a MIC was not achievable at the maximum concentration that could be obtained for a compound, the MIC was arbitrarily set at the next theoretically higher concentration so that FICI and isobolograms could be calculated.

Dose reduction index

The dose reduction index (DRI) describes the difference between the effective dose of a compound in combination in comparison to its individual dose. DRI was calculated using the following formula (2):

$$DRI = \frac{\text{MIC of ionophore or adjuvant alone}}{\text{MIC of ionophore or adjuvant in combination}} \quad (2)$$

Evaluation of DRI is important clinically because it can allow the dose of a drug to be reduced (and hence the risk of toxicity) without changing the efficacy.[25]

Statistical analysis

Paired Student's *t*-tests were used to investigate differences between the MIC of the drug compound when used alone and when in combination. If two concentrations of ionophores or adjuvants resulted in the same MIC, the lowest concentration was chosen for analysis. Prism v7.02 (GraphPad; San Diego, CA, USA) was used for all analyses and for plotting of isobolograms. Significance was set at $P < 0.05$.

Results

Antimicrobial activity of narasin in the presence of adjuvants

The interactions between narasin and the three adjuvants are shown in Table 1 (only the best MIC combinations with the lowest FICI are presented). When narasin was combined with NAC, the only additive effects were seen against *S. aureus*. A combination of narasin and NAC against ATCC SA 29213 revealed a FICI from 0.63 to 0.75 with a two- to four-fold dose reduction in narasin MIC and two- to eight-fold dose reduction in NAC MIC ($P < 0.01$). An isobologram analysis (Figure 2a) showed a curve of additive interaction (below the indifference line) with the corresponding MIC values for both narasin and NAC against ATCC SA 29213. No additive effects were seen for narasin and NAC against the Gram-negative organisms.

On the one hand, when narasin was combined with Tris-EDTA, additive effects were seen against *P. aeruginosa* but not against *S. aureus* or *P. mirabilis*. A FICI of 0.75 was obtained for both ATCC PA 27853 and ATCC PAO1. A MIC was not attainable for narasin alone against both Gram-negative ATCC strains, whereas a narasin MIC of 128 μg/mL was achieved in combination with Tris-EDTA along with a four-fold reduction in the MIC of Tris-EDTA from 6,000/1,900 to 1,500/470 μg/mL ($P < 0.05$). The additive effects of the narasin/Tris-EDTA combination against both Gram-negative ATCC strains is clearly shown in the isobologram (Figure 2c). On the other hand, there was indifference in the interaction of narasin and Tris-EDTA against ATCC SA 29213 and the *P. mirabilis* isolate.

When narasin was combined with disodium EDTA, additive effects were seen against the Gram-negative ATCC strains with a FICI of 0.75. There was an achievable MIC for narasin at 128 μg/mL when combined with disodium EDTA against ATCC PA 27853 and ATCC PAO1. Furthermore, the MIC of disodium EDTA in combination

Table 1. (a-c) Minimal inhibitory concentration (MIC), fractional inhibitory concentration index (FICI) and dose reduction index (DRI) of the combination effect of (a) narasin and *N*-acetylcysteine (NAC), (b) narasin and Tris-EDTA and (c) narasin and disodium EDTA

(a)

	MIC individual (μg/mL)		Best MIC combination (μg/mL)				DRI	
Isolates	Narasin	NAC	Narasin	NAC	FICI	Interpretation	Narasin	NAC
ATCC SA 29213	0.125	5,000	0.063	625	0.63	Additive	2	8
ATCC PA 27853	>128	2,500	>128	2,500	2	Indifference	1	1
ATCC PAO1	>128	2,500	>128	2,500	2	Indifference	1	1
Proteus mirabilis	>128	5,000	>128	2,500	1.5	Indifference	1	2

(b)

	MIC individual (μg/mL)		Best MIC combination (μg/mL)				DRI	
Isolates	Narasin	Tris/EDTA	Narasin	Tris/EDTA	FICI	Interpretation	Narasin	Tris-EDTA
ATCC SA 29213	0.25	1,500/470	0.25	1,500/470	2	Indifference	2	1
ATCC PA 27853	>128	6,000/1,900	128	1,500/470	0.75	Additive	2	4
ATCC PAO1	>128	6,000/1,900	128	1,500/470	0.75	Additive	2	4
Proteus mirabilis	>128	3,000/930	>128	3,000/930	2	Indifference	1	1

(c)

	MIC individual (μg/mL)		Best MIC combination (μg/mL)				DRI	
Isolates	Narasin	EDTA	Narasin	EDTA	FICI	Interpretation	Narasin	EDTA
ATCC SA 29213	0.25	470	0.25	240	1.5	Indifference	2	2
ATCC PA 27853	>128	7,500	128	1,900	0.75	Additive	2	4
ATCC PAO1	>128	7,500	128	1,900	0.75	Additive	2	4
Proteus mirabilis	>128	930	>128	930	2	Indifference	1	1

For MIC values > 128, the value of 256 was arbitrarily set for the calculation of FICI and DRI.
Testing against ATCC type strains (*Staphylococcus aureus* ATCC 29213, *Pseudomonas aeruginosa* ATCC 27853 and *P. aeruginosa* biofilm producer ATCC PAO1) and a canine otitis externa clinical isolate of *Proteus mirabilis*. Best MIC combination indicates the combination of narasin and NAC or Tris-EDTA or disodium EDTA that achieved the lowest FICI. The MICs of Tris-EDTA are represented as Tris/EDTA.

with narasin reduced from 7,500 to 1,900 μg/mL indicating DRI = 4 ($P < 0.05$). Additive interaction is further illustrated in Figure 2d. No interaction of narasin and disodium EDTA was found in both ATCC SA 29213 and *P. mirabilis*.

The MIC of narasin without the presence of Tris-EDTA or disodium EDTA was arbitrarily set at 256 μg/mL (twice the concentration of the highest tested concentration) to add clarity in the isobologram analyses (Figure 2c and d).

Antimicrobial activity of monensin in the presence of adjuvants

The antimicrobial combination effects of monensin and the three adjuvants *in vitro* are shown in Table 2 (only the best MIC combinations with the lowest FICI are presented). When monensin was combined with NAC there was an additive effect against *Staphylococcus*. The FICI of ATCC SA 29213 fell between 0.53 and 0.63 with the highest dose reduction of monensin MIC from 4 to 0.125 μg/mL (DRI = 32) and NAC MIC from 5,000 to 625 μg/mL (DRI = 8) ($P < 0.05$). Figure 2b demonstrates an additive to synergistic isobologram analysis with the corresponding MIC values of monensin and NAC against ATCC SA 29213.

When monensin was combined with disodium EDTA, there was a weak additive effect against the Gram-positive organism ATCC SA 29213 with a FICI of 1 and a two-fold dose reduction (DRI = 2) in both compounds. Moreover, isobologram analysis did not yield a substantial curve below the indifference line (data not shown).

The combination of monensin and Tris-EDTA did not enhance the susceptibility of any of the isolates. No additive effects were seen with the combination of monensin with NAC, Tris-EDTA or disodium EDTA against the Gram-negative organisms.

Discussion

In the present study, the combination of narasin with either Tris-EDTA or disodium EDTA showed additive effects against the Gram-negative ATCC strains, *P. aeruginosa* 27853 and *P. aeruginosa* biofilm producer PAO1. An achievable narasin MIC of 128 μg/mL against *P. aeruginosa* in the presence of disodium EDTA or Tris-EDTA is a very significant finding. This is a crucial discovery if narasin was to be considered as a potential treatment for canine OE. Previous study has shown that narasin is effective against Gram-positive organisms, but was not effective against Gram-negative organisms.[18] In order to cover the spectrum of organisms that are typically involved in canine OE, an adjuvant would be required that enhanced activity of narasin against Gram-negative bacteria. Both Tris-EDTA and disodium EDTA appeared to be suitable adjuvants for this purpose with both reducing the MIC for the *Pseudomonas* strains. By contrast,

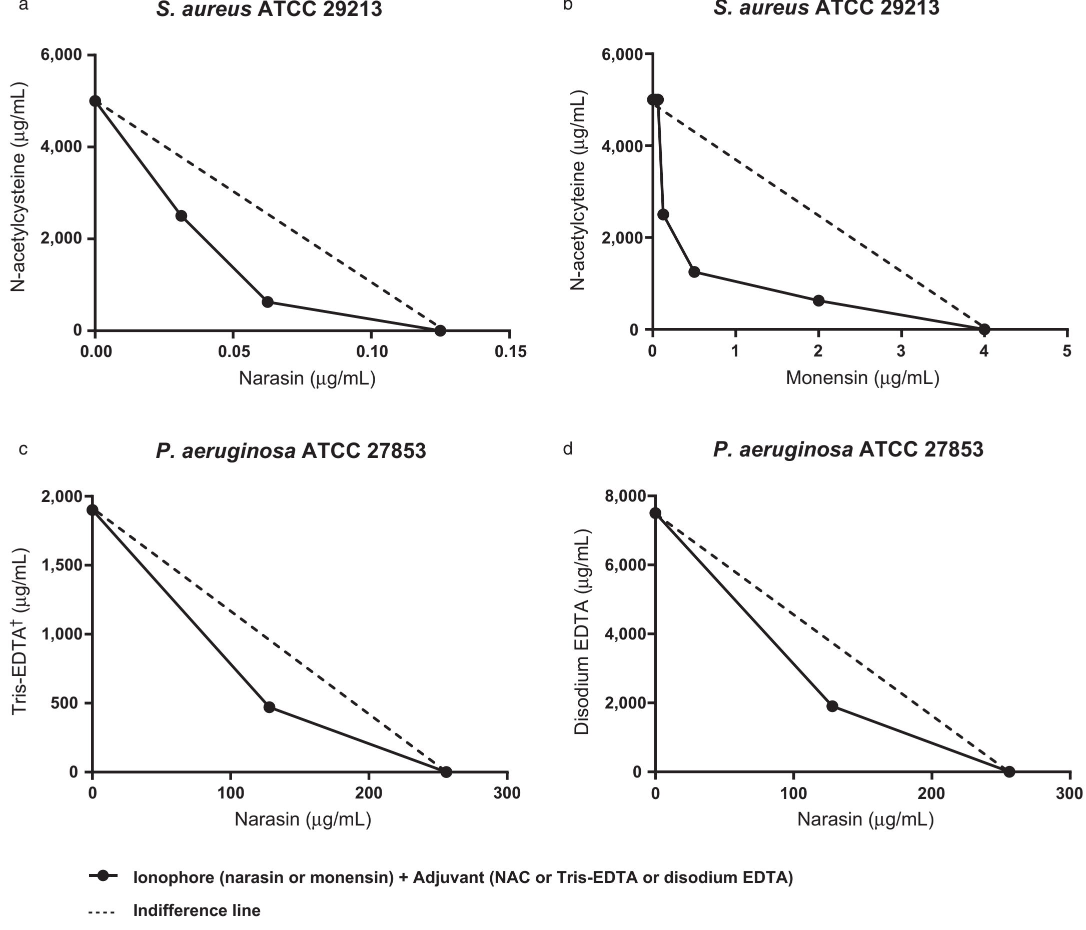

Figure 2. Isobologram analyses.
Minimum inhibitory concentrations (MIC) of narasin or monensin are plotted on the *x*-axis and the MICs of *N*-acetylcysteine (NAC), Tris-EDTA or disodium EDTA on the *y*-axis. The curves below the indifference line (dotted line) represent additive ($1 \geq$ FICI > 0.5) or synergistic (FICI ≤ 0.5) whereas the curves above the indifference line indicate no interaction ($1 <$ FICI ≤ 4) or antagonistic (FICI > 4). Additive and synergistic activity were observed in the combination of narasin + NAC (a) and monensin + NAC (b), respectively, against *Staphylococcus aureus* ATCC 29213. Additive interaction was found in the combination of narasin + Tris-EDTA (c) and narasin + disodium EDTA (d) against *Pseudomonas aeruginosa* ATCC 27853. ATCC PAO1 gave identical results to ATCC 27853 (data not shown).

additive effects against Gram-negative organisms were not observed when monensin was combined with Tris-EDTA or disodium EDTA. Likewise, no significant enhancement in anti-staphylococcal activity was seen when either narasin or monensin were combined with Tris-EDTA or disodium EDTA.

Polyether ionophores narasin and monensin are highly lipophilic enabling them to penetrate the porous peptidoglycan cell wall layer of a Gram-positive bacteria and cause an imbalance of transmembrane concentration gradients by chelating reversibly with NA^+ and K^+.[26] This mechanism affects cellular function and leads to bacterial cell disruption in Gram-positive bacteria.[16] However, Gram-negative bacteria are inherently resistant to ionophores due to the double-layered outer cell wall structure that prevents permeation of ionophores into the inner cell membrane.[14] Therefore, disodium EDTA can be used to chelate and remove divalent cations, Ca^{2+} and Mg^{2+} which disrupts the integrity of the lipopolysaccharide outer cell wall membrane to allow the penetration of ionophores into the inner cell wall of a Gram-negative bacteria.[27] Tris-buffer can further enhance the chelating activity of disodium EDTA.[28]

In the narasin/Tris-EDTA combination, the MIC of disodium EDTA against *Pseudomonas* was 470 μg/mL in comparison to a MIC of 1,900 μg/mL when the disodium EDTA was combined with narasin alone. This four-fold reduction in MIC indicates that a lower concentration of disodium EDTA was required in the presence of Tris-buffer. Our findings are in agreement with various studies demonstrating that Tris-EDTA and disodium EDTA potentiate the activity of other

Table 2. (a-c) Minimal inhibitory concentration (MIC), fractional inhibitory concentration index (FICI) and dose reduction index (DRI) of the combination effect of (a) monensin and *N*-acetylcysteine (NAC), (b) monensin and Tris-EDTA and (c) monensin and disodium EDTA for various bacterial isolates

(a)

	MIC individual (μg/mL)		Best MIC combination (μg/mL)				DRI	
Isolates	Monensin	NAC	Monensin	NAC	FICI	Interpretation	Monensin	NAC
ATCC SA 29213	4	5,000	0.125	2,500	0.53	Additive	32	2
ATCC PA 27853	>16	2,500	>16	2,500	2	Indifference	1	1
ATCC PAO1	>16	2,500	>16	2,500	2	Indifference	1	1
Proteus mirabilis	>16	5,000	>16	5,000	2	Indifference	1	1

(b)

	MIC individual (μg/mL)		Best MIC combination (μg/mL)				DRI	
Isolates	Monensin	Tris/EDTA	Monensin	Tris/EDTA	FICI	Interpretation	Monensin	Tris/EDTA
ATCC SA 29213	4	1,500/470	2	1,500/470	1.5	Indifference	2	1
ATCC PA 27853	>16	3,000/930	>16	3,000/930	2	Indifference	1	1
ATCC PAO1	>16	3,000/930	>16	3,000/930	2	Indifference	1	1
Proteus mirabilis	>16	1,500/470	>16	1,500/470	2	Indifference	1	1

(c)

	MIC individual (μg/mL)		Best MIC combination (μg/mL)				DRI	
Isolates	Monensin	EDTA	Monensin	EDTA	FICI	Interpretation	Monensin	EDTA
ATCC SA 29213	4	470	2	240	1	Additive	2	2
ATCC PA 27853	>16	7,500	>16	7,500	2	Indifference	1	1
ATCC PAO1	>16	7,500	>16	7,500	2	Indifference	1	1
Proteus mirabilis	>16	470	>16	470	2	Indifference	1	1

For MIC values > 16, the value of 32 was arbitrarily set for the calculation of FICI and DRI.
ATCC type strains (*Staphylococcus aureus* ATCC 29213, *Pseudomonas aeruginosa* ATCC 27853 and *P. aeruginosa* biofilm producer ATCC PAO1) and a canine otitis externa clinical isolate of *Proteus mirabilis*. Best MIC combination indicates the combination of monensin and NAC or Tris-EDTA or disodium EDTA that achieved the lowest FICI. The MICs of Tris-EDTA are represented as Tris/EDTA.

antimicrobial agents against *P. aeruginosa* and other Gram-negative bacteria.[29–31]

An enhancement in anti-staphylococcal activity was seen when both narasin and monensin were combined with NAC. However, this is less important in terms of antimicrobial activity because both narasin and monensin have inherent high-level efficacy against Gram-positive organisms. Despite this, the combination of active agents with an adjuvant may provide other additional benefits such as allowing a lower dose of each drug to be used in a clinical setting. This might be beneficial if any of the agents proved to be toxic or irritant in canine ears. For example, NAC was shown to result in conductive hearing loss when injected into the tympanic bullae of guinea pigs at concentrations >20,000 μg/mL.[32] In the present study, the MIC of NAC was reduced up to eight-fold (625 μg/mL) when combined with narasin or monensin; the MIC of narasin and monensin were reduced up to four-fold (0.03 μg/mL) and 32-fold (0.125 μg/mL), respectively, in the presence of NAC.

Further *in vivo* studies would be required to assess the safety and efficacy of an ionophore/adjuvant combination in the treatment of canine OE. However, the data reported in this study provide a promising starting point for the development of a new class of otic formulation. Such a formulation would certainly adhere to the principles of good antimicrobial stewardship. Many of the currently available otic preparations for dogs contain critically important antimicrobial agents such as fluoroquinolones or aminoglycosides. These classes are reserved for serious or life-threatening infection in human and veterinary medicine, and should be used sparingly. *Pseudomonas aeruginosa* isolated from the canine ear and skin infections has been reported to be resistant to enrofloxacin (≤72.2%), marbofloxacin (≤33.3%) and polymyxin B (7%).[33–36] MRSP strains isolated from otitis/pyoderma have demonstrated resistance to gentamicin (92.3%) with simultaneous resistance to four or more different antimicrobial classes,[37] and multidrug resistance to at least one or more fluoroquinolones (52%).[38]

Further studies are warranted to investigate the combination of narasin with Tris-EDTA or disodium EDTA in animal models to assess their suitability as a treatment for canine otitis associated with Gram-positive and Gram-negative organisms. Monensin would seem less suitable for this purpose because, even in the presence of adjuvants, it had no antimicrobial efficacy against Gram-negative organisms.

The combination of NAC with either narasin or monensin could represent a suitable treatment for staphylococcal infections due to the additive effects observed; NAC also would have the advantage of providing anti-biofilm activity. A 1–2% NAC flushing solution has been recommended as part of OE management to remove biofilm physically before application of topical otic treatment to aid the therapeutic success of the latter.[39,40]

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In conclusion, the *in vitro* findings of an additive effect of narasin combined with Tris-EDTA or disodium EDTA, and additive effects in narasin or monensin combined with NAC, provide valuable *in vitro* data to support the further investigation of these combinations as potential pharmaceutical preparations for the treatment of canine OE.

Acknowledgement

Wei Yee Chan is supported by the Ministry of Higher Education of Malaysia and Universiti Putra Malaysia.

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Résumé

Contexte – L'émergence de résistances aux antimicrobiens représente un risque pour la santé humaine et animale. Une bonne utilisation des antimicrobiens est essentielle pour prolonger la durée de vie des antibiotiques actuels et de nouvelles stratégies sont nécessaires pour combattre les infections chez l'homme et l'animal.

Hypothèses/Objectifs – Déterminer les interactions *in vitro* des ionophores (narasine ou monensine) et des adjuvants antimicrobiens (NAC *N-acetylcystéine*, Tris-EDTA ou disodium EDTA) contre les souches bactériennes pathogènes associées aux otites canines externes (OE).

Sujets/Isolats – Les souches de l'ATCC (American Type Culture Collection) *Staphylococcus aureus* 29213, *Pseudomonas aeruginosa* 27853 et *P. aeruginosa* productrice de biofilm PAO1, et une souche clinique de *Proteus mirabilis* issue d'un cas d'OE canine ont été testées.

Matériels et méthodes – Une méthode de microdilution 2D en damier a été utilisée, permettant un calcul de FICI (fractional inhibitory concentration index), de l'index de réduction de dose (DRI) et des marqueurs d'isobologrammes.

Résultats – L'association de narasine, soit avec Tris-EDTA soit avec disodium EDTA, a produit des effets additifs (FICI = 0.75) contre *P. aeruginosa* ATCC 27853 et *P. aeruginosa* producteur de biofilm ATCC PAO1. Un effet additif (FICI = 0.53–0.75) a été trouvé contre *S. aureus* ATCC 29213 lorsque la narasine ou la monensine étaient combinés avec NAC. Le plus haut DRI (32 fois) a été trouvé avec monensine/NAC là ou le MIC était diminué de 4 à 0.125 μg/mL.

Conclusions et importance clinique – L'association de la narasine avec le Tris-EDTA ou le disodium EDTA est une stratégie prometteuse pour inhiber les éléments de résistance des bactéries Gram-négatives. Ces nouvelles associations peuvent potentiellement être utiles dans une approche multimodale pour le traitement des infections mixtes des OE canines.

Resumen

Introducción – la aparición de resistencia a los antimicrobianos representa un grave riesgo para la salud humana y animal. Una razonable administración de antimicrobianos es esencial para prolongar la vida útil de los antibióticos existentes, y se requieren nuevas estrategias para combatir las infecciones en seres hunanos y animales.

Hipótesis/Objetivos – determinar la interacción *in vitro* de los ionóforos (narasina o monensina) con adyuvantes antimicrobianos (N-acetilcisteína (NAC), Tris-EDTA o EDTA disódico) contra cepas bacterianas que representan patógenos asociados con la otitis externa canina (OE).

Animales/aislados – cepas de la American Type Culture Collection (ATCC) *Staphylococcus aureus* 29213, *Pseudomonas aeruginosa* 27853 y *P. aeruginosa* productor de biopelículas PAO1, y un aislado clínico de *Proteus mirabilis* de un caso de OE canina.

Métodos y materiales – se usó un método de tablero de cuadrículas de microdilución 2D, que permite calcular el índice de concentración inhibitoria fraccional (FICI), el índice de reducción de dosis (DRI) y el trazado de isobologramas.

Resultados – la combinación de narasina con Tris-EDTA o EDTA disódico produjo efectos aditivos (FICI = 0,75) frente a *P. aeruginosa* ATCC 27853 y *P. aeruginosa* productor de biopelículas ATCC PAO1. Se encontró un efecto aditivo (FICI = 0,53-0,75) frente a *S. aureus* ATCC 29213 cuando se combinaron narasina o monensina con NAC. El DRI más alto (32 veces) se encontró con monensina/NAC donde la MIC se redujo de 4 a 0,125 μg/ml.

Conclusiones e importancia clínica – la combinación de narasina con Tris-EDTA o EDTA disódico es una estrategia prometedora para inhibir los elementos de resistencia intrínseca de las bacterias Gram-negativas. Estas nuevas combinaciones podrían ser útiles como un enfoque multimodal para tratar infecciones mixtas en la OE canina.

Zusammenfassung

Hintergrund – Das Auftreten von antimikrobieller Resistenz bedeutet ein schwerwiegendes Gesundheitsrisiko für Mensch und Tier. Eine gute antimikrobielle Stewardship ist essentiell, um die Lebensdauer existierender Antibiotika zu verlängern, außerdem sind neue Strategien nötig, um Infektionen bei Mensch und Tier zu bekämpfen.

Hypothese/Ziele – Die Bestimmung der *in vitro* Interaktion von Ionophoren (Narasin oder Monensin) mit antimikrobiellen Adjuvantien (*N*-Acetylcystein (NAC), Tris-EDTA oder Disodium EDTA) gegenüber Bakterienstämmen, die Pathogene darstellen, die bei einer Otitis externa (OE) des Hundes gerne auftreten.

Tier/Isolate – Es wurden Stämme aus der American Type Culture Collection (ATCC) von *Staphylococcus aureus* 29213, *Pseudomonas aeruginosa* 27853 and *P. aeruginosa* Biofilm Produzent PAO1, sowie ein klinisches Isolat von *Proteus mirabilis* von einem Hund mit OE getestet.
Methoden und Materialien – Es wurde eine 2D Mikrodilutionsschachbrettmethode verwendet, welche die Kalkulation des fraktionellen Hemmstoffkonzentrationsindex (FICI), des Dosisreduktionsindex (DRI) und ein Plotting des Isobologramms erlaubte.
Ergebnisse – Die Kombination von Narasin mit entweder Tris-EDTA oder Disodium EDTA zeigte additive Wirkung (FICI = 0,75) gegen *P. aeruginosa* ATCC 27853 und *P. aeruginosa* Biofilm Produzent ATCC PAO1. Eine additive Wirkung (FICI = 0,53-0,75) wurde gegen *S. aureus* ATCC 29213 gefunden, wenn Narasin oder Monensin mit NAC kombiniert worden waren. Die höchste DRI (32-fach) wurde mit Monensin/NAC gefunden, wodurch die MIC von 4 auf 0,125 µg/mL reduziert wurde.
Schlussfolgerungen und klinische Bedeutung – Die Kombination von Narasin mit Tris-EDTA oder Disodium EDTA ist eine vielversprechende Strategie, um die Elemente der intrinsischen Resistenz von Gram-negativen Bakterien zu inhibieren. Diese neuen Kombinationen könnten möglicherweise als multimodale Herangehensweise nützlich sein, um Mischinfektionen bei der OE des Hundes zu behandeln.

要約
背景 – 抗菌薬耐性の出現は、人間と動物の深刻な健康リスクを表している。既存の抗生物質のライフスパンを延ばすためには、優れた抗菌薬管理が不可欠であり、人間や動物の感染症と闘うには新しい戦略が必要である。
仮説/目的 – 本研究の目的は、犬外耳炎(OE)に関連する病原体を表す細菌株に対するイオノフォア(ナラシンまたはモネンシン)および抗菌アジュバント(N-アセチルシステイン(NAC)、Tris-EDTAまたはEDTA二ナトリウム)のin vitroにおける相互作用を決定することである。
被験動物/分離株 – 黄色ブドウ球菌American Type Culture Collection(ATCC)29213、緑膿菌ATCC 27853および緑膿菌バイオフィルム産生株ATCC PAO1、および犬外耳炎症例からProteus mirabilisの臨床分離株を検査した。
材料と方法 – 2D微量希釈チェッカーボード法を使用して、分別阻害濃度指数(FICI)、線量低減指数(DRI)の計算およびアイソボログラム法のプロッティングを可能にした。
結果 – ナラシンとTris-EDTAまたはEDTA二ナトリウムの併用は、緑膿菌ATCC 27853および緑膿菌バイオフィルム産生株ATCC PAO1に対して相加効果(FICI = 0.75)をもたらした。ナラシンまたはモネンシンをNACと併用した場合、黄色ブドウ球菌ATCC 29213に対して相加効果(FICI = 0.53-0.75)を認めた。最も高いDRI(32倍)は、MICが4から0.1251g/ mLに減少したモネンシン/ NACで検出された。
結論と臨床的重要性 – ナラシンとTris-EDTAまたは二ナトリウムEDTAの併用は、グラム陰性菌固有の耐性要素を抑制する有望な戦略である。これらの新しい併用法は、犬外耳炎の混合感染症治療のマルチモーダルアプローチとして有用である可能性がある。

摘要
背景 — 抗菌素耐药问题会带来人类和动物严重的健康风险。良好的抗菌素管理对于延长现有抗生素的产品寿命至关重要,需要采取新的策略来对抗人和动物的感染。
假设/目的 — 确定离子型抗生素(那拉菌素或莫能菌素)与抗微生物佐剂(N-乙酰半胱氨酸(NAC),Tris-EDTA或EDTA二钠)相互作用后,抗犬外耳炎(OE)病原体的体外效果。
动物/分离株 — 测试美国菌种保藏中心(ATCC)的金黄色葡萄球菌29213,铜绿假单胞菌27853和铜绿假单胞菌生物膜形成物PAO1,以及从犬OE病例中分离出的变形杆菌。
方法和材料 — 采用2D微稀释棋盘法,计算分级抑制浓度指数(FICI)、剂量降低指数(DRI)并绘制等效线图。
结果 — 那拉菌素与Tris-EDTA或EDTA二钠的组合,对铜绿假单胞菌ATCC 27853和铜绿假单胞菌生物膜形成物ATCC PAO1具有协同作用(FICI = 0.75)。 当那拉菌素或莫能菌素与NAC联用时,发现对金黄色葡萄球菌ATCC 29213同样具有协同作用(FICI = 0.53-0.75)。 莫能菌素/ NAC的DRI最高(32倍),MIC从4降至0.125µg/mL。
结论和临床意义 — 对抑制革兰氏阴性细菌的固有耐药因素,那拉菌素联合Tris-EDTA或EDTA二钠是一种有前途的策略。 这些新颖的联合,可作为犬OE混合感染的多模式治疗方案。

Resumo
Contexto – O surgimento de resistência antimicrobiana representa um sério risco à saúde humana e animal. Boas práticas de administração de antimicrobianos são essenciais para prolongar a vida útil dos antibióticos existentes, e novas estratégias são necessárias para combater infecções no homem e nos animais.
Hipótese/Objetivos – Determinar a interação *in vitro* de ionóforos (narasina ou monensina) com adjuvantes antimicrobianos (N-acetilcisteína (NAC), Tris-EDTA ou EDTA dissódico) contra cepas bacterianas associados à otite externa canina (OE).
Animal/isolados – As cepas padrão registradas na *American Type Culture Collection* (ATCC) de *Staphylococcus aureus* 29213, *Pseudomonas aeruginosa* 27853, *P. aeruginosa* PAO1 produtora de biofilme, e um isolado clínico de *Proteus mirabilis* oriundo de um caso de OE canino foram testados.

Métodos e materiais – Utilizou-se o método de *checkerboard* em microdiluição 2D, permitindo o cálculo do índice de concentração inibitória fracionária (FICI), índice de redução da dose (DRI) e plotagem de isobologramas.
Resultados – A combinação de narasina com Tris-EDTA ou EDTA dissódico prossuiu efeitos aditivos (FICI = 0,75) contra *P. aeruginosa* ATCC 27853 e *P. aeruginosa* ATCC PAO1 produtora de biofilme. Um efeito aditivo (FICI = 0,53-0,75) foi encontrado contra *S. aureus* ATCC 29213 quando narasina ou monensina foram combinadas com NAC. O maior DRI (32 vezes) foi encontrado com monensina / NAC, onde a CIM foi reduzida de 4 para 0,125 μg / mL.
Conclusões e importância clínica – A combinação de narasina com Tris-EDTA ou EDTA dissódico é uma estratégia promissora para inibir os elementos intrínsecos de resistência de bactérias Gram-negativas. Essas novas combinações poderiam ser potencialmente úteis como uma abordagem multimodal no tratamento de infecções mistas nas OEs caninas.

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Chapter 6: Biofilm production by pathogens associated with canine otitis externa, and the antibiofilm activity of ionophores and antimicrobial adjuvants

Statement of Authorship

Title of Paper	Biofilm production by pathogens associated with canine otitis externa, and the antibiofilm activity of ionophores and antimicrobial adjuvants
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Chan, W. Y., Hickey, E. E., Page, S. W., Trott, D. J., & Hill, P. B. (2019). Biofilm production by pathogens associated with canine otitis externa, and the antibiofilm activity of ionophores and antimicrobial adjuvants. *Journal of Veterinary Pharmacology and Therapeutics, 42*, 682-692. doi:10.1111/jvp.12811

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Name of Principal Author (Candidate)	Wei Yee, Chan		
Contribution to the Paper	Performed literature search, planned and conducted the experiments, analysis on all samples, interpreted data, wrote manuscript and acted as corresponding author. .		
Overall percentage (%)	80%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Signature		Date	08/08/19

Name of Co-Author	Peter B. Hill		
Contribution to the Paper	Principal supervisor, provided guidance on content and editing of manuscript		
Signature		Date	06/08/19

Received: 21 June 2019 | Revised: 13 August 2019 | Accepted: 21 August 2019
DOI: 10.1111/jvp.12811

ORIGINAL ARTICLE

WILEY

Biofilm production by pathogens associated with canine otitis externa, and the antibiofilm activity of ionophores and antimicrobial adjuvants

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Funding information
This work was supported by ARC Linkage [grant numbers LP130100736] with Luoda Pharma Pty. Ltd as a partner organization.

Abstract

Otitis externa (OE) is a frequently reported disorder in dogs associated with secondary infections by *Staphylococcus*, *Pseudomonas* and yeast pathogens. The presence of biofilms may play an important role in the resistance of otic pathogens to antimicrobial agents. Biofilm production of twenty *Staphylococcus pseudintermedius* and twenty *Pseudomonas aeruginosa* canine otic isolates was determined quantitatively using a microtiter plate assay, and each isolate was classified as a strong, moderate, weak or nonbiofilm producer. Minimum biofilm eradication concentration (MBEC) of two ionophores (narasin and monensin) and three adjuvants (N-acetylcysteine (NAC), Tris-EDTA and disodium EDTA) were investigated spectrophotometrically (OD_{570nm}) and quantitatively (CFU/ml) against selected *Staphylococcus* and *Pseudomonas* biofilm cultures. Concurrently, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of planktonic cultures were assessed. 16/20 of the *S. pseudintermedius* clinical isolates were weak biofilm producers. 19/20 *P. aeruginosa* clinical isolates produced biofilms and were distributed almost equally as weak, moderate and strong biofilm producers. While significant antibiofilm activity was observed, no MBEC was achieved with narasin or monensin. The MBEC for NAC ranged from 5,000–10,000 µg/ml and from 20,000–80,000 µg/ml against *S. pseudintermedius* and *P. aeruginosa*, respectively. Tris-EDTA eradicated *P. aeruginosa* biofilms at concentrations ranging from 6,000/1,900 to 12,000/3,800 µg/ml. The MBEC was up to 16-fold and eightfold higher than the MIC/MBC of NAC and Tris-EDTA, respectively. Disodium EDTA reduced biofilm growth of both strains at concentrations of 470 µg/ml and higher. It can be concluded that biofilm production is common in pathogens associated with canine OE. NAC and Tris-EDTA are effective antibiofilm agents *in vitro* that could be considered for the treatment of biofilm-associated OE in dogs.

KEYWORDS
biofilms, canine otitis externa, ionophores, N-acetylcysteine, *Pseudomonas aeruginosa*, *Staphylococcus pseudintermedius*, Tris-EDTA

Darren J. Trott and Peter B. Hill are Joint senior authors.

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1 | INTRODUCTION

Otitis externa (OE) is one of the most common disorders in dogs, accounting for 4.5% to 16% of dogs presented to veterinary practices as a primary complaint or as a secondary condition that was unnoticed by the owner (Grono, 1969; Hill et al., 2006). The treatment and management of OE in dogs can be frustrating and unpredictable due to the multifactorial aetiology of the disease. Predisposing and primary causes (i.e. ear conformation, otic parasites, allergies and endocrinopathies) and secondary bacterial and yeast infections can lead to perpetuating factors (i.e. pathologic changes in ear canals) that result in chronic and relapsing OE (August, 1988; Rosser, 2004; Saridomichelakis, Farmaki, Leontides, & Koutinas, 2007). In recent years, the presence of antibiotic-resistant bacteria (Dégi et al., 2013; Penna, Thomé, Martins, Martins, & Lilenbaum, 2011) and bacterial biofilms have been recognized as possible causes of unresolved OE (Griffin & Aniya, 2017; Nuttall, 2016; Pye, Yu, & Weese, 2013).

Biofilm formation by *Staphylococcus* and *Pseudomonas* otic pathogens may play an important role in bacterial persistence and resistance. Biofilms are initially formed when monolayers of planktonic bacteria attach irreversibly to a living or nonliving surface, followed by production of a three-dimensional extracellular matrix consisting mainly of exopolysaccharides (EPS). Maturation of the biofilm is followed by dispersion of bacteria, allowing further spread of infection (Jamal et al., 2018; Kostakioti, Hadjifrangiskou, & Hultgren, 2013). Biofilm forming ability is, therefore, considered as a virulence factor contributing to antibiotic resistance and the spread of nosocomial infections (Osland, Vestby, Fanuelsen, Slettemeås, & Sunde, 2012). Biofilm formation by pathogens such as *Staphylococcus aureus*, *Staphylococcus pseudintermedius*, *Pseudomonas aeruginosa* and *Escherichia coli* can complicate infections in situations such as wounds, otitis media, cystic fibrosis and surgical implant placement, resulting in prolonged treatment and recovery (Costerton, Stewart, & Greenberg, 1999; Høiby, Bjarnsholt, Givskov, Molin, & Ciofu, 2010; Pompilio et al., 2015). Bacterial biofilms are ten to a thousand times more resistant to antibiotics compared with the planktonic state of the same bacterial strain (Mah & O'Toole, 2001; Olson, Ceri, Morck, Buret, & Read, 2002). Possible resistant mechanisms include impaired penetration of drugs by extracellular polymeric substances (EPS), reduced growth rate, expression of multidrug resistance pumps and presence of persister cells (Mah & O'Toole, 2001; Römling & Balsalobre, 2012; Spoering & Lewis, 2001; Stewart, 2002).

We have previously reported the *in vitro* efficacy of repurposed ionophores and antimicrobial adjuvants on pathogens associated with canine OE (Chan et al., 2018a; Chan et al., 2018b; Chan, Khazandi, Hickey, Page, Trott, et al., 2018). The ionophores narasin and monensin, both anticoccidial agents widely used in production animals, were shown to be effective against Gram-positive canine otic pathogens including methicillin-resistant *S. pseudintermedius* (MRSP), but were not effective against Gram-negative bacteria such as *P. aeruginosa* (Chan et al., 2018a, 2018). Further studies of the antimicrobial adjuvants N-acetylcysteine (NAC), Tris-EDTA and disodium EDTA revealed activity against both Gram-positive and Gram-negative bacteria (Chan, Khazandi, Hickey, Page, Trott, et al., 2018). A synergistic effect was seen against *P. aeruginosa* when narasin was combined with either Tris-EDTA or disodium EDTA (Chan et al., 2019, submitted). As chronic OE in dogs can be complicated by the presence of biofilms, this study aimed to determine the ability of *S. pseudintermedius* and *P. aeruginosa* isolates from cases of canine OE to form biofilm, and to explore the antibiofilm activity of ionophores and adjuvants against these pathogens.

2 | MATERIALS AND METHODS

2.1 | Bacterial strains and growth conditions

Twenty *S. pseudintermedius* isolates (16 methicillin-susceptible *S. pseudintermedius* (MSSP) and 4 MRSP); and 20 *P. aeruginosa* isolates from cases of canine OE were collected from diagnostic laboratories throughout Australia. These organisms were speciated using biochemical testing and MALDI-TOF mass spectrometry (Bruker). American Type Culture Collection (ATCC) strains *S. aureus* 29213 and *P. aeruginosa* 27853 were used as internal quality control strains in the evaluation of biofilm formation of clinical isolates. *P. aeruginosa* strain PAO1 (biofilm-producing reference strain) was a positive control for biofilm formation assessment and was also used in the preliminary studies of the minimum biofilm eradication concentration (MBEC) assay. Isolates were subcultured on 5% Sheep Blood Columbia Agar (Thermo Fisher Scientific) and incubated at 36 ± 1°C for 24 hr. Purity was confirmed by inspecting the overnight culture on an agar plate.

2.2 | Phenotypic characterization of biofilm production

Biofilm production was determined quantitatively using a microtiter plate assay as previously described by Stepanovic et al. (2007), with some modification. Briefly, each isolate was suspended in 3 ml of tryptic soy broth (TSB) (Becton Dickson Pty Ltd) supplemented with 1% glucose. After incubation at 36 ± 1°C for 24 hr, turbidity of each isolate was adjusted to 0.5 McFarland standard (approximately 1.5×10^8 CFU/ml) and diluted 1:100 in TSB + 1% glucose. Then, 200 µl of bacterial suspension was inoculated into flat-bottomed 96-well microtiter plates (Nunclon™ Delta Surface; Thermo Fisher Scientific) in quadruplicate and incubated without shaking, overnight at 36 ± 1°C. After incubation, the content in each well was gently discarded and washed three times with 300 µl phosphate-buffered saline (PBS, Oxoid™), followed by heat fixing at 60°C for 1 hr. Next, the adherent biofilm layer was stained with 150 µl of 0.5% crystal violet (Oxoid™) for 15 min at room temperature. Excess crystal violet solution was then removed, and the plate was washed three times with 300 µl of PBS and resolubilised with 150 µl of 95% ethanol for 30 min at room temperature. The optical density (OD) of any adherent biofilm stained with crystal violet was measured at 570 nm using a

microtiter spectrophotometer (xMark™, Bio-Rad Laboratories Inc., TYO). All isolates were tested three times in quadruplicate. The TSB + 1% glucose was used as a negative control (eight replicates in each test), and *P. aeruginosa* strain PAO1 was used as the positive control. Isolates were classified as zero, weak, moderate or strong biofilm producers based on the calculation of the average value of OD_{570} of isolates and OD cut off value (ODc) of negative controls with the following interpretation (Stepanovic et al., 2007):

- ODc = average OD_{570} of negative control + (3 × standard deviation of negative control);
- No (zero) biofilm producer = OD_{570} (isolate) ≤ ODc;
- Weak biofilm producer = ODc < OD_{570} (isolate) ≤ 2 × ODc;
- Moderate biofilm producer = 2 × ODc < OD_{570} (isolate) ≤ 4 × ODc;
- Strong biofilm producer = OD_{570} (isolate) > 4 × ODc.

Based on the results of this testing, two isolates of *S. pseudintermedius* that were moderate biofilm producers, and two isolates of *P. aeruginosa* that were strong biofilm producers were selected for *in vitro* biofilm antimicrobial susceptibility testing.

2.3 | Antimicrobial agents

2.3.1 | Ionophores

Technical grade narasin (>98% purity) and monensin A sodium salt (>98% purity) were purchased from BioAustralis, Smithfield. Stock solutions of narasin, and monensin were prepared at 100 times the maximum testing concentration in dimethyl sulfoxide (DMSO ≥ 99.9%, Sigma-Aldrich) and stored at −80°C until used. For *in vitro* biofilm antimicrobial susceptibility testing of *S. pseudintermedius* clinical isolates, narasin and monensin stock was prepared at the concentration of 1.6 mg/ml. For testing of *P. aeruginosa* biofilm-producing reference strain and clinical isolates, narasin and monensin stock were prepared at 12.8 mg/ml and 6.4 mg/ml, respectively.

2.3.2 | Adjuvants

N-acetylcysteine (NAC) was purchased from Sigma-Aldrich. Tris-HCL (tromethamine-hydrochloride) and disodium EDTA (ethylenediaminetetraacetic acid) were purchased from Promega. These adjuvant working solutions were prepared fresh on the day of the *in vitro* biofilm antimicrobial susceptibility testing of *S. pseudintermedius* and *P. aeruginosa* clinical isolates. NAC was prepared at 80,000 μg/ml in cation-adjusted Mueller Hinton Broth (CAMHB) (Becton Dickinson Pty Ltd). Prior to the preparation of Tris-EDTA working solution, 1 M Tris-HCL and 0.5 M disodium EDTA stock solutions were prepared in MilliQ water and stored at room temperature. Tris-EDTA was then prepared at a concentration of 400 mM (48,500 μg/ml) Tris-HCL with 40 mM (15,000 μg/ml) disodium EDTA by mixing and diluting 1 M Tris-HCL and 0.5 M disodium EDTA in CAMHB and then buffered to pH 8.0 with sodium hydroxide (AnalaR®, Merks Pty. Ltd). Disodium EDTA alone was prepared in CAMHB at 80 mM (30,000 μg/ml). All working solutions were stored at 2–8°C.

2.3.3 | Control antimicrobial agent

Enrofloxacin, 99.8% purity (Sigma-Aldrich), was used as a control antimicrobial agent for biofilm antimicrobial susceptibility testing. Enrofloxacin stock was prepared at 1.6 mg/ml and 6.4 mg/ml for the testing of *S. pseudintermedius* and *P. aeruginosa* clinical isolates, respectively. Stock solution was stored at −80°C until use.

2.4 | Biofilm antimicrobial susceptibility testing

The biofilm antimicrobial susceptibility testing was performed using methods previously described with some modification (Ceri et al., 1999; Harrison et al., 2010) to determine minimum biofilm eradication concentration (MBEC). Biofilm-producing reference strain PAO1 was tested for susceptibility to narasin and monensin during the preliminary studies. Two moderate biofilm producer isolates of *S. pseudintermedius*, and two strong biofilm producer isolates of *P. aeruginosa* were selected postevaluation of biofilm production as described earlier. These clinical isolates were used to determine the susceptibility of the bacterial biofilms to narasin, monensin, NAC, Tris-EDTA and disodium EDTA. The MBEC™ biofilm inoculator (MBEC™ Physiology & Genetics Assay; Innovotech), a device consisting of a sterile 96-peg lid on a 96-well microtiter plate, was used in this study. Briefly, bacterial inocula were suspended in TSB, adjusted to 1.0 McFarland standard, diluted 1:30 and 150 μl was dispensed into each well of the MBEC™ inoculator. Biofilms were formed on the pegs after 24 hr incubation (36 ± 1°C) in an orbital mixer incubator (OM11, Ratek Instruments Pty. Ltd.) at 100 rpm. The bacterial biofilms (peg lid) were then challenged with the antimicrobial agents that had been prepared in CAMHB and incubated for 24 hr at 36 ± 1°C. The antimicrobial agents were two fold serially diluted from the stock or working solutions, for example. 80,000–157 μg/ml for NAC. Following the antimicrobial challenge, the peg lid was rinsed twice in PBS and exposed biofilms were then disrupted into TSB recovery medium by 10 min sonication (160TD, Soniclean Pty. Ltd.). Negative growth controls containing only TSB, and positive controls containing TSB and bacterial biofilms were included in each test. All isolates were tested in duplicate.

2.5 | Determination of minimum biofilm eradication concentrations (MBEC) and biofilm growth (CFU/ml)

According to Ceri et al. (1999), MBEC is the lowest concentration of an antimicrobial agent that prevents visible growth from occurring in the recovery medium after overnight incubation. In this study, MBEC was assessed both spectrophotometrically and quantitatively. After biofilms were sonicated into TSB recovery medium, 100 μl was removed from each well and a tenfold serial dilution was performed in PBS. Each dilution was then spot plated (10 μl) in triplicate on SBA plates. The remaining recovery medium and SBA spot plates were then incubated overnight at 36 ± 1°C. MBEC of the biofilms in the

TABLE 1 Classification of biofilm production of *Staphylococcus pseudintermedius* and *Pseudomonas aeruginosa* clinical isolates of canine otitis externa and reference strains

Biofilm production	Clinical isolates			Reference strains		
	S. pseudintermedius (*n* = 20)		*P. aeruginosa* (*n* = 20)	*S. aureus* ATCC 29213 (*n* = 1)	*P. aeruginosa* ATCC 27853 (*n* = 1)	*P. aeruginosa* biofilm producer PAO1 (*n* = 1)
	MSSP (*n* = 16)	MRSP (*n* = 4)				
No biofilm	0	0	1			
Weak	12	4	6	1	1	
Moderate	4	0	7			
Strong	0	0	6			1

Note: MSSP = methicillin-susceptible *S. pseudintermedius*; MRSP = methicillin-resistance *S. pseudintermedius*; ODc = average OD_{570} of negative control + (3 × standard deviation of negative control); no biofilm producer = OD_{570} (isolate) ≤ ODc; weak biofilm producer = ODc < OD_{570} (isolate) ≤ 2 × ODc; moderate biofilm producer = 2× ODc < OD_{570} (isolate) ≤ 4 × ODc and strong biofilm producer = OD_{570} (isolate) > 4 × ODc.

recovery plate was determined spectrophotometrically with an optical density at 650 nm (OD_{650nm}) < 0.1 or OD_{650nm} ≤ negative control. The number of colonies on SBA spot plates was counted to determine CFU/ml. Quantitatively, MBEC was the lowest concentration of antimicrobial agent required to eradicate the biofilm growth (CFU/ml = 0). A graph was plotted showing the antimicrobial concentrations and Log_{10} CFU/ml for each isolate using GraphPad Prism v7.0.

2.6 | Determination of planktonic minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

Planktonic cells were shed from the surface of the peg biofilms during the antimicrobial challenge. Thus, minimum inhibitory concentration (MIC) of the planktonic bacterial growth was assessed spectrophotometrically at OD_{650nm} after 24 hr of antimicrobial challenge. MIC was determined as the lowest concentration of an antimicrobial agent with OD_{650nm} < 0.1 or OD_{650nm} ≤ negative control. Thereafter, 20 µl aliquot of planktonic culture from each well was transferred into 180 µl of CAMHB in a fresh 96-well microtiter plate, incubated overnight at 36 ± 1°C. MBC of the planktonic bacterial growth was then determined spectrophotometrically as the lowest concentration of an antimicrobial agent with OD_{650nm} < 0.1 or OD_{650nm} ≤ negative control.

2.7 | Statistical analysis

GraphPad Prism v7.0 was used to perform Kruskal–Wallis (nonparametric) tests with Dunn's multiple comparisons to compare the differences in growth of biofilm (Log_{10} CFU/ml) exposed to different concentrations of an antimicrobial agent in each isolate. Significance was set at $p < .05$.

3 | RESULTS

3.1 | Biofilm production

The production of biofilm by *S. pseudintermedius* and *P. aeruginosa* clinical otitis externa isolates is shown in Table 1. The majority (16/20) of the *S. pseudintermedius* clinical isolates, including all MRSP, were weak biofilm producers. Biofilm production by *P. aeruginosa* was seen in 19/20 clinical isolates, but it was more variable and ranged from weak to strong. *P. aeruginosa* strain PAO1 was a consistently strong biofilm producer and both *S. aureus* ATCC 29213 and *P. aeruginosa* ATCC 27853 were weak producers.

3.2 | Susceptibility of biofilms to antimicrobial agents

Table 2 summarizes MBEC results of narasin, monensin, Tris-EDTA, disodium EDTA and enrofloxacin against biofilms of *S. pseudintermedius* and *P. aeruginosa* clinical isolates.

3.2.1 | Susceptibility of biofilms to narasin and monensin

No MBEC was achieved with the highest tested concentrations of narasin and monensin (128 and 64 µg/ml, respectively) against either the *Pseudomonas* reference strain (Figure 1a,b) or the clinical isolates (Figure 3a,b). However, in comparison to the untreated PAO1 biofilms, 0.25–8 µg/ml of monensin caused a significant reduction ($p < .05$–$p < .001$) in biofilm growth (Figure 1b). Also, one of the *P. aeruginosa* clinical isolates had biofilm growth reduced in the presence of 2–4 µg/ml of monensin ($p < .01$–$p < .001$) (Figure 3b). At the highest concentrations of 16 µg/ml, narasin and monensin were unable to achieve a MBEC against the staphylococcal clinical isolates (Figure 2a and b), but 4–16 µg/ml of narasin showed a significant reduction ($p < .01$–$p < .001$) in both *S. pseudintermedius* biofilms compared with the untreated group (Figure 2a).

3.2.2 | Susceptibility of biofilms to N-acetylcysteine (NAC)

NAC was an effective antibiofilm agent against both staphylococcal and *Pseudomonas* isolates in this study. The MBEC of NAC ranged from 5,000 to 10,000 µg/ml against biofilms of *S. pseudintermedius* (Figure 2c) and 20,000 to 80,000 µg/ml against biofilms of *P. aeruginosa* clinical isolates (Figure 3c). Furthermore, a significant decrease in the growth of *S. pseudintermedius* and *P. aeruginosa* biofilms were

TABLE 2 Summary of minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and minimal biofilm eradication concentration (MBEC) of narasin, monensin, N-acetylcysteine (NAC), Tris-EDTA, disodium EDTA and enrofloxacin against planktonic and biofilm growth of two moderate biofilm producer *Staphylococcus pseudintermedius* and two strong biofilm producer *Pseudomonas aeruginosa* clinical isolates of canine otitis externa

Antimicrobial agents	*S. pseudintermedius* (*n* = 2) (μg/ml; mM concentrations in parentheses)			*P. aeruginosa* (*n* = 2) (μg/ml; mM concentrations in parentheses)		
	MIC^P	MBC^P	MBEC	MIC^P	MBC^P	MBEC
Narasin	0.125	1	>16	>128	>128	>128
Monensin	2	>16	>16	>64	>64	>64
NAC	2,500–5,000	5,000	5,000–10,000	5,000	5,000	20,000–80,000
Tris-EDTA	1,500/470 (12.5/1.25)	12,000/3,800 (100/10)	>48,500/15,000 (>400/4)	1,500/470 (12.5/1.25)	6,000/1,900–12,000/3,800 (50/5–100/10)	6,000/1,900–12,000/3,800 (50/5–100/10)
Disodium EDTA	470 (1.25)	3,800 (10)	>30,000 (>80)	470 (1.25)	30,000 (80)	> 30,000 (>80)
Enrofloxacin	0.25	2	4	4	32	> 64

Note: MIC^P or MBC^P = MIC or MBC indicated in planktonic growth.

observed in the presence of lower NAC concentrations at 2,500 μg/ml, in comparison to the growth control without NAC treatment ($p < .05$).

3.2.3 | Susceptibility of biofilms to Tris-EDTA and disodium EDTA

Biofilms of *P. aeruginosa* clinical isolates were highly susceptible to Tris-EDTA with MBEC ranging from 6,000/1,900–12,000/3,800 μg/ml (50/5 to 100/10 mM) (Figure 3d). No MBEC was achieved against biofilms of *S. pseudintermedius* isolates (Figure 2d), but there was a significant reduction in biofilm growth (approximately 4 Log_{10} CFU/ml) at concentrations of 1,500/470 μg/ml (12.5/1.25 mM) and above in comparison with the control, which was not exposed to Tris-EDTA ($p < .05$–$p < .0001$) (Figure 2d). For disodium EDTA, no MBEC was obtained for either staphylococcal or *Pseudomonas* clinical isolate biofilms (Figures 2e and 3e). However, the biofilm growth reduced significantly when challenged with disodium EDTA at concentrations of 470 μg/ml (1.25 mM) or above ($p < .01$–$p < .0001$).

3.2.4 | Susceptibility of biofilms to enrofloxacin

The MBEC of enrofloxacin was 4 μg/ml against *S. pseudintermedius* biofilms (Figure 2f). Biofilms of *P. aeruginosa* clinical isolates were reduced significantly by concentrations of enrofloxacin ranging from 16 to 64 μg/ml ($p < .01$–$p < .0001$), but not eradicated (Figure 3f).

3.3 | Susceptibility of planktonic cultures to antimicrobial agents

MICs and MBCs for narasin, monensin, NAC, Tris-EDTA, disodium EDTA and enrofloxacin against planktonic bacterial cultures are shown in table 2. Narasin and monensin showed antibacterial effects against *S. pseudintermedius* with MIC at 0.125 μg/ml and 2 μg/ml, respectively but were not effective against *P. aeruginosa*. NAC was bactericidal at 5,000 μg/ml for both *S. pseudintermedius* and *P. aeruginosa* planktonic cells. The growth of both Gram-positive and Gram-negative planktonic cultures were inhibited in the presence of 1,500/470 μg/ml of Tris-EDTA but, up to an eightfold increase in concentration (12,000/3,800 μg/ml) was needed to achieve MBC. The MIC of disodium EDTA against *S. pseudintermedius* and *P. aeruginosa* planktonic cultures was both 470 μg/ml but, the MBC of disodium EDTA against *P. aeruginosa* (30,000 μg/ml) was approximately eight times higher than *S. pseudintermedius*. However, bactericidal activity was not indicated in both Tris-EDTA and disodium EDTA as the MBC to MIC ratio was >4. Enrofloxacin MICs and MBCs were within the expected range according to CLSI guidelines (CLSI, 2018).

4 | DISCUSSION

In this study, all *S. pseudintermedius* clinical OE isolates produced biofilm, but 80% of these isolates (MSSP = 12, MRSP = 4) were weak biofilm producers regardless of their resistance profile. In contrast,

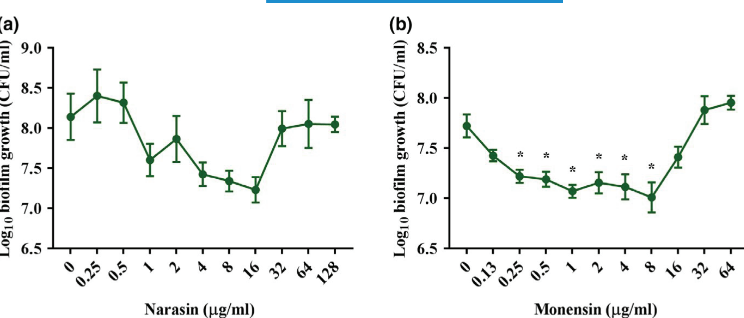

FIGURE 1 The antibiofilm activity of ionophores narasin (a) and monensin (b) against *Pseudomonas aeruginosa* biofilm producer PAO1. All data shown are the growth of biofilms in Log_{10} CFU/ml ± *SEM*. Data points that are statistically different from the untreated control (0 μg/ml on the *x*-axis) are denoted with an asterisk (*). The reduction in efficacy seen at ≥ 32 μg/ml is likely due to precipitation of the active compounds [Colour figure can be viewed at wileyonlinelibrary.com]

a previous study found that only 39.3% of *S. pseudintermedius* (previously identified as *S. intermedius*) isolated from canine OE were biofilm producers, but no further classification of biofilm formation was undertaken (Moreira et al., 2012). Another study revealed that 98% (n = 140) of *S. pseudintermedius* isolated from dogs with skin, wound and surgical infections were biofilm producers with the majority (61%) being strong biofilm producers (Singh, Walker, Rousseau, & Weese, 2013). Our study agrees with that of Singh et al. (2013) in that there was no difference in the ability to produce biofilm between MSSP and MRSP. For *P. aeruginosa* canine OE isolates (n = 20) in our study, 95% were biofilm producers, evenly distributed between weak (30%), moderate (35%) and strong (30%) biofilm production. This contrasts with a similar study by Pye et al. (2013) in which only 40% (33/83) of *P. aeruginosa* canine otic isolates produced biofilms (8.4% (7/83) weak, 18.1% (15/83) moderate and 13.3% (11/83) strong). In humans, the majority (77.5%) of *P. aeruginosa* isolates formed weak (42.5%), moderate (27.5%) or strong (5%) biofilms (Lima et al., 2018). In a study of *P. aeruginosa* isolates from cases of cystic fibrosis, 68% (n = 74) formed biofilms with the majority (64.9%) being weak biofilm producers (Perez, Costa, Freitas, & Barth, 2011). Collectively, our data, along with that from other literature, clearly indicate that *S. pseudintermedius* and *P. aeruginosa* produce biofilms that could complicate the pathogenesis of OE and its treatment and management.

In our study, N-acetylcysteine (NAC) was an effective agent against *S. pseudintermedius* and *P. aeruginosa* biofilms. We demonstrated that NAC has the ability to inhibit planktonic bacterial growth and further eradicate biofilms formed by both Gram-positive and Gram-negative canine otic isolates. The NAC MIC ranged from 2,500 to 5,000 μg/ml and only a twofold increase in the concentration (MBEC = 5,000–10,000 μg/ml) was needed to eradicate *S. pseudintermedius* biofilms. A much higher concentration of NAC ranging from 20,000 to 80,000 μg/ml (MBEC) was required against *P. aeruginosa* biofilms, a 4- to 16-fold higher concentration than the NAC MIC or MBC. However, it is important to note that >20,000 μg/ml of NAC may cause conductive hearing loss in guinea pigs when it is injected directly to fill the middle ear via the tympanic membrane (Saliba, El Fata, Ouelette, & Robitaille, 2010). In agreement with a study by May, Conklin, and Bemis (2016), canine *Staphylococcus* and *Pseudomonas* otic isolates were susceptible to NAC with MICs ranging from 5,000–20,000 μg/ml. Recently, the use of 1 to 2% (10,000 – 20,000 μg/ml) NAC flushing solution has been recommended for the removal of biofilm physically with or without an ear cleaner prior to application of topical otic treatment (Griffin & Aniya, 2017; Nuttall, 2016). As 20,000 μg/ml of NAC eradicated biofilms of all *S. pseudintermedius* and some *P. aeruginosa* isolates in our study, this seems to be a reasonable recommendation.

NAC is a known nonantibiotic, mucolytic agent with antimicrobial properties. A number of studies have shown that NAC decreases biofilm formation by inhibiting bacterial adherence, reducing production of EPS, promoting disruption of mature biofilm and reducing the sessile cell viability (El-Feky et al., 2009; Marchese et al., 2003; Olofsson, Hermansson, & Elwing, 2003). A systemic review by Dinicola, De Grazia, Carlomagno, and Pintucci (2014) analysed NAC as an effective and safe agent to increase the permeability of antibiotics and eradicate preformed mature bacterial biofilms in *Helicobacter pylori* infection, vascular catheter- and ureteral stent-related infections. In a study by Eroshenko, Polyudova, and Korobov (2017), the growth and biofilm formation of Gram-positive skin pathogens including *S. aureus* were completely inhibited at 25,000 μg/ml of NAC. In a study using confocal laser scanning microscopy with an insertion of fluorescing GFP plasmid, *P. aeruginosa* PAO1 biofilm was shown to be disrupted and inhibited by NAC at 10,000 μg/ml (Zhao & Liu, 2010).

This study demonstrated that Tris-EDTA has antibiofilm activity against *P. aeruginosa* otic isolates where biofilms were eradicated at 6,000/1,900–12,000/3,800 μg/ml. The MBEC concentrations were four to eightfold higher than the Tris-EDTA MIC to inhibit the planktonic cultures. However, Tris-EDTA did not eradicate biofilms of *S. pseudintermedius* but significantly reduced the biofilm growth at concentrations of 1,500/470 μg/ml and above. Similar observations were found for both *S. pseudintermedius* and *P. aeruginosa* biofilms when treated with disodium EDTA alone. Biofilms were not eradicated but reduced significantly in the presence of 470 μg/ml of EDTA. A previous study found that 1 mM (380 μg/ml) of EDTA disrupted the growth of 24 hr preformed biofilms in comparison to growth control of Gram-positive and Gram-negative otic isolates (Khazandi et al., 2019). In fact, Tris can enhance the metal chelating activity of disodium EDTA to permeabilise the outer membrane and further dispersed EPS in biofilms in Gram-negative bacteria (Liu et al., 2017; Vaara, 1992). Liu et al. (2017) also reported that 30 mg/ml (30,000 μg/ml) EDTA significantly reduced EPS formation and

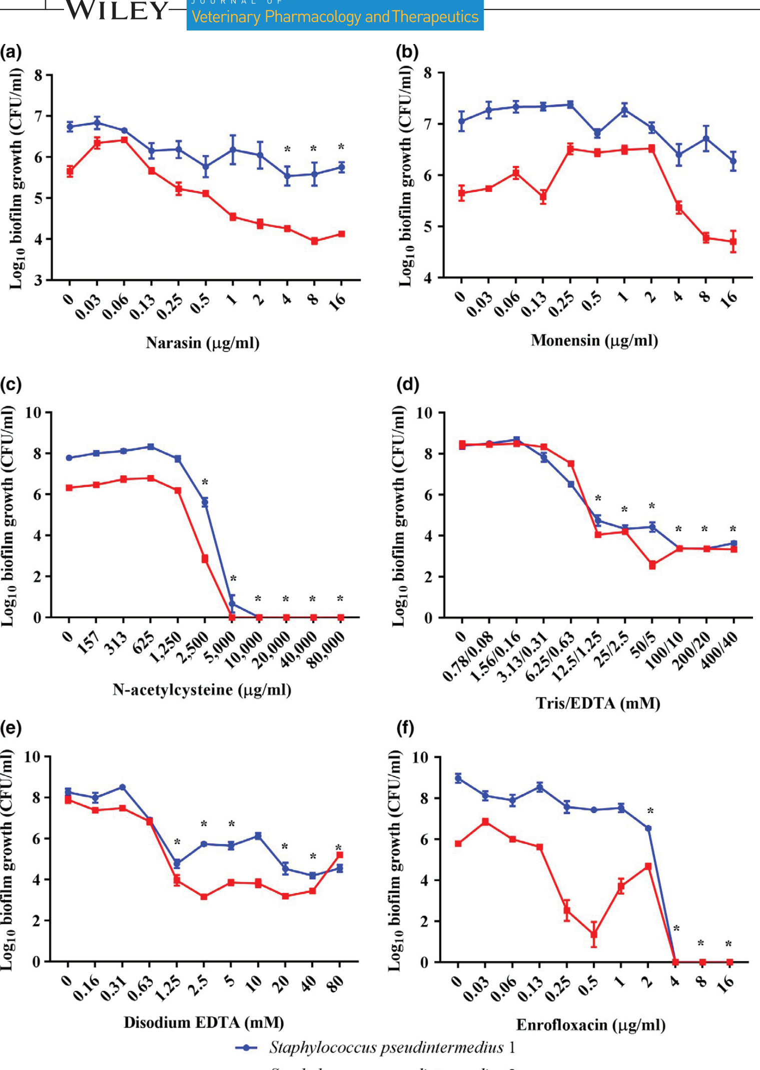

FIGURE 2 The antibiofilm activity of two ionophores narasin (a) and monensin (b); three antimicrobial adjuvants, N-acetylcysteine (c), Tris-EDTA (d) and disodium EDTA (e) and a control, enrofloxacin (f) against two moderate biofilm producers *Staphylococcus pseudintermedius* clinical otitis isolates. All data shown are the growth of biofilms in Log_{10} CFU/ml ± *SEM*. Data points that are statistically different from the untreated control (0 μg/ml on the *x*-axis) are denoted with an asterisk (*), represented by *S. pseudintermedius* 1 [Colour figure can be viewed at wileyonlinelibrary.com]

viable bacterial cells in biofilms as well as decreasing the thickness, average dispersal distance and textural entropy of *Pseudomonas* biofilms. Another study demonstrated that 50 mM (18,600 μg/ml) of EDTA reduced *P. aeruginosa* PAO1 biofilms from approximately 10^8 to 10^6 CFU and further reduction to 10^2 CFU in the presence of Tris (Tris-EDTA) (Banin, Brady, & Greenberg, 2006). Additionally, Banin et al. (2006) found that EDTA induced dispersal detachment and lysis of PAO1 biofilms *in vitro*. Furthermore, previous studies showed that EDTA or Tris-EDTA was an effective adjuvant that enhances the antibiofilm activity of primary antimicrobial agents. Antibiofilm activities of commercial antiseptics such as Octenillin®, Prontosan® and Betadine® against *S. aureus* and *P. aeruginosa* biofilms increased by 20,000-fold, 4000-fold and 200-fold, respectively in the presence of EDTA (Lefebvre, Vighetto, Di Martino, Garde, & Seyer, 2016). Pye, Singh, and Weese (2014) demonstrated that Triz-EDTA® aqueous flush increased the susceptibility of *P. aeruginosa* biofilms (MIC_{90}) to gentamicin by 16-fold and neomycin by twofold in cases of canine OE. Hence, further study is warranted to test the combination of Tris-EDTA or disodium EDTA with other antimicrobial agents for the presence of synergistic antibiofilm activity.

Even though narasin and monensin were not able to eradicate biofilms, we found that these ionophores capable of reducing biofilm growth and formation. *S. pseudintermedius* biofilms decreased in the presence of 4–16 μg/ml narasin while *P. aeruginosa* biofilms were reduced by 2–4 μg/ml of monensin. Our findings agree with previous studies on the ability of ionophores to reduce biofilm formation in different pathogens. Hickey et al. (2018) demonstrated that narasin (0.5–16 μg/ml) and monensin (2–32 μg/ml) reduced preformed ATCC 29213 *S. aureus* biofilms. In another study, biofilm produced by *S. epidermidis* was reduced up to 90% in the presence of monensin at 4 μg/ml (Stefańska, Stępień, Huczyński, & Tyski, 2015). In a study of *Clostridium perfringens* biofilms tolerance to narasin at 1 μg/

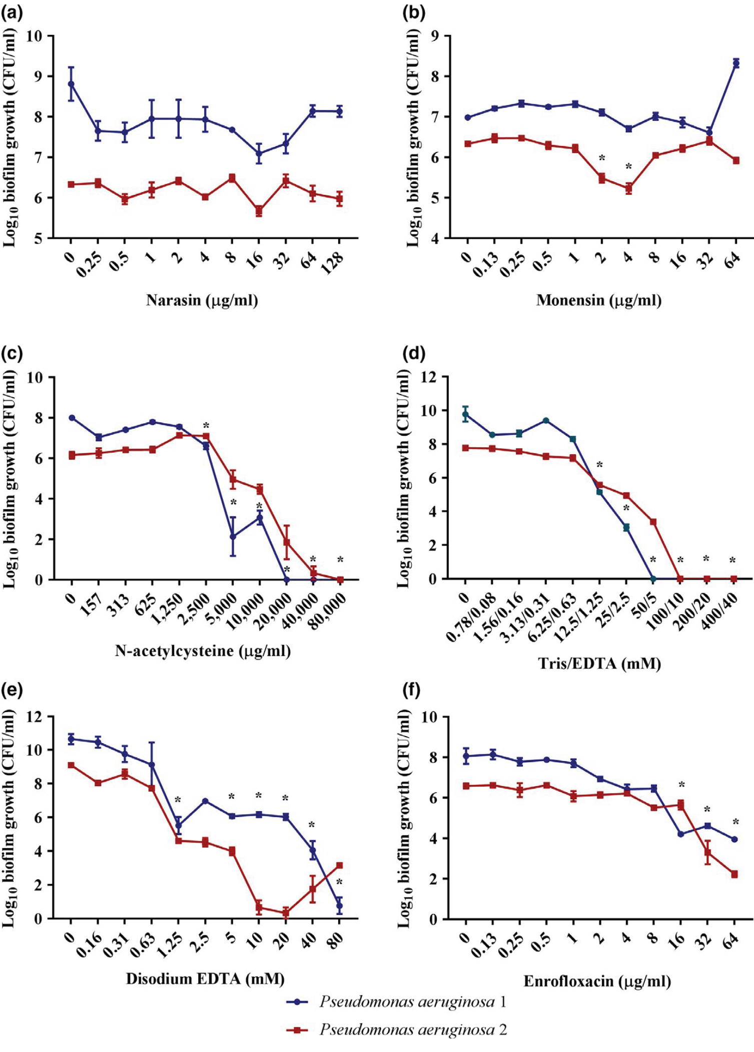

FIGURE 3 The antibiofilm activity of two ionophores narasin (a) and monensin (b); three antimicrobial adjuvants, N-acetylcysteine (c), Tris-EDTA (d) and disodium EDTA (e) and a control, enrofloxacin (f) against two strong biofilm producers *Pseudomonas aeruginosa* clinical otitis isolates. All data shown are the growth of biofilms in Log_{10} CFU/ml ± *SEM*. Data points that are statistically different from the untreated control (0 µg/ml on the *x*-axis) are denoted with an asterisk (*), represented by *P. aeruginosa* 1 or 2 [Colour figure can be viewed at wileyonlinelibrary.com]

ml and monensin at 4 µg/ml, biofilms were more efficiently reduced by ionophores with prolonged exposure at 24 hr compared with 6 hr (Charlebois, Jacques, & Archambault, 2014). Ionophores are highly lipophilic ion carriers that readily penetrate the porous peptidoglycan cell wall of Gram-positive bacteria, but not the two-layered cell wall of Gram-negative bacteria (Callaway et al., 2003). They interfere with the ion transport systems of bacteria by catalysing an electroneutral cation-proton exchange across the cell barrier and abolishing the ionic gradient of the bacterial cell membrane (Butaye, Devriese, & Haesebrouck, 2003; Pressman, 1976). However, their precise mode of action in relation to biofilm disruption remains to be determined. As the susceptibility of biofilm to ionophores varies among bacterial strains, ionophores, in common with other antimicrobial agents, do not represent a universally effective antibiofilm agent.

Topical therapy is the most common treatment modality used to treat canine otitis externa. Topical therapy can deliver a high concentration of antimicrobial in the mg/ml concentration range, and this can overcome apparent *in vitro* resistance to concentrations in the µg/ml range (Nuttall, 2013). In a recent study, the concentrations of a polyvalent topical agent were 100 to 1,000 fold higher than the MIC90s for *S. pseudintermedius* and *M. pachydermatis* (Nuttall & Forster, 2015). Thus, it is possible to apply a high concentration of an antimicrobial agent in an otic formulation to achieve therapeutic efficacy against biofilms. The data from our study show that *S. pseudintermedius* and *P. aeruginosa* biofilms can be eradicated *in vitro* with fourfold higher concentration of MIC or MBC of NAC (20,000 µg/ml or 20 mg/ml) and up to eightfold higher concentration of Tris-EDTA MIC or MBC (12,000/3,800 µg/ml or 1.2/0.38 mg/ml) to disrupt *P. aeruginosa* biofilms.

Previous studies have demonstrated that combining NAC with various antibiotics such as fluoroquinolones, aminoglycosides (e.g. neomycin, streptomycin, kanamycin, spectinomycin, gentamicin

and tobramycin), erythromycin and imipenem can lead to antagonism against various bacterial strains, including *P. aeruginosa* (Goswami & Jawali, 2010; Parry & Neu, 1977; Rodríguez-Beltrán et al., 2015; Saggers & Lawson, 1966). Likewise, when Tris-EDTA combined with enrofloxacin against *P. aeruginosa* canine otic isolates (n = 31), there was a reduction in antibacterial efficacy with fourfold or higher MIC in 39% of the isolates (Pye et al., 2014). However, we have shown in a previous study (in press) that such antagonism does not occur when NAC or Tris-EDTA is combined with ionophores (Chan et al., in press).

5 | CONCLUSION

This study has demonstrated that the majority of the *S. pseudintermedius* and *P. aeruginosa* canine otic isolates used in these experiments were biofilm producers. The ability to form biofilms by otic pathogens needs to be taken into consideration in the treatment and management of OE. We have also shown that NAC and Tris-EDTA are two effective antibiofilm agents *in vitro*. These agents could be either used individually prior to the application of ear treatment or could be incorporated into a polypharmaceutical broad-spectrum topical ear preparation. However, further in vivo study is warranted to ensure safety and efficacy at the high concentrations of these agents that are needed to overcome biofilm resistance.

ACKNOWLEDGMENT

Wei Yee Chan is supported by the Ministry of Higher Education of Malaysia and Universiti Putra Malaysia.

CONFLICTS OF INTEREST

Stephen W. Page is a director of Luoda Pharma Pty. Ltd. Darren J. Trott has received research funding from Luoda Pharma, Neoculi, Zoetis, Bayer, Merial, Virbac and Elanco. All other authors have declared no conflict of interest.

AUTHOR CONTRIBUTIONS

WYC, EEH, SWP, DJT and PBH involved in the conception and design of research; WYC and EEH performed experiments and analysed data of biofilm production; WYC performed experiments and analysed data of MBEC, MIC and MBC assay; WYC and PBH interpreted results of experiments; WYC drafted manuscript and prepared figures and tables; WYC, EEH, SWP, DJT and PBH edited and revised manuscript; WYC, EEH, SWP, DJT and PBH approved the final version of manuscript.

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How to cite this article: Chan WY, Hickey EE, Page SW, Trott DJ, Hill PB. Biofilm production by pathogens associated with canine otitis externa, and the antibiofilm activity of ionophores and antimicrobial adjuvants. *J vet Pharmacol Therap*. 2019;42:682–692. https://doi.org/10.1111/jvp.12811

Chapter 7: General discussion and Conclusion

7.1 Major findings

The emergence of antimicrobial resistance (AMR) due to resistant superbugs is one of the biggest threats to human and animal health today (O'Neill, 2016). Misuse and overuse of antimicrobials in human and animal medicine accelerate the emergence and spread of AMR. New antimicrobial therapies are needed to combat AMR. However, the development of new antibiotics is not able to keep pace with the emergence of AMR. Major pharmaceutical companies have exited the field of antibiotic research and development due to a poor return on investment and lack of incentives (Blaskovich, 2019). Since 1980, only a few new classes of antibiotics with narrow-spectrum activity against Gram-positive bacteria (lipopeptides) and *Mycobacterium tuberculosis* (diarylquinolines) have been discovered and approved for use. Therefore, prudent use of antimicrobials is essential to reduce resistance selection and to preserve the efficacy of existing antimicrobials.

Otitis externa (OE) is one of the most commonly diagnosed dermatological diseases in dogs with a prevalence of up to 20% (Miller et al., 2013). In recent years, increasing reports of zoonotic transmission of pathogens of canine origin, including resistant pathogens have raised a serious public health concern (Pomba et al., 2017). Resistant pathogens typically associated with canine OE are methicillin-resistant *Staphylococcus pseudintermedius* (MRSP), multidrug-resistant *Staphylococcus pseudintermedius* (MDRSP) and multidrug-resistant *Pseudomonas aeruginosa* (MDRPA). Moreover, biofilm formation by *S. pseudintermedius* and *P. aeruginosa* may play a vital role in bacterial persistence and resistance. Infections with these organisms are typically treated with critically important antimicrobial classes for human medicine such as fluoroquinolones (e.g. marbofloxacin and marbofloxacin), aminoglycosides (e.g. gentamicin) and polymyxins (e.g. polymyxin B), which may not represent good antimicrobial stewardship.

Repurposing of existing drug compounds with known pharmacological and toxicological profiles (e.g. polyether ionophores) for new purposes is one strategy that has been

developed to address the global issue of the diminished antimicrobial pipeline and address the challenges in AMR and antimicrobial stewardship (Strittmatter, 2014). Furthermore, antimicrobial adjuvants can be used to enhance the spectrum of activity of existing antimicrobial agents to further combat Gram-negative and drug-resistant bacterial infections (Domalaon et al., 2018; Wright, 2016). Antimicrobial-adjuvant combination therapy has an added advantage in reducing the onset and development of resistance in comparison to a monotherapy (Worthington & Melander, 2013). Therefore the work in this thesis has addressed current concerns in AMR and explored a number of options for drug repurposing and antimicrobial-adjuvant combination therapy for the treatment of bacterial and yeast otitis externa in dogs. Detailed discussions have been included in the relevant papers in chapters 2 to 6, and only the main findings are summarised here.

In this research, two polyether ionophores, narasin and monensin were investigated as potential treatments for bacterial and yeast infections in canine otitis externa. Their efficacy was evaluated *in vitro* against pathogens associated with canine OE (Chapters 2 and 3). These drugs are only licensed for animal use, and they are conventionally used as rumen modulators and anticoccidial agents in production animals (Butaye et al., 2003; Callaway et al., 2003). Also, polyether ionophores are listed by the World Health Organisation as one of the antimicrobial classes currently not used in humans (WHO, 2019). To date, there is little evidence for the development of bacterial resistance or co-selection for resistance to other classes of antimicrobials by the ionophores (Butaye et al., 2000; Callaway et al., 2003; Subbiah et al., 2016). All the above features of ionophores make them potential candidates for repurposing and addressing the challenges in AMR and antimicrobial stewardship.

Narasin and monensin had antimicrobial activity against Gram-positive otic pathogens including MDRSP but lacked activity against Gram-negative pathogens (Chapters 2 and 3). In these studies, methicillin-susceptible *S. pseudintermedius* (n=17), MDRSP (n=13) and β-haemolytic *Streptococcus* spp. (n=20) were more susceptible to narasin, with MIC_{50} and MIC_{90} of 0.125 μg/ml, 32-fold lower than the MIC_{50} and MIC_{90} of monensin (4 μg/ml). Furthermore,

narasin demonstrated anti-yeast activity against some *Malassezia pachydermatis* isolates at a higher concentration of 128 µg/ml, which was not achieved by monensin. As expected, both Gram-negative otic isolates, *Pseudomonas aeruginosa* and *Proteus mirabilis* were not susceptible to narasin or monensin. Gram-negative bacteria have inherent resistance to hydrophobic molecules with a molecular weight of > 600 such as narasin and monensin and thus, prevent their penetration through the two-layered cell wall structure of Gram-negative bacteria (Butaye et al., 2003). On the other hand, ionophores (being lipophilic ion carriers) can easily permeate into the porous peptidoglycan layer of Gram-positive bacteria and disrupt the normal intracellular ionic and pH gradients (Callaway et al., 2003). These findings support the possibility that narasin and monensin could be developed as topical agents to treat staphylococcal ear infections in dogs, including those associated with methicillin resistance. However, the lack of Gram-negative activity in both ionophores would prevent their use in Gram-negative infections.

In chapter 4, we explored various potential adjuvants to enhance the antimicrobial activity of ionophores against Gram-negative bacteria. One non-antibiotic mucolytic agent, N-acetylcysteine (NAC), and two metal chelating agents, Tris-EDTA and disodium EDTA, demonstrated intrinsic antimicrobial activities against Gram-positive bacteria including MRSP, Gram-negative bacteria and yeast otic isolates. Tris-HCL did not show any antimicrobial activity against *P. aeruginosa* otic isolates, but it was proven to potentiate the chelating activity of disodium EDTA in which the MIC range for disodium EDTA in the presence of Tris-HCL (Tris-EDTA) was two to eight times lower than when disodium EDTA was used alone. On the other hand, monoglycerides (monolaurin and monocaprin) had limited to no antimicrobial activity, and thus, these drugs were not chosen for further susceptibility testing against otic pathogens. Additionally, polymyxin B nonapeptide (PMBN), a derivative of polymyxin B was not expected to have activity against the Gram-negative pathogens. As PMBN demonstrated intrinsic antibacterial activity against Gram-negative pathogens in our study, the high likelihood for selective resistance against polymyxin in the future negates its acceptability.

Therefore, NAC, Tris-EDTA and disodium EDTA could represent promising adjuvants to potentiate the efficacy of ionophores against Gram-negative and multidrug-resistant bacterial infections.

In chapter 5, the drug interactions between narasin or monensin and NAC, Tris-EDTA or disodium EDTA were tested in combination against Gram-positive and Gram-negative ATCC reference strains using a checkerboard assay (see appendix 2). We showed in chapters 2 to 4 that the susceptibility of the reference strains to the test compounds was consistent with the clinical isolates and therefore these strains were used as proxies for canine OE isolates in this interaction study. An additive effect was shown with the combination of narasin and Tris-EDTA or disodium EDTA against *P. aeruginosa* strains. *P. aeruginosa* strains were not susceptible to narasin alone, but in the presence of Tris-EDTA or disodium EDTA, there was an achievable narasin MIC of 128 μg/ml. Hence, Tris-EDTA and disodium EDTA could be regarded as "resistance breakers" by potentiating the antimicrobial activity of narasin against *P. aeruginosa* strains. These two chelating agents interfere with the lipopolysaccharide content and enhance the permeability of the outer membrane of *P. aeruginosa* to allow the uptake of narasin (Gray & Wilkinson, 1965). Another additive effect was found with the combinations of monensin or narasin with NAC against the *Staphylococcus aureus* strain. The combination of monensin and NAC against *S. pseudintermedius* clinical isolates produced an additive effect as well (see appendix 3). In the presence of NAC, the monensin MIC was significantly reduced 16- to 32-fold against *S. aureus* and *S. pseudintermedius* isolates. The enhancement of anti-staphylococcal activity of ionophores in the presence of NAC may provide additional benefits by allowing the dose of each agent to be reduced if they were used in a clinical setting. This would be important if any toxic or irritant effects were seen if they were applied to canine ears. Other combinations did not produce any positive interactions or antagonism. Hence, a combination of narasin with Tris-EDTA or disodium EDTA may represent a promising strategy to inhibit the intrinsic resistance elements of Gram-negative bacteria. These novel

combinations could be potentially useful as a multimodal approach to treat mixed infections in canine OE.

In the final chapter, the ability of *S. pseudintermedius* and *P. aeruginosa* canine OE isolates to produce biofilms was investigated (see additional data in appendix 4). The majority (16/20) of *S. pseudintermedius* isolates including four MRSP were weak biofilm producers, and the remaining were moderate biofilm producers. On the other hand, 19 out of 20 *P. aeruginosa* isolates were biofilm producers, evenly distributed among weak (30%), moderate (35%) and strong (30%) biofilm production. In contrast to our findings, in two previous studies, only 39.3% (11/27) and 40% (33/83) of *S. pseudintermedius* and *P. aeruginosa* otic isolates, respectively, were reported to produce biofilms (Moreira et al., 2012; Pye et al., 2013). These differences are likely to represent methodological and technical differences in experimentation. However, our findings add to the existing evidence that biofilm production is common in pathogens associated with canine OE. The presence of bacterial biofilms is a common cause of persistent infections and development of AMR (Costerton et al., 1999).

Therefore, ionophores and adjuvants were further examined for their potential to eradicate pre-formed *S. pseudintermedius* and *P. aeruginosa* biofilms (Chapter 6). NAC is already known to be an effective antibiofilm agent against both Gram-positive and Gram-negative otic isolates. In our study, *S. pseudintermedius* and *P. aeruginosa* biofilms were eradicated by NAC at a concentration of 5,000 μg/ml and 20,000 μg/ml, respectively. Tris-EDTA eradicated *P. aeruginosa* biofilms at concentrations between 6,000/1,900 and 12,000/3,800 μg/ml, but only reduced *S. pseudintermedius* biofilms. The presence of 470 μg/ml disodium EDTA had the effect of reducing biofilms but not eradication of biofilms. Once again, the presence of Tris was proven to enhance the chelating activity of disodium EDTA to permeabilise the outer membrane and disperse the exopolysaccharides in biofilms of Gram-negative bacteria (Vaara, 1992). Therefore, NAC and Tris-EDTA are two antibiofilm agents *in vitro* that could be considered for the treatment of biofilm-associated canine OE.

Both narasin and monensin were shown to have high-level efficacy against Gram-positive pathogens in chapters 2 and 3. Conversely, when *S. pseudintermedius* isolates formed biofilms, they became non-susceptible to the ionophores (Chapter 7). Furthermore, we observed that both *S. pseudintermedius* and *P. aeruginosa* otic isolates were ≥16 times more resistant to enrofloxacin when tested in preformed biofilms compared to their planktonic states. These findings agree with previous studies that bacteria in biofilm cells can become 10 to 1000 times more resistant to antimicrobial agents (Mah & O'Toole, 2001; Olson et al., 2002). Thus, a combination therapy including an antibiofilm agent would need to be considered if ionophores were taken forward as potential treatments for infections in cases of canine OE.

7.2 Future directions

In this research, the *in vitro* studies in antimicrobial and antibiofilm susceptibility testing, and drug interaction studies between ionophores (narasin and monensin) and adjuvants (NAC, Tris-EDTA and disodium EDTA) serve as a fundamental basis for the further development of novel otic formulations. If these drugs were to be used as topical agents, further evaluation of their safety would be required. *In vitro* cytotoxicity assessment is not necessarily predictive of the safety of a topical agents *in vivo*, but our research group has previously investigated the *in vitro* cytotoxicity profiles of narasin and monensin against mammalian hepatic, fibroblast and kidney cell lines (Hickey et al., 2018). Likewise, the cytotoxicity profiles of disodium EDTA has been examined by Khazandi et al. (2019). However, the cytotoxic effects of these agents when used in combination may need to be evaluated if evidence is provided that systemic absorption from the ear canal could occur (for example, in the case of ulceration). Furthermore, the assessment of dermal toxicity and irritation by these agents in the healthy canine ear canal is warranted.

Future studies aim to develop a novel otic formulation containing an ionophore and an antimicrobial adjuvant (chelating or antibiofilm agent) for bacterial, yeast and biofilm-associated otitis externa in dogs. Following on from this work, the following studies and further development are planned:

1. Combinations of the active agents will be compounded into various vehicles and formulations suitable for application to an ear canal.
2. The otic formulations will be tested for chemical stability using high-performance liquid chromatography (HPLC).
3. Stable formulations will be further tested in vitro to ensure susceptibility of the otic pathogens is consistent with that seen when the drugs are used in solution.
4. Subsequently, the safety of the formulations will be tested in normal ear canals of dogs. Video otoscopy will be performed and a measurement scale such as 0-3 otitis index score will be used to assess erythema, swelling, ulceration and exudate of the ear canal following application of the formulations in healthy dogs (Nuttall & Bensignor, 2014). Other conditions of the ear canal including pain, pruritus, wax production, desquamation, and tympanic membrane integrity will be monitored as well.
5. Following the safety assessment in healthy dogs, the efficacy of the new otic preparation will be evaluated in a pilot trial involving spontaneous clinical cases of canine otitis externa.

7.3 Conclusions

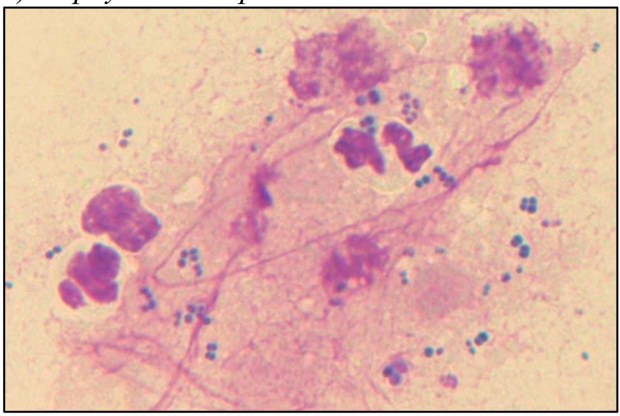
This thesis contains novel work that has contributed significant new findings to the literature. The concept of drug repurposing is relatively new to veterinary medicine and the studies contained herein provide an example of how this could be applied to an important area of clinical medicine. The work is also important, because it addresses one of the biggest health problems facing the human population – antimicrobial resistance. Antibiotic stewardship is pivotal to preserving the longevity of existing antibiotics, and studies such as these pave the way for possible development of new therapeutics that do not endanger the value of critically

important drugs. Commercial development of the agents described in these studies would represent a high impact outcome for these *in vitro* findings. Our hope is that in the future, canine OE could be treated effectively with agents that are not critically important for human health applications, thus prolonging the value of antibiotics for generations to come.

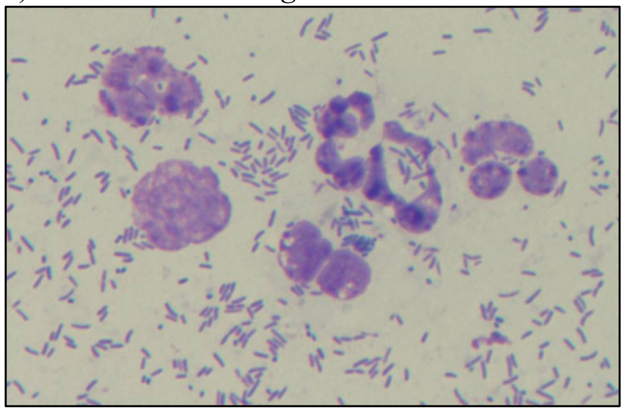
This thesis also generated some key outcomes that have direct relevance to current veterinary clinical practice. The findings of biofilm-forming potential of staphylococci and *Pseudomonas* spp. associated with canine OE could have a significant impact on the effectiveness of antimicrobial therapy. NAC and Tris-EDTA were found to be promising antimicrobial adjuvants with intrinsic broad-spectrum antimicrobial and antibiofilm activities *in vitro* against Gram-positive and Gram-negative biofilm-producers in canine OE.

Appendix 1: Common pathogens associated with canine otitis externa

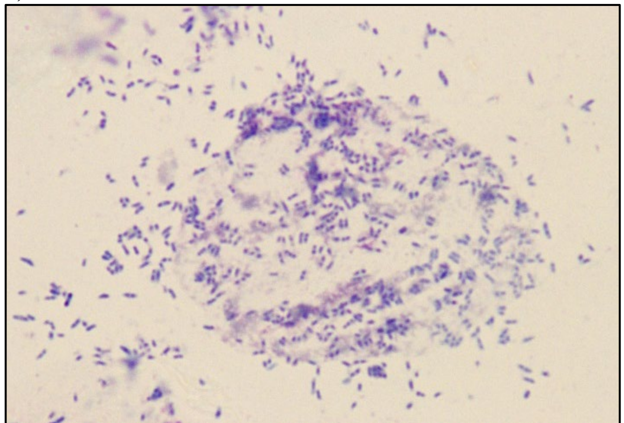
a) *Staphylococcus pseudintermedius*



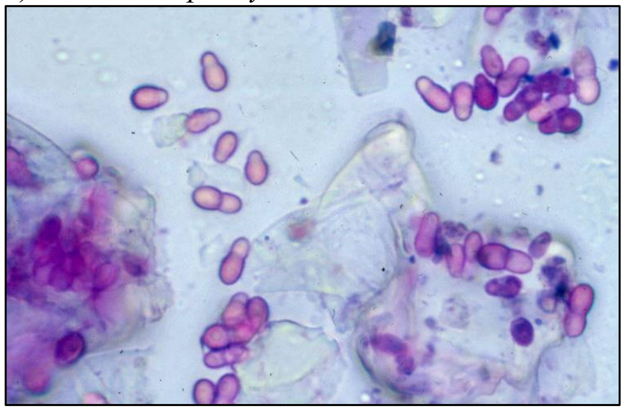
b) *Pseudomonas aeruginosa*



c) *Proteus mirabilis*

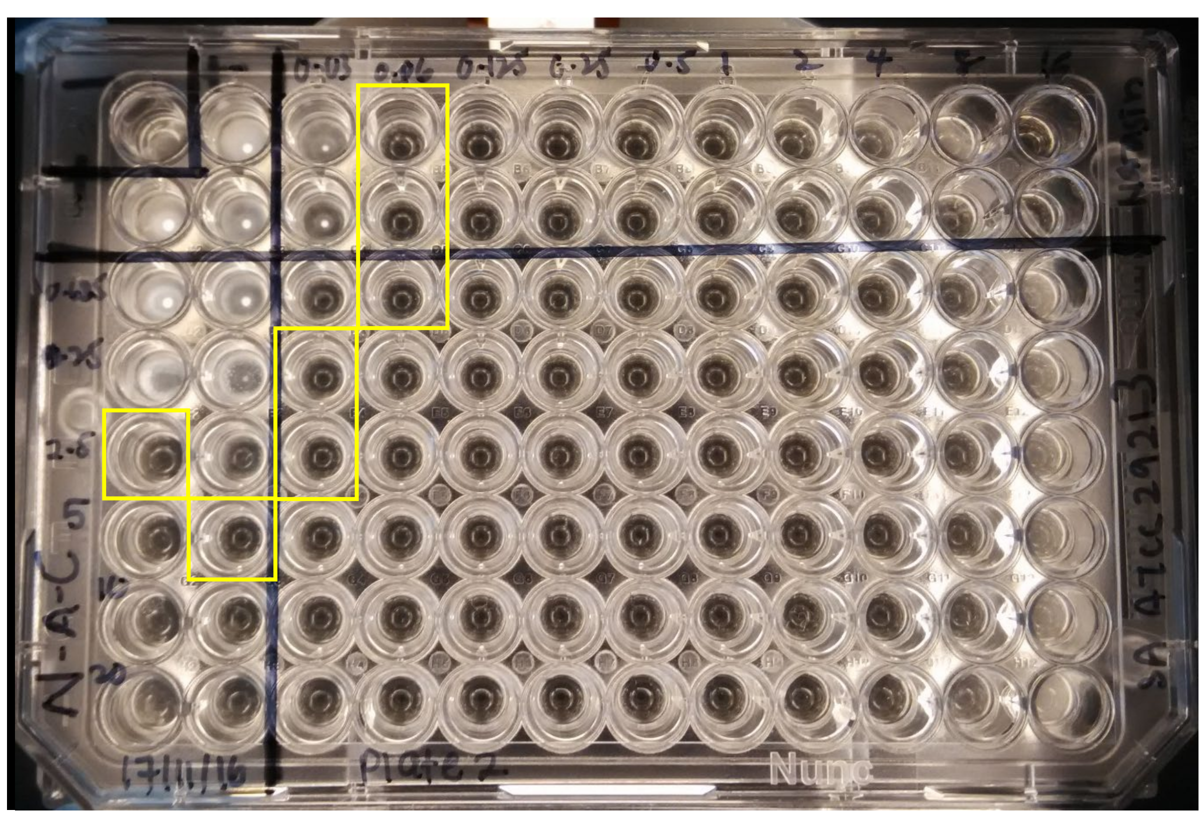


d) *Malassezia pachydermatis*

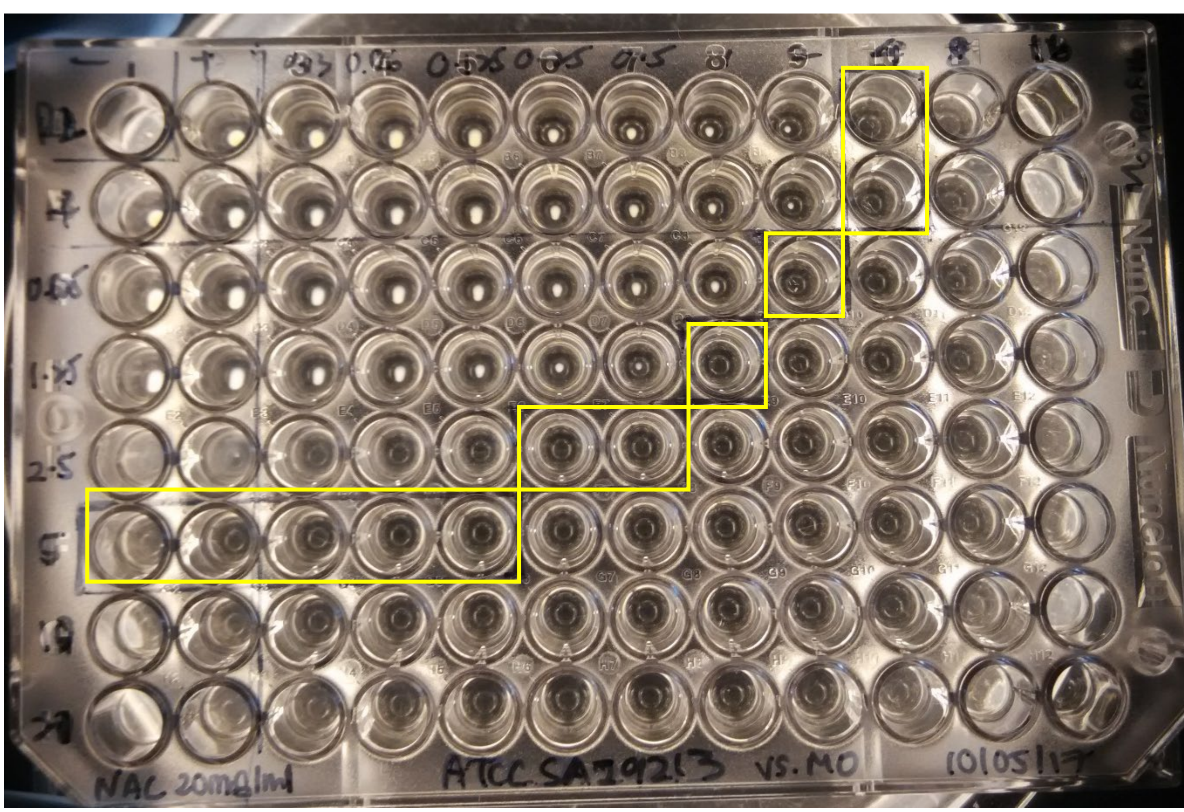


Images courtesy of P.B. Hill.

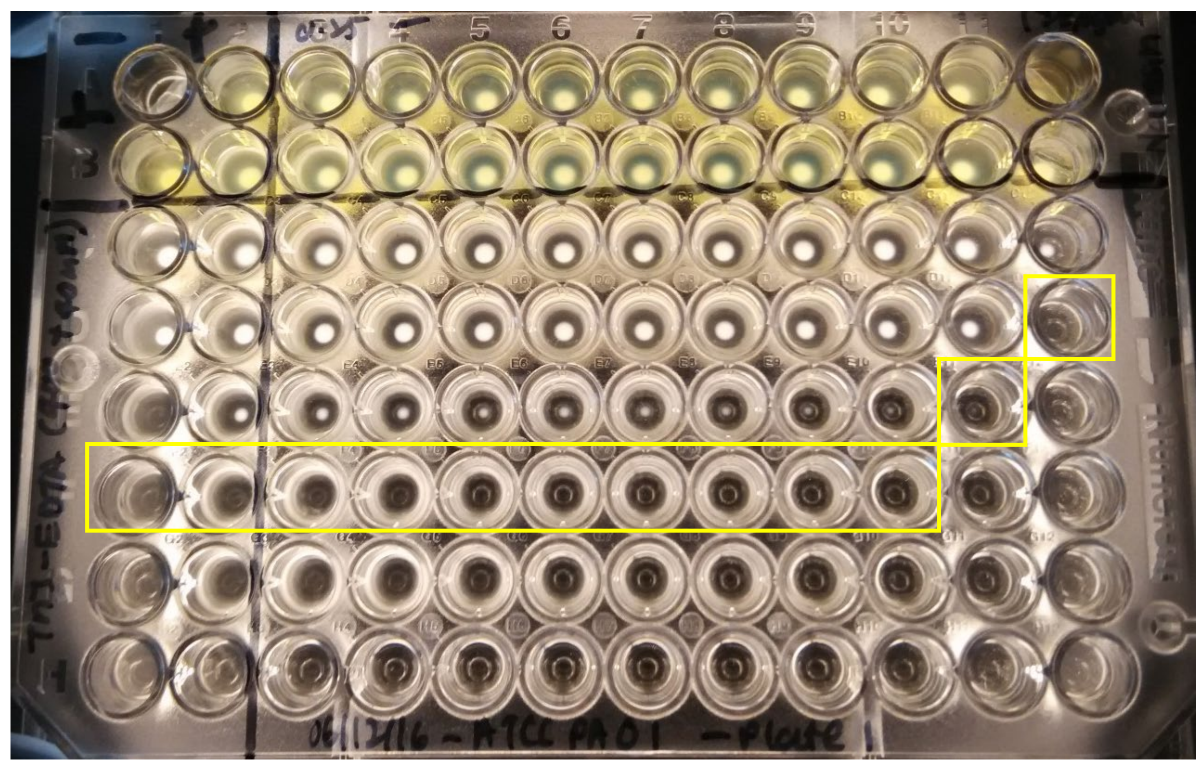
Appendix 2: Examples of checkerboard assay in which narasin or monensin tested in combination with N-acetylcysteine, Tris-EDTA or disodium against *Staphylococcus aureus* ATCC 29213, *Pseudomonas aeruginosa* ATCC 27853 and *Pseudomonas aeruginosa* PAO1. The yellow boxes outline the wells when no visible growth can be seen. See page 100 for further illustration.



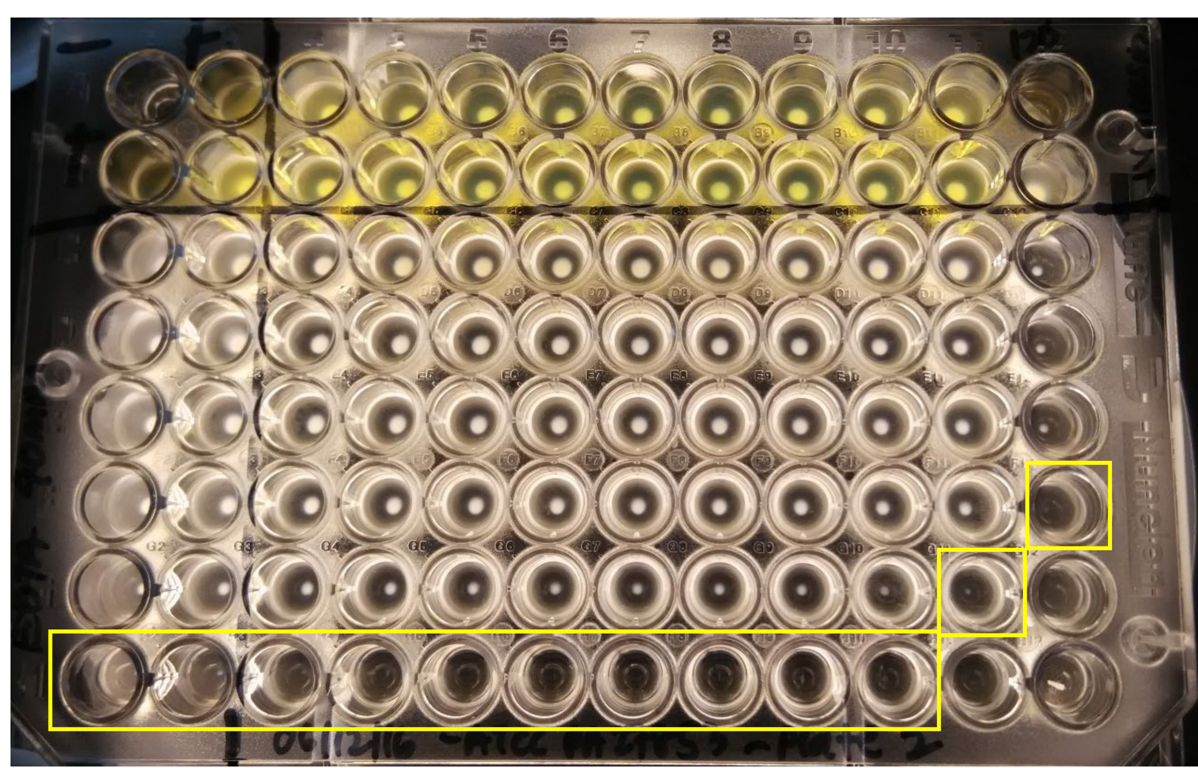
a) Narasin and N-acetylcysteine against *Staphylococcus aureus* ATCC 29213



b) Monensin and N-acetylcysteine against *Staphylococcus aureus* ATCC 29213



c) Narasin and Tris-EDTA against *Pseudomonas aeruginosa* PAO1



d) Checkerboard assay of narasin and disodium EDTA against *Pseudomonas aeruginosa* ATCC 27853

Appendix 3: Additional data of minimum inhibitory concentration (MIC), fractional inhibitory concentration index (FICI) and dose reduction index (DRI) of the combination effect of monensin (MO) and N-acetylcysteine (NAC). The susceptibility to the test compounds was consistent between the ATCC reference strains and clinical isolates from cases of canine otitis externa.

Isolates		MIC Individual (µg/ml)		MIC Combination (µg/ml)		FICI	Interpretation	DRI	
		MO	NAC	MO	NAC			MO	NAC
Gram-positive reference strain	SA ATCC 29213	4	5,000	2	625	0.625	Additive	2	8
				1	1,250	0.53		4	4
				0.125	2,500	0.53		32	2
Clinical otic isolates	SP 1	2	2,500	1	625	0.75	Additive	2	4
				0.5	1,250	0.75		4	2
	SP 2	2	5,000	0.5	1,250	0.75	Additive	4	4
				0.125	2,500	0.56		16	2
Gram-negative reference strains	PA ATCC 27853	>16	2,500	>16	2,500	2	Indifference	1	1
	ATCC PAO1	>16	2,500	>16	2,500	2	Indifference	1	1
Clinical otic isolates	PA 5	>64	2,500	>64	2,500	2	Indifference	1	1
	PA 7	>64	2,500	>64	2,500	2	Indifference	1	1
	PM 8	>16	5,000	>16	5,000	2	Indifference	1	1
	PM 15	>64	5,000	>64	5,000	2	Indifference	1	1

Notes:

For MIC values >16, and > 64, value of 32, 128 and were respectively assumed for calculation of FICI and DRI.

ATCC= American Type Culture Collection

SA= *Staphylococcus aureus*

SP = *Staphylococcus pseudintermedius*

PA = *Pseudomonas aeruginosa*

PAO1 = Biofilm producer reference strain

PM = *Proteus mirabilis*

Appendix 4: Summary results of three studies of biofilm growth evaluation of *Staphylococcus pseudintermedius* and *Pseudomonas aeruginosa* canine otitis externa isolates.

1. *Staphylococcus pseudintermedius*

#	Isolates	Study 1: 04/11/16	Study 2: 09/11/16	Study 3: 11/11/16	Summary	Cat. #	Isolates
N	Negative control	Negative control	Negative control	Negative control	Negative control	0	Negative control
P	Positive control	+++, Strong	+++, Strong	+++, Strong	Positive control	3	Positive control
C	ATCC SA 29213	0 , No biofilm	++, Moderate	+, Weak	0 to ++	1	ATCC SA 29213
1	N13/1/568*	0 , No biofilm	+, Weak	+, Weak	0 to +	1	N13/1/568*
2	N13/1/570	+, Weak	++, Moderate	++, Moderate	+ to ++	2	N13/1/570
3	N13/1/614	+, Weak	+, Weak	+, Weak	+	1	N13/1/614
4	N13/1/652	+, Weak	++, Moderate	++, Moderate	+ to ++	2	N13/1/652
5	N13/1/752	+, Weak	++, Moderate	++, Moderate	+ to ++	2	N13/1/752
6	S13/1/55	+, Weak	+, Weak	+, Weak	+	1	S13/1/55
7	S13/1/73	+, Weak	+, Weak	+, Weak	+	1	S13/1/73
8	S13/1/77	0 , No biofilm	+, Weak	+, Weak	0 to +	1	S13/1/77
9	S13/1/78	0 , No biofilm	+, Weak	+, Weak	0 to +	1	S13/1/78
10	S13/1/79	+, Weak	+, Weak	+, Weak	+	1	S13/1/79
11	Q13/1/44	++, Moderate	++, Moderate	+, Weak	+ to ++	2	Q13/1/44
12	Q13/1/56	+, Weak	+, Weak	+, Weak	+	1	Q13/1/56
13	Q13/1/57	0 , No biofilm	+, Weak	+, Weak	0 to ++	1	Q13/1/57
14	Q13/1/24*	+, Weak	+, Weak	++, Moderate	+ to ++	1	Q13/1/24*
15	V13/2/172	0 , No biofilm	+, Weak	+, Weak	0 to +	1	V13/2/172
16	V13/2/173*	0 , No biofilm	+, Weak	+, Weak	0 to +	1	V13/2/173*
17	V13/2/347	0 , No biofilm	+, Weak	+, Weak	0 to +	1	V13/2/347
18	V13/2/422	+, Weak	+, Weak	+, Weak	+	1	V13/2/422
19	V13/2/456	+, Weak	+, Weak	+, Weak	+	1	V13/2/456
20	W13/1/11*	0 , No biofilm	+, Weak	+, Weak	0 to +	1	W13/1/11*

No biofilm	0	None
Weak	1	All above except these four isolates below
Moderate	2	N13/1/570, N13/1/652, N13/1/752, Q13/1/44
Strong	3	None

Notes:

Categorisation of biofilm production was based on OD_{570nm} values in which the interpretation has been explained in chapter 6 and the following classifications (Stepanovic, Vukovic, & Hola, 2007) are used for clearer presentation in this table:
0 (no biofilm producer),
+ or 1 (weak biofilm producer),
++ or 2 (moderate biofilm producer),
+++ or 3 (strong biofilm producer).

2. *Pseudomonas aeruginosa*

#	Isolates	Study 1: 04/11/16	Study 2: 09/11/16	Study 3: 11/11/16	Summary	Cat. #	Isolates
N	Negative control	Negative control	Negative control	Negative control	Negative control	0	Negative control
P	Positive control	++, Moderate	+++, Strong	+++, Strong	Positive control	3	Positive Control
C	ATCC PA 27853	+ , Weak	0 , No biofilm	+, Weak	0 to +	1	ATCC PA 27853
1	PA 002	++, Moderate	+, Weak	+++, Strong	+ to +++	2	PA 002
2	PA 003	+, Weak	+, Weak	+, Weak	+	1	PA 003
3	PA 004	++, Moderate	++, Moderate	++, Moderate	++	2	PA 004
4	PA 005	+++, Strong	++, Moderate	+++, Strong	++ to +++	3	PA 005
5	PA 006	+++, Strong	+++, Strong	++, Moderate	++ to +++	3	PA 006
6	PA 007	+ , Weak	+ , Weak	+ , Weak	+	1	PA 007
7	PA 008	+ , Weak	+ , Weak	++, Moderate	+ to ++	1	PA 008
8	PA 009	++, Moderate	+ , Weak	+ , Weak	+ to ++	1	PA 009
9	PA 010	+++, Strong	++, Moderate	+++, Strong	++ to +++	3	PA 010
10	PA 012	++, Moderate	+++, Strong	+++, Strong	++ to +++	3	PA 012
11	PA 013	++, Moderate	++, Moderate	+ , Weak	+ to ++	2	PA 013
12	PA 014	++, Moderate	+ , Weak	+++, Strong	++ to +++	2	PA 014
13	PA 015	0 , No biofilm	0 , No biofilm	+ , Weak	0 to +	0	PA 015
14	PA 016	++, Moderate	+ , Weak	0 , No biofilm	0 to ++	1	PA 016
15	PA 017	++, Moderate	+ , Weak	++, Moderate	+ to ++	2	PA 017
16	PA 018	++, Moderate	++, Moderate	++, Moderate	++	2	PA 018
17	PA 020	+++, Strong	+++, Strong	+++, Strong	+++	3	PA 020
18	PA 022	+ , Weak	+ , Weak	+ , Weak	+	1	PA 022
19	PA 023	++, Moderate	++, Moderate	++, Moderate	++	2	PA 023
20	PA 024	+++, Strong	+++, Strong	+++, Strong	+++	3	PA 024

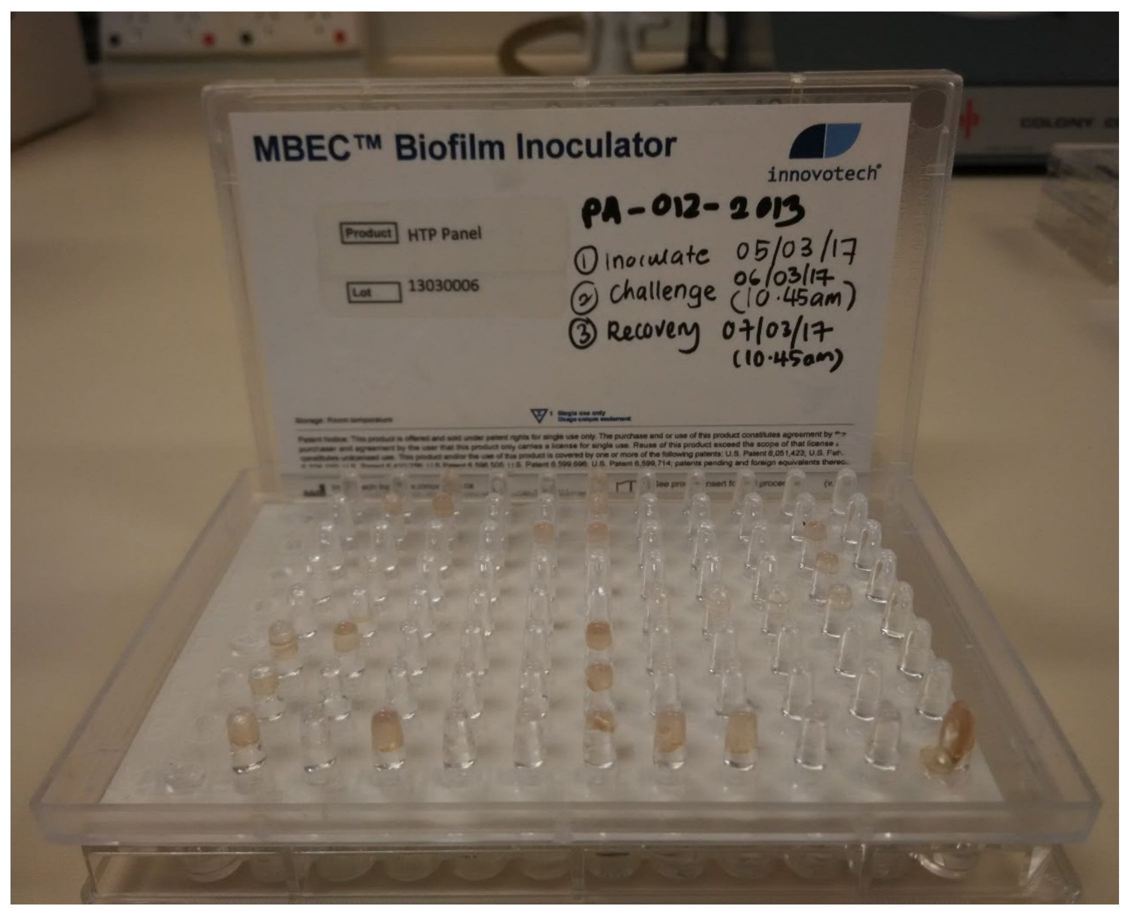
No biofilm	0	PA015
Weak	1	ATCC PA 27853, PA 003, PA 007, PA 008, PA 009, PA 016, PA 022
Moderate	2	PA 002, PA 004, PA 013, PA 014, PA 017, PA 018, PA 023
Strong	3	PA 005, PA 006, PA 010, PA 012, PA 020, PA 024

Notes:

Categorisation of biofilm production was based on OD_{570nm} values in which the interpretation has been explained in chapter 6 and the following classifications (Stepanovic et al., 2007) are used for clearer presentation in this table:

0 (no biofilm producer),
+ or 1 (weak biofilm producer),
++ or 2 (moderate biofilm producer),
+++ or 3 (strong biofilm producer).

Appendix 5: An example of biofilms formed on the pegs of MBEC™ Inoculator (Calgary Biofilm Device)



Appendix 6: Supporting papers

These publications were not a specific focus of my PhD studies, but they were undertaken during my period of candidature and they provide evidence of further collaboration and supervision.

Veterinary Dermatology

Vet Dermatol 2017; **28**: 333–e71 DOI: 10.1111/vde.12435

Survival of *Staphylococcus pseudintermedius* in modified Romanowsky staining solutions

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Background – Stains that are used regularly for patient-side diagnosis to rapidly identify bacterial and fungal infections could become contaminated by common pathogens, such as *Staphylococcus pseudintermedius,* during slide immersion.

Hypothesis/Objectives – To determine whether the inoculation of *S. pseudintermedius* into modified Romanowsky type stains (Quick Dip®) results in viable bacterial contamination and whether this is influenced by the addition of organic debris (canine hair and skin).

Methods – A clinical isolate of *S. pseudintermedius* was inoculated into clean and organically contaminated Quick Dip® solutions (methanol fixative, eosin, methylene blue), and positive (broth) and negative (bleach) controls. Each solution was tested for the presence of viable bacteria by counting the number of colony forming units (CFU/mL) at various time points. Solutions also were examined under high power microscopy to count the number of visible bacteria at each time point.

Results – *Staphylococcus pseudintermedius* was able to survive in the clean and contaminated Quick Dip® stains for at least one hour, but by 24 h no viable bacteria remained. Survival of the bacteria was not supported in the fixative at any time point. *Staphylococcus pseudintermedius* remained visible under high power microscopy for up to 2 weeks in all organically contaminated solutions of the Quick Dip® set.

Conclusions and clinical importance – *Staphylococcus pseudintermedius* only remains viable in eosin and methylene blue for short periods of time, but the prolonged visibility of dead organisms could theoretically lead to the misdiagnosis of cytology samples.

Introduction

One of the most commonly used staining procedures in clinical cytology is the sequential application of methanol fixative, eosin and methylene blue (a modification of the Romanowsky–Giemsa stain, commonly referred to as DiffQuik® or Quick Dip®). These stains are used to rapidly identify bacterial and fungal infections for patient-side diagnosis. The recommended staining procedure for these stains involves dipping glass slides into the staining solutions, rather than adding stains to the slides. Therefore, during staining of specimens for cutaneous cytological examination it is theoretically possible that bacteria or fungi on slides could be transferred to the staining solutions, leading to contamination. This would become increasingly likely if staining solutions were used frequently and not often replaced. If the stain contaminants were able to survive for a prolonged period, samples subsequently stained may be misdiagnosed. Therefore, it is advisable to determine whether or not common bacteria are able to survive in such stains as viable contaminants.

Accepted 20 January 2017
Sources of Funding: This project was funded by the School of Animal and Veterinary Sciences, University of Adelaide.
Conflict of Interest: No conflicts of interest have been declared.

Some stains are known to have bactericidal properties whereas others have no disinfectant action and allow growth of bacteria.[1,2] Previous studies have shown that *Pseudomonas aeruginosa* can survive for variable periods of time in eosin and methylene blue stains of the Quick Dip® set,[1] and that Merck's methylene blue has minimal disinfectant action.[2] However, both gentian and crystal violet stains have significant disinfectant action against Gram-positive bacteria, including the *Staphylococcus* genus.[2] In several circumstances, contamination of stains has led to false positive results. For example, fungal contamination of Grocott light-green counterstain solution led to the misdiagnosis of yeast infections in two separate cases.[3] Gram-stained clinical samples of cerebrospinal fluid resulted in false positive diagnosis of Gram-negative bacilli due to bacterial contamination of the piped deionized water that was used to make the Gram stain solutions.[4] Previous studies also have highlighted misdiagnosis and unnecessary therapy following contamination of other media, including Gram-negative bacilli in Amies transport medium used for the transport of swabs,[5] and fungal contaminants in Hanks' solution.[6]

Staphylococcus pseudintermedius is the most common cutaneous pathogen in the dog, regularly isolated from cases of pyoderma and otitis externa.[7,8] This pathogen is commonly stained using Romanowsky-type stains. There have been no previously published data on whether or not this common pathogen is able to survive in and

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contaminate staining solutions. *Staphylococcus pseudintermedius* is a common contaminant of the environment, contaminating clothing in veterinary hospitals,[9] and household areas commonly used by pets.[10] Although it is unknown if stains are bactericidal to *S. pseudintermedius,* previous research indicates that bleach is bactericidal, even in low concentrations,[8] and that oils derived from natural products can inhibit growth.[7]

The aim of this study was to determine whether the inoculation of *S. pseudintermedius* into eosin and methylene blue stains could result in viable bacterial contamination and whether this is influenced by the addition of organic debris (canine hair and skin).

Materials and methods

Bacteria and inoculum size

A methicillin-sensitive isolate of *S. pseudintermedius* originating from a dog with otitis externa was used for this study (isolate number V13/2/347). The isolate was stored in brain heart infusion broth (Becton Dickinson Pty Ltd; Sydney, NSW, Australia) with 20% glycerol (Ajax Finechem; Cheltenham, Victoria, Australia) at −80°C. Fresh cultures were grown on 5% Columbia sheep blood agar (Thermo Fisher Scientific; Melbourne, Victoria, Australia) and incubated at 37°C for 18 h. Fresh pure colonies were suspended in 7 mL of phosphate buffered saline (PBS) pH 7.3 (Thermo Fisher Scientific) to generate a 0.5 McFarland standard with a corresponding optical density at 600 nm of 0.1 (0.08–0.13) as determined by a spectrophotometer (BioPhotometer plus, Eppendorf; Macquarie Park, Australia). Preliminary studies found that this suspension contained approximately 1.5×10^8 colony forming units (CFU)/mL. An inoculation volume was calculated by determining the concentration of solution required to be able to visualize and count bacteria under 1000× oil-immersion magnification. It was concluded that a solution with 7×10^6 CFU/mL allowed sufficient visualization of bacteria after 1 h of incubation at room temperature in Mueller–Hinton broth. This concentration was achieved for each experiment by inoculating 2.86 mL of solution (controls, fixative, eosin, methylene blue) with a 140 μL aliquot of a 0.5 McFarland standard suspension to make up a 3 mL solution.

Staining and control solutions

The positive control for this experiment was cation-adjusted Mueller–Hinton broth (Becton Dickinson Pty Ltd). The negative control was sodium hypochlorite (White King bleach, 42 g/L, Pental Products; Shepparton, Victoria, Australia). The staining system used was Quick Dip® (Point of Care Diagnostics Scientific; Artarmon, New South Wales, Australia). The staining set comprised methanol (>99% w/v) as a fixative, Quick Dip® 1 stain (eosin Y <0.5% w/v, disodium phosphate <5.0% w/v, monosodium phosphate <5.0% w/v and preservatives) and Quick Dip® 2 stain (methylene blue <1.0% w/v, methylene azures <0.1% w/v, disodium phosphate <5.0% w/v, monosodium phosphate <5.0% w/v and preservatives).

Quick Dip® solutions were used both in a clean state (fresh out of the bottle) and after contamination with organic debris as might occur following repeated cutaneous sampling. The organic debris was sourced from a fresh frozen canine cadaver. The skin (epidermis and dermis) and hair were cleaned with 70% ethanol and rinsed with PBS to eliminate any surface bacteria. In the organically contaminated experiments, each solution was contaminated with 0.01 g of hair and 0.03 g of skin.

Experimental design

Each of the solutions (controls, fixative, eosin and methylene blue) were inoculated in triplicate with the *S. pseudintermedius* isolate and tested for the presence of viable or visible bacteria at 5 min, 1 h and 24 h. Solutions were left at room temperature, out of direct sunlight, and covered but not tightly sealed. The organically contaminated solutions were left for an additional 2 weeks to determine if organic debris aided survival of the bacteria over an extended duration.

Viability and visibility of bacteria

In order to determine the viability of *S. pseudintermedius* at each time point, 100 μL of each solution was removed and serially diluted 10-fold up to ten times, with sterile PBS. Preliminary experiments were performed to determine the dilution end-points for each solution. Ten microlitres of the original solution and each dilution was then spot-plated in triplicate, as described previously, onto Mueller–Hinton agar (Thermo Fisher Scientific) and incubated at 37°C for 20 h.[11] The viability was then determined by counting the number of CFUs in each spot.

In order to determine whether the bacteria present were microscopically visible at each time point, 100 μL of each solution was air-fixed onto a microscope slide. Glass slides were then stained using Quick Dip® stains according to the manufacturer's recommendations. To quantify the number of visible bacteria, three representative high power fields (HPF) were examined under 1000× oil-immersion magnification and each individual coccus was counted. To verify the integrity of the peptidoglycan cell wall a Gram stain also was performed. A 100 μL sample from the fixative solution at 2 weeks was air-dried on a glass slide, Gram-stained and examined under 1000× oil-immersion magnification.

Statistical analysis

Because the data were not normally distributed, nonparametric analysis was used. The Kruskal–Wallis test was used to compare the bacterial count across different solutions and time points. Where significant differences were observed, *post hoc* comparisons between multiple solutions and different time points were performed using Dunn's multiple comparisons test. All statistical analysis was completed using GraphPad Prism software (GraphPad Prism for Mac v6.00, GraphPad Software; San Diego, CA, USA; www.graphpad.com).

Results

Viability of bacteria

The survival of *S. pseudintermedius* in clean and organically contaminated staining solutions is shown in Supporting Information Figures S1 and S2, respectively. In both clean and organically contaminated solutions, the broth (positive control) supported growth of the bacteria at all time points. whereas the bleach (negative control) and fixative showed no growth at any time point (Supporting Information S1 and S2, respectively). The number of bacteria in the clean broth at 1 h and 24 h was significantly higher than at 5 min ($P < 0.05$), and the number of bacteria in the organically contaminated broth at 24 h and 2 weeks was significantly higher than at 5 min and 1 h ($P < 0.05$), indicating growth of *S. pseudintermedius*. In both the clean and organically contaminated solutions, the eosin and methylene blue stains supported survival of *S. pseudintermedius* at 5 min and 1 h, but by 24 h there were no viable bacteria remaining. In both clean and contaminated solutions, bacterial survival in methylene blue at 5 min was significantly higher than in eosin ($P < 0.05$), but not significantly different to the broth ($P > 0.05$). Bacterial survival in methylene blue at 5 min and 1 h was significantly higher than the eosin at 5 min and 1 h ($P < 0.05$) in both clean and contaminated solutions.

Visibility of bacteria

The number of bacteria visible per HPF under high power microscopy for the clean and organically contaminated staining solutions is shown in Supporting Information Figures S3 and S4, respectively. Photomicrographs of the

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visible bacteria are shown in Supporting Information Figure S5. In both the clean and contaminated solutions, bacteria were visible at each time point in broth, fixative, eosin and methylene blue (Figures S3, S4 and S5), whereas no bacteria were visible in the bleach at any time point. In the organically contaminated broth solutions, the number of bacteria per HPF was too numerous to count at both 24 h and 2 weeks, so an estimate of 1000 was used for analysis. There also was no significant difference in the number of bacteria visible at 24 h across the fixative, eosin and methylene blue ($P > 0.05$) in the clean solutions. Bacteria that were visible at 2 weeks remained Gram-positive.

Discussion

This study demonstrated that *S. pseudintermedius* can survive for variable periods of time in Quick Dip® stains. *Staphylococcus pseudintermedius* survived for 1 h in both the clean and organically contaminated eosin and methylene blue stains. After inoculation, the number of bacteria in the eosin and methylene blue stains decreased over time, until 24 h, when no viable bacteria remained. Although the bacteria were not viable at 24 h, they remained visible for an extended duration in the eosin, methylene blue and methanol fixative. Although the results suggest that *S. pseudintermedius* are unable to grow in the Quick Dip® set, it is theoretically possible that dead, 'preserved' bacteria could accumulate in the stains and fixative, which could then lead to misdiagnosis. This would only occur if dead bacteria were able to adhere to the cellular substrate on the glass slides during immersion and rinsing. It is not known to what extent this might occur in a clinical setting. It is possible that the dead bacteria would be rinsed off the slide during the staining process, but further studies would be needed to document if complete removal is achieved.

In a previous study,[1] it was determined that *P. aeruginosa* was not visible at any time point when placed in the methanol fixative. The results of the present study differed, because *S. pseudintermedius* was visible at all time points in the clean and organically contaminated fixative (as well as in the other solutions). This result might be explained by the different cell wall structure of Gram-positive and Gram-negative bacteria. In Gram-positive bacteria, the cell wall has a thick peptidoglycan layer. During Gram-staining, the alcohol decolourization step causes only minor damage to cell wall integrity.[12] This provides a possible explanation as to why *S. pseudintermedius* remained visible for 2 weeks in the methanol fixative. In Gram-negative bacteria, the outer membrane lipid bilayer is particularly sensitive to disruption caused by ethanol and other organic solvents,[13] and hence, it is severely damaged by alcohol decolourization. This may explain why *P. aeruginosa* was no longer visible after inoculation in methanol. In this experiment, Gram staining of the visible bacteria seen at 2 weeks revealed retention of the purple, crystal violet–iodine complex, and hence that the peptidoglycan layer was still intact after 2 weeks inoculation in methanol. The difference in visibility of *S. pseudintermedius* and *P. aeruginosa* in the methanol fixative was mirrored in the eosin and methylene blue stains. *Staphylococcus pseudintermedius* remained visible in the eosin and methylene blue stains for a prolonged period of time after bacteria were dead, compared to the previous study in which *P. aeruginosa* was not visible in the same staining solutions after bacteria were dead. Additionally, *S. pseudintermedius* survived significantly better in methylene blue than eosin, whereas *P. aeruginosa* survived significantly better in eosin than methylene blue. It is possible that these differing results are also due to the fundamental differences in cell wall structure between the two genera.

Staphylococcus pseudintermedius was visible in methanol at all time points, despite methanol's bactericidal properties. Methanol is classified as a denaturing fixative as it removes water from cells and alters the tertiary structure of proteins while preserving their secondary structure.[14] Methanol exposure can damage cells by making cell membranes more permeable, disrupting the cytoskeleton,[15] affecting bacterial cell morphology and detaching surface ultrastructures, proteins and lipopolysaccharides.[16] All of these factors may provide an explanation for why bacteria were preserved after inoculation in methanol, but unable to grow at any time point. The results indicate that the methanol fixative is as bactericidal as household bleach, which is known to be bactericidal to *S. pseudintermedius*.[8] Bleach contains sodium hypochlorite, which is rapidly bactericidal due to the damage it inflicts simultaneously on many cellular components. This includes causing a loss of ATP, a reduction in DNA replication and protein transport across membranes, and damage to essential proteins.[17] Radicals derived from sodium hypochlorite cause lipid peroxidation, leading to destruction of bacterial cells.[16] This is a likely explanation for why bacteria were no longer viable or visible after inoculation in bleach.

Organic contamination did not aid the survival of bacterial organisms. In both the clean and organically contaminated eosin and methylene blue stains, the bacteria were no longer viable at 24 h. It is unknown whether organic contamination of stains prolonged the visibility of bacteria, because a 2 week comparison cannot be made with the clean experiment, as it was not performed. It was hypothesized that contamination of stains with organic debris would prolong survival of bacteria, due to the expected formation of a biofilm[18] and the presence of cell wall-associated proteins on the surface of *S. pseudintermedius.* These proteins aid adherence to fibrinogen, fibronectin and the organic extracellular matrix proteins.[19] It is possible that the stains proved too bactericidal, and hence, formation of a biofilm was not possible. It is important to note that the level of organic contamination used in this experiment would not occur in a clinical setting. The amount of canine hair and skin in each of the solutions was significantly greater than the amount that could be transferred into staining solutions during immersion of samples. This large quantity was used to maximize the chance for bacterial survival. However, it should also be noted that in real clinical samples, the organic material likely would already be contaminated with *S. pseudintermedius* which might alter its ability to survive.

If the Quick Dip® procedure is followed correctly and the fixative is used, it is unlikely that any bacteria would remain viable in the methanol to then contaminate the eosin and methylene blue stains. However, slides are only placed in each of the Quick Dip® solutions for 10 s and it is unknown whether the fixative is bactericidal over this short period of time. However, a short duration in the methanol fixative would still significantly reduce the concentration of viable bacteria on the slide. If bacteria were still viable after immersion in the fixative it is possible that they may contaminate the eosin and methylene blue stains for at least 1 h. The likelihood of this happening in sufficiently high concentrations to lead to misdiagnosis is remote. To determine the likelihood of stains becoming contaminated with bacteria in a concentration similar to this experiment, it is important to consider the inoculum size. This experiment was designed to allow sufficient CFU/mL to enable the visualization of bacteria under high-power microscopy. The staining solutions had a concentration of 7×10^6 CFU/mL at the time of inoculation. Based on the typical number of organisms on clinical samples taken from sites of infection, the chance of bacteria accumulating to this concentration in a clinical setting is extremely unlikely. The likelihood of contamination can be further reduced by frequently replacing Quick Dip® solutions.

In summary, this study has shown that *S. pseudintermedius* can remain viable in methylene blue and eosin for short periods of time, but the prolonged visibility of dead organisms could theoretically lead to the misdiagnosis of cytology samples. The extent to which this might occur in a clinical setting is unknown.

Acknowledgements

We would like to thank Elizabeth Hickey for her assistance with laboratory procedures.

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Supporting Information

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

Figure S1. Survival of *Staphylococcus pseudintermedius* in positive and negative control solutions and clean Quick Dip® stains at different incubation times.

Figure S2. Survival of *Staphylococcus pseudintermedius* in positive and negative control solutions and Quick Dip® stains contaminated with organic debris (hair and skin).

Figure S3. Visibility of *Staphylococcus pseudintermedius* in positive and negative control solutions and clean Quick Dip® stains at different incubation times.

Figure S4. Visibility of *Staphylococcus pseudintermedius* in positive and negative control solutions and Quick Dip® stains contaminated with organic debris (hair and skin).

Figure S5. *Staphylococcus pseudintermedius* stained with Quick Dip® stains, visible under high power microscopy (1000×) oil immersion at 5 min (left) and 24 h (right) in contaminated solutions.

Résumé

Contexte – Les colorations qui sont utilisées pour le diagnostic au chevet du patient pour l'identification rapide d'infections bactériennes et fongiques pourraient être contaminées par des pathogènes tels que *Staphylococcus pseudintermedius* au cours de l'immersion des lames.

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Hypothèses/objectifs – Déterminer si l'inoculation de *S. pseudintermedius* dans des colorants de type Romanovsky modifié (Quick Dip®) résulte en une contamination bactérienne viable et si celle-ci est influencée par l'addition de débris organiques (poils et peau de chien).
Méthodes – Une souche clinique de *S. pseudintermedius* a été inoculée dans des solutions de Quick Dip® (méthanol, éosine et bleu de méthylène) saines et contaminées par des débris organiques, et des solutions de contrôle positives (diluant) et négatives (eau de javel). Chaque solution était testée pour la présence de bactérie viable par comptage du nombre de CFU (colony forming unit/mL) à différents temps. Les solutions étaient également examinées sous microscope à haute résolution pour déterminer le nombre de bactérie visible à chaque temps.
Résultats – *Staphylococcus pseudintermedius* était capable de survivre dans les colorants de Quick Dip® propres et contaminés pendant 24 heures mais après 24h aucune bactérie viable ne persistait. La survie des bactéries n'était pas possible dans le fixateur quelque-soit l'instant. *Staphylococcus pseudintermedius* restait visible à fort grossissement microscopique jusqu'à 2 semaines dans toutes les solutions contaminées du kit.
Conclusions et importance clinique – *Staphylococcus pseudintermedius* reste visible seulement dans l'éosine et le bleu de méthylène sur de courtes périodes mais la visualisation prolongée d'organismes morts pourraient théoriquement entrainer des erreurs de diagnostic des échantillons de cytologie.

Resumen

Introducción – Las tinciones que se utilizan con regularidad para el diagnóstico rápido e identificación en pacientes de infecciones bacterianas y fúngicas pueden contaminarse con patógenos comunes, como *Staphylococcus pseudintermedius*, durante la inmersión de preparaciones.
Hipótesis/Objetivos – Determinar si la inoculación de *S. pseudintermedius* en tinciones de tipo Romanowsky modificadas (Quick Dip®) puede dar lugar a contaminación bacteriana viable y si esto puede estar influido por la adición de restos orgánicos (pelo y piel caninos).
Métodos – Un aislado clínico de *S. pseudintermedius* se inoculó en soluciones limpias y contaminadas orgánicamente de Quick Dip (fijador de metanol, eosina, azul de metileno) y en controles positivos (caldo de cultivo) y negativos (lejía). Cada solución fue probada para detectar la presencia de bacterias viables contando el número de unidades formadoras de colonias (UFC/ml) a varios tiempos. Las soluciones también se examinaron con microscopía de alta resolución para contar el número de bacterias visibles en cada tiempo.
Resultados – *Staphylococcus pseudintermedius* fue capaz de sobrevivir en soluciones de tinción Quick Dip® limpias y contaminadas durante al menos una hora, pero a las 24 h no quedaron bacterias viables. No hubo supervivencia de bacterias en el fijador en ningún momento. *Staphylococcus pseudintermedius* permanecieron visibles con microscopía de alta resolución hasta 2 semanas en todas las soluciones orgánicamente contaminadas de la batería Quick Dip®.
Conclusiones y importancia clínica – *Staphylococcus pseudintermedius* sólo permanece viable en eosina y azul de metileno por períodos cortos de tiempo, pero la visibilidad prolongada de los organismos muertos podría teóricamente crear un diagnóstico erróneo de las muestras citológicas.

Zusammenfassung

Hintergrund – Färbelösungen, die regelmäßig zur Diagnose am Patienten Verwendung finden, um bakterielle und Pilzinfektionen rasch zu identifizieren, können während der Objektträger eingelegt wird mit gewöhnlichen pathogenen Keimen verunreinigt werden, wie z.B. mit *Staphylococcus pseudintermedius*.
Hypothese/Ziele – Eine Bestimmung, ob die Inokulation mit *S. pseudintermedius* in modifizierten Romanowsky Färbungen (Quick Dip®) in einer unterschiedlichen bakteriellen Kontamination resultiert und ob diese durch die Zugabe von organischem Material (Haar und Haut von Hunden) verstärkt wird.
Methoden – Ein klinisches Isolate eines *S. pseudintermedius* wurde in saubere und organisch kontaminierte Quick Dip Lösungen (Methanolfixierung, Eosin, Methylenblau) inokuliert, sowie in eine Positivkontrolle (Brühe) und eine Negativkontrolle (Bleiche). Jede Lösung wurde auf das Vorkommen verschiedener Bakterien getestet, indem die Anzahl der Kolonie-bildenden Einheiten (CFU/mL) zu verschiedenen Zeitpunkten gezählt wurden. Die Farblösungen wurden im Hochleistungsmikroskop betrachtet, um die Anzahl der sichtbaren Bakterien zum jeweiligen Zeitpunkt zu bestimmen.
Ergebnisse – *Staphylococcus pseudintermedius* konnte mindestens eine Stunde in einer sauberen und verunreinigten Quick Dip® Färbung überleben, aber nach 24h gab es keine überlebensfähigen Bakterien mehr. Das Überleben der Bakterien wurde in der Fixierlösung zu keinem Zeitpunkt ermöglicht. *Staphylococcus pseudintermedius* blieb im Hochleistungsmikroskop bis zu 2 Wochen lang in allen Quick Dip® Lösungen, die mit organischem Material kontaminiert waren, sichtbar.
Schlussfolgerungen und klinische Bedeutung – *Staphylococcus pseudintermedius* bleibt nur in Eosin und Methylenblaulösungen für kurze Zeit überlebensfähig, aber die langfristige Sichtbarkeit der toten Organismen könnte theoretisch zur falschen Interpretation der zytologischen Proben führen.

要約

背景 – 細菌および真菌感染症を迅速に院内診断するために定期的に使用される染色液は、スライド浸漬中に*Staphylococcus pseudinterinterus*などの一般的な病原体によって汚染される可能性がある。

仮説/目的 – *S. pseudintermedius*を改変Romanowsky染色(Quick Dip)に接種することにより、生存細菌による汚染がもたらされるかどうか、およびそれが有機屑(犬の被毛や皮膚)添加の影響を受けるかどうかを調べること。

方法 – *S. pseudintermedius*の臨床分離株を、クリーンだが有機屑に汚染されたQuick Dip溶液(メタノール固定剤、エオシン、メチレンブルー)、陽性コントロール(培養液)および陰性コントロール(漂白剤)に接種した。様々な時点でのコロニー形成単位数(CFU / mL)を測定することにより、各溶液の生存細菌の有無について調べた。また、溶液を高倍率顕微鏡下で検査して、各時点での可視細菌数を計測した。

結果 – *Staphylococcus pseudintermedius*は、クリーンで汚染されたQuick Dip染色液中で少なくとも1時間は生存することができたが、24時間までに生存した細菌はいなかった。いずれの測定時点においても、固定中に生存細菌は認められなかった。有機屑に汚染されたすべてのQuick Dip溶液中について、*Staphylococcus pseudintermedius*は最大2週間にわたって高倍率顕微鏡下で観察可能のままであった。

結論および臨床的な重要性 – *Staphylococcus pseudintermedius*は、エオシンおよびメチレンブルー溶液中で短期間のみ生存可能であった。しかし、死んだ細菌が長期間観察可能であったことより、理論的には、細胞診の誤診につながる可能性がある。

摘要

背景 — 快速鉴定细菌和真菌感染的染液,经常用于临床诊断。在玻片浸泡过程中,可能会被常见病原菌污染,如假中间型葡萄球菌。

假设/目的 — 探讨改良的罗曼诺夫斯基染液(Quick Dip®)接种假中间型葡萄球菌后,是否会导致活菌污染;以及这种污染是否受有机碎片(如犬的毛发和皮肤)的影响。

方法 — 将临床分离的假中间型葡萄球菌的菌株,接种到清洁的和有机物污染的Quick Dip溶液中(包括甲醇固定剂、曙红、亚甲蓝),并设置阳性(肉汤)和阴性(漂白剂)对照。在不同时间点计数菌落形成单位(CFU/ml),测试每种溶液中的存活菌数。在高倍显微镜下观察,计算各种溶液在每个时间点的可见细菌数量。

结果 — 假中间型葡萄球菌能够在清洁的和污染的Quick Dip染液中存活至少1小时,但没有活菌能维持到24小时。在任何时间点,固定剂中均不存在活菌。在高倍显微镜下观察,假中间型葡萄球菌在所有有机物污染的Quick Dip溶液中均保持可见,且长达2周。

结论与临床意义 — 假中间型葡萄球菌在曙红和亚甲蓝溶液中,仅在短时间内保持存活;但死亡菌体长期可见,理论上可能会导致细胞学检测样本的误诊。

Resumo

Contexto – Os corantes rotineiramente utilizados para identificação rápida e ambulatorial de infecções fúngicas e bacterianas podem se tornar contaminados por patógenos comuns, tais como o *Staphylococcus pseudintermedius*, durante a imersão da lâmina.

Hipótese/Objetivos – Determinar se a inoculação de *Staphylococcus pseudintermedius* em corantes do tipo Romanowsky modificadas (Quick Dip®) resulta em contaminação por bactérias viáveis e se isto pode ser influenciado pela adição de debris orgânicos (pele e pelos de cães).

Métodos – Um isolado clínico de *S. pseudintermedius* foi inoculado em soluções de Quick Dip® (fixador de metanol, eosina, azul de metileno) limpas e contaminadas por matéria orgânica, e em controles positivos (meio de cultura) e negativos (hipoclorito de sódio). Cada solução foi testada para a presença de bactérias viáveis a partir da contagem do número de unidades formadoras de colônias (UFC/Ml) em vários tempos. As soluções foram também examinadas em microscópios de alta magnificação para a contagem do número de bactérias visíveis em cada tempo.

Resultados – *Staphylococcus pseudintermedius* foi capaz de sobreviver nos corantes de Quick Dip® limpos e contaminados por matéria orgânica por ao menos uma hora, mas em aproximadamente 24 horas, as bactérias não se mantiveram viáveis. Não foi possível a sobrevivência das bactérias na solução fixadora em nenhum tempo avaliado. *S. pseudintermedius* permaneceu visível ao microscópio de alta magnificação por até duas semanas em todas as soluções contaminadas do kit Quick Dip®.

Conclusões e importância clínica – *Staphylococcus pseudintermedius* somente se mantém viável em eosina e azul de metileno por curtos períodos de tempo, mas a visibilidade prolongada de microrganismos mortos poderia, teoricamente, levar ao diagnóstico errôneo de amostras de citologia.

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RESEARCH ARTICLE

Bioluminescent murine models of bacterial sepsis and scald wound infections for antimicrobial efficacy testing

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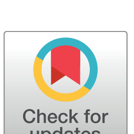

OPEN ACCESS

Citation: Ogunniyi AD, Kopecki Z, Hickey EE, Khazandi M, Peel E, Belov K, et al. (2018) Bioluminescent murine models of bacterial sepsis and scald wound infections for antimicrobial efficacy testing. PLoS ONE 13(7): e0200195. https://doi.org/10.1371/journal.pone.0200195

Editor: Michael R. Hamblin, Massachusetts General Hospital, UNITED STATES

Received: February 1, 2018

Accepted: June 7, 2018

Published: July 16, 2018

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Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: This study was funded by Australian Research Council (ARC; arc.gov.au) Linkage Project LP130100736 to SWP and DJT, with Luoda Pharma as the Partner Organization, and by an ARC Linkage grant (LP110200770) to SWP and DJT, with Neoculi Pty Ltd as the Partner Organization. The funders had no role in study design, data collection and analysis, decision to

Abstract

There are very few articles in the literature describing continuous models of bacterial infections that mimic disease pathogenesis in humans and animals without using separate cohorts of animals at each stage of disease. In this work, we developed bioluminescent mouse models of partial-thickness scald wound infection and sepsis that mimic disease pathogenesis in humans and animals using a recombinant luciferase-expressing *Staphylococcus aureus* strain (Xen29). Two days post-scald wound infection, mice were treated twice daily with a 2% topical mupirocin ointment for 7 days. For sepsis experiments, mice were treated intraperitoneally with 6 mg/kg daptomycin 2 h and 6 h post-infection and time to moribund monitored for 72 h. Consistent bacterial burden data were obtained from individual mice by regular photon intensity quantification on a Xenogen IVIS Lumina XRMS Series III biophotonic imaging system, with concomitant significant reduction in photon intensities in drug-treated mice. Post-mortem histopathological examination of wounds and bacterial counts in blood correlated closely with disease severity and total flux obtained from Xen29. The bioluminescent murine models provide a refinement to existing techniques of multiple bacterial enumeration during disease pathogenesis and promote animal usage reduction. The models also provide an efficient and information-rich platform for preclinical efficacy evaluation of new drug classes for treating acute and chronic human and animal bacterial infections.

publish, or preparation of the manuscript. The Partner Organizations (Luoda Pharma and Neoculi Pty Ltd) provided support in the form of salary for SWP but did not have any additional role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. The specific roles of SWP are articulated in the 'author contributions' section.

Competing interests: I have read the journal's policy and the authors of this manuscript have the following competing interests: Dr Stephen W Page is Director of Luoda Pharma, Caringbah, NSW 2229, Australia and Neoculi Pty Ltd, Burwood, VIC 3125, Australia. This does not alter our adherence to PLOS ONE policies on sharing data and materials.

Introduction

Infection of skin wounds and sepsis caused by pathogenic bacteria that are resistant to multiple classes of antimicrobials account for massive morbidity and mortality in humans and animals worldwide [1–3], with coagulase-positive *Staphylococcus* spp. a leading cause [4–7]. The increasing global prevalence and spread of clinically-relevant antibiotic-resistant bacterial pathogens, particularly methicillin-resistant *S. aureus* (MRSA) in hospitals, among hospital workers, veterinarians and within the community is of major public health concern and poses significant impact on health-care costs in many countries [2, 8–11]. Therefore, new drug classes are urgently needed to address this problem. However, animal models for detailed investigation of disease progression in a scenario that mimic bacterial infections in animals and humans often involve using separate cohorts of animals at each stage of the disease process. These assays are laborious, time consuming and involve costly microbiology techniques and materials required for harvesting, plating and enumeration of bacteria derived from infected mice, which are sometimes inconsistent from one day to another.

The development of reproducible and reliable animal infection models that will lead to reduction and refinement of animal usage as well as having the potential to reduce labour and material costs is a significant advance, allowing efficient preclinical evaluation of the efficacy of new drug classes for treating acute and chronic bacterial infections in humans and animals. Bioluminescence is a powerful technique that has been used widely as a reporter system in various *in vitro* and *in vivo* studies, including evaluation of acute and chronic bacterial infections and investigation of antibacterial activities of antibiotics and detection of bacterial resistance to antimicrobials [12–17]. As part of our novel antimicrobial discovery program, reliable and consistent animal infection models to assess preclinical efficacy of drugs designed to treat acute sepsis and/or chronic skin wounds, are of paramount importance. Accordingly, we have developed and optimised bioluminescent models of partial-thickness scald wounds and sepsis in mice by infection with a recombinant luciferase-expressing *Staphylococcus aureus* strain (Xen29) [18].

Materials and methods

Bacterial strain and growth conditions

For this study, a bioluminescent derivative of *S. aureus* ATCC12600 carrying a modified *lux* operon from *Photorhabdus luminescens* (Xen29) [18], purchased from PerkinElmer, was used. The strain was routinely cultured on Horse Blood Agar (HBA) plate supplemented with 200 μg/ml kanamycin and incubated at 37˚C for 18 h before being subcultured into Luria Bertaini (LB) broth supplemented with 200 μg/ml kanamycin and grown to A_{600} = 0.5 (equivalent to approx. 1.5×10^8 colony-forming units (CFU)/ml). Bacteria at this density were centrifuged, washed twice in phosphate-buffered saline (PBS) and resuspended to the appropriate density for scald wound infections or sepsis experiments.

Ethics statements

For partial-thickness scald injury experiments, 6- to 8-week-old male BALB/c mice, weighing between 20 g to 22 g, were used. For sepsis experiments, outbred 5 to 6-week-old male CD1 (Swiss) mice (weighing between 25 g to 32 g), were used. Mice had access to food and water ad libitum throughout the experiments. All mice were obtained from the Laboratory Animal Services breeding facility of the University of Adelaide. The Animal Ethics Committee of The University of Adelaide (approval numbers S-2015-150 and S-2015-151) reviewed and approved all animal experiments. The study was conducted in compliance with the Australian

Code of Practice for the Care and Use of Animals for Scientific Purposes (8th Edition 2013) and the South Australian Animal Welfare Act 1985.

Partial-thickness scald injury and infection experiments

A 69 mm^2, second-degree, partial thickness burn was created on the dorsal skin of mice as described previously [19] and by a modification of the procedure of Bjorn *et al.* [12], as follows: Mice were anaesthetised by intraperitoneal (IP) administration of xylazine (10 μg/g; Ilium), ketamine (100 μg/g; Ilium) and buprenorphine (0.05 μg/g; Reckitt Benckiser; for pain relief) in 500 μl of 0.9% saline. The dorsum of each mouse was shaved and then placed into a water tight container with the exposed dorsal skin sealed against an aperture using a rubber grommet to prevent leakage and then partially submerged (with breathing holes above the water) into a 65˚C water bath for 45 seconds. This was followed by partially submerging the mouse in a cold running water bath (15˚C) for 45 seconds to stop the burning process. This procedure creates a highly reproducible, partial thickness burn characterised by the appearance of a blister, oedema and the absence of tissue damage to the underlying fascia and muscle tissues. To minimise pain and discomfort post-procedure, buprenorphine was administered 12 h and 24 h post-procedure. In addition, 500 μl of 0.9% saline was administered IP to mice that showed weight loss at 24 h post-infection. Digital images of wounds were taken daily for macroscopic analysis of burns using optimized protocols [19].

Mice were placed in individually ventilated cages as 3 treatment groups comprising 6 mice per group, as follows: (i) wounded but not infected; (ii) wounded and infected at day 2 post-wounding with bioluminescent *S. aureus* Xen29 (1×10^7 CFU in 10 μl PBS) but not treated, or (iii) wounded and infected at day 2 post-wounding with Xen29 (1×10^7 CFU in 10 μl PBS) and then treated from 24 h post-infection, as described below). All mice were imaged immediately after infection on a Xenogen IVIS Lumina XRMS Series III live animal biophotonic imaging system (Caliper Life Sciences). Signals were collected from a defined region of interest (ROI) using the contour ROI tool and total flux intensities (photons/s) analyzed using Living Image Software 4.5. Starting from 24 h post-infection, one group of infected mice were treated twice daily with a total of 200 μl of a 2% mupirocin (as Bactroban®) ointment (equivalent to 4 μg of mupirocin) for 7 days and all mice subjected to bioluminescence imaging daily to quantify bacterial burden. Digital photographs of infected wounds were taken daily and analyzed for macroscopic healing of wounds using the ImageProPlus program (Media Cybernetics Inc., Bethesda, MD, U.S.A.). Power analysis was performed targeting 20% reduction in bacterial luminescence and wound area which would be considered biologically significant and clinically relevant; a sample size of 6 executes this protocol with 95% power using the statistics package G Power 3.1.7.

At the conclusion of the experiment (8 days after commencement of mupirocin treatment), 100 μl blood was withdrawn from each mouse by submandibular bleeding, mice were humanely killed and wounds were collected. Half of each wound was resuspended in 200 μl PBS, vortexed rigorously 3 times over 10 minutes and serially diluted in PBS to assess bacterial load by plating on LB+kanamycin agar. The remaining half was formalin fixed and processed using routine protocols for use in histological assessment of bacterial load and wound healing using Haematoxylin and Eosin stain. Microscopic wound length was determined following standardized methods of manually measuring the distance between wound margins by drawing below the epidermis or clot between the burn wound margins. Microscopic dermal wound gape was determined by measuring between the dermal wound margins. The percentage of the burn wound that had re-epithelialized was determined by measuring the portions of the wound that were covered with epidermis as a percentage of the entire wound. Blinded

measurements of histological slides by two independent assessors were performed. Gram stain of the histological section at end point of the experiment also confirmed presence of *S. aureus* in infected healing burn wounds following established protocols.

Sepsis experiments

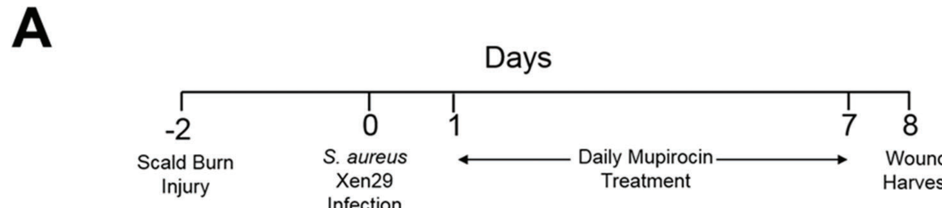
In order to obtain the optimal challenge dose for *S. aureus* sepsis, four groups of Swiss mice (n = 3 per group were initially challenged IP with approx. 5×10^6 CFU, 1×10^7 CFU, 2.5×10^7 CFU, or 5×10^7 CFU of *S. aureus* Xen29 in 200 μl PBS containing 3% porcine gastric mucin type III (Sigma Aldrich; Cat No M1778) over a 12 h period. The mice that received 2.5×10^7 CFU of Xen29 produced consistent and reliable infection over the 12 h period; infection of mice with the lower doses were inconsistent, while mice that received the 5×10^7 CFU dose succumbed to infection rapidly and became moribund within 6 h (data not shown). Therefore, the 2.5×10^7 CFU dose was chosen as the optimal dose for subsequent experiments.

For the sepsis challenge and drug treatment experiments, two groups of Swiss mice (n = 6 mice per group) were challenged IP with approx. 2.5×10^7 CFU of Xen29. At 2 h post-infection, approx. 50 μl of blood was withdrawn from the submandibular plexus of all mice for bacterial enumeration after which they were subjected to bioluminescence imaging in both ventral and dorsal positions on the IVIS Lumina XRMS Series III system. Immediately thereafter, group 1 mice received only the drug vehicle (20% (v/v) DMSO in PEG400) IP, while group 2 received daptomycin (as cubicin) at 6 mg/kg IP prepared in drug vehicle. The clinical conditions of all mice were closely monitored, and at 4 h and 6 h post-infection, approx. 50 μl blood was again withdrawn, followed by bioluminescent imaging. After imaging at 6 h post-infection, a second dose of drug vehicle or daptomycin was administered. Mice were further monitored frequently for signs of distress and those that had become moribund or showed any evidence of distress were humanely euthanized by cervical dislocation. At 10 and 16 h post-infection, living mice were further subjected to bioluminescent imaging. In all experiments, signals were collected from a defined ROI using the contour ROI tool and total flux intensities (photons/s) analyzed using Living Image Software 4.5. Correlation of bioluminescence with bacterial CFU in blood at 2 h, 4 h and 6 h post-infection was assessed by the Spearman rank test using Prism GraphPad 7.0c software. Differences in median survival times (time to moribund) for mice between groups were analyzed by the log-rank (Mantel-Cox) tests. Differences in luminescence signals between groups were compared by multiple *t*-tests.

Results

For both partial-thickness scald injury and sepsis experiments, consistent and reproducible bacterial burden data were obtained from individual mice by regular quantification of photon intensity on a Xenogen IVIS Lumina XRMS Series III live animal biophotonic imaging system. The bacterial burden data were also consistent with bacterial viable counts. A timeline representative of the scald injury experiments is shown in Fig 1A.

For the scald injury experiment, significant reduction in photon intensities in mupirocin-treated mice became apparent 2 days post-treatment (day 4) and reduced to background levels from 4 days post treatment (day 6), which remained until conclusion of the experiment (Fig 1B and 1C). Post-mortem analyses showed the bacterial burden in wounds correlated strongly with total flux obtained from bioluminescent signals of Xen29 (Fig 1D). Representative digital images of healing wounds are shown in Fig 2A, illustrating reproducible wounds at day 0 of the experiment. As a result of scalding the circular wounds developed a white eschar with a surrounding hyperemic zone. Surface wound area was measured in all groups using planimetric analysis of digital images (Fig 2A). As expected, the appearance of non-infected burn



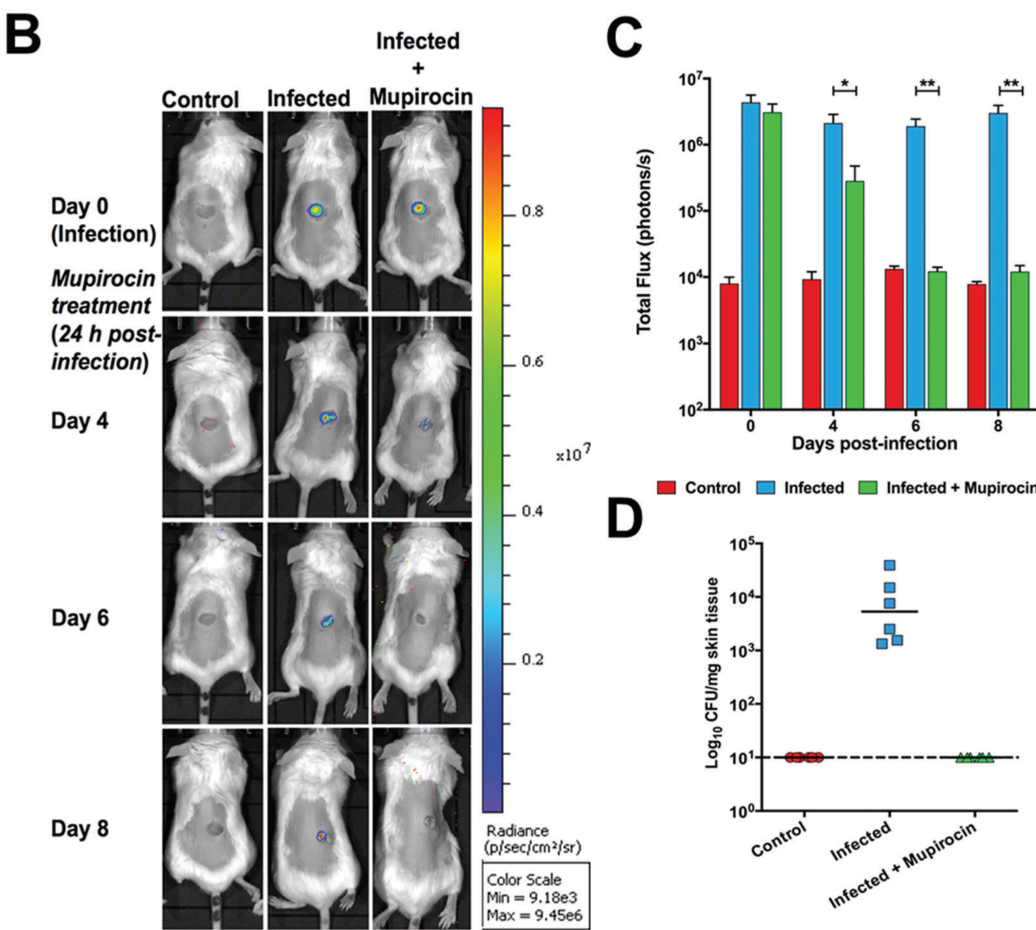
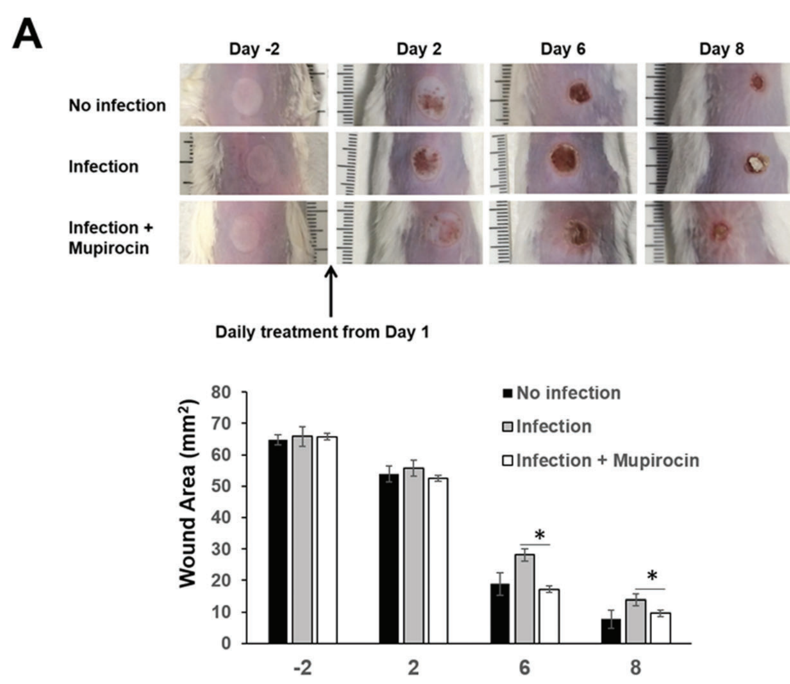


Fig 1. Biophotonic imaging of burn wounds. (**A**) Timeline of scald injury experiments. (**B**) Dorsal images of representative BALB/c mice challenged with approx. 1×10^7 CFU of bioluminescent *S. aureus* ATCC 12600 (Xen29). Mice were subjected to bioluminescent imaging on IVIS Lumina XRMS Series III system at the indicated times. (**C**), Quantification of photon intensities of bacterial burden showing significant reduction in photon intensities in mupirocin-treated mice. Total flux (means±SEM photons/s; n = 6 mice). $^{*}p<0.05$; $^{**}p<0.01$; multiple *t*-tests). (**D**), Total bacterial counts from tissues of control, infected but not treated, and infected + mupirocin-treated mice at the conclusion of the experiment, showing strong correlation with total photon intensities obtained from each treatment group.----- denotes limit of detection; —— denotes geometric mean counts.

https://doi.org/10.1371/journal.pone.0200195.g001



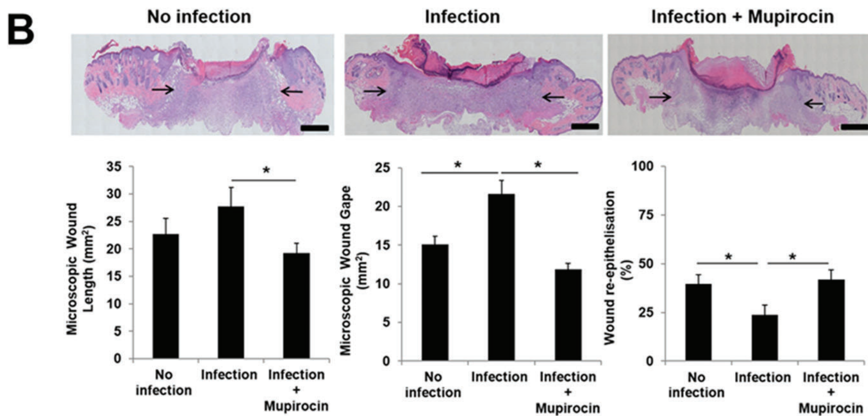


Fig 2. Analysis of wound healing in non-infected, infected but not treated, and infected + mupirocin-treated scald burn wounds. (**A**), Representative images and graphical analysis of scald wounds over a time-course of 10 days illustrating macroscopic differences in rate of healing between non-infected, infected but not treated, and infected + mupirocin-treated mouse scald wounds. (**B**), Representative haematoxylin and eosin-stained sections of partial-thickness scald wounds in mice with non-infected, infected but not treated, and infected + mupirocin-treated wounds. Microscopic analysis of scald wounds at day 10 post-wounding suggests that mupirocin treatment of scald wounds is effective in treating *S. aureus* infected wounds leading to significantly decreased wound length, dermal gape and significantly increased wound re-epithelisation compared to infected wounds. Arrows indicate dermal wound gape distance. Magnification ×4 stitched image. Scale bar = 100 μm. Results represent means and SEM, n = 6 wounds per mice group with a single time-point.

https://doi.org/10.1371/journal.pone.0200195.g002

wounds was clinically superior with decreased inflammation and quicker wound re-epithelization of wounds from day 6 of the experiment. Infection of burn wounds with *S. aureus* slowed down the rate of wound healing, as illustrated by larger macroscopic wound area at day 6 of the experiment and presence of the scab at day 8 macroscopically, however no statistical significance was observed in wound length between infected and non-infected burn wounds on microscopic assessment of histological sections (Fig 2A and 2B). The main indicators of delayed wound healing in infected burn wounds were significantly increased dermal wound gape and significantly reduced wound re-epithelization compared to both non-infected and infected and mupirocin treated wounds at day 8 of the experiment (Fig 2A and 2B). Infected and mupirocin treated wounds appeared paler however more raised at later time-points of the experiment. Macroscopic assessment of mupirocin treated wounds showed significant improvement in healing with smaller wound area macroscopically at both day 6 and 8 of the experiment (Fig 2A). In agreement with these findings, histological assessment of these wounds revealed significantly smaller wound length and dermal gape and higher rate of wound re-epithelization compared to infected burn wounds, and appeared similar to the healing profile of non-infected control mice (Fig 2B). These findings suggest mupirocin treatment is effective in improving the healing of *S. aureus* infected partial thickness scald wounds.

The robustness of the bioluminescent model to measure the therapeutic potential of systemically-administered drugs was assessed in an IP sepsis model using *S. aureus* Xen29 challenge and subsequent daptomycin treatment, as described in Methods. In these experiments, treatment of mice with 6 mg/kg daptomycin at 2 and 6 h post-Xen29 challenge significantly reduced bacterial burden (Fig 3) and total flux (Fig 4A). The loss of photon counts correlated with 100% survival of mice (Fig 4B) and complete bacterial clearance from blood (Fig 4C). Strong correlation of bacterial counts with total photon intensities was obtained at 4 h ($p<0.05$) and 6 h ($p<0.01$) from each treatment group (S1 Fig).

Discussion and conclusions

Bioluminescent models have been used widely to evaluate the efficacy of antimicrobials in treating acute and chronic bacterial infections and to assess bacterial resistance to antimicrobials [13–15, 18, 20–22]. In this study, we have successfully used a well-described *S. aureus* strain ATCC12600 carrying a re-engineered *lux* operon from *P. luminescens* [18] to establish reliable working mouse models of bacterial scald wound infection and sepsis as robust *in vivo* assays to accurately mimic disease pathogenesis in animals and humans. The two models provide a platform for evaluating the efficacy of new drugs or immunotherapeutic agents against serious bacterial pathogens.

We previously developed the second-degree, partial thickness burn model described here [19]. This model results in approximately 7% of total body surface area burn, and closely represents human second-degree burns in clinical and pathologic aspects as healing is reliant on wound re-epithelization rather than contracture often seen with 3rd degree burn wounds in rodents [12, 23]. To our knowledge, this is the first *in vivo* report of real-time monitoring of *S. aureus* infection and treatment after a second-degree, partial thickness burn. The burn model can be reliably applied to evaluate efficacy of therapeutic agents in the healing evolution of deep second-degree burns (in which the dermis remains intact) without the risk associated with a full thickness skin defect.

The use of bioluminescent models to assess pathogenesis of *S. aureus* in deeper host tissues and for drug efficacy evaluation is also well documented. These include bioluminescent thigh [13, 24], intravenous [25] and IP [26] models. In this study, we have successfully optimised a similar working model of *S. aureus* IP sepsis and kidney infection for efficacy evaluation of new antibiotics in the pipeline of our novel antimicrobial program.

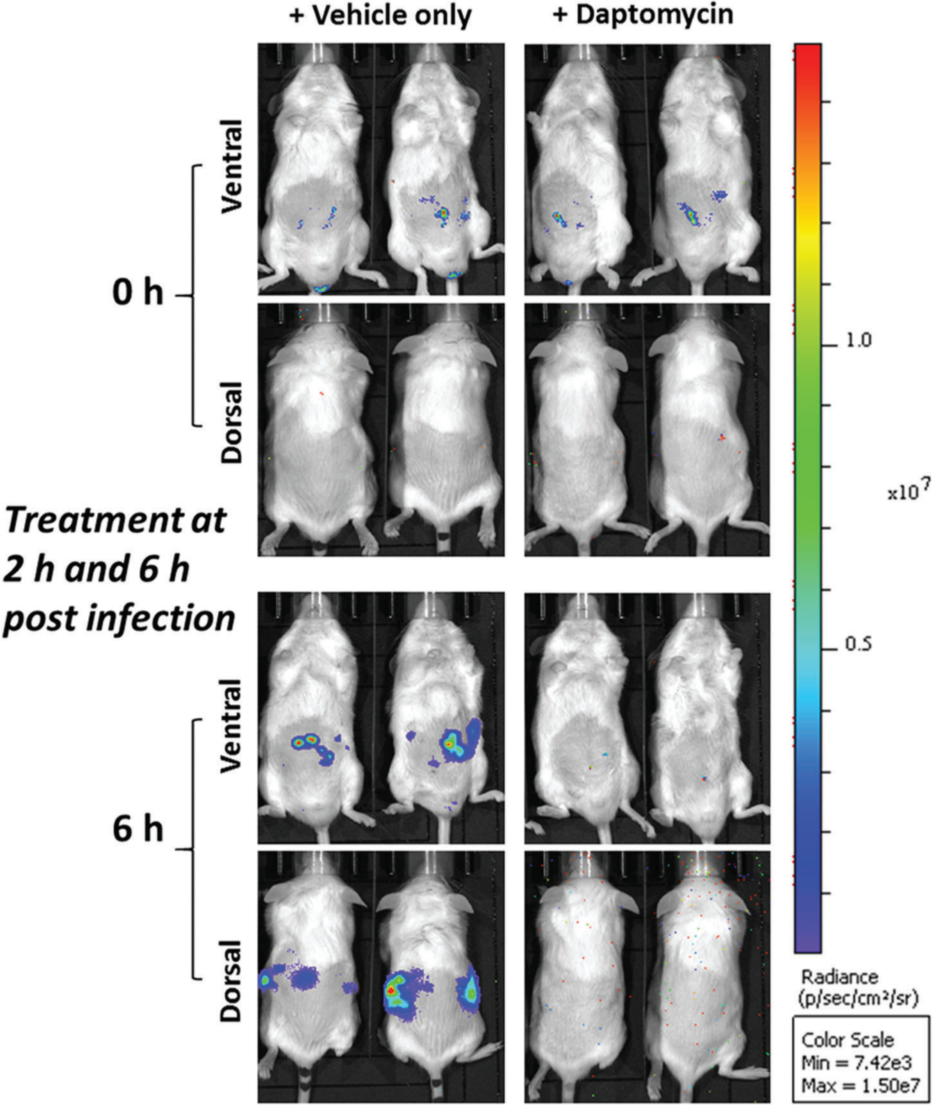
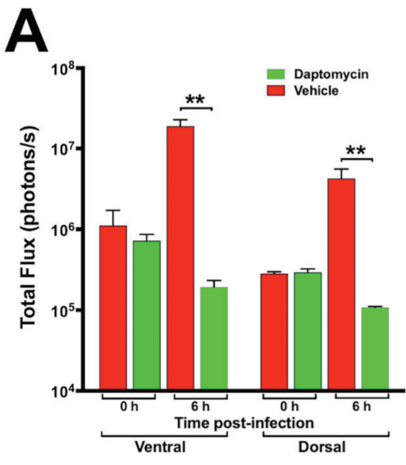
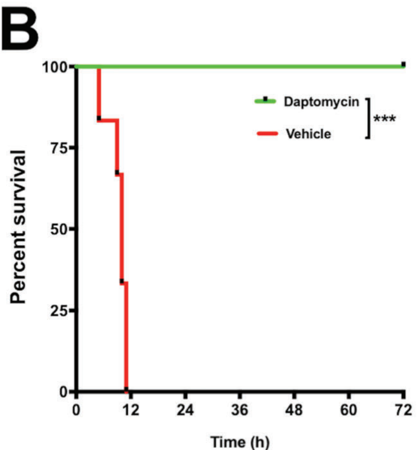


Fig 3. Biophotonic ventral and dorsal images of 2 representative CD1 male mice challenged IP with approx. 2.5 × 10^7 CFU of bioluminescent *S. aureus* ATCC 12600 (Xen29) and then administered the drug vehicle only or daptomycin IP at 2 and 6 h post-infection. Mice were subjected to bioluminescent imaging on IVIS Lumina XRMS Series III system at the indicated times.

https://doi.org/10.1371/journal.pone.0200195.g003

The bioluminescent models described here have several additional advantages over conventional methods, satisfying many aspects of humane animal experimentation. The models promote significant reduction in the number of mice required for long-term studies involving multiple sampling, thereby reducing labour, time and costs associated with harvesting, plating and bacterial enumeration for pathogenesis assessments. Bacterial photon emission from individually-infected mice are consistent and reproducible, and total flux correlates very strongly


A
Daptomycin
Vehicle
**
**
Total Flux (photons/s)
10^8
10^7
10^6
10^5
10^4
0 h
6 h
0 h
6 h
Time post-infection
Ventral
Dorsal


B
Daptomycin
Vehicle
***
Percent survival
100
75
50
25
0
0
12
24
36
48
60
72
Time (h)

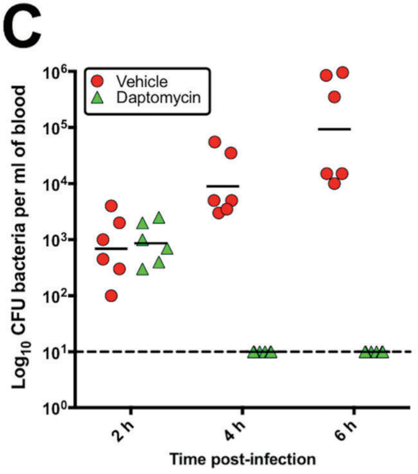

C
Vehicle
Daptomycin
Log10 CFU bacteria per ml of blood
10^6
10^5
10^4
10^3
10^2
10^1
10^0
2 h
4 h
6 h
Time post-infection

Fig 4. Luminescence signal comparison between groups of CD1 mice challenged IP with *S. aureus* ATCC 12600 (Xen29). (**A**), Quantification of photon intensities on a Xenogen IVIS Lumina XRMS Series III live animal biophotonic imaging system, showing significant reduction in photon intensities in daptomycin-treated mice. Total flux (means±SEM photons/s; n = 6 mice). $^{**}p<0.01$; multiple *t*-tests). (**B**), Survival times for CD1 male mice (n = 6) challenged IP with approx. 2.5×10^7 CFU of bioluminescent *S. aureus* Xen29 and administered the drug vehicle only or daptomycin (6 mg/kg) IP at 2 and 6 h post-infection. Differences in median survival times (time to moribund) for mice between groups were analyzed by the log-rank (Mantel-Cox) tests. ***, $P<0.001$. (**C**), Total bacterial counts from blood of control and infected + daptomycin-treated mice at 2, 4 and 6 h post-infection.----- denotes limit of detection; —— denotes geometric mean counts.

https://doi.org/10.1371/journal.pone.0200195.g004

with total bacterial burden. The fact that a single animal can be sequentially observed for the entire duration of an experiment is an important refinement to existing techniques by eliminating the inherent day-to-day variability in bacterial viable counts associated with random selection and sacrifice of mice. Therefore, the model described here allows for standardization and systematization of techniques and data collection necessary to underpin reliable and reproducible analysis.

There is a potential limitation to using bioluminescence to monitor chronic bacterial infections. Bioluminescence is a product of metabolic activity, as such bacteria that are in stationary phase, such as those in late stage biofilms may not emit sufficient light to establish a correlation with total colony-forming units. In this scenario, a combination of biophotonic imaging and bacterial enumeration at termination of experiments is likely to improve data interpretation.

In conclusion, bioluminescent models of bacterial sepsis and wound infection have been shown to be valuable research tools that can provide a more accurate representation of stages of infection and biofilm formation, thereby promoting better scientific understanding of disease pathogenesis. The models also support preclinical assessment of potential new wound therapies or novel antibacterial agents, and have reduced welfare implications for laboratory animals.

Supporting information

S1 Fig. *S. aureus* burden in blood of mice correlates with photon intensity. Correlation of bioluminescence with bacterial CFU counts in blood at 2 h, 4 h and 6 h post-infection was assessed by the Spearman rank test using Prism GraphPad 7.0c software. Positive correlation (r) and statistical significance were obtained at 4 h ($p<0.05$) and 6 h ($p<0.01$) post-infection. (TIFF)

Acknowledgments

We wish to thank Dr Agatha Labrinidis of the Centre for Advanced Microscopy and Microanalysis at Adelaide Microscopy, The University of Adelaide for assistance with *in vivo* bioluminescence imaging. We also wish to thank Ms Amanda Ruggero and Ms Krishna Kathawala at the University of South Australia for technical assistance and drug formulation, respectively.

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Resources: Sanjay Garg, Henrietta Venter, Stephen W. Page, Allison J. Cowin, Darren J. Trott.

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Journal of Pharmaceutical Sciences 108 (2019) 1007-1016

Contents lists available at ScienceDirect

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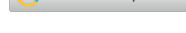
Pharmaceutical Nanotechnology

Formulation Optimization of Chitosan-Stabilized Silver Nanoparticles Using *In Vitro* Antimicrobial Assay



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ARTICLE INFO

Article history:
Received 30 April 2018
Revised 28 August 2018
Accepted 14 September 2018
Available online 19 September 2018

Keywords:
chitosan
silver
chitosan-silver nanoparticles
antibacterial activity
minimum inhibitory concentration
minimum bactericidal activity
biofilm
minimum biofilm eradication concentration assay

ABSTRACT

Antimicrobial resistance at the infected site is a serious medical issue that increases patient morbidity and mortality. Silver has antibacterial activity associated with some dose-dependent toxicity. Silver nanoparticles, due to larger surface area, have antibacterial properties, which make them useful in the treatment of infections. Chitosan-stabilized silver nanoparticles (CH-AgNP) were formulated and evaluated for minimal inhibitory concentration and minimal bactericidal concentration testing against *Staphylococcus aureus* ATCC 29213, *S aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922, and 20 methicillin-resistant *S aureus* isolates. Minimum biofilm eradication concentration study was used to evaluate the biofilm reduction, and *in vitro* antimicrobial checkerboard assays were performed. The effective optimum ratio of AgNP:chitosan solution was 1:4. Minimal inhibitory concentration and minimal bactericidal concentration ranges of CH-AgNP were 4 to 14 times lower compared to AgNP alone against methicillin-resistant *S aureus* isolates. Minimum biofilm eradication concentration values of CH-AgNP for ATCC PA-01, *P aeruginosa* isolate 1, and *P aeruginosa* isolate 2 were found to be >84.59 μg/mL, 42.29 μg/mL, and 21.15 μg/mL, respectively. Thus, CH-AgNP is a potential formulation for wound treatment and management of infected sites associated with antimicrobial resistance.

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Introduction

Globally, the emergence of bacterial resistance has highlighted an urgent need to combat the challenge of bacterial infection at wound sites. Antimicrobial resistance at the infected site is identified as a serious medical issue that increases morbidity as well as mortality. Moreover, it prolongs the period of hospitalization and has incurred much financial loss to the global economy. For example, methicillin-resistant *Staphylococcus aureus* (MRSA) infection accounts for delayed wound healing in human infections.[1] It is more prevalent in hospitals and nursing homes where people with open wounds and weak immune systems are more susceptible to the risk of hospital-acquired infection. Furthermore, bacterial biofilms, producing extracellular polymeric substance matrix,[2] which adds to the challenge for wound healing as biofilms are 100 times more tolerant to antimicrobial agents compared to bacteria in planktonic form.[3]

Antimicrobial agents arrest the growth or kill microorganisms on a surface or in an infected site. Usually, these compounds are used for different applications at a specific concentration, which retards microbial growth and directly kills bacterial cells.[4] Antimicrobial potency is possessed by both organic as well as inorganic chemical agents. Commonly used organic chemical agents include quaternary ammonium salts and chlorinated phenols. The inorganic antimicrobial agents involve the use of silver and copper ions. Before the introduction of antibiotics, silver-based preparations were majorly used for the prevention of skin infections during the early 20th century.[5,6] Advancement in nanotechnology plays an

Conflict of interest: The authors do not have conflict of interest. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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https://doi.org/10.1016/j.xphs.2018.09.011
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important role in treating these infections due to bacterial resistance. Nanoscale metal particles such as silver nanoparticles (AgNP) have emerged as novel therapeutic candidates because of their advantage in having larger surface area and exclusive antibacterial properties.[7]

Silver being a metal possesses a high melting point, making it suitable for high thermal processes over organic compounds.[8] Silver-based products with low concentrations of silver ions are readily available in the market.[9] Some of the examples of marketed preparations include creams, gels, wound dressings, catheters, dental material, medical devices, and implants.[10] Nanoparticles of silver possess excellent properties including surface-enhanced plasmon resonance effect, good chemical stability, and antimicrobial properties. The antibacterial properties of silver nanoparticles are dependent on their size and distribution.[11] Nanoparticles of silver can be prepared by the chemical reduction process[12] using agents such as citrate, glucose, ethylene glycol, or sodium borohydride. The antimicrobial effectiveness of nanoparticles is governed by parameters such as particle number, size, zeta potential, polydispersity index. Particle size and the surface chemistry play a significant role with respect to the type of biological response.[13] However, the mechanism of AgNP is not fully understood. Silver mainly interacts with the components of the bacterial cell wall, cytoplasm, and the r-DNA.[14-16] Stabilizing agents play a pivotal role in preventing the growth and accumulation of generated silver nanoparticles.[17] Different stabilizing agents such as polyvinyl alcohol, citrate, and chitosan at different concentration affects the size and shape of silver nanoparticles required for targeted drug delivery.[18] When chitosan interacts with silver nanoparticles, it can modify the surface chemistry of silver nanoparticles in terms of their charge, stage of agglomeration, and antimicrobial potential.[19,20] Formulation of silver-based nanoparticles linked with chitosan can provide higher therapeutic potential in the development of a wound healing formulation.

Chitosan, a naturally occurring polysaccharide is obtained from animal sources such as the exoskeleton of invertebrates including crustaceans, insects,[21] lobsters, krill and crabs, as well as scales of fish.[22] Chitosan is also biocompatible, biodegradable, haemostatic, anti-infective and acts as a wound healing accelerator.[23] Structurally, chitosan is a zwitterion with 1 amino group and 2 hydroxyl groups in the repeating glucosides residue.[24] Chitosan has a carbohydrate backbone resembling cellulose, involving β-1, 4-linked D-glucosamine moieties with variable degree of acetylation. Chemically, chitosan can be defined as a linear copolymer of β-(1–4) linked 2-acetamido-2-deoxy-β-D-glucopyranose and 2-amino-2-deoxy-β-D-glycopyranose, obtained after deacetylation of its parent polymer chitin.[25] The primary amino groups on the chitosan molecule offer high affinity sites for bonding in the presence of favourable conditions.[26] Chitosan due to its antifungal properties has potential in other industries such as the food industry.[27] Use of submicron chitosan dispersion maintains shelf life and quality during storage.[28] Chitosan in nano emulsion form shows better antifungal effects compared to its conventional form[29] and can be used as biofungicide.[30] Submicron chitosan dispersion acts as plant growth enhancer.[31] It enhances the production of plant defense related enzymes.[32,33] Moreover, it has been effectively used in extending the postharvest shelf life of fruits,[34]

Chitosan provides ample opportunities for research in the development of wound healing products.[35] Several researchers have worked on the antimicrobial potential of chitosan and proved that it inhibits growth of bacterial colonies. Silver also shows antibacterial activity associated with some dose dependent toxicity. Therefore, to compensate for the toxicity of silver nanoparticles, a combination of chitosan with silver nanoparticles was proposed in this study and antibacterial assays were undertaken against ATCC reference bacterial strains and 20 MRSA isolates. Antimicrobial assays of chitosan and silver have been undertaken independently, however, the combination of these 2 agents has not been explored. There is need for optimisation of chitosan and AgNP concentrations into a single formulation using *in vitro* antimicrobial checkerboard assays. Optimisation of chitosan stabilised silver nanoparticles (CH-AgNP) may also minimise side effects of silver and add the beneficial effects of chitosan. The aim of the present investigation was to formulate and optimise chitosan stabilised silver nanoparticles and to evaluate its antibacterial potential against ATCC strains, MRSA isolates and a biofilm producing strain of *Pseudomonas aeruginosa* (ATCC PA-01).

Materials and Methods

Materials

Silver nitrate ($AgNO_3$) (purity 99.7%) was purchased from Merck Specialities Pvt Ltd. (Mumbai, India). Sodium borohydride ($NaBH_4$) (purity ≥98%) was purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). Chitosan (high molecular weight) (degree of deacetylation ≥90%) was purchased from Sisco Research Laboratories Pvt Ltd. (Mumbai, India). Other chemicals including cation adjusted Mueller Hinton broth (CAMHB) and Tryptic soy broth (TSB) were purchased from Becton Dickinson Pty Ltd. (Cockeysville, MD). Phosphate buffer saline (PBS) was procured from Oxoid™ (Basingstoke, UK). Sheep blood agar (SBA) plates were purchased from Thermo Fischer Scientific (Melbourne, Victoria, Australia). Ninety-six well microtiter plates were purchased from Nunclon™ Delta Surface; Thermo Fisher Scientific (Roskilde, Denmark). MBEC™ biofilm inoculator was purchased from Innovotech (Edmonton, AB, Canada). Glacial acetic acid was procured from Chem-Supply Pty. Ltd. (Gillman, South Australia). Milli-Q water (Milli-Q assembly; Sartorius, Goettingen, Germany) used during the experimental procedure was prepared in-house using laboratory facility of the University of South Australia (Adelaide, Australia). All chemicals and reagents were used as received from the supplier without any prior modification.

Methods

Synthesis of Silver Nanoparticles by Chemical Reduction Method

Silver nanoparticles (AgNP) were formulated using the method as described by Kim et al.[36] with some modifications. Briefly, a 100 mL solution of 1 mM silver nitrate and 300 mL solution of 2 mM sodium borohydride were prepared in Milli-Q water. Both the solutions were chilled in a beaker at a temperature below 5°C and mixed manually. After mixing silver nitrate and sodium borohydride, silver ions were reduced and clustered together to form a transparent nanoparticle solution. The yellow colored solution gave absorption at 392 nm. The solution was stirred occasionally until stable yellow color was obtained. Using freeze-drying approach, AgNP in the solution form was converted into free-flowing powder. For this, the sample was kept in a deep freezer maintained at −80°C for 24 h (MDF-U74V-PE; Panasonic Healthcare Co., Ltd., Gunma, Japan). The sample was then freeze dried using a lyophilizer (Labconco) at −44°C and 8 × 10^{-3} M bar for 36 h. After freeze drying, the sample was weighed and dispersed with Milli-Q water with the aid of Ultrasonicator (Soniclean 500T; Soniclean Pty. Ltd., Thebarton, Australia) to achieve a stock solution with concentration of 38.279 μg/mL. The concentration of elemental silver was determined by inductively coupled plasma optical emission spectrometry (ICP-OES) (Optima 3300 RL; Perkin Elmer) with 1.5 mL/min sample flow rate, 20 s sample flush time and 2.5 mL/min sample flush rate.

Characterisation of Nanoparticles

UV-Visible Spectroscopy

The estimation of absorption of light due to color of AgNP was measured using ultraviolet-visible (UV-VIS) spectrophotometer (Thermo Fisher Scientific, Basingstroke, UK) by scanning over a wavelength ranging between 200 to 800 nm using a 1 cm quartz cuvette.

Transmission Electron Microscopy

The size and surface morphology of the AgNP were examined using transmission electron microscopy (TEM), (JEOL, JEM-2100F-HR; Jeol Ltd., Tokyo, Japan) and CH-AgNP was analyzed using TEM (Tecnai-20; Philips, Amsterdam, the Netherlands). The sample solution was placed over the TEM copper grid (Formvar with carbon coating on 200 mesh Copper) supplied by ProSciTech (QLD, Australia) and air dried overnight. The microscope equipped with a field emission gun was operated at accelerating voltage of 200 kV. Bright field images were recorded with Gatan Orius SC1000 (832). Images were captured using Gatan digital micrograph.

Dynamic Light Scattering/Photon Correlation Spectroscopy

Dynamic light scattering (DLS) also referred as photon correlation spectroscopy (PCS) is the most common method for particle size estimation in colloidal suspension. Particle size was measured using DLS method (SZ 100; Horiba Scientific, Tokyo, Japan) at 25°C and scattering angle of 90 degrees.[37]

Zeta Potential

The surface charge of the AgNP was evaluated by estimation of zeta potential (Zeta sizer Nano, ZS90; Malvern Instruments Ltd., Malvern, UK) at 25°C with the dual scattering angle using U shaped disposable zeta cell.

In Vitro *Antimicrobial Study*

Bacterial Strains and Growth Conditions

Twenty methicillin-resistant *Staphylococcus aureus* (MRSA) and 2 *Pseudomonas aeruginosa* isolates were used for the study. MRSA isolates were kindly provided by Prof G. Coombs (PathWest Laboratory Medicine, Murdoch, Western Australia) and these isolates represented the most common sequence types of both hospital-acquired (HA) and community-associated (CA) MRSA isolates. *Pseudomonas aeruginosa* clinical isolate was obtained from a case of canine otitis externa infection. American Type Culture Collection (ATCC) reference strains were used for preliminary testing and were included as controls during testing of clinical isolates. The Gram-positive bacterial strains used were *Staphylococcus aureus* ATCC 29213 and *Staphylococcus aureus* ATCC 25923. Gram-negative bacterial strains used were *Pseudomonas aeruginosa* ATCC 27853, *Pseudomonas aeruginosa* ATCC PA-01 (biofilm producer) and *Escherichia coli* ATCC 25922. Both clinical isolates and references strains were sub cultured using SBA plates and incubated at 37°C for 16 to 18 h.

Minimal Inhibitory Concentrations

Broth micro-dilution assays were performed as per the Clinical Laboratory Standards Institute (CLSI) guidelines.[38] Concisely, a bacterial suspension for each isolate was prepared in PBS and the turbidity of the solution was adjusted to 0.5 McFarland standard followed by 1:20 dilution. The stock solutions of AgNP, chitosan, physical mixture of chitosan solution and AgNP (PM) and CH-AgNP were 2-fold serially diluted with CAMHB in 96 well microtiter plates to achieve an antimicrobial concentration range of 0.075-38.28 μg/mL, 32-16,384 μg/mL, 0.198-101.5 μg/mL and 0.165-84.59 μg/mL, respectively. Finally, 10 μL of the bacterial suspension was added to achieve a final bacterial concentration of 5×10^5 colony forming unit per mL (CFU/mL) in the wells. All isolates and reference strains were tested in duplicates. Negative growth controls contained only CAMHB and positive growth controls contained CAMHB and bacterial suspension. Minimal inhibitory concentration (MIC) values were determined after overnight incubation at 37°C and recorded as the lowest concentration of each test solution which completely inhibited the growth of the microorganisms in the wells as observed visually.

Minimal Bactericidal Concentrations

Minimal bactericidal concentration (MBC) is defined as the lowest concentration of antimicrobial agent required to kill 99.95% of microorganism in the inoculum. From the microtiter plate used to determine MIC concentration, an aliquot of 10 μL was taken from the MIC wells as well as from each remaining well which did not show turbidity when observed visually. The solution was spotted over SBA plates and incubated overnight at 37°C. The number of colonies were counted on each spot and MBC was determined as per CLSI guidelines.[39] For an inoculum with the final concentration of 5×10^5 CFU/mL, the dilution of test solution with equal and less than 11 colonies in each 10 μL sample was concluded as the MBC value.

Synergy Testing Using Checkerboard Assay Method

Checkerboard assays were performed to find the interaction activity of the combination of AgNP and chitosan solution in 0.25% v/v acetic acid solution. ATCC reference strains were used in the assay method as a model. The bacterial suspension was prepared as per the method described previously for determination of MIC. A stock solution of chitosan solution and AgNP were prepared at a concentration of 16,384 μg/mL and 38.279 μg/mL, respectively. Chitosan solution and AgNP stock solutions were 2-fold serially diluted in CAMHB in 2 different 96-well microtiter plates. As per the standard protocol diluted AgNP and chitosan solution were then transferred to another microtiter plate to yield a mixture in ratio 1:1. Further, 10 μL of inoculum was added to the wells and incubated overnight at 37°C. MICs for chitosan solution, AgNP and the combination were determined. Fractional inhibitory concentration index (FICI) was calculated using the following formula (Eq. 1).

$$FICI = \frac{A}{MIC_A} + \frac{B}{MIC_B} \tag{1}$$

Where, A and B were the MICs of AgNP and chitosan respectively in the combination. MIC_A and MIC_B were the MICs of AgNP and chitosan alone, respectively. The antimicrobial agents are considered to have synergistic activity if the FICI value is less than 0.5. The effect is considered to be additive or partial synergistic, if the FICI value is more than 0.5 but less than or equal to 1.0 (FICI >0.5 but ≤1). The effects are considered to be indifferent when the value lies between 1.0 and 4.0. The agents are considered to possess antagonistic activity if the value of FICI is ≥4.0.[40] After FICI interpretation, the strain with synergistic or additive effect was used to determine the best possible combination ratio of AgNP and chitosan to synthesize the CH-AgNP.

Preparation of Physical Mixture Containing AgNP and Chitosan Solution

Chitosan stock solution (16,384 μg/mL) was prepared by dissolving accurately weighed chitosan in 1% v/v acetic acid solution maintained at 300 rpm using magnetic stirrer (MSH-30D; Thermoline Scientific, New South Wales, Australia) at room temperature. The physical mixture (PM) of AgNP (38.279 μg/mL) and chitosan solution (16,384 μg/mL) was prepared by mixing them in high speed vortex mixer for 5 min (Super Mixer; LAB-LINE instruments, Inc., Melrose Park, IL). One part of AgNP (38.279 μg/mL) was mixed with four part of chitosan solution (16,384 μg/mL). The

final stock concentration of PM was 101.05 μg/mL, determined by ICP-OES (Optima 3300 RL; Perkin Elmer).

Preparation of Chitosan Stabilised Silver Nanoparticles

Chitosan stabilised silver nanoparticles (CH-AgNP) were prepared as per the method described by Cavassin et al.[41] with some modifications. Briefly, 20 mL of chitosan solution having concentration 1 mg/mL was prepared in 1% v/v acetic acid solution. Thirty milliliter of 1 mM silver nitrate solution and 1 mL of 0.1 M sodium borohydride was prepared in Milli-Q water. Sodium borohydride solution was maintained at a temperature below 5°C. Silver nitrate solution was stirred at 300 rpm using magnetic stirrer (MSH-30D; Thermoline Scientific, New South Wales, Australia) at room temperature. Chitosan solution was added to this solution followed by sodium borohydride. The solution was stirred continually to obtain a yellow colored solution. The color change indicated the formation of chitosan stabilized silver nanoparticles. Final stock concentration was 84.586 μg/mL, determined by ICP-OES (Optima 3300 RL; Perkin Elmer). The developed formulation was characterized by particle size, zeta potential and was used for determination of MIC, MBC against 5 ATCC strains, 20 methicillin-resistant *Staphylococcus aureus* (MRSA) isolates obtained from G coombs and minimum biofilm eradication concentration (MBEC) assay was performed biofilm producer reference strain PA-01 and 2 *Pseudomonas aeruginosa* isolates (*P aeruginosa* isolate 1 and *P aeruginosa* isolate 2).

Minimum Biofilm Eradication Concentration Assay

The biofilm susceptibility testing was performed using methods previously described by Ceri et al.[42-44] Biofilm producer reference strain PA-01 and 2 *Pseudomonas aeruginosa* isolates (*P aeruginosa* isolate 1 and *P aeruginosa* isolate 2) which showed moderate to strong biofilm producing activity were used. Briefly, the bacterial inoculum was suspended in TSB and adjusted to a turbidity 1.0 McFarland standard. The solution was diluted to 1:30 and 150 μL of inoculum was dispensed into each well of MBEC™ biofilm inoculator. Biofilms were formed over the peg lid after an incubation of 24 h at 37°C in the shaking incubator at 100 rpm (Ratek orbital mixer incubator; Adelab, Thebarton, South Australia). The biofilm peg lid was rinsed with PBS and placed in 96 well microtiter plate containing AgNP, chitosan solution and CH-AgNP which were 2-fold serially diluted in CAMHB as the method described above in MIC section. After exposing the test samples for 24 h at 37°C, the peg lid was rinsed twice with PBS for 1-2 min and transferred to a fresh 96-well microtiter plate containing TSB to recover biofilms by sonication for 10 min. Optical density at 650 nm (OD_{650}) was read concurrently to obtain MIC and MBC, post challenge by the test samples and MBEC post biofilms recovery. Finally, the growth of biofilms was quantified by 10-fold serial dilutions of each well containing recovered biofilms. These dilutions were spotted in triplicates (10 μL) on SBA plates and incubated overnight at 37°C. The number of colonies on SBA plates was counted and CFU/mL was determined. A graph was plotted between the antimicrobial concentrations and Log_{10} transformed CFU/mL for each test sample. MBEC was the lowest concentration of antimicrobial agent required to eradicate the biofilm growth (CFU/mL = 0).

Results and Discussion

Characterization of the AgNP

The characteristic color of the colloidal silver nanoparticle solution was due to surface plasmon resonance. The clear yellow colored colloidal silver nanoparticle solution after the synthesis is shown in Figure 1a. Figure 1b shows its absorption band at 392 nm using UV-VIS spectroscopy. This absorption band is characteristic of spherical silver nanoparticles due to their surface plasmon resonance. Particle shape, size and the functional group significantly affect the surface plasmon resonance.[45-47] In general, as the particles become larger, the plasmon peak shifts to longer wavelengths and broadens.[48] The stability of the prepared solution was confirmed by measuring the absorption at up to 10 serial dilutions. The absorption spectra of the serially diluted solution represent equal absorption maxima at 392 nm as seen in Figure 1c compared to the spectra of the original yellow colored solution of silver nanoparticles. This confirms that the silver nanoparticles were not clustered together or agglomerated with other particles to form larger particles.

Particle size and zeta potential are essential characteristics of the nanoparticles.[49] Particle size was found to be 22.8 nm by DLS technique whereas particle size was found 11.8 nm by TEM analysis. The difference in size could be attributed to the fact that the DLS technique is not very effective in differentiation between a single particle or an agglomerate. In comparison, TEM analysis measures the diameter of a single particle. DLS measurement shows hydrodynamic diameter, whereas TEM measurement shows the size of dry particles.[50] Size and shape of the synthesized silver nanoparticles was characterized by TEM in Figure 2a, which indicated the spherical shape of nanoparticles with an average diameter of 11.8 nm nanoparticles that were also highly monodispersed. Thus, the particle size was considered to be 11.8 nm for AgNP. Zeta

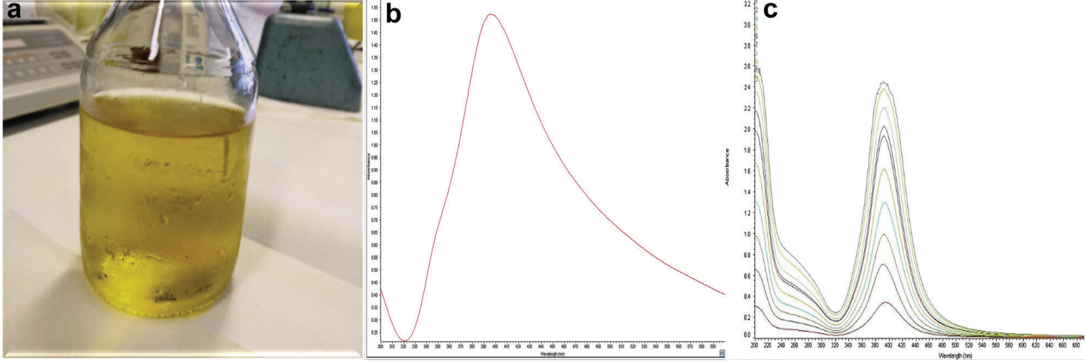

Figure 1. (a) Yellow-colored colloidal silver nanoparticle solution, (b) UV absorption spectra of silver nanoparticle solution at 392 nm, and (c) UV absorption spectra of diluted AgNP solution at 392 nm.

potential is an important parameter while considering the stability of the nanoparticles. A zeta potential value of -30.97 ± 0.8 mV indicated the stability of the nanoparticles. A zeta potential value of +30 to -30 mV is considered to reflect stability.[49]

Synergy Testing Using Checkerboard Assay Method

In the present study, the *in vitro* antimicrobial interaction activity between AgNP and chitosan solution was evaluated using the reference ATCC strains. Individual MIC and combination MIC for both AgNP and chitosan solution are shown in Table 1. Fractional inhibitory concentration index (FICI)[40] were calculated using formula 1. Based on the FICI value, it can be interpreted that there was no difference in inhibitory activity against *S aureus* ATCC 29213, *S aureus* ATCC 25923 and *P aeruginosa* ATCC 27853. But, an additive effect was seen in the case of *P aeruginosa* ATCC PA-01 and *E coli* ATCC 25922.

Sondi et al.[51] studied *E coli* using agar dilution and growth curve methods and showed that Gram-negative bacteria are more prone to positively charged AgNP. The negative charge in the lipopolysaccharide membrane of Gram-negative bacteria has a greater affinity to the positive charges of silver compared to the cell of membrane of Gram-positive bacteria, resulting in the production of cell membrane pores. Shrivastava et al.[52] studied 2 antimicrobial-susceptible strains, *S Aureus* ATCC 25923 and *E coli* ATCC 25922, and 2 antimicrobial-resistant strains, *E coli* and *S typhus* by agar dilution and growth curve methods to show improved antimicrobial activity of AgNP against Gram-negative bacteria as compared to Gram-positive bacteria.

It can be observed that there is an additive effect in the case of *P aeruginosa* ATCC PA-01 and *E coli* ATCC 25922. After FICI interpretation, the strain with synergistic or additive effects was used to find the best combination ratio of AgNP and chitosan solution to synthesize the CH-AgNP. Results showed that 1:1 to 1:16 of AgNP:chitosan showed an additive effect of AgNP and chitosan solution at a different ratio. The dose reduction index (DRI) for AgNP was in the range of 2 to 8 times. There was 4 to 8 times reduction in the dose when the ratio of AgNP:chitosan was in the range of 1:4 to 1:16. However, there was a 2 times dose reduction in the case of chitosan solution. Based on checkerboard assay,[53,54] 1:16 weight ratio of AgNP:chitosan showed the lowest dose reduction index (DRI). However, the amount of chitosan in 1:16 weight ratio produced a highly viscous solution which led to the handling issue in MIC testing as well negating further development of CH-AgNP. There was no difference in DRI value with 1:8 and 1:4 weight ratio of AgNP: Chitosan solution. Therefore, the weight ratio of 1:4 (AgNP: chitosan) was considered for the development of CH-AgNP.

Characterization of PM

The zeta potential of the PM was evaluated using nanopartica (SZ 100; Horiba Scientific). Zeta potential of PM was found to be +49.5 ± 2.05 mV. Positive zeta potential value indicates influence of chitosan in PM.

Characterization of CH-AgNP

Particle size and zeta potential of the CH-AgNP were evaluated using TEM (Tecnai-20; Philips) and nanopartica (SZ 100; Horiba Scientific) respectively. Particle size estimated by DLS was 27.1 nm where as it was found to be 11.9 nm by TEM for CH-AgNP as shown in Figure 2b. Particle size of CH-AgNP was found similar to that of AgNP as per TEM data. As discussed in characterisation of AgNP, TEM measurement was considered for estimation of particle size. The measurements of particle size confirm the development of particles in nano dimensions using optimised technique. Zeta potential value signifies the degree of electrostatic repulsion among the particles which in turn is responsible for the monodispersed particles. Zeta potential of CH-AgNP was found to be +50.1 ± 2.99 mV which was significantly higher than zeta potential value of AgNP with value -30.97 ± 0.8 mV. This could be due to positive charge of chitosan in CH-AgNP. This change in the zeta potential may be responsible for the difference in antimicrobial activity of formulation (CH-AgNP). The results of CH-AgNP conclude the nanoparticles in monodisperse system.

Determination of MIC and MBC

Table 2 shows the MIC values of AgNP was significantly lower as compared to chitosan solution for all reference strains. Moreover, the PM showed lower MIC and MBC values when compared to AgNP and the chitosan solution individually. The MBC value of AgNP was slightly higher than MBC of PM for *E coli* ATCC 25922. The MIC value for CH-AgNP was lower than PM except for *S aureus* ATCC 25923. All reference strains were most susceptible to CH-AgNP with lowest MIC range from 1.32 to 2.64 μg/mL.

Effect of AgNP against various microorganisms depends on several factors such as size, shape, stability and concentration of

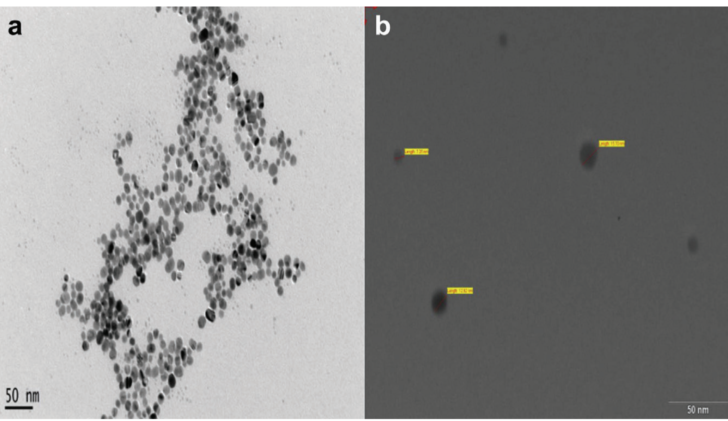

Figure 2. TEM image of (a) silver nanoparticles and (b) CH-AgNP on 200# copper grids.

Table 1
Results of Checkerboard Assay of AgNP and Chitosan Combination and Its Interpretation

Isolates	MIC Individual (μg/mL)		MIC Combination (μg/mL)		AgNP: Chitosan (Weight Ratio)	FICI	Interpretation	Dose Reduction Index (DRI)	
	AgNP	Chitosan	AgNP	Chitosan				AgNP	Chitosan
ATCC SA 29213	19.1395	1024	19.1395	1024	-	2	Indifference	1	1
ATCC SA 25923	9.57	512	9.57	512	-	2	Indifference	1	1
ATCC PA 27853	19.1395	1024	19.1395	1024	-	2	Indifference	1	1
ATCC PA-01	9.57	1024	1.196	512	1:16	0.62	Addition	8	2
			2.392	512	1:8	0.75		4	2
			4.785	256	1:2	0.75		2	4
ATCC EC 25922	19.1395	1024	9.57	256	1:1	0.75	Addition	2	4
			4.785	512	1:4	0.75		4	2

AgNP.[55] A previous study[56] reported that the MICs of chitosan nanoparticles loaded with silver nitrate for *E coli* ATCC 25922 and *S aureus* ATCC 25923 were 3 μg/mL and 6 μg/mL, respectively while our study used a comparatively similar formulation of CH-AgNP that yielded lower MIC values (from 1.32 to 2.64 μg/mL and 2.64 μg/mL, respectively). This lower MIC could be attributed to the lower particle size and the lower zeta potential of CH-AgNP compared to chitosan nanoparticle loaded with silver nitrate by Du et al.[56] having values 90.29 nm and +92.05 mV respectively. Antibacterial studies on Gram-positive bacteria reported by Li et al.[57] indicated that the MBC of AgNP for *S aureus* ATCC 6538P was 20 μg/ml. As the concentration was increased to 50 μg/mL with 6 to 12 h of exposure, it leads to DNA condensation followed by cell wall breakdown respectively and suggested the damaging effect on the bacterial cell membrane could be due to AgNP. The MBC value of AgNP against *E coli* ATCC 8739 was found by Li et al.[58] to be 10 μg/mL in antibacterial studies on Gram-negative bacteria. Bacterial cell membrane permeability was lost at 10 μg/mL of AgNP. Our study showed that MBC range of AgNP for Gram-positive ATCC strains was slightly higher than for Gram-negative ATCC strains with values of 9.57-19.14 μg/mL and 4.79-19.14 μg/mL, respectively. However, these were not significantly different. Comparison with the studies of Li et al.[57] and Li et al.,[58] showed that the MBC of AgNP for *S aureus* ATCC 6538P (Gram-positive) was 20 μg/mL and for *E coli* ATCC 8739 (Gram-negative) it was 10 μg/mL. The difference in the mechanism of action of AgNP against Gram-negative and Gram-positive bacteria is likely due to structural differences in the bacterial cell wall and cell membrane permeability.[59]

Determination of MIC and MBC Against MRSA Isolates

The present study evaluated the *in vitro* antimicrobial activity of AgNP, chitosan solution, PM and CH-AgNP against 20 MRSA isolates (Table 3).

Table 3 shows that MIC_{90} value of AgNP for all 20 MRSA isolates was significantly lower compared to chitosan solution. Moreover, the PM showed lower MIC values compared to AgNP and chitosan solution individually. The MIC value for CH-AgNP for all 20 MRSA isolates was the lowest compared to all other formulations. MIC values were increasing in the following order: CH-AgNP < PM < AgNP < chitosan solution. Thus, CH-AgNP was found to be most effective in inhibiting the growth of all 20 MRSA isolates. In this study, the MIC_{50} and MIC_{90} of CH-AgNP against MRSA isolates were both 2.64 μg/mL which was lower compared to the study by Cavassin et al.[41] where the MIC_{50} and MIC_{90} were 3.4 μg/mL and 6.7 μg/mL, respectively. Similarly, Ansari et al.[14] studied antimicrobial activities of AgNP against *S aureus* ATCC 25923, methicillin-sensitive *S aureus* (MSSA), and (MRSA) using 3 different methods. It was explained that irrespective of the bacterial resistance mechanism, AgNP showed MIC and MBC range of 12.5-50 μg/mL and 25-100 μg/mL respectively. The reason for lower MIC values in our study could be attributed to a lower particle size of 11.9 nm. In the study by Ayala et al.[60] antimicrobial effect of AgNP against MRSA was size dependent. Three different sizes of AgNP that is 10 nm, 30-40 nm and 100 nm were studied and MIC_{90} values of 10.79 mg/mL, 4.17 mg/mL and 1.37 mg/mL respectively were obtained. Based on this study, it was concluded that 10 nm particle size had better antimicrobial activity against MRSA irrespective of bacterial resistance mechanism and had a non-toxic effect on HeLa cell lines compared to the study by Ayala et al., the particle size of CH-AgNP in our study was found to be 11.9 nm and the MIC value of 2.64 μg/mL was obtained. Thus, it can be concluded that only particle size is not contributing to the lower MIC values but the additive effect from the combination of chitosan with AgNP could lead to lower MIC values along with the critical role of particle size.

From the results shown in Table 4, it can be observed that the MBC_{90} value of AgNP for all 20 MRSA isolates was significantly lower as compared to chitosan solution and PM. Whereas, individual MBC value of AgNP, chitosan solution, PM and CH-AgNP may vary because of the different characteristics of each bacterial strains. MBC_{90} value for CH-AgNP against all 20 MRSA isolates was the lowest compared to all other samples. MBC_{90} values increases in the order: CH-AgNP < AgNP < PM < Chitosan solution. Thus, CH-AgNP were found to be the most effective bactericidal agent against all 20 MRSA isolates. Therefore, it is clear from the results that CH-AgNP had a bactericidal effect against all 20 MRSA isolates.

Table 2
MIC and MBC Values of Silver Nanoparticles, Chitosan Solution, Physical Mixture of AgNP and Chitosan Solution, and Chitosan-Stabilized Silver Nanoparticles Against 5 ATCC Reference Strains

Reference Strains	AgNP (μg/mL)		Chitosan Solution (μg/mL)		PM (μg/mL)		CH-AgNP (μg/mL)	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
S aureus ATCC 29213	9.57	9.57	256	256	3.158	3.158	2.643	5.287
S aureus ATCC 25923	9.57	19.14	512	512	1.5789	1.5789-3.158	2.643	ND
P aeruginosa ATCC 27853	9.57	19.14	512	512	3.158	3.158	2.643	ND
P aeruginosa ATCC PA-01	9.57	ND	1024	8192	3.158	50.525	2.643	ND
E coli ATCC 25922	4.79	4.79	512	512	3.158	3.158	1.322-2.643	ND

ND, not determined.

Table 3
MIC Values of Each Test Sample Against 20 MRSA Isolates

Sr. No.	MRSA Isolates (MLST)	AgNP (μg/mL)	Chitosan Solution (μg/mL)	PM (μg/mL)	CH-AgNP (μg/mL)
1	AUS2-MRSA (ST239)	19.14	256-512	3.158	2.643
2	AUS3-MRSA (ST239)	19.14	256	3.158	2.643
3	Bengal Bay PVL$^+$-CA-MRSA (ST772)	38.28	256	1.5789	2.643
4	Classic-HA-MRSA (ST250)	38.28	256	1.5789	2.643
5	Irish 1-HA-EMRSA (ST8)	19.14-38.28	256	1.5789-3.158	2.643
6	Irish 2-HA-EMRSA (ST8)	19.14	256	3.158	2.643
7	NY Japan-HA-MRSA (ST5)	38.28	256	3.158	2.643
8	QLD PVL$^+$-HA-EMRSA (ST22)	19.14	256	1.5789-3.158	2.643
9	ST 398-CA-MRSA-V (ST398)	19.14-38.28	256	3.158	2.643
10	Taiwan PVL$^+$-CA-cMRSA (ST59)	19.14	256	3.158	2.643
11	UK 15-HA-EMRSA (ST22)	19.14	256	3.158	2.643
12	UK 15 PVL$^+$-HA-EMRSA (ST22)	19.14-38.28	256	3.158	2.643
13	UK 16-HA-EMRSA (ST36)	9.57-19.14	256	3.158	2.643
14	UK 17-HA-EMRSA (ST247)	19.14	256	3.158	2.643
15	USA 300-MRSA (ST8)	9.57	256	3.158	2.643
16	WA1-CA-MRSA (ST1)	9.57	256	3.158	2.643
17	WA2-CA-MRSA (ST78)	9.57	256	3.158	2.643
18	WA3-CA-MRSA (ST5)	9.57	256	3.158	2.643
19	WA 84-CA-MRSA (ST45)	19.14	256	3.158	2.643
20	WSSP-PVL negative-CA MRSA (ST30)	9.57	256	3.158	2.643
MIC range		9.57-38.28	256-512	1.5789-3.158	2.643
MIC_{50}		19.14	256	3.158	2.643
MIC_{90}		38.28	256	3.158	2.643

MRSA, methicillin-resistant *S aureus*; MLST, multilocus sequence type; PVL, panton-valentine leukocidin status; CA, community acquired; HA, health care—associated; EMRSA, epidemic methicillin-resistant *S aureus*.

Minimum Biofilm Eradication Concentration Study

MBEC study was performed using a biofilm producing reference strain *P aeruginosa* ATCC PA-01 and 2 *P aeruginosa* clinical isolates (*P aeruginosa* isolate 1 and *P aeruginosa* isolate 2) with moderate to strong biofilm production. Three different samples, AgNP, chitosan solution and CH-AgNP were tested for this study. Table 5 shows the MBEC value of AgNP, chitosan solution and CH-AgNP for 3 different isolates. Our result indicated that no MBEC was achieved with AgNP, but chitosan solution inhibited biofilm production at 8192 μg/mL against all tested isolates. Whereas, CH-AgNP had MBEC range of 21.15-42.29 μg/mL against 2 *P aeruginosa* clinical isolates but no MBEC observed with the reference strain. This can be due to the difference in biofilm producing potential of bacteria. Susceptibility of the bacteria to a different antimicrobial agent may vary depending upon the type of bacterial strains. Kalishwarlal et al.[61] studied the effect of AgNP against biofilm production by both *P aeruginosa* and *S epidermidis* and explained that AgNP at 100 nM (100 μg/mL) concentration reduced biofilm formation by 95%-98%. This may be due to the easy entry of AgNP into the biofilm via water channels present for nutrition exchange. Habash et al.[62] studied different size of AgNP (10, 20, 40, 60, 100 nm) against *P aeruginosa* biofilms where 10 nm size of AgNP had MBEC range from 1.25 μg/mL to 5.0 μg/mL indicating better biofilm eradication capabilities compared to results of

Table 4
MBC Values of Each Test Sample Against 20 MRSA Isolates

Sr. No.	MRSA Isolates (MLST)	AgNP (μg/mL)	Chitosan Solution (μg/mL)	PM (μg/mL)	CH-AgNP (μg/mL)
1	AUS2-MRSA (ST239)	38.28	512	3.158-50.525	5.287
2	AUS3-MRSA (ST239)	38.28	16,384	101.05	2.643
3	Bengal Bay PVL$^+$-CA-MRSA (ST772)	38.28	16,384	101.05	2.643-5.287
4	Classic-HA-MRSA (ST250)	38.28	256	1.5789	2.643-5.287
5	Irish 1-HA-EMRSA (ST8)	38.28	16,384	50.525-101.05	5.287
6	Irish 2-HA-EMRSA (ST8)	38.28	256	3.158	5.287
7	NY Japan-HA-MRSA (ST5)	38.28	256	3.158	5.287
8	QLD PVL$^+$-HA-EMRSA (ST22)	38.28	256	1.5789-3.158	5.287
9	ST 398-CA-MRSA-V (ST398)	38.28	16,384	3.158	5.287
10	Taiwan PVL$^+$-CA-cMRSA (ST59)	38.28	16,384	50.525	5.287
11	UK 15-HA-EMRSA (ST22)	38.28	16,384	101.05	5.287
12	UK 15 PVL$^+$-HA-EMRSA (ST22)	38.28	16,384	101.05	5.287
13	UK 16-HA-EMRSA (ST36)	38.28	16,384	3.158	5.287
14	UK 17-HA-EMRSA (ST247)	38.28	256-512	3.158	5.287
15	USA 300-MRSA (ST8)	19.14	16,384	101.05	5.287
16	WA1-CA-MRSA (ST1)	38.28	16,384	3.158	5.287
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20	WSSP-PVL negative-CA MRSA (ST30)	38.28	256	3.158	5.287
MBC range		19.14-38.28	256-16,384	1.5789-101.05	2.643-5.287
MBC_{50}		38.28	256	3.158	5.287
MBC_{90}		38.28	16,384	101.05	5.287

MRSA, methicillin-resistant *S aureus*; MLST, multilocus sequence type; PVL, panton-valentine leukocidin status; CA, community acquired; HA, health care—associated; EMRSA, epidemic methicillin-resistant *S aureus*.

Table 5
Minimum Biofilm Eradication Concentration of AgNP, Chitosan Solution, and CH-AgNP Against ATCC PA-01 and Two *P aeruginosa* Isolates

Isolates	AgNP (μg/mL)	Chitosan Solution (μg/mL)	CH-AgNP (μg/mL)
ATCC PA-01	>38.28	8192	>84.586
P aeruginosa isolate 1	>38.28	8192	42.293
P aeruginosa isolate 2	>38.28	8192	21.15

our study (21.15-42.29 μg/mL for chitosan-stabilised AgNP). The higher MBEC values may be due to slightly higher particle size compared to size of AgNP determined by Habash et al.[62]

Figures 3a-3c show graphical representation of Log_{10} transformed CFU/mL against the concentration of the sample tested. MBEC value of CH-AgNP for ATCC PA-01, *P aeruginosa* isolate 1 and *P aeruginosa* isolate 2 was found to be >84.59 μg/mL, 42.29 μg/mL, and 21.15 μg/mL respectively. Though no MBEC value was achieved, there was a gradual decrease in the biofilm production for ATCC PA-01 as shown in Figure 3a. Similarly, biofilm production was observed for *P aeruginosa* isolate 1 and *P aeruginosa* isolate 2, but, there was complete biofilm eradication at 42.29 μg/mL and 21.15 μg/mL respectively. Moreover, this gradual decrease in the biofilm production began at 5.287 μg/mL in following order *P aeruginosa* isolate 2 < *P aeruginosa* isolate 1 < ATCC PA-01 for all 3 isolates.

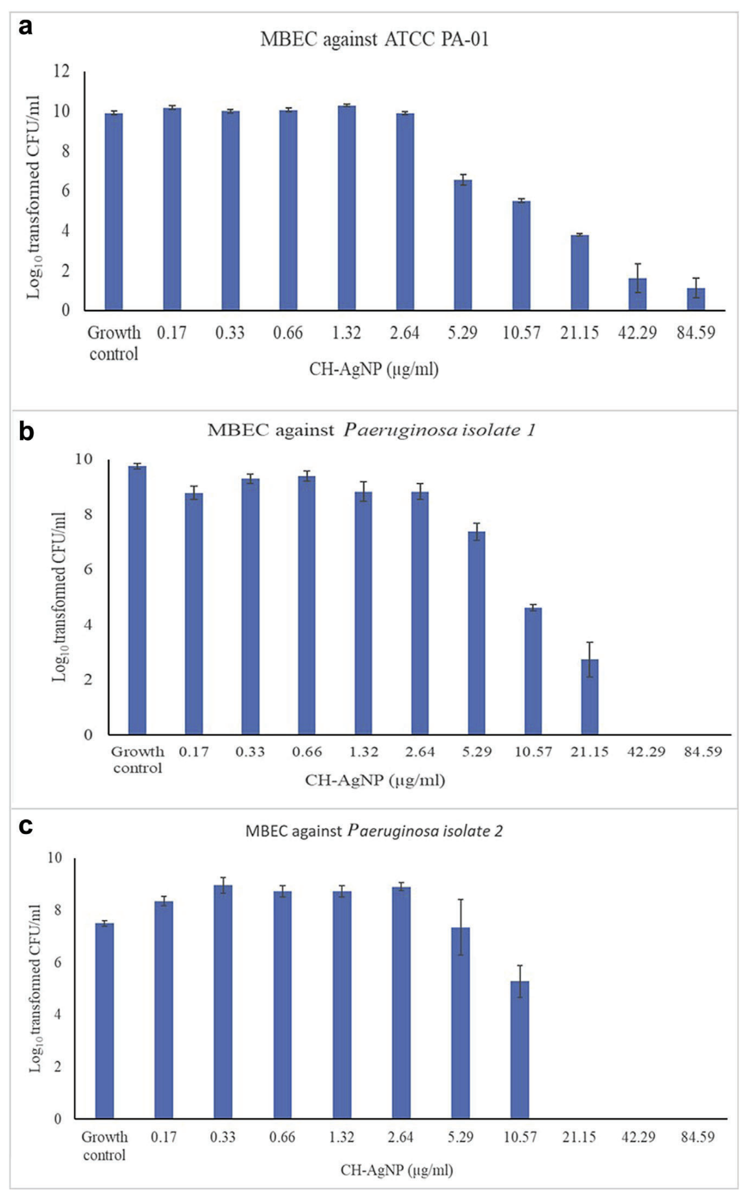

Figure 3. Minimum biofilm eradication concentration study of CH-AgNP against (a) ATCC PA-01, (b) *P aeruginosa isolate 1*, and (c) *P aeruginosa isolate 2*.

Conclusion

In the present study, the effective optimum ratio of AgNP and chitosan solution was identified using antimicrobial checkerboard assay. The effective optimum ratio of AgNP:chitosan solution was 1:4. Based on optimized ratio, CH-AgNP were developed by chemical reduction method and characterised for particle size and zeta potential by TEM and zeta sizer. CH-AgNP has the most effective antimicrobial activity against both Gram-negative and Gram-positive ATCC reference bacterial isolates as well as MRSA compared to AgNP, chitosan solution and PM. MIC and MBC range of CH-AgNP were 4 to 14 times lower compared to AgNP against MRSA isolates. MBEC value of CH-AgNP for ATCC PA-01, *P aeruginosa* isolate 1 and *P aeruginosa* isolate 2 was found to be >84.59 μg/mL, 42.29 μg/mL and 21.15 μg/mL respectively. The developed nano formulation with the smaller particle size has tremendous potential in eradicating the biofilms and inhibiting the growth of bacteria commonly found in the wound bed. In conclusion, the developed formulation of chitosan stabilised silver nanoparticles may lead to the development of newer antimicrobial formulations for wound treatment and management.

Acknowledgments

Authors would like to acknowledge Dr. Manjunath Ghate, Director, Institute of Pharmacy, Nirma University for providing the facilities to carry out the research work and supported in this collaborative work, which is a part of the Doctor of Philosophy (PhD) research work of Chintan Pansara, to be submitted to Nirma University, Ahmedabad, India. The authors are highly thankful to Department of Science and Technology, Fund for improvement of S&T infrastructure (FIST) (Grant no.: SR/FST/LSI-607/2014), Government of India for providing equipment facility. The authors at Institute of Pharmacy, Nirma University would also like to thank Prof. Sanjay Garg, Director, Center for Pharmaceutical Innovation and Development (CPID), University of South Australia for providing facilities for formulation development. Sincere thanks to Prof. G. Coombs, PathWest Laboratory Medicine, Western Australia, for providing 20 MRSA isolates.

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ORIGINAL RESEARCH
published: 25 April 2019
doi: 10.3389/fmicb.2019.00837

In vitro Antimicrobial Activity of Robenidine, Ethylenediaminetetraacetic Acid and Polymyxin B Nonapeptide Against Important Human and Veterinary Pathogens

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OPEN ACCESS

Edited by:
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Reviewed by:
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Elias Adel Rahal, American University of Beirut, Lebanon

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Specialty section:
This article was submitted to Antimicrobials, Resistance and Chemotherapy, a section of the journal Frontiers in Microbiology

Received: *23 October 2018*
Accepted: *01 April 2019*
Published: *25 April 2019*

Citation:
Khazandi M, Pi H, Chan WY, Ogunniyi AD, Sim JXF, Venter H, Garg S, Page SW, Hill PB, McCluskey A and Trott DJ (2019) In vitro Antimicrobial Activity of Robenidine, Ethylenediaminetetraacetic Acid and Polymyxin B Nonapeptide Against Important Human and Veterinary Pathogens. Front. Microbiol. 10:837. doi: 10.3389/fmicb.2019.00837

The emergence and global spread of antimicrobial resistance among bacterial pathogens demand alternative strategies to treat life-threatening infections. Combination drugs and repurposing of old compounds with known safety profiles that are not currently used in human medicine can address the problem of multidrug-resistant infections and promote antimicrobial stewardship in veterinary medicine. In this study, the antimicrobial activity of robenidine alone or in combination with ethylenediaminetetraacetic acid (EDTA) or polymyxin B nonapeptide (PMBN) against Gram-negative bacterial pathogens, including those associated with canine otitis externa and human skin and soft tissue infection, was evaluated *in vitro* using microdilution susceptibility testing and the checkerboard method. Fractional inhibitory concentration indices (FICIs) and dose reduction indices (DRI) of the combinations against tested isolates were determined. Robenidine alone was bactericidal against *Acinetobacter baumannii* [minimum inhibitory concentrations (MIC) mode = 8 μg/ml] and *Acinetobacter calcoaceticus* (MIC mode = 2 μg/ml). Against *Acinetobacter* spp., an additivity/indifference of the combination of robenidine/EDTA (0.53 > FICIs > 1.06) and a synergistic effect of the combination of robenidine/PMBN (0.5 < FICI) were obtained. DRIs of robenidine were significantly increased in the presence of both EDTA and PMBN from 2- to 2048-fold. Robenidine exhibited antimicrobial activity against *Escherichia coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*, in the presence of sub-inhibitory concentrations of either EDTA or PMBN. Robenidine also demonstrated potent antibacterial activity against multidrug-resistant Gram-positive pathogens and all Gram-negative pathogens isolated from cases of canine otitis externa in the presence of EDTA. Robenidine did not demonstrate antibiofilm activity against Gram-positive and Gram-negative bacteria. EDTA facilitated biofilm biomass degradation for both Gram-positives

and Gram-negatives. The addition of robenidine to EDTA was not associated with any change in the effect on biofilm biomass degradation. The combination of robenidine with EDTA or PMBN has potential for further exploration and pharmaceutical development, such as incorporation into topical and otic formulations for animal and human use.

Keywords: robenidine, combination, antimicrobial, canine otitis externa, EDTA

INTRODUCTION

The widespread occurrence of multidrug-resistant (MDR) pathogens is problematic in both human and animal medicine (Morehead and Scarbrough, 2018). In particular, ESKAPE pathogens (*Enterococcus* spp., *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp.) deserve global attention due to the development of MDR (Santajit and Indrawattana, 2016) and increased mortality among patients (Pendleton et al., 2013). The other worrisome factor is the potential of bacteria to form biofilms that are extremely resistant to antimicrobials (Chambers and Deleo, 2009). In the past, resistance could be combated by the development of new drugs active against antimicrobial-resistant bacteria. However, the pharmaceutical industry has reduced its research efforts for the discovery and development of novel antibacterial drugs (Theuretzbacher et al., 2018). Adding to this global issue, the only novel antimicrobial classes that have been introduced in the last 20 years are the lipopeptides (daptomycin), oxazolidinones (linezolid and tedizolid) and the lipoglycopeptides (dalbavancin, oritavancin, and telavancin), which predominantly have a Gram-positive spectrum of activity (Wilcox, 2005; Saravolatz et al., 2009; Zhanel et al., 2012). The lack of novel antimicrobial development has resulted in attempts to safeguard critically important antimicrobials (antimicrobial stewardship) and a search for alternatives to treat MDR infections, including those in animals (Chan et al., 2018a; Hickey et al., 2018).

The use of critically important antimicrobials (WHO, 2017) for veterinary applications may also contribute to the development of antimicrobial resistance. For example, MDR strains of *P. aeruginosa* (MDRPA) and methicillin-resistant strains of coagulase-positive *Staphylococcus* spp. and coagulase-negative *Staphylococcus* spp., are now widespread in veterinary medicine, particularly as a cause of infections such as canine otitis, dermatitis and bovine mastitis (Beck et al., 2012; Abraham et al., 2017; Heward et al., 2018; Khazandi et al., 2018). Otitis externa is one of the most common infectious diseases in dogs, and it can be caused by both Gram-positive and Gram-negative organisms, as well as fungi. It is typically treated by topical administration of antimicrobials, such as aminoglycosides and fluoroquinolones that are critically important for human medicine (Paterson, 2016). Otitis externa treatment failures are often due to the development of antimicrobial resistance in key target pathogens, for example methicillin-resistant *Staphylococcus pseudintermedius* (MRSP) and MDRPA (Martin Barrasa et al., 2000; Heward et al., 2018). Development of antimicrobial resistance in these companion animal pathogens is a potential public health concern with documented transmission of MDRPA and MRSP occurring between humans and dogs within households (Lozano et al., 2017; Fernandes et al., 2018).

One approach that promotes antimicrobial stewardship and minimizes the likelihood of cross-resistance development and transmission between different host species is the repurposing of existing drugs for new applications. For example, monensin and narasin (polyether ionophores used as anticoccidials in animals, but not in humans), and closantel (a salicylanilide anthelmintic) have both been shown to be active against MRSP and methicillin-resistant *S. aureus* (MRSA) (Rajamuthiah et al., 2015; Chan et al., 2018a,b; Hickey et al., 2018). Robenidine is licensed as an anticoccidial agent and has been used safely worldwide since the early 1970s for control of coccidiosis in poultry and rabbits (Kantor et al., 1970; Bampidis et al., 2019). Recently, our laboratory reported that robenidine had antimicrobial activity against MRSA, vancomycin-resistant enterococci and *Streptococcus pneumoniae*, but no activity against Gram-negative bacteria unless robenidine was tested in combination with sub-inhibitory concentrations of polymyxin B nonapeptide (PMBN) (Abraham et al., 2016). The fact that robenidine only displays activity against Gram-negative organisms in the presence of PMBN is a good indication that robenidine acts on the cytoplasmic membrane of Gram-negative organisms, but is unable to breach the permeability barrier of the outer membrane (OM) (Arzanlou et al., 2017) in the absence of a membrane permeabilizer.

The spectrum of activity of antimicrobial agents can be extended by combining them with adjuvants. Two such agents, ethylenediaminetetraacetic acid (EDTA) and polymyxin B nonapeptide (PMBN), were selected for further investigation in this study. EDTA is a prescription medicine in humans given intravenously or intramuscularly for the treatment of lead poisoning (Selander, 1969), and is a component of many topically applied ointments, eye drops and ear cleaners (Guardabassi et al., 2010). EDTA is a bacteriostatic compound that permeabilizes the outer membrane of Gram-negative bacteria by chelating Ca^{2+} and Mg^{2+} cations (Vaara, 1992). In addition, EDTA has demonstrated antibiofilm activities against existing biofilms as well as preventing biofilm formation (Finnegan and Percival, 2015). PMBN derived from polymyxin B, whilst lacking antibacterial activity (except against *Pseudomonas* spp.), is able to render Gram-negative bacteria more susceptible to antimicrobials by increasing their outer membrane permeability without affecting bacterial cell viability (Schneider et al., 2017). It has been reported that the combination of PMBN with novobiocin or erythromycin

administered intraperitoneally successfully treated mice infected with Gram-negative pathogens (Ofek et al., 1994; Allam et al., 2017).

Our aims in this study were to evaluate the *in vitro* antimicrobial and antibiofilm activities of robenidine either alone or in the presence of EDTA or PMBN against Gram-negative bacteria predominantly associated with otitis externa of animals and skin infections of humans, and assess the activity of the most effective combination/s against field strains of canine otitis externa pathogens including *P. aeruginosa*, *Proteus mirabilis*, *S. pseudintermedius* and beta-haemolytic streptococci. We hypothesized that either EDTA or PMBN would increase the antimicrobial activity of robenidine against Gram-negative bacteria through outer membrane permeabilization.

MATERIALS AND METHODS

Antimicrobial Agents

Analytical grade robenidine was provided by Neoculi Pty Ltd., Burwood, VIC, Australia. The compound was stored in a sealed container in the dark at 4°C at the Infectious Diseases Laboratory, Roseworthy campus, The University of Adelaide. Polymyxin B nonapeptide (PMBN), ampicillin, apramycin, enrofloxacin, and gentamicin were purchased from Sigma-Aldrich (Australia). Stock solutions (25.6 mg/ml of PMBN in DMSO, 12.8 mg/ml of ampicillin in PBS, 12.8 mg/ml of apramycin in DMSO, 3.2 mg/ml of enrofloxacin in $^1/_2$ volume of water to which was added NaOH dropwise to facilitate dissolution and 12.8 mg/ml of gentamicin in Milli-Q water) were prepared and stored in 1 ml aliquots at −80°C. They were defrosted immediately prior to use. EDTA (disodium salt) was purchased from Chem-Supply Pty Ltd., South Australia and was dissolved in Milli-Q water to 200 mM.

Bacterial Strains

Escherichia coli ATCC 25922, *E. coli* ATCC 11229, *P. aeruginosa* ATCC 27853, *P. aeruginosa* PA01, *Pseudomonas putida* ATCC 17428, *P. mirabilis* ATCC 43071, *K. pneumoniae* ATCC 13883, *A. baumannii* ATCC 19606, and *A. baumannii* ATCC 12457 were used for preliminary susceptibility testing and combination experiments. *S. aureus* ATCC 29213 and *S. pneumoniae* ATCC 49619 were used as internal quality controls. A variety of bacterial organisms from both human and canine infections were investigated in this study (n = 119 isolates in total). Twenty-eight clinical *Acinetobacter* spp. isolates were obtained from cases of human skin and soft tissue infections, including 18 *Acinetobacter baumannii* and 10 *A. calcoaceticus*, kindly provided by Ms Jan Bell (Institute of Medical and Veterinary Science, South Australia). It is notable that *A. baumannii* ST2 producing OXA-23 have been reported in both humans and animals, representing a possible zoonotic lineage (van der Kolk et al., 2019). Ninety-one clinical isolates were obtained from cases of canine otitis externa, including seven methicillin-susceptible *S. pseudintermedius* (MSSP), 13 multidrug- and methicillin-resistant *S. pseudintermedius* (MRSP) (Saputra et al., 2017), 20 beta-haemolytic *Streptococcus* spp., 30 *P. aeruginosa* (10 of them resistant to gentamicin and 21 *P. mirabilis* isolates). These isolates were obtained from the bacterial collection of the national survey of antimicrobial resistance in animals conducted in Australia. Swab samples from dogs with signs of otitis externa were collected by veterinarians and submitted to government, private or university diagnostic laboratories throughout Australia. After routine bacterial identification and the removal of confidential information, the participating veterinary diagnostic laboratories submitted the bacteria and their clinical information to PC2 Laboratories, Australian Centre for Antimicrobial Resistance Ecology, School of Animal and Veterinary Sciences, University of Adelaide, Roseworthy Campus, Roseworthy, SA, Australia, for further study. Thus, animal ethics approval was not required in this study. These organisms were identified to species level using biochemical testing and MALDI-TOF mass spectrometry (Bruker, Preston, VIC, Australia).

Antimicrobial Susceptibility Testing

Minimum inhibitory concentrations (MIC) were determined for robenidine, EDTA and PMBN in round bottom 96-well microtiter trays (Thermo Fisher Scientific, Australia), using the modified broth micro-dilution method recommended by the Clinical and Laboratory Standards Institute (CLSI, 2015). Testing concentrations were as follows: robenidine- 256–0.25 μg/ml; EDTA- 3800–45 μg/ml; PMBN- 32–0.06 μg/ml. Luria Bertani (LB) broth (Oxoid, Australia) was applied for MIC testing in lieu of cation-adjusted Mueller–Hinton broth as robenidine has been previously shown to chelate calcium ions. Furthermore, a twofold serial dilution of robenidine was performed in 100% DMSO, with 1 μl dispensed to each well due to the hydrophobicity of the compound (Abraham et al., 2016). The MIC for ampicillin or gentamicin against each isolate was determined for each test to serve as an internal quality control. The MICs of isolates were determined by visual reading and using an EnSpire Multimode Plate Reader 2300 atA_{600nm}. MIC_{50}, MIC_{90}, and MIC range for robenidine and EDTA were calculated against clinical isolates of *P. aeruginosa* and *P. mirabilis*, *S. pseudintermedius* and β-haemolytic *Streptococcus* spp., MIC range and MIC mode were calculated for *A. baumannii* and *A. calcoaceticus*.

Minimum Bactericidal Concentration (MBC) Determination

The MBC of robenidine alone or in combination with EDTA or PMBN against Gram-positive and Gram-negative bacteria was determined. Briefly, 10 μl aliquots from each duplicate well from the MIC assays (starting from the MIC for each compound) were inoculated onto a sheep blood agar (SBA) plate and incubated at 37°C. Plates were examined at 24 separate intervals for a period of 2 days, the MBC was recorded as the lowest concentration of each test compound at which a 99.95% colony count reduction was observed on the plate (CLSI, 1999).

Synergy Testing by Checkerboard Microdilution, Isobolograms and Dose Reduction Analysis

To assess the potential activity of robenidine, MICs against a range of Gram-negative ATCC strains as well as clinical isolates of canine otitis externa pathogens were performed in the presence or absence of 23.2–7,500 μg/ml (0.06–20 mM) EDTA and 0.25–128 μg/ml PMBN in a slightly modified standard checkerboard assay as described previously (Hwang et al., 2012). Briefly, antimicrobial stock solutions for robenidine and PMBN were prepared at a concentration of 12.8 mg/ml in DMSO. The antimicrobial stock solution for EDTA was prepared at a concentration of 200 mM in Milli-Q water. Then, a twofold serial dilution of each antimicrobial stock solution was prepared in its appropriate solvent (e.g., DMSO for robenidine and Milli-Q water for EDTA) from wells 12 to 3 (from 12.8 to 0.25 mg/ml for robenidine and PMBN; and 100 to 0.06 mM for EDTA). A 1 μl aliquot of the first compound from each combination was dispensed along the abscissa (from row A to G) of the 96-well microplate, while the second compound was dispensed along the ordinate (from column 12 to column 3) using an electronic multichannel pipette followed by 89 μl of LB broth. Each well of the plate was inoculated with an aliquot of 10 μl bacterial suspension at a concentration of $1-5 \times 10^6$ colony forming units (CFU) per ml. Subsequently, the plate was incubated at 37°C for 24 h. The fractional inhibitory concentration index (FICI) described the results of the combinations, and was calculated utilizing the following formula:

$$\text{FICI of combination} = \text{FICA} + \text{FICB}$$

FIC A is the MIC of robenidine in the combination/MIC of robenidine alone, FIC B is the MIC of the adjuvant (EDTA or PMBN) in the combination/MIC of the adjuvant alone. The results indicate synergism when the corresponding FICI $\leq$ 0.5, additivity when 0.5 $<$ FICI $\leq$ 1, indifference when 1 $<$ FICI $\leq$ 4 and antagonism when the FICI $>$ 4. In this study, the FIC for robenidine and PMBN against Gram-negative bacteria in the combination was calculated to be zero (e.g., $1 \div >256 = 0$) when robenidine or PMBN did not show any antibacterial activity alone against Gram-negative bacteria at the highest concentration tested (e.g., 256 μg/ml), but antimicrobial activity was observed when the compounds were tested in combination.

The results of the checkerboard experiments are illustrated by isobolograms, as follows: The MIC of drug A is marked on the *x*-axis of an isobologram and the MIC of drug B on the *y*-axis, with the line connecting the two marks representing the indifferent line (no interaction) (Tallarida, 2006). The MIC values of the combination located below the indifference line indicate additive (1 $\geq$ FICI $>$ 0.5) or synergistic (FICI $\leq$ 0.5) interactions. Values that are found above the indifferent line indicate indifferent (1 $<$ FICI $\leq$ 4) or antagonistic (FICI $>$ 4) interactions (Hwang et al., 2012).

The dose reduction index (DRI) shows the difference between the effective doses in combination in comparison to its individual dose. DRI was calculated as follows:

$$\text{DRI} = \text{MIC of drug alone}/\text{MIC of drug in combination}$$

Robenidine and PMBN did not show any antimicrobial activity against the majority of Gram-negative bacteria tested, the highest concentration of each compound tested against each isolate was included in the DRI equation as its MIC [e.g., the MIC of robenidine alone against *E. coli* was >256 (μg/ml) and its MIC in combination with EDTA was 1 (μg/ml); DRI = 256/1].

Dose reduction indices is very important clinically when the dose reduction is associated with a toxicity reduction without changing efficacy (Eid et al., 2012). Commonly, a DRI higher than 1 is considered beneficial.

Time-Dependent Killing Assays

Time kill assays were performed (in duplicate) for the robenidine ± EDTA assays as described previously (CLSI, 1999) with slight modifications. Briefly, colonies of each bacterium (*P. aeruginosa* ATCC 27853, *P. aeruginosa* PA01, a clinical isolate of *P. aeruginosa* from canine otitis externa, *A. baumannii* ATCC 19606, human clinical isolates of *A. baumannii* B10 and *A. baumannii* B11) from overnight SBA plates were separately emulsified in normal sterile saline and adjusted to $A_{600\text{nm}} = 0.10$ (equivalent to approximately 5×10^7 CFU/ml). Subsequently, the bacterial suspensions were further diluted 1:10 in sterile saline. The robenidine or EDTA were serially diluted in 100% DMSO or Milli-Q water at 100× the final desired concentration and a 100 μl aliquot of appropriate concentrations added to each 10 ml preparation. Robenidine or EDTA solution was prepared in 10 ml volumes at MIC and 2× MIC concentration in LB broth. After adding inoculum dose to each tube, duplicate cultures were incubated at 37°C, with samples withdrawn at 0, 0.5, 1, 2, 4, and 24 h, serially diluted tenfold and plated on SBA overnight at 37°C for bacterial enumeration. According to CLSI, an antimicrobial agent is considered bactericidal if it causes a $\geq 3 \times \log_{10}$ (99.95%) reduction in CFU/ml after 18–24 h of incubation, and the combination is considered synergistic when it causes a $\geq 2 \times \log_{10}$ reduction in CFU/ml (Tängdén et al., 2014).

Antibiofilm Susceptibility Testing

The minimum biofilm eradication concentration (MBEC) was determined for robenidine and EDTA using the MBEC™ High-throughput assay system (MBEC™ BioProducts, Innovotech, Canada) consisting of a lid with 96 pegs and a 96-well microtiter plate as previously described (Ceri et al., 2001; Harrison et al., 2010). Briefly, biofilms of *P. aeruginosa* PA01, two clinical isolates of *P. aeruginosa* isolates and two clinical isolates of *S. pseudintermedius* were formed by inoculating 150 μl of 10^7 CFU/ml of each bacterial suspension in the MBEC™ device. The inoculated device was aerobically incubated on an orbital shaker at 37°C (OM11, Ratek Instruments Pty Ltd., Australia) for 24 h to produce equivalent (Uniform) biofilms on all pegs. Biofilms of *P. aeruginosa* and *S. pseudintermedius* were exposed to challenge plates

containing a serial concentration of robenidine (from 0.125 to 128 μg/ml) or EDTA (1–32 mM) and incubated at 37°C for 24 h. Following antimicrobial challenge, the biofilms were rinsed twice with phosphate buffered saline (pH = 7) and disrupted via sonication (Soniclean, Model 160TD, Australia) for 10 min into the recovery medium. Viable cell counts were determined for recovered cells (colony-forming units per peg) after preparing a serial dilution and plating 10 μl in duplicates of each dilution onto plate count agar. Viable counts were then expressed as a percentage of the mean CFU of growth controls. MBEC was defined as the lowest concentration of antimicrobial agent that eradicates the biofilms recovered from the antimicrobial challenge.

Checkerboard Microdilution Assay for Antibiofilm Activity of Robenidine

A slightly modified standard checkerboard assay was used to determine the activity of robenidine in the presence or absence of 37.2–12,000 μg/ml (1–32 mM) EDTA as described previously (Hwang et al., 2012). Briefly, the MBEC^{TM} High-throughput assay system (MBEC^{TM} BioProducts, Innovotech, Canada) was used for the preparation of Gram-positive and Gram-negative biofilm producing bacteria as described above for antibiofilm susceptibility testing. The antimicrobial stock solution for EDTA was prepared at a concentration of 128 mM in Milli-Q water and robenidine was prepared at 12.8 mg/ml in DMSO. Then, a twofold serial dilution of each antimicrobial stock solution was prepared in its appropriate solvent from wells 12 to 3 (from 12.8 to 0.25 mg/ml for robenidine and 128 to 1 mM for EDTA). A 2 μl aliquot of robenidine compound from each concentration was dispensed along the abscissa (from row A to H) of the 96-well microplate, while 100 μl of EDTA was dispensed along the ordinate (from column 12 to column 3) using an electronic multichannel pipette followed by 98 μl of LB broth. Subsequently, the plate was incubated at 37°C for 24 h.

In vitro Cytotoxicity Assays

A panel of adherent mammalian cell lines, HaCat (human immortalized keratinocytes), HEK 293 (human embryonic kidney) and MDCK (normal Madin Darby Canine Kidney) were assayed for *in vitro* cytotoxicity of robenidine alone or in combination with EDTA or PMBN. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and 1% PenStrep (100 U/mL Penicillin and 100 μg/mL Streptomycin) at 37°C with 5% CO_2. Cells were serially passaged at ∼80% confluence ∼ every 4 days. Assays were performed in duplicate in 96 well plates seeded with ∼25,000 cells per well. After 24 h, media was removed, washed once with medium without antimicrobials and replaced with fresh media to which robenidine in the presence or absence of EDTA and PMBN were added same concentrations used for antimicrobial susceptibility testing. Briefly, the antimicrobials were prepared by performing a twofold serial dilution at 100× of tested concentration in DMSO. Subsequently, a 1 μl aliquot of each concentration was transferred to a sterile 96-well plate containing fresh DMEM with either 10% FBS. After mixing four times, the media aliquots with different concentrations of antimicrobial were transferred to each well of the 96-well plate seeded with cells, using wells containing 1–2% DMSO only as control. To determine the effect of FBS on the cytotoxicity of each compound, DMEM with 40% FBS containing different concentrations of antimicrobial was prepared as described above. After 24 h of exposure, WST-1 reagent (Cell Proliferation Assay reagent, Roche) at a concentration of 10% was added to each well. Absorbance at $A_{450\ nm}$ on a Multiskan Ascent 354 Spectrophotometer (Labsystems) was measured after 1 h of incubation. The IC_{50} value was determined for each compound against each cell line via non-linear regression (three parameters) using GraphPad Prism v6 software.

RESULTS

Antimicrobial Activity of Robenidine Against Gram-Negative Control Strains

Robenidine did not demonstrate any antimicrobial activity against Gram-negative control strains (*E. coli* ATCC 25922, *E. coli* ATCC 11229, *P. aeruginosa* ATCC 27853, *P. mirabilis* ATCC 43071 and *K. pneumoniae* ATCC 13883) at the highest concentrations (256 μg/ml) tested except for *A. baumannii* ATCC 19606 (32 μg/ml) and *A. baumannii* ATCC 12457 (64 μg/ml).

Antimicrobial Activity of Robenidine Against Human Clinical *Acinetobacter* spp.

The MIC results of robenidine against *A. baumannii* and *A. calcoaceticus* isolated from human clinical cases were demonstrated at concentrations ranging from 8 to 64 μg/ml (MIC mode = 8 μg/ml) for 18 *A. baumannii* and 1–8 μg/ml (MIC mode = 2 μg/ml) for 10 *A. calcoaceticus.* The ratio of MBC/MIC values for both *Acinetobacter* spp. was either 2× or 4× their MICs.

Combination of Robenidine With EDTA or PMBN Against Gram-Negative Control Strains

The presence of EDTA in combination with robenidine was associated with a notable increase in the potency and spectrum of activity against Gram-negative control strains. The results of MIC and DRI values for the combination of robenidine and EDTA against *E. coli* ATCC 25922, *E. coli* ATCC 11229, *P. aeruginosa* ATCC 27853, *P. mirabilis* ATCC 43071, *K. pneumoniae* ATCC 13883, *A. baumannii* ATCC 19606, and *A. baumannii* ATCC 12457 are presented in **Table 1**. The combination of robenidine and EDTA resulted in a synergistic interaction against the standard isolates of *E. coli* as well as against *P. aeruginosa* ATCC 27853, *P. putida* ATCC 17428, *P. aeruginosa*, and *K. pneumoniae* ATCC 13883. An additive/indifferent interaction was recorded against *P. mirabilis*,

TABLE 1 | The MIC (μg/ml) values for robenidine, EDTA and the combination effect of EDTA on the MIC of robenidine for Gram-negative control strains and a human clinical *A. calcoaceticus* isolate.

Isolates	MIC (μg/ml; mM concentrations in parentheses)			Combination Effect (FICI)[b]	DRI[c]
	Single drug		Combination		
	EDTA	ROB[a]	EDTA:ROB		EDTA:ROB
E. coli ATCC 25922	3800 (10)	>256	950 (2.5):4	Synergism (0.25)	4:64
E. coli ATCC 11229	950 (2.5)	>256	228 (0.6):8	Synergism (0.25)	4:32
P. putida ATCC 17428	1900 (5)	>256	950 (2.5):1.25	Synergism (0.5)	2:256
P. aeruginosa PA01	1900 (5)	>256	950 (2.5):1.25	Synergism (0.5)	2:256
P. aeruginosa ATCC 27853	3800 (10)	>256	1900 (5):1.25	Synergism (0.5)	2:256
P. mirabilis ATCC 43071	228 (0.6)	>256	228 (0.6):1.25	Additivity (1)	1:256
K. pneumoniae ATCC 13883	11400 (30)	>256	3800 (10):128	Synergism (0.33)	3:2
A. baumannii ATCC 19606	380 (1)	32	190 (0.5):4	Additivity (0.62)	2:8
A. baumannii ATCC 12457	190 (0.5)	64	95 (0.25):2	Additivity (0.53)	2:32
A. calcoaceticus	228 (0.6)	4	228 (0.6):0.125	Indifference (1.06)	1:32

[a]ROB, robenidine; [b]the results indicate synergism when the corresponding FICI ≤ 0.5; additivity when 0.5 < FICI ≤ 1, indifference when 1 < FICI ≤ 4 and antagonism when the FICI > 4; [c]DRI, dose reduction index.

TABLE 2 | The MIC (μg/ml) values for robenidine, PMBN and the combination effect of PMBN on the MIC of robenidine for Gram-negative control strains and a human clinical *A. calcoaceticus*.

Isolates	Antimicrobial concentration (μg/ml)			Combination Effect (FICI)[c]	DRI[d]
	Single drug		Combination		
	PMBN[a]	ROB[b]	PMBN:ROB		EDTA:ROB
E. coli ATCC 25922	>32	>256	6:8	Synergism (<0.5)	5:32
E. coli ATCC 11229	>32	>256	6:8	Synergism (<0.5)	5:32
P. putida ATCC17428	4	>256	1:1	Synergism (0.25)	4:256
P. aeruginosa ATCC27853	2	>256	0.75:1	Synergism (0.25)	4:256
P. mirabilis ATCC 43071	>32	>256	NA[e]	No effect	NA
K. pneumoniae TCC13883	>32	>256	0.5:4	Synergism (<0.5)	64:64
A. baumannii ATCC19606	>32	32	1:2	Synergism (0.07)	32:16
A. baumannii ATCC 12457	>32	64	1:2	Synergism (0.03)	32:32
A. calcoaceticus	>32	4	1.5:0.5	Synergism (0.12)	21:8

[a]PMBN, polymyxin B nonapeptide; [b]ROB, robenidine; [c]the results indicate synergism when the corresponding FICI ≤ 0.5; additivity when 0.5 < FICI ≤ 1, indifference when 1 < FICI ≤ 4 and antagonism when the FICI > 4; [d]DRI, dose reduction index; [e]NA, no antimicrobial activity was recorded.

A. baumannii, and *A. calcoaceticus* control strains. DRIs of robenidine were significantly increased in the presence of EDTA from 2- to 256-fold (**Table 1**).

The results of MIC, FICI, and DRI values for the combination of robenidine and PMBN against *E. coli* ATCC 25922, *E. coli* ATCC 11229, *P. aeruginosa* ATCC 27853, *P. mirabilis* ATCC 43071, *K. pneumoniae* ATCC 13883, *A. baumannii* ATCC 19606, and *A. baumannii* ATCC 12457 are presented in **Table 2**. The combination of robenidine and PMBN resulted in a synergistic interaction against all the isolates tested except *P. mirabilis* ATCC 43071 (**Table 2**). DRIs of robenidine were significantly increased in the presence of PMBN from 8- to 256-fold (**Table 2**).

Isobologram analyses were carried out for the combination of EDTA and robenidine against *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853 and *P. mirabilis* ATCC 43071, and for the combination of PMBN and robenidine against *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853. Dose-effect curves for drugs with different maxima and the corresponding isobole combination are presented in **Figure 1**. Isoboles of the combination of EDTA and robenidine against *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 indicated synergism. Similarly, isoboles of the combination of PMBN and robenidine against *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 also indicated synergism.

Antimicrobial Activity of Robenidine Against Canine Otitis Externa Pathogens

MIC range, MIC_{50}, MIC_{90} (μg/ml) values of robenidine, gentamicin, apramycin, and ampicillin against quality control strains (*S. aureus* ATCC 29213, *P. aeruginosa* ATCC 27853, *E. coli* ATCC 25922) and clinical isolates from otitis externa cases in dogs [*S. pseudintermedius* (n = 20), beta-haemolytic streptococci (n = 20), *P. mirabilis* (n = 21), and *P. aeruginosa* (n = 30)] are presented in **Table 3**.

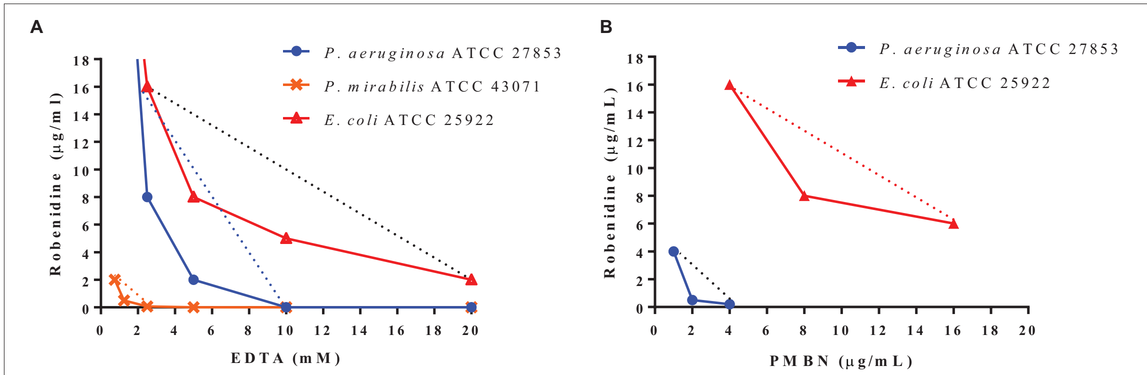

FIGURE 1 | Isobologram analyses. Minimum inhibitory concentrations of **(A)** EDTA and **(B)** PMBN are plotted on *x*-axis and minimum inhibitory concentration values of robenidine on *y*-axis. The curves represent the combinations against *E. coli* ATCC 25922, *P. mirabilis* ATCC 43071 and *P. aeruginosa* ATCC 27853 including the indifference line (.......) for each isolate.

TABLE 3 | The MIC range, MIC_{50}, MIC_{90} (µg/ml) values of robenidine, gentamicin, apramycin, and ampicillin against control strains, including *P. aeruginosa* ATCC 27853, *E. coli* ATCC 25922 *S. aureus* ATCC 29213, *S. pneumoniae* ATCC 49619 and clinical isolates from otitis externa cases in dogs, including *S. pseudintermedius* (*n* = 20), beta-haemolytic *Streptococci* (*n* = 20), *P. mirabilis* (*n* = 21), and *P. aeruginosa* (*n* = 30).

	Value	MIC (µg/ml)			
		Robenidine	Gentamicin	Apramycin	Ampicillin
P. aeruginosa (*n* = 30)	MIC range	>256	0.25–64	16–64	–a
	MIC_{50}	>256	32	32	–
	MIC_{90}	>256	64	64	–
P. mirabilis (*n* = 21)	MIC range	>256	4–> 128	16–64	–
	MIC_{50}	>256	8	32	–
	MIC_{90}	>256	32	64	–
S. pseudintermedius (*n* = 20)	MIC range	1–4	1–64	1–16	0.03–32
	MIC_{50}	2	2	8	0.125
	MIC_{90}	2	2	16	8
Beta-haemolytic streptococci (*n* = 20)	MIC range	4–16	8–16	4–128	0.06–0.5
	MIC_{50}	8	8	64	0.125
	MIC_{90}	8	8	128	0.25
Quality control strains					
S. aureus ATCC 29213	MIC	2	0.5	4	1
P. aeruginosa ATCC 27853	MIC	–	2	16	–
E. coli ATCC 25922	MIC	–	0.5	8	4
S. pneumoniae ATCC 49619	MIC	–	–	–	0.125

[a]*Antimicrobial activity was not tested.*

Combination of Robenidine With EDTA Against Canine Otitis Externa Pathogens

Minimum inhibitory concentrations and DRI values for the combination of robenidine and EDTA against 30 *P. aeruginosa*, 21 *P. mirabilis*, 20 *S. pseudintermedius*, and 20 beta-haemolytic streptococci isolated from canine otitis externa cases are shown in **Table 4**. The clinical isolates of *P. aeruginosa* including 10 antimicrobial-resistant isolates, showed a synergistic interaction with the combination of robenidine and EDTA. An additivity interaction (95.3%) was recorded against clinical isolates of *P. mirabilis* in the combination of robenidine and EDTA. The DRIs of robenidine for *P. aeruginosa* and *P. mirabilis* isolates increased between 64- and 2048-fold, and the DRIs of EDTA increased two and fourfold. Additionally, additive and indifferent activity of the combination robenidine and EDTA was observed against clinical isolates of MRSP, MSSP and beta-haemolytic streptococci.

Robenidine demonstrated antibacterial activity against Gram-positive bacteria, with MIC values ranging from 1 to 16 µg/ml against *S. pseudintermedius* and beta-haemolytic streptococci, respectively. The lowest level of interaction was recorded for the combination of robenidine and EDTA against *S. pseudintermedius* and beta-haemolytic streptococci. However,

TABLE 4 | The MIC range, MIC_{50}, MIC_{90} (μg/ml) and DRI values for robenidine, EDTA alone and their combination against 30 *Pseudomonas aeruginosa*, 21 *Proteus mirabilis*, 20 *Staphylococcus pseudintermedius*, and 20 beta-haemolytic streptococci isolated from otitis externa cases in dogs.

Isolates	Value	Antimicrobial concentration (μg/ml; mM concentrations in parentheses)				Combination Effect[b] (percentage)	DRI[c]	
		Single drug		Combination				
		EDTA	ROB[a]	EDTA	ROB		EDTA	ROB
P. aeruginosa [*n* = 30; MSSP (20), MRSP (10)]	MIC range	750–4500 (2–12)	>256	380–1500 (1–4)	0.125–4	Synergism (100%)	2–3	64–2048
	MIC_{50}	3000 (8)	>256	1500 (4)	0.25		2	1024
	MIC_{90}	3000 (8)	>256	1500 (4)	2		2	128
	MBC/MIC	BS[d]	NA[e]	750 (2)	≥2	–		
P. mirabilis (*n* = 21)	MIC range	190–750 (0.5–2)	>256	190–380 (0.5–1)	0.125–4	Additivity (95.3%) Synergism (4.7%)	1–2	64–2048
	MIC_{50}	380 (1)	>256	380 (1)	0.125		2	2048
	MIC_{90}	750 (2)	>256	380 (1)	0.5		2	512
	MBC/MIC	BS	NA	750 (2)	≥2	–		
S. pseudintermedius [*n* = 20; MSSP (7), MRSP (13)]	MIC range	95–380 (0.25–1)	1–4	95 (0.25)	0.25–2	Additivity (100%)	1–4	2–8
	MIC_{50}	190 (0.5)	2	95 (0.25)	1		2	2
	MIC_{90}	190 (0.5)	2	95 (0.25)	1		2	2
	MBC/MIC	BS	1.5	750 (2)	2	–		
Beta-haemolytic streptococci (*n* = 20)	MIC range	190–750 (0.5–2)	4–16	95–380 (0.25–1)	1–8	Additivity (100%)	1–2	2–8
	MIC_{50}	380 (1)	8	190 (0.5)	2		2	4
	MIC_{90}	750 (2)	8	380 (1)	4		1	2
	MBC/MIC	BS	1.75	750 (2)	2	–		

[a]*ROB, robenidine;* [b] *the results indicate synergism when the corresponding FICI* ≤ 0.5*; additivity when* $0.5 < FICI \leq 1$*, indifference when* $1 < FICI \leq 4$ *and antagonism when the FICI* > 4*;* [c]*DRI, dose reduction index;* [d]*BS, bacteriostatic compound;* [e]*NA, no antimicrobial activity was recorded.*

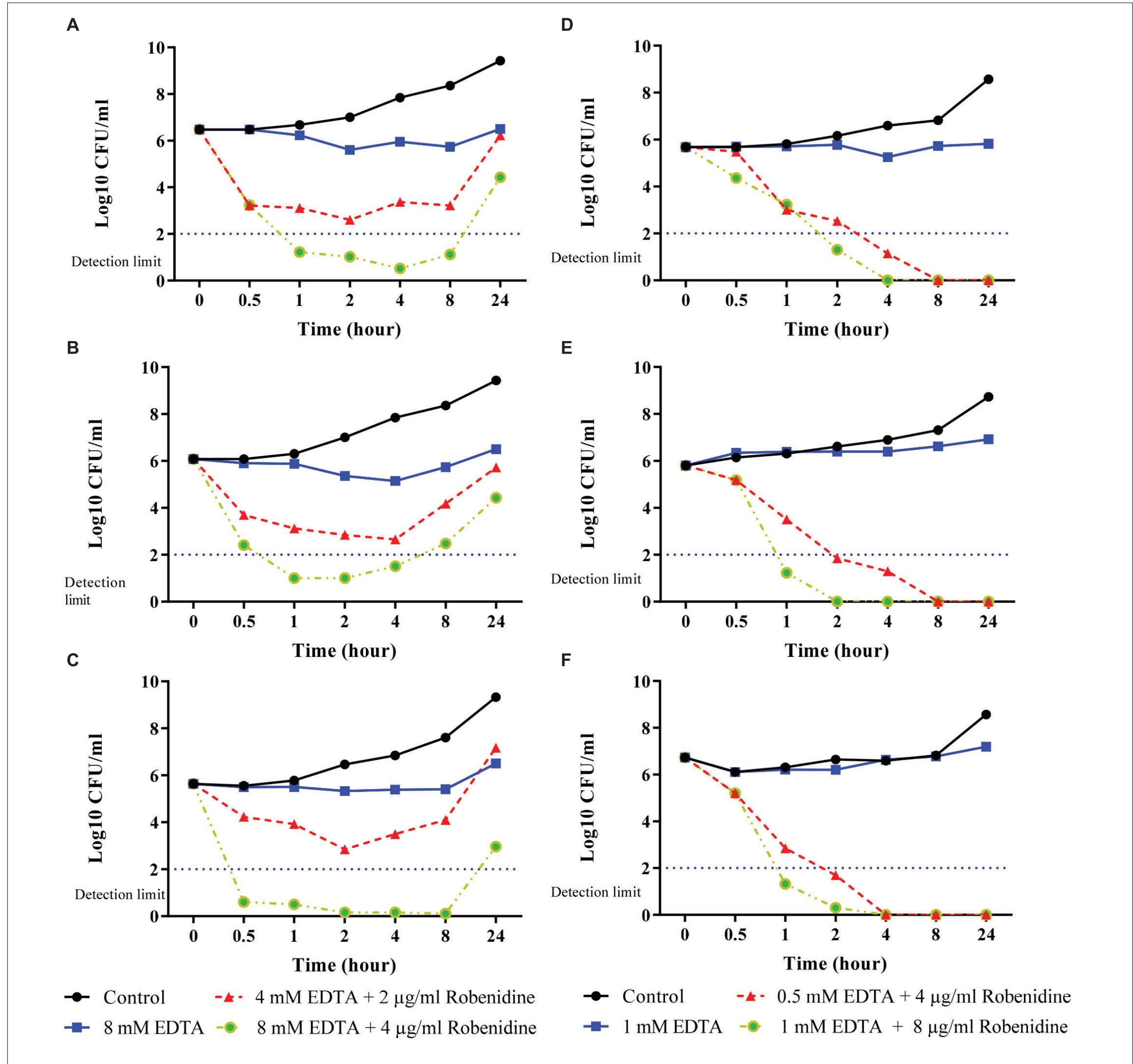

FIGURE 2 | Time kill curves of EDTA and the combination of robenidine and EDTA against **(A)** *Pseudomonas aeruginosa* ATCC 27853, **(B)** *P. aeruginosa* PA01, **(C)** a clinical isolate of *P. aeruginosa* from dog **(D)** *Acinetobacter baumannii* ATCC 19606, **(E)** a clinical isolate of *A. baumannii* B10 from human and **(F)** a clinical isolate of *A. baumannii* B11 from human. Control represents bacteria incubated in the absence of EDTA and robenidine. Bactericidal activity of robenidine in the combination with EDTA was defined as a reduction in the numbers of viable bacteria of ≥3 log_{10} CFU/ml at any incubation time tested.

the dose reduction for beta-haemolytic streptococci ranged from 2- to 16-fold for robenidine and twofold for EDTA.

Time Kill Kinetics of Drug Combinations Against *P. aeruginosa* and *A. baumannii*

Time kill curves for robenidine in the presence of EDTA at the concentration of MIC_{90} (2 μg/ml robenidine + 1,500 μg/ml or 4 mM of EDTA) and 2× MIC_{90} (4 μg/ml robenidine + 3,000 μg/ml or 8 mM of EDTA) were obtained for *P. aeruginosa* ATCC 27853, *P. aeruginosa* PA01 and a clinical isolate of *P. aeruginosa* from a canine otitis externa case are presented in **Figures 2A–C**. The combination of robenidine and EDTA at MIC_{90} significantly reduced the colony count of *P. aeruginosa* isolates (about 3 log_{10}) over 0.5, 1, 2, and 4 h with a synergistic effect in comparison to the control growth and EDTA alone. However, at 24 h, bacterial regrowth was observed to almost the same level as the sample treated with EDTA alone. Further reductions of the bacteria (greater than 5 log_{10} CFU/ml reduction) at 0.5 h were recorded when

the EDTA concentration increased from MIC_{90} (3,000 μg/ml or 4 mM) to 2× MIC_{90} (3,000 μg/ml or 8 mM) in comparison to control and EDTA alone. A minimum of a 5 log_{10} reduction was still evident at 4 h incubation, however, after 24 h the numbers of bacteria present had increased. However, this reduction (approximately 5 log_{10} reduction) remained consistent in comparison to growth control.

Time kill curves for robenidine in the presence of EDTA at the concentration of MIC (4 μg/ml robenidine + 188 μg/ml or 0.5 mM of EDTA) and 2× MIC (8 μg/ml robenidine + 376 μg/ml or 1 mM of EDTA) for *A. baumannii* ATCC 19606, two human clinical isolates of *A. baumannii* (B10 and B11) from canine otitis externa are presented in **Figures 2D–F**. The combination of robenidine and EDTA at both MIC and 2× MIC significantly reduced the colony counts of *A. baumannii* ATCC 19606 and two clinical isolates of *A. baumannii* over 1 and 2 h with a synergistic effect in comparison to the control growth and EDTA alone. After 8 and 4 h, bacteria were eliminated for tested isolates in both MIC and 2× MIC, respectively.

Antibiofilm Activity of Robenidine Alone and in the Presence of EDTA

Preformed biofilms of *P. aeruginosa* PA01, two clinical isolates of *P. aeruginosa* and two clinical isolates of *S. pseudintermedius* were tested against robenidine and EDTA to determine their activities. Robenidine at concentration of up to 128 μg/ml did not show any antibiofilm activity against *P. aeruginosa* and *S. pseudintermedius* isolates in comparison to enrofloxacin as a positive control (**Figures 3A,B**). However, 1 mM concentration of EDTA demonstrated a significantly effect in disrupting the 24 h preformed biofilms in comparison to growth control against both Gram-positive and Gram-negative bacteria (**Figure 3C**). EDTA was more effective against the biofilms when the concentration of EDTA increased to 16 mM. However, the presence of robenidine in combination with EDTA was not associated with any change in the antibiofilm activity of EDTA against both Gram-positive and Gram-negative bacteria. The results of the antibiofilm activity of the EDTA in the present of robenidine against *P. aeruginosa* PA01 are shown in **Figure 3D**.

Robenidine Cytotoxicity to Mammalian Cell Lines

The cytotoxicity profile of robenidine in the presence and absence of EDTA and PMBN was evaluated in a panel of different cultured mammalian cells using the WST-1 Cell Proliferation Assay reagent (Roche). The results of the *in vitro* cytotoxicity measurements show IC_{50} values of 12 μg/ml for robenidine, IC_{50} values of 3.4 mM for EDTA, while PMBN gave IC_{50} values of >32 μg/ml against all the cell lines tested (**Table 5**). We found that the *in vitro* cytotoxicity measurements show IC_{50} values of 12 μg/ml for robenidine in the presence of either 3.4 mM for EDTA or 32 μg/ml for PMBN (**Table 5**). Real-time cell viability measurements using HaCaT and HEK 239 cell lines also confirmed no measurable effect on cell viability for robenidine at either 12 μg/ml up to 24 h post-treatment alone or in the presence of either 3.4 mM for EDTA or 32 μg/ml for PMBN. Real-time cell viability showed that the combination of 8 μg/ml robenidine with 4 Mm EDTA was not toxic during the first 12 h of assays. Importantly, the toxicity of robenidine alone or in the presence of either EDTA or PMBN was significantly reduced from 12 μg/ml to higher than 32 μg/ml for all tested cell lines when the amount of FBS was increased from 10 to 40% (**Table 5**).

DISCUSSION

Bacterial pathogens have developed numerous resistance strategies against antimicrobial agents used in both humans and animals. A major challenge in successful treatment of bacterial infections is the emergence and rapid global spread of multidrug-resistant clones that are refractory to current antimicrobial therapy. To address this problem, we have examined and repurposed robenidine as a new class of antibacterial agent. To evaluate the potential of robenidine as an antibacterial agent, we previously assessed its potency, metabolic stability, pharmacokinetic and safety profiles, in a mouse PK study and a series of *in vitro* efficacy and cell toxicity studies (Abraham et al., 2016; Ogunniyi et al., 2017). We identified that robenidine had a predominantly Gram-positive spectrum of activity, and that the site of action was likely to be the cytoplasmic membrane (Ogunniyi et al., 2017) hence this compound should potentially have an antimicrobial effect on Gram-negative organisms. The Gram-positive selective activity of robenidine is most likely to be a result of the inability of this compound to traverse the outer membrane of Gram-negative organisms (Arzanlou et al., 2017). In the present study, we extended our analyses by assessing *in vitro* efficacy against a range of clinical human and animal Gram-negative bacterial isolates in the presence or absence of sub-inhibitory concentrations of EDTA and PMBN.

We found that robenidine showed antimicrobial activity against *Acinetobacter* spp. even in the absence of OM permeabilisation, and its MICs were reduced 8- to 32-fold in the presence of EDTA and PMBN. This result is quite surprising as the permeability of the OM of *A. baumannii* is estimated to be only 1–8% that of *E. coli* as *A. baumannii* lacks the general, non-specific trimeric porins found in *E. coli* (Nikaido, 2003; Zgurskaya et al., 2015). The general architecture of the OM between *A. baumannii* and other Gram-negative bacteria is the same, however, lipid A in *A. baumannii* is acylated with C12 and C14 fatty acids, compared with C10 and C12 fatty acids in *E. coli* (Zgurskaya et al., 2015). As a result, the hydrophobic core of *A. baumannii* is expected to be thicker and lipid A should occupy a larger area per lipid. These features are likely to make the OM of *A. baumannii* more hydrophobic and could be responsible for the susceptibility of this organism to amphiphilic antimicrobials such as novobiocin and tetracycline (Krishnamoorthy et al., 2017). Similarly, robenidine is an amphiphilic molecule and the same differences in the OM of *A. baumannii* could increase its susceptibility to robenidine.

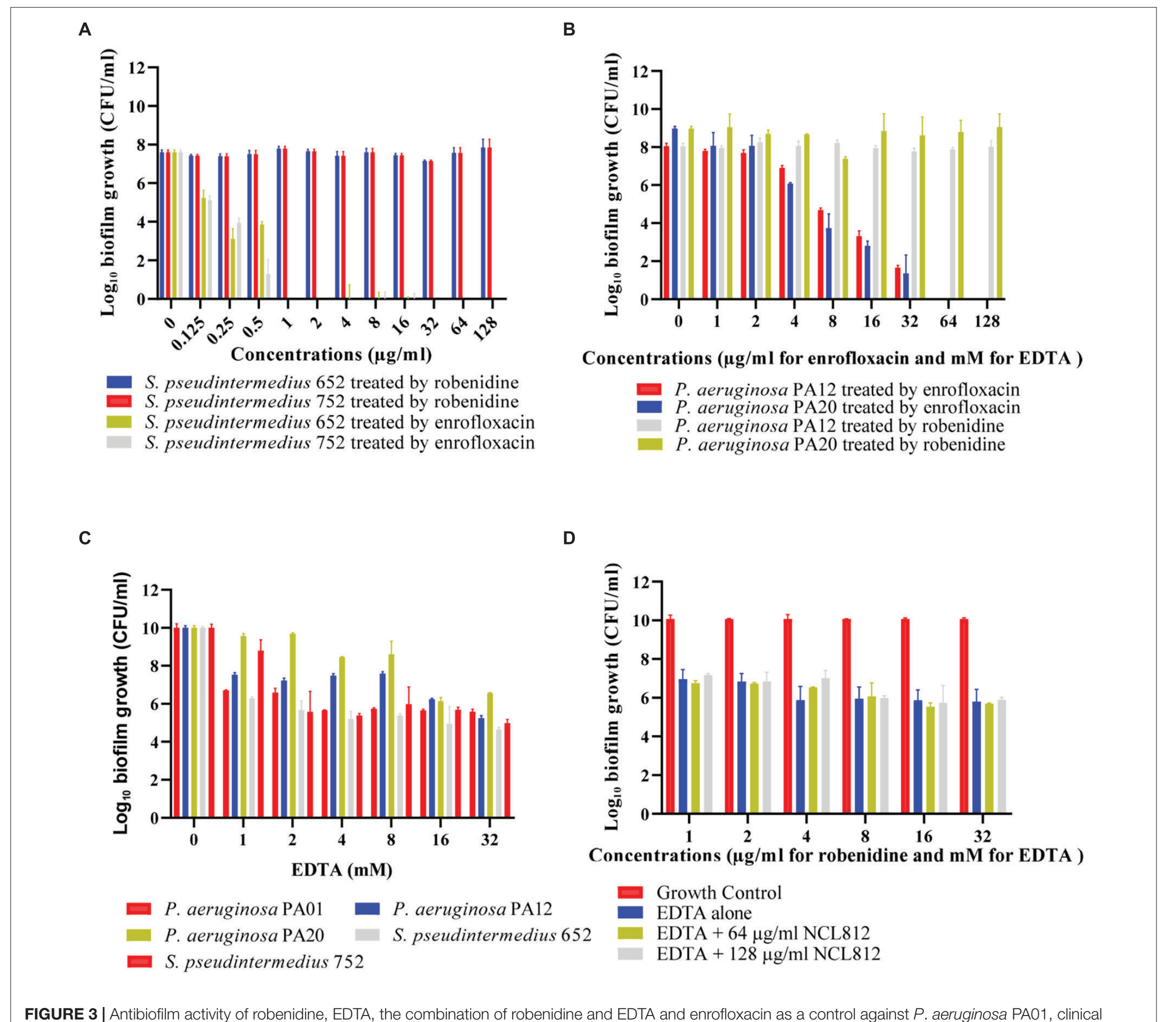

FIGURE 3 | Antibiofilm activity of robenidine, EDTA, the combination of robenidine and EDTA and enrofloxacin as a control against *P. aeruginosa* PA01, clinical isolates of *P. aeruginosa* (PA12 and PA20) and *S. pseudintermedius* (652 and 752) from dogs. **(A)** antibiofilm activity of robenidine against *S. pseudintermedius* isolates (652 and 752), **(B)** antibiofilm activity of robenidine against *P. aeruginosa* PA01 and clinical isolate of *P. aeruginosa*, **(C)** antibiofilm activity of EDTA against *P. aeruginosa* PA01, two clinical isolates of *P. aeruginosa* (PA12 and PA20) and *S. pseudintermedius* (652 and 752), and **(D)** antibiofilm activity of the EDTA in the present of robenidine against *P. aeruginosa* PA01.

Given these encouraging results for antibacterial activity against *Acinetobacter* spp., the safety and efficacy of robenidine could be further explored in animal models of *Acinetobacter* infection (Paluchowska et al., 2017; Gorla et al., 2018) prior to further clinical development. In addition, using an appropriate formulation can improve the potency and safety of robenidine as a novel treatment for infections caused by *A. baumannii* (Paluchowska et al., 2017; Gorla et al., 2018) and *A. baumannii-calcoaceticus* complex (Clark et al., 2016; Ozvatan et al., 2016), which are reported to be emerging pathogens worldwide (Gales et al., 2001). Our results show that EDTA would be a suitable adjuvant for topical delivery but not systemic use due to the high concentrations of Ca^{2+} and Mg^{2+} in blood, while PMBN does not have this limitation and could be included as a possible adjuvant in both topical and systemic *Acinetobacter* infection models.

Robenidine in the presence of sub-inhibitory concentrations of EDTA or PMBN also displayed improved antibacterial activity against a variety of ESKAPE isolates (*S. aureus*, *E. coli*, *K. pneumoniae*, *A. baumannii*, and *P. aeruginosa*). In the case of PMBN, it resulted in a 4- to 256-fold increase in the susceptibility of tested Gram-negative ATCC strains of ESKAPE pathogens in combination with robenidine, inhibiting growth at robenidine concentrations as low as 0.125 µg/ml, whilst the MIC

TABLE 5 | IC_{50} data for robenidine, EDTA, PMBN and robenidine in the combination with either EDTA or PMBN against the HaCaT, HEK 293, and MDCK cell lines in the presence of 10 or 40% FBS in DMEM.

Agent	IC_{50} values (μg/ml for robenidine and PMBN; mM for EDTA)									
	ROB[a]		EDTA		PMBN		ROB:EDTA		ROB:PMBN	
FBS	10%[b]	40%[c]	10%	40%	10%	40%	10%	40%	10%	40%
HaCaT	14	>32	3.8	3.8	>32	>32	12:3	>32:3	12:>32	>32:>32
HEK 293	12	>32	3.4	3.4	>32	>32	12:3	>32:3	12:>32	>32:>32
MDCK	12	>32	3.4	3.4	>32	>32	12:3	>32:3	12:>32	>32:>32

[a]*ROB, robenidine,* [b]*DMEM with 10% FBS used for cytotoxicity,* [c]*DMEM with 40% FBS used for cytotoxicity.*

of PMBN was reduced 4- to 64-fold when used in combination for *P. aeruginosa*. Our cytotoxicity results showed that robenidine (IC_{50} = 12 μg/ml) was not toxic at the MIC_{90} (0.5–4 μg/ml) of the tested pathogens, with IC_{50}/MIC ratio ranging from sixfold (Gram-negative pathogens) to threefold (Gram-positive pathogens) in the presence of EDTA. The MIC (0.5–8 μg/ml) obtained for robenidine in the presence of PMBN against Gram-negative pathogens was not toxic against all tested cell lines, with IC_{50}/MIC ratio ranging from approximately 2- to 24-fold. In this study, we found that toxicity of robenidine was significantly reduced in the presence of serum, possibly due to the interaction between robenidine and serum. This serum impact was observed on the MIC values of robenidine with 10% serum (fourfold increase) and 50% serum (no antimicrobial activity), which was reported in a previous study (Abraham et al., 2016). This suggests the probable high level of serum protein binding with robenidine may significantly reduce its toxicity and robenidine would be likely to be safe when applied as a topical or otic treatment. However, testing in animal models would be required to confirm efficacy and safety. In addition, the use of EDTA in topical treatments containing robenidine also is expected to be safe. EDTA-tromethamine solution consisting of 250 mM EDTA and 50 mM tromethamine has previously been used for the treatment of otitis externa, dermatitis and cystitis without any toxicity or other side effects observed (Farca et al., 1997). Given the substantial reduction in MICs and toxicity of robenidine in the presence of either EDTA or PMBN, the *in vivo* activity of these combinations for topical and systemic treatment of ESKAPE pathogen infections could be evaluated in mouse models of infection.

We found that robenidine has no activity against biofilms formed by Gram-positive or Gram-negative bacteria. However, in this study EDTA demonstrated antibiofilm activity against both Gram-positive and Gram-negative species at a concentration of 1 mM that is in agreement with previous studies (Al-Bakri et al., 2009; Finnegan and Percival, 2015). Our results demonstrate that the presence of robenidine does not affect the antibiofilm activity of EDTA. Many pathogens are able to form biofilms making them less susceptible to various classes of antimicrobials (Chambers and Deleo, 2009). There is an urgent need for antimicrobials that can either kill planktonic cells or eradicate biofilms. Together, our results show that the combination of EDTA and robenidine is a suitable antimicrobial combination with activity against both Gram-positive and Gram-negative species and their biofilm formation.

Commercially available otic products typically contain antifungal, antibiotic and anti-inflammatory agents, such as Surolan® (polymyxin B-miconazole-prednisolone), Aurizon® (marbofloxacin-clotrimazole-dexamethasone) and Otomax® (gentamicin-clotrimazole-betamethasone) (Rougier et al., 2005; Rigaut et al., 2011). These otic products share antimicrobial agents used in human medicine, increasing the likelihood of cross-resistance development and transmission between different host species. In addition, the response to these otic products varies due to the emergence of antimicrobial resistance in canine otic pathogens. Polymyxin B resistance was reported in 100% of *S. pseudintermedius* and *Proteus* spp. and 7% of *P. aeruginosa* from cases of canine otitis externa in Australia (Bugden, 2013) and between 9.6 and 27% of canine otitis/pyoderma isolates were resistant to marbofloxacin (Rubin et al., 2008; Arais et al., 2016). Resistance to gentamicin was found in 43.3% *P. aeruginosa* otitis isolates (Mekic et al., 2011). It is notable that there is no study that demonstrates an otic product with 100% cure rate. For instance, cure rates of 58.3% for Aurizon® and 41.2% for Surolan® were observed in one study (Rougier et al., 2005). We found that the new combination of robenidine and EDTA has potential for development as a topical treatment of canine otitis externa with mixed bacterial infections. In our study, EDTA acted as an adjuvant that potentiates the activity of robenidine against Gram-negative bacteria with additional inhibitory activity against biofilm-forming bacteria. The use of an antimicrobial and an antimicrobial adjuvant as a two-drug combination antimicrobial therapy such as robenidine and EDTA has the benefit of reducing the onset of resistance development compared to monotherapy (Worthington and Melander, 2013). Recently, we reported that EDTA has anti-fungal activity against *Malassezia pachydermatis* isolated from canine otitis externa (Chan et al., 2018c) which is an advantage to the use of combination therapy of robenidine and EDTA for canine otitis externa. This combination is an approach to promote antimicrobial stewardship by eliminating the likelihood of cross-resistance development and transmission of resistance determinants of public health significance between dogs and humans.

In our study, robenidine demonstrated noteworthy activity against thirteen multidrug- and methicillin-resistant *S. pseudintermedius* and 20 β-haemolytic streptococci isolates

from clinical cases of canine otitis externa. This is in agreement with our previous study that reported robenidine was effective against clinical MRSA and *S. pneumoniae* strains at concentrations ranging from 1–2 μg/ml and 2–8 μg/ml, respectively (Abraham et al., 2016; Ogunniyi et al., 2017). The finding that robenidine in the presence of EDTA demonstrated antibacterial activity against the Gram-negative canine otitis externa pathogens, *P. aeruginosa* and *P. mirabilis* is in agreement with our previous findings for robenidine tested against two strains each of *E. coli* and *P. aeruginosa* in the presence of PMBN (Abraham et al., 2016). However, our results showed that low concentrations of robenidine in combination with PMBN or EDTA improved potency and spectrum of activity, specifically targeting Gram-negative pathogens. These results suggest that in addition to having excellent activity against Gram-positive organisms, robenidine in combination with EDTA or PMBN has potential as a broad-spectrum topical treatment, particularly against pathogens that have become resistant to multiple classes of currently registered antimicrobial agents.

CONCLUSION

The results of our study demonstrate that robenidine is not suitable as a sole antimicrobial agent for the treatment of Gram-negative pathogen infections due to the lack of activity against the majority of Gram-negative isolates except for *A. baumannii* and *A. calcoaceticus*. However, we demonstrated *in vitro* efficacy against all selected Gram-negative organisms when robenidine was tested in combination with EDTA or PMBN, including against multidrug-resistant strains. Therefore, robenidine may be an appropriate candidate as a component of a combination preparation for the treatment of otitis externa in dogs. This study provides proof of concept of drug repurposing in the field of veterinary otology and would represent a good example of antimicrobial stewardship when the compound is ultimately developed and used clinically in dogs. Finally, the additive and synergistic effects of robenidine in combination with EDTA or PMBN provide an important and novel development pathways for treatment of additional antimicrobial-resistant Gram-negative pathogens in animals and humans.

AUTHOR CONTRIBUTIONS

MK contributed to the study design, MIC and combination testing, kill time, biofilm assay, analyzed results, and wrote the preliminary manuscript. HP contributed towards kill time assay, MIC testing, biofilm assay, and data analysis. WC performed testing on the robenidine and EDTA combination, biofilm assay, and data analysis. JS participated in cell cytotoxicity assays. AO contributed to data analysis and manuscript editing. HV and PH contributed to interpretation, analysis, and discussion. AM and SG contributed to discussion, writing and editing. SP conceived the study's design, and contributed to the writing and editing, and provided financial support for the study. DT contributed to study design, and participated in writing, editing, and discussion, and provided financial support for the study. All authors read and approved the submitted version of the manuscript, in addition to contributing to manuscript revision.

FUNDING

This work was supported by ARC Linkage (ARC LP110200770) with Neoculi Pty Ltd., as a partner organization.

ACKNOWLEDGMENTS

The authors would like to thank Ms. Amanda Ruggero, Ali Khazandi, Ms. Lora Bowes, and Ms. Anh Hong Nguyen at the University of South Australia for their technical assistance.

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Conflict of Interest Statement: SP is a director of Neoculi Pty Ltd. DT has received research funding from Neoculi Pty Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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AUSTRALIA'S PREMIER VETERINARY SCIENCE TEXT

SMALL ANIMALS

SMALL ANIMALS

Lesion distribution in cases of canine atopic dermatitis in South Australia

M Graham, WY Chan and P Hill*

Objective To determine the lesion distribution patterns in different breeds of dogs affected by atopic dermatitis in South Australia.

Methods The presence or absence of erythematous skin lesions in 267 cases of canine atopic dermatitis (CAD) was recorded across 36 anatomical sites. Breeds represented by ≥ 9 dogs were included in the analysis. The percentage of dogs showing lesions at each of the body sites was calculated and illustrated on colour-coded diagrams. Variations in affected body sites within and between breeds were compared using Kruskal-Wallis ANOVA and Dunn's multiple comparison tests.

Results The prevalence of skin lesions at different body sites differed significantly both in the population as a whole and within breeds. The sites affected in ≥ 75% dogs were the dorsal and ventral aspects of the front and hind paws. The sites affected in 50–74% were the medial pinnae, axillae, ventral chest, abdomen and perineum. Sites affected in 25–49% of dogs included the face, periocular region and forelimb. Remaining body sites were affected in < 25% of dogs. Analysis at the breed level revealed some differences from this standard distribution pattern.

Conclusion The results of this study confirmed the typical lesion distributions seen in CAD and highlighted some subtle differences in breeds commonly seen in South Australia. This will be useful for clinical practitioners in prioritising differential diagnoses for pruritic skin conditions.

Keywords atopic dermatitis; dogs; South Australia

Abbreviations CAD, canine atopic dermatitis; GR, Golden Retriever; GSD, German Shepherd Dog; SBT, Staffordshire Bull Terrier; WHWT, West Highland White Terrier

Aust Vet J 2019;97:262–267 doi: 10.1111/avj.12828

Canine atopic dermatitis (CAD) is an inflammatory and pruritic allergic skin disease with strong breed predispositions and characteristically presents with clinical signs associated with IgE antibodies directed against environmental allergens.[1] Signs may include erythema and pruritus of the face, paws, axillae and ventral abdomen, but none of the clinical signs are pathognomonic and a definitive diagnosis cannot be made on preliminary examination, because of the diversity of the presenting signs.[2] This often presents a diagnostic challenge for general practitioners.[3]

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Assessment of skin lesion distribution is a critical component of dermatological diagnosis. A clinical diagnosis of CAD is made by fulfilling a strongly associated set of clinical criteria while ruling out similar pruritic conditions.[2,3] Two such sets of criteria by Willemse[4] and Favrot et al.[5] have been widely accepted by the veterinary community when diagnosing suspected allergic skin diseases.

Intradermal allergy tests or IgE serology can be used to confirm a state of IgE-mediated hypersensitivity and identify allergens that can be used for allergen-specific immunotherapy.[3]

Two different systems have been validated to determine the severity of lesions in dogs presented with CAD. They include the Canine Atopic Dermatitis Lesion Index (CADLI) and Canine Atopic Dermatitis Extent and Severity Index (CADESI-03 and -04), both of which can be used to grade the severity of lesions and determine the efficacy of a given treatment protocol.[6,7] The most frequently affected areas of the body are highlighted in both indices and include the head, pinnae, front paws, hind paws, ventral thorax, axillae, ventral abdomen and inguinal region (CADLI), as well as the cubital fossa, caudal/lateral elbow, flank and flexural carpus (CADESI-04).

The clinical phenotype of atopic dermatitis varies among breeds, regions and countries. Previous studies have demonstrated that breeds such as the Labrador Retriever, Golden Retriever (GR), German Shepherd Dog (GSD), West Highland White Terrier (WHWT), Boxer, Cocker Spaniel, Bichon Frise, Shar-pei and Scottish Terrier are genetically predisposed to CAD.[8–12] The clinical presentation in some of these breeds has been documented briefly by Picco et al.,[9] and more extensively by Wilhem et al.,[13] who demonstrated the clinical phenotypes in nine often affected breeds of dog to highlight the variability of this allergic skin disease. Those studies however, were completed in countries other than Australia and did not include several breeds of dog commonly owned in Australia such as the Staffordshire Bull Terrier (SBT), Australian Kelpie and Maltese Terrier.

The primary aim of this study was to determine the common lesion distribution patterns in different breeds of dog presented to the dermatology referral service at the University of Adelaide's Companion Animal Health Centre (South Australia). Secondary aims were to determine the age of onset, the prevalence of secondary infection and the significance of food versus environmental allergens.

This study intended to provide clinically useful information that can be used by general practitioners when diagnosing CAD. Breed-specific phenotypes that were identified will be invaluable in allowing differential diagnoses to be prioritised.

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Materials and methods

Study design and population

The cases for this study were identified from those presenting to the dermatology referral service at the University of Adelaide's Companion Animal Health Centre (South Australia) between 2010 and 2018. Cases were obtained from the Cornerstone practice management software by searching for the following terms and codes: 'atopic dermatitis', 'allergic dermatitis – precise cause not determined', 'intradermal skin test' and 'IgE serology test' or dogs that had been prescribed the anti-allergic drug oclacitinib (Apoquel®, Zoetis).

Diagnosis of CAD

CAD was diagnosed according to standard methodology and criteria.[3] Briefly, parasitic skin conditions were ruled out by microscopic examination of appropriate skin samples and/or the current administration of effective ectoparasiticides. Dogs were assessed for the presence of concurrent skin infections using appropriate cytological samples. Dogs found to have evidence of secondary skin infections with either *Malassezia pachydermatitis* or staphylococci were still included in the study because these are considered to be part of the overall disease process in CAD and are known to be triggered by microbiome dysbiosis. Furthermore, the inclusion of these cases reflected a more accurate representation of real-world clinical practice. The possibility of food-induced CAD was investigated (when possible) by performing a 6–8-week elimination diet trial using either a commercial hydrolysed diet or a home-cooked diet.

Assessment of lesions

Information on the distribution of skin lesions was obtained from the dogs' medical records. All medical records contained a written description of the dog's lesions and their distribution, as well as a lesion distribution diagram. All dogs had been assessed by a single clinician who was a board-certified veterinary dermatologist (PH). All lesions that could be attributable to CAD, including erythema, scaling, lichenification, hyperpigmentation, excoriation and self-induced alopecia, were recorded for the purposes of this study. No attempt was made to grade the severity of skin lesions – they were simply reported as present or absent.

Contrary to the 8 sites of the CADLI model or the 20 sites of the CADESI-04 model (which do not cover the entire body surface), 36 anatomical sites were chosen for the purposes of this study (Supplementary Table 1). The aim was to document the presence or absence of skin lesions at any site on the dogs.

Analysis of skin lesion distribution

Data on skin lesion distribution were extracted from the medical records and transferred into an Excel spreadsheet. Breeds of dog that were represented by ≥ 9 individuals within the total population were included in the analysis of the clinical breed phenotypes. This was an arbitrary cut-off chosen by the authors, who considered that fewer representatives than this may not have generated an accurate picture. Silhouettes were drawn for each breed with the outline of the 36 anatomical sites in order to graphically present the results. The percentage of animals affected at each of the 36 anatomical sites was calculated and colour-coded as follows: white, 0–25% of dogs affected; pink, 25–50% of dogs affected; light-red, 50–75% of dogs affected; bright red, 75–100% of dogs affected.

Using this system, a series of colour-coded diagrams depicting lesion distribution were created to highlight the specific clinical presentation of CAD in the entire population as well as in selected breeds.

Additional information collected from the medical record included the dog's age, breed and sex, together with results of microscopic examinations to determine secondary bacterial or yeast infections.

Statistical analysis

Microsoft Excel and Graphpad Prism were used to calculate descriptive and analytical statistics. The frequency of lesions in a particular anatomical site was calculated for each breed of dog. Differences in frequency between the same anatomical site in different breeds, and between different anatomical sites within the same breed, were compared using Kruskal-Wallis ANOVA and Dunn's multiple comparison tests. Significance was set at $P \leq 0.05$.

Results

A total of 314 dogs were identified using the search criteria. Of these, 26 were removed from the study population because they were not examined by a specialist dermatologist; 3 dogs were removed because of an exclusive diagnosis of food-induced allergic skin disease; 4 dogs were removed because the medical record clearly stated that a diagnosis of CAD was highly unlikely; 14 other dogs were also removed because of a definitive diagnosis of a different condition. Of the remaining 267 dogs, 146 were male and 121 were female; 107 had a positive intradermal allergy test or IgE serological test and 160 had a clinical diagnosis without an allergy test being performed. In those cases, the owners of the dogs preferred to pursue symptomatic anti-allergy treatment rather than allergy testing; 39 dogs undertook a hypoallergenic food trial and 228 did not. Analysis of these subgroups did not reveal any statistical significance, so the population was examined as a whole.

Breeds represented by ≥ 9 dogs included the SBT (n = 45), Labrador Retriever (n = 24), GSD (n = 16), Maltese Terrier (n = 14), GR (n = 11), Boxer (n = 10), Australian Kelpie (n = 9), Beagle (n = 9) and WHWT (n = 9).

The frequency of affected body sites in the total population and in the nine individual breeds is shown in Supplementary Table 1. The frequency of affected sites in all breeds combined is illustrated in Figure 1. Figure 2 graphically depicts the frequency of affected sites in descending order. The lesion distributions in the nine individual breed are shown in Figure 3.

The sites affected in ≥ 75% of all dogs were the dorsal and ventral aspects of the front and hind paws (Figure 2). The sites affected in 50–74% were the medial pinnae, axillae, ventral chest, abdomen and perineum. Sites affected in 25–49% of dogs included the face, periocular region and forelimb. Remaining body sites were affected in < 25% of dogs. Several differences from this standard distribution pattern were noted at the breed level.

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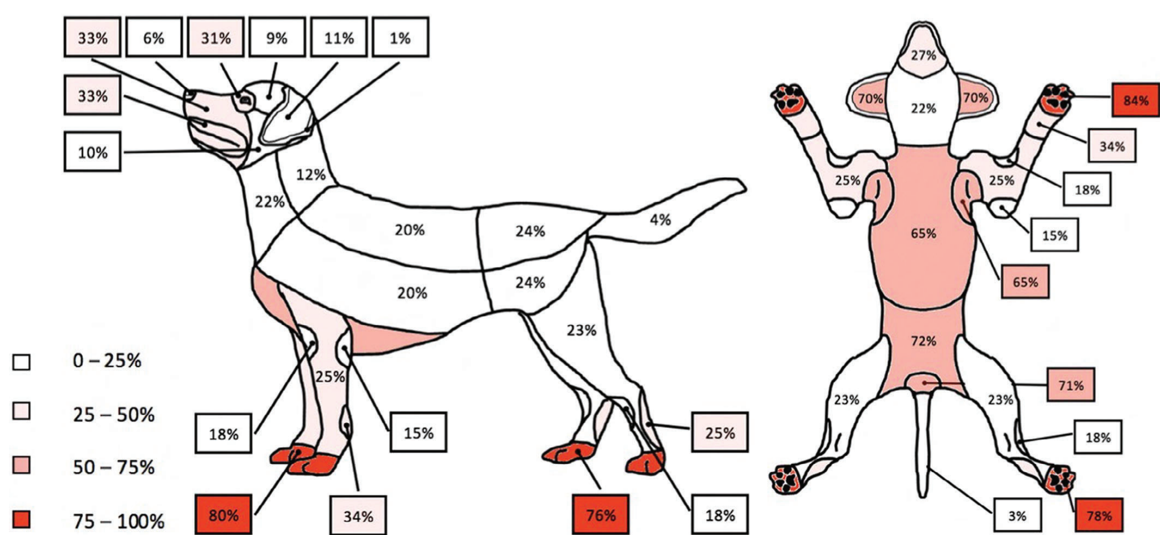

Figure 1. Skin lesion distribution diagrams for the total population. Each colour corresponds to the percentage of animals affected in that specific anatomical site.

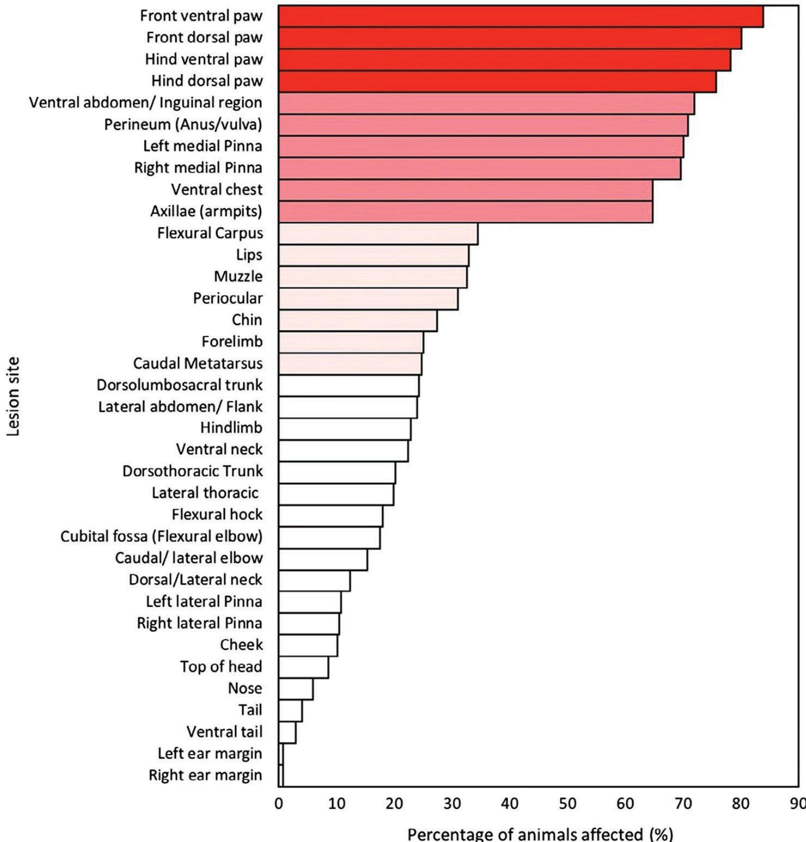

Figure 2. Bar graph representing the frequency of erythematous skin lesions across the 36 anatomical sites for the total population in descending order.

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The frequency of skin lesions in the Beagle was higher on the front paws (90%) than the hind paws (67%), with the flexural carpus and caudal metatarsus (67%) also often affected. The frequency of skin lesions in the Boxer was notably high on the ventral neck (50%) and over the dorsum (30%), but lesions of the face were also particularly prevalent (60%). In the GSD the trunk, thorax, abdomen, lips and flexural carpus was affected in > 38% of cases and the periocular region was affected in 31% of cases. The medial pinna was the most affected area for the GR but the face, forelimb and ventral paws also recorded a high frequency of skin lesions. The Kelpie was significantly different from the Beagle (P = 0.0105), Boxer (P= 0.0452), GSD (P = 0.0214), GR (P = 0.0051) and Maltese (P = 0.0102), as it did not have a high frequency of lesions on the medial pinnae. However, skin lesions were most commonly recorded on the face, limbs, paws, ventrum and flank. The lateral pinnae of the Kelpie also had the same frequency of skin lesions as the medial pinnae (33%). In Labradors, a predilection for the perineum and paws (92%) was observed and the forelimb, flexural carpus (both 42%) and hindlimb (38%) were also commonly affected. Aside from the pinnae, paws and ventrum, skin lesions were frequently seen on the face, periocular region and limbs of the Maltese Terrier. The SBT presented often with skin lesions on the ventral neck and face, with the flank (29%) also frequently affected. The WHWT was the most widely affected dog analysed in the study (average 23 body sites affected). In contrast to the standard lesion distribution, erythema was prevalent on the ventral neck (56%), face (44%), periocular region (67%) and the dorsum (44%), with the flank, forelimb and hindlimb also common sites (33%).

The mean age of onset of erythematous lesions was 3.88 years in all dogs (median age, 3 years). However, the Maltese Terrier (8.2 years) and WHWT (7.7 years) did not follow this trend. The age of onset of erythematous lesions in the Maltese Terrier differed significantly from the total population (P = 0.0085) and suggested that this breed is affected later in life. The prevalence of secondary staphylococcal infection was 26% and 23% of the population was affected by a secondary *Malassezia* overgrowth. The GSD (68.8%) was significantly more affected by secondary bacterial infections compared with the rest of the population (P = 0.0077). The WHWT was affected by a secondary bacterial infection in 55.6% of cases, but this was found not to be significant (P > 0.05).

Discussion

The present study evaluated the distribution of skin lesions in 267 dogs with CAD (Figure 1) and nine individual breeds of dogs represented by ≥ 9 dogs (Figure 3). In the total population of dogs, the paws were the most frequently affected area (> 75%), followed by the medial pinnae, axillae and ventrum (> 50%), with the face, periocular region and forelimbs affected in > 25% of cases. The least commonly affected sites included the tail, ear margins, nose, cheek and top of the head. These findings are consistent with previous reports[4,5,11,12,14] and are considered characteristic of CAD. In the studies previously conducted, it was shown that the distal limbs are most often affected, followed by the axillae, ventrum, medial pinnae and then the face. That finding was confirmed in the present study. Favrot et al. found that the dorsolumbar area and the ear margins were highly associated with non-atopic conditions,[5] which was certainly the case in our study as the ear margins were affected in only 1% of cases. However, although not commonly affected, the dorsothoracic trunk and dorsolumbosacral trunk were affected in 20% and 24% of cases, respectively.

Appraising the individual breed lesion distribution diagrams (Figure 3), some appear similar to each other such as the Labrador Retriever, GR and Beagle, or the GSD and the WHWT. Some breeds, however, appear distinctly different from each other such as the Kelpie and the Boxer. A similar study conducted in 2011 documented lesion distribution in nine breeds of dogs with CAD presenting to 34 veterinary dermatologists working in 15 different countries.[13] The Boxer, GSD, GR, Labrador Retriever and WHWT were also included in that study. The findings of the present study were similar in nature, apart from a few key exceptions, highlighted in Table 1. The reason for these differences cannot be ascertained from the present study. They could reflect geographical or genetic differences or could be related to the different methodologies used in the two studies. It is interesting to note that the GSD, Boxer and WHWT were all affected on the dorsum in over 30% of cases. Skin lesions over this site are typically regarded as more characteristic of flea allergy dermatitis rather than atopic dermatitis.[3]

The Australian Kelpie, Beagle, Maltese Terrier and SBT have not often been mentioned in the previous literature concerning CAD.[8] Of these dogs, only the SBT was previously considered to have a breed predisposition to CAD.[15,16] However, an Australian study based in New South Wales conducted in 2016 reported that the Australian Kelpie and Maltese Terrier had a significantly decreased odds ratio of developing CAD, whereas the Beagle and SBT had a significantly increased odds ratio.[8] The reason for these differences is not known, but they too could reflect geographical or methodological differences. In the present study, the SBT presented with a typical distribution of erythematous skin lesions. The paws were most frequently affected, followed by the ventrum, medial pinnae and face, whereas the only aspects of the limbs affected in > 25% of cases were the flexural carpus and caudal metatarsus. Interestingly, the flank and ventral neck were also affected in 30–40% of cases. The Maltese Terrier also presented with some uncharacteristic skin lesions, as in 29% of cases erythema was observed on the dorsum and flank. The periocular region was affected in 43% of cases, 12% more frequent when compared with the rest of the population. The ventrum and medial pinnae were affected in > 75% of cases of CAD in the Beagle; 67% of cases presented with lesions on the hind paws and in 90–100% of cases the front paws were affected. The Beagle was the breed most affected by erythematous lesions on the flexural carpus and caudal metatarsus (both 67%), a frequency 30–40% greater than the total population.

The Australian Kelpie was found to differ significantly from the generic model of CAD. Predilection sites observed included the ventral chest (78%), followed by the ventral abdomen and paws (all 67%), with the axillae, perineum, forelimb and caudal metatarsus affected in 56% of cases. All other aspects of the limbs were affected in 33–44% of cases. The frequency of lesions on the lateral pinnae (33%) was the same as for the medial pinnae, a comparison that was found to be significantly different in the total study population

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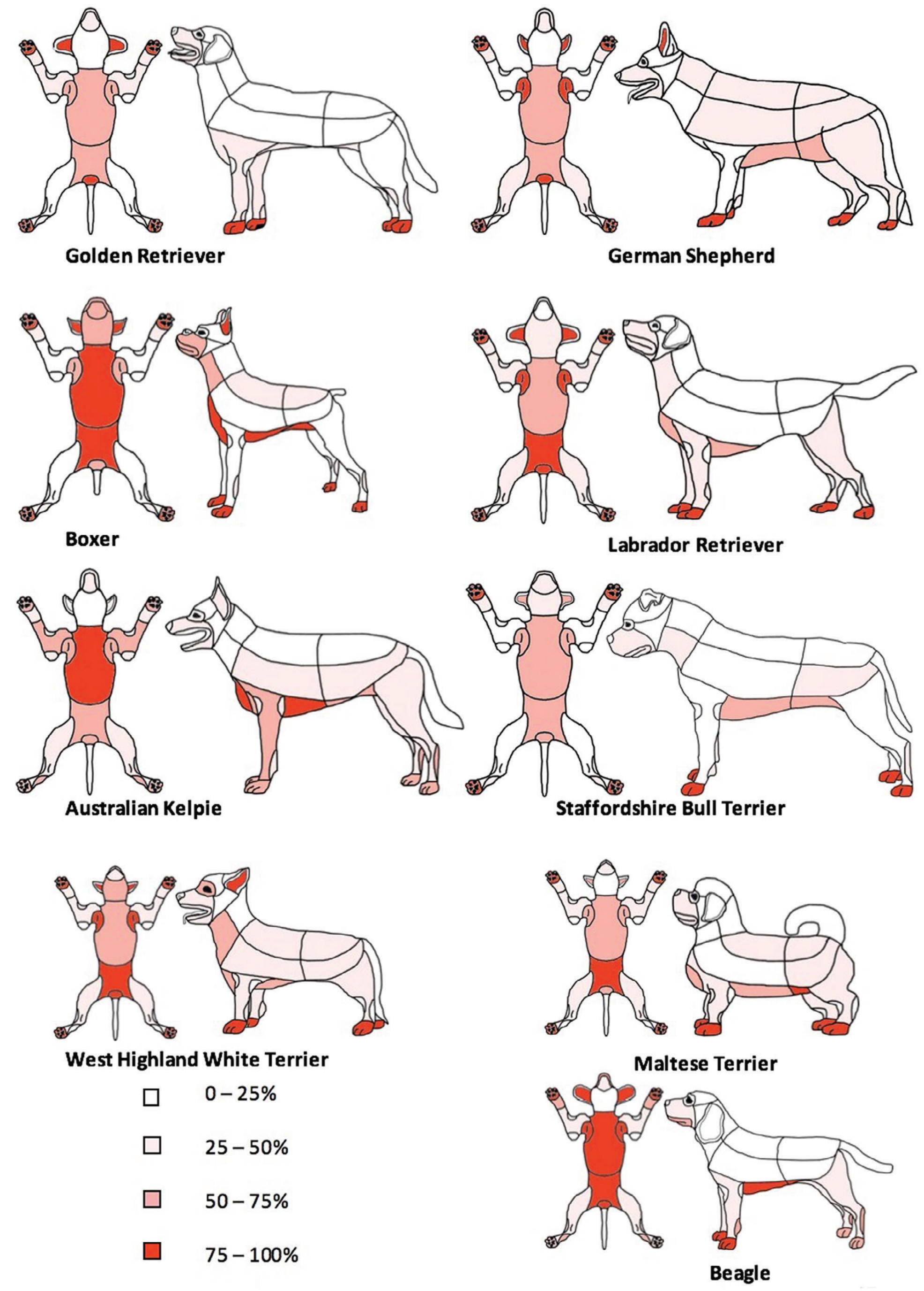

Figure 3. Lesion distribution diagrams for the Golden Retriever, German Shepherd Dog, Boxer, Labrador Retriever, Australian Kelpie, Staffordshire Bull Terrier, West Highland White Terrier, Maltese Terrier and Beagle. Each colour corresponds to the percentage of affected animals within each breed.

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Table 1. **Similarities and differences noted in skin lesion distribution between the present study and that of Wilhem et al[13]**

Breed	Similarities	Differences in the present study
Boxer	Limbs, axillae, ventral abdomen, perineum and paws	Face, ventral chest and dorsum were more frequently affected
GSD	Face, paws and ventrum	Ventral neck, limbs, ventral abdomen less affected Dorsum more affected
GR	Face, limbs, paws, axillae, ventral abdomen	Ventral chest, perineum more commonly affected
Labrador Retriever	Limbs, paws, ventral abdomen and trunk	Ventral chest, perineum more commonly affected
WHWT	Pinnae, limbs, axillae, ventral abdomen, perineum, dorsum and trunk	Ventral neck and periocular region more frequently affected

GR, Golden Retriever; GSD, German Shepherd Dog; WHWT, West Highland White Terrier.

($P < 0.0001$). Because of the high number of comparisons made between body sites in the statistical analysis (Dunn's multiple comparisons test), there are too many associations to present in this study. For example, in the SBT, out of 630 body site comparisons, significant differences were identified between 231 sites. However, in the Kelpie, no significant comparisons were observed, despite a significantly different ANOVA result ($P = 0.0003$). This may reflect the low statistical power caused by the small sample size.

Conclusion

Assessment of skin lesion distribution is a critical component of dermatological diagnosis. The results of this study confirmed the typical lesion distributions seen in CAD and highlighted some subtle differences in breeds commonly found in South Australia. This data should be helpful to clinicians when dogs are presented with pruritic skin disease that is suspected to be CAD. First, the typical lesion distribution can be compared with that seen in other skin disorders. Second, knowledge of the variations to this generic pattern that occur in different breeds will allow clinicians to modify their index of suspicion and prioritise the differential diagnosis list more accurately.

Acknowledgment

Wei Yee Chan was supported by the Ministry of Higher Education of Malaysia and Universiti Putra Malaysia

Conflicts of interest and sources of funding

The authors declare no known conflicts of interest. This study was funded by The University of Adelaide clinical research project fund

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site: http://onlinelibrary.wiley.com/doi/10.1111/avj.12828/suppinfo.

Supplementary Table 1 Frequency of erythematous skin lesions in 36 anatomical sites for individual breeds and the total population

(Accepted for publication 20 February 2019)

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Veterinary Dermatology

Vet Dermatol 2019; **30**: 524–e159 DOI: 10.1111/vde.12794

Antimicrobial activity of thyme oil, oregano oil, thymol and carvacrol against sensitive and resistant microbial isolates from dogs with otitis externa

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Background – Multidrug-resistant pathogens present a major global challenge in antimicrobial therapy and frequently complicate otitis externa in dogs.

Hypothesis/Objectives – *In vitro* efficacy of oregano oil, thyme oil and their main phenolic constituents against bacterial and fungal isolates associated with canine otitis externa were investigated. It was hypothesized that the main phenolic components would have greater antimicrobial activity compared to the relative essential oil.

Methods and materials – Antimicrobial susceptibility testing was performed using broth microdilution with spot-plating technique to determine minimum inhibitory and bactericidal/fungicidal concentrations (MICs, MBCs and MFCs). A time–kill kinetics assay was performed to confirm the bactericidal and fungicidal activity of the oils and their phenolic constituents. One hundred bacterial and fungal isolates, including meticillin-susceptible *Staphylococcus pseudintermedius* (n = 10), meticillin-resistant *S. pseudintermedius* (n = 10), β-haemolytic *Streptococcus* spp. (n = 20), *Pseudomonas aeruginosa* (n = 20; including 10 isolates resistant to one or two antimicrobials), *Proteus mirabilis* (n = 20) and *Malassezia pachydermatis* (n = 20) from dogs with otitis externa were used.

Results – Oregano oil, thyme oil, carvacrol and thymol exhibited antibacterial activity against all bacterial and fungal isolates tested. MIC_{90} values ranged from 0.015 to 0.03% (146–292 μg/mL) for the Gram-positive bacteria and *P. mirabilis*. For *P. aeruginosa* and *M. pachydermatis*, MIC_{90} values ranged from 0.09 to 0.25% (800–2,292 μg/mL).

Conclusions and clinical significance – Oregano oil, thyme oil, carvacrol and thymol showed good *in vitro* bactericidal and fungicidal activity against 100 isolates from dogs with otitis externa, including some highly drug-resistant isolates. These essential oils and their main phenolic constituents have the potential to be further investigated *in vivo* for the treatment of canine otitis externa.

Introduction

The emergence of antimicrobial resistance is a major worldwide medical concern in both humans and animals. This global issue has been reported to cause approximately 700,000 deaths annually and is expected to increase to 10 million by 2050 at a cost of more than 100 trillion Australian dollars.[1] Antimicrobial-resistant pathogens could be combated with the development of new classes of antimicrobial agents. However, due to the low investment return, the pharmaceutical industry has reduced its research efforts for the discovery and development of novel antimicrobials.[2] The lack of novel synthetic drug development and the widespread occurrence of antimicrobial-resistant pathogens provide an opportunity for alternatives to conventional treatments.

Otitis externa, one of the most common dermatological diagnoses, affects approximately 20% of the pet dog population worldwide.[3] Topical and oral antimicrobial agents are commonly used for treating otitis externa.[4] However, these treatments have increasingly been ineffective in recent years due to emergence and spread of antimicrobial resistant bacteria such as meticillin-resistant *Staphylococcus pseudintermedius* (MRSP) and multidrug-resistant (MDR, resistance to at least three antimicrobial classes) *Pseudomonas aeruginosa*.[5,6] These resistant pathogens could be a potential public health concern because there are cases reporting the transmission of MRSP from dogs to humans,[7] and transmission of MDR *P. aeruginosa* in the human–dog–environment interface, possibly due to the high degree of genome similarity among the isolates.[8,9] The potential for bilateral transmission indicates that canine otitis externa can

Accepted 5 August 2019
Sources of Funding: This study was self-funded.
Conflict of Interest: No conflicts of interest have been declared.

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become a significant public health concern. Hence, there is a pressing need for the discovery of novel antimicrobial agents or alternative treatments for otitis externa, particularly that caused by MDR zooanthroponotic pathogens.

Essential oils, such as rosemary (*Rosmarinus officinalis*) and clove (*Syzygium aromaticum*) have been highlighted as one potential approach to combat antimicrobial-resistant pathogens.[10] The curative properties of herbs and spices have been well-known since ancient times; however, current attention is drawn towards scientific studies including treatment of infectious diseases.[11] Herbal products, including plant-based essential oils are reported by the U.S. Food and Drug Administration (FDA) as substances that are generally recognized as safe (GRAS) for human consumption.[12] Previous studies also have showed that plant essential oil exhibited potent antifungal activities.[13,14]

Thyme oil and oregano oil, and their main component have been reported for antimicrobial activities against both bacteria and fungi.[15,16] One study determined antimicrobial activity for these essential oils against microbes associated with otitis externa in animals.[17] However, to the best of the authors' knowledge, no study has looked at their main phenolic components against a range of common clinical otitis externa microbes, including sensitive and resistant strains. Therefore, *in vitro* efficacy of oregano oil, thyme oil and their main phenolic constituents against bacterial and fungal isolates associated with canine otitis externa were investigated. It was hypothesized that the main phenolic components would have a stronger antimicrobial activity compared to the relative essential oil.

Methods and materials

Bacterial and fungal isolates

A total of 100 bacterial and fungal isolates from cases of canine otitis externa were kindly provided by the Australian Centre for Antimocrbial Resistance Ecology, The University of Adelaide, including: meticillin-susceptible *S. pseudintermedius* (MSSP, n = 10); MRSP (n = 10); β-haemolytic *Streptococcus* spp. (n = 20); *P. aeruginosa* (n = 20, including six isolates resistant to gentamicin, two isolates resistant to ciprofloxacin, one isolate resistant to cefepime, and one isolate resistant to both ciprofloxacin and cefepime (Table S3); *Proteus mirabilis* (n = 20); and *Malassezia pachydermatis* (n = 20). All tested bacterial and fungal isolates in this study were obtained from a collection (frozen in brain heart infusion with 20% glycerol at −80°C until tested) of a national survey of antimicrobial resistance in animals conducted in Australia.[5] Isolates had been identified to species level using biochemical testing and Matrix-Assisted Laser Desorption/Ionization-Time Of Flight mass spectrometry (Bruker; Preston, Victoria, Australia) before antimicrobial susceptibility testing.[18–20]

The isolates were regrown one day before antimicrobial activity testing. Bacterial isolates were sub-cultured on 5% sheep blood agar (SBA, Thermo Fisher Scientific; Melbourne, Victoria, Australia) and incubated at 36 ± 1°C for 24 h, whereas fungal isolates were inoculated on Sabouraud's dextrose agar (SDA) supplemented with 1% Tween 80 (Sigma-Aldrich; Sydney, New South Wales, Australia) and incubated at 35 ± 1°C for up to 72 h. For quality control for minimum inhibitory concentration (MIC) testing, *Escherichia coli* American Type Culture Collection (ATCC) 25922, *Staphylococcus aureus* ATCC 29213, *P. aeruginosa* ATCC 27853, *Candida albicans* ATCC 90028 and *C. krusei* ATCC 6258 were used as described previously.[18]

Essential oils and antimicrobial agents

Essential oils from oregano (*Thymus capitatus*, W282812) containing 60–75% phenols, and thyme (*Thymus vulgaris*, W306509) containing ≥40% phenols, as well as their major phenolic compounds, carvacrol (≥98% of purity, W224502) and thymol (≥98.5% of purity, T0501) were purchased from Sigma-Aldrich (St Louis, MO, USA). Ampicillin, gentamicin and amphotericin B (Sigma-Aldrich) were selected where applicable to monitor the quality control isolates to test system performance.

Antibacterial susceptibility testing

Two-fold serial dilutions of the essential oils and their phenolic compounds were performed in 100% dimethyl sulfoxide (DMSO). Subsequently, a 1 μL aliquot of each concentration was transferred to each corresponding well in the challenge plate using an electronic multichannel pipette because the compounds are hydrophobic. Concentration of essential oils and the phenolic compounds tested were converted to μg/mL based on the densities reported in the sample label. The assay was performed in a total volume of 100 μL with test concentrations ranging from 0.002 to 1% (18–9,760 μg/mL) for all samples. Preliminary testing found that 1% DMSO in the final concentration did not exhibit antimicrobial activity (data not shown). MIC tests involving ampicillin and gentamicin against *S. pseudintermedius*, *P. aeruginosa* and *P. mirabilis* were performed according to CLSI standards[19] in cation-adjusted Mueller–Hinton broth (CAMHB, Becton Dickinson Pty Ltd; Sparks, MA, USA) from 0.03 to 64 μg/mL. MICs were determined for streptococcal isolates using CAMHB supplemented with 4% lysed horse blood. All plates were incubated for 20–24 h in ambient air at 36 ± 1°C and growth of bacteria assessed after 24 h, both visually and using optical density (OD) readings from a microplate reader (Eppendorf BioPhotometer plus; Hamburg, Germany) at a wavelength of 600 nm. MICs were recorded as the lowest concentration that completely inhibited growth, disregarding any single colony or faint haze caused by the inoculum;[21] the values at which 50% (MIC_{50}) and 90% (MIC_{90}) of the isolates were inhibited were calculated.

In order to determine minimum bacterial concentrations (MBCs), 10 μL aliquots were taken from all wells above the MIC and spotted onto SBA after the determination of the MIC. MBC was determined as the lowest concentration where 99.9% of the bacterial inoculum was eradicated after incubating plates for 24 and 48 h.

Kill kinetics assay

Kill kinetic assays on luminescent *P. aeruginosa* ATCC PAO1 (Xen 41) and luminescent *S. aureus* ATCC 12600 (Xen 29) were performed (in duplicate) as described for the MIC determination, with the exception that the starting concentration of each compound was 0.5% (4,585–5,250 μg/mL). For each bacterium, the kill kinetics assay was performed using 200 μL volumes in a black round-bottom 96-well microtitre plate (Sarstedt 82.1582.001, Sarstedt; Adelaide, South Australia, Australia). The plate was covered with a Breathe-Easy sealing membrane (Z380059, Sigma-Aldrich) and incubated for 20 h overnight at 36 ± 1°C in a Cytation 5 Cell Imaging Multi-Mode Reader (BioTek; Winooski, VT, USA).[22]

Time-dependent killing assays

Time–kill studies on clinical isolates of *P. aeruginosa* and *S. pseudintermedius* were performed (in duplicate) using the CLSI macrodilution broth method.[21] Briefly, each essential oil or its phenolic component was prepared in 9.9 mL volumes at MIC, and 2 × MIC in CAMHB. A few colonies of *P. aeruginosa* and *S. pseudintermedius* from an overnight SBA were suspended in 3 mL of normal saline and adjusted to 600 nm = 0.10 [equivalent to approximately 5×10^7 colony forming unit (cfu/mL)]. After adding 200 μL bacterial suspension to each tube, the tubes were incubated in a shaking incubator at 36 ± 1°C, for 24 h. Duplicate cultures were incubated at 36 ± 1°C, with samples withdrawn at 0, 0.25, 0.5, 1, 2, 4 and 24 h. Serial 10-fold dilutions (using sterile saline) were grown on SBA for bacteria enumeration.

Table 1. Minimum inhibitory concentration (MIC) range, MIC_{50} and MIC_{90} (% values) (μg/mL) of oregano oil, carvacrol, thyme oil and thymol against 100 microbial isolates from dogs with otitis externa

Organism	Value	Concentration, % values (μg/mL[†])			
		Oregano	Carvacrol	Thyme	Thymol
Staphylococcus pseudintermedius [n = 20; MSSP(10), MRSP (10)]	MIC range	0.015–0.03 (140–281)	0.015–0.03 (146–292)	0.015–0.03 (137–275)	0.01–0.02 (100–200)
	MIC_{50}	0.015 (140)	0.015 (146)	0.03 (275)	0.02 (200)
	MIC_{90}	0.03 (281)	0.015 (146)	0.03 (275)	0.02 (200)
β-haemolytic *Streptococcus* spp. (n = 20)	MIC range	0.03 (281)	0.015–0.03 (146–292)	0.03 (275)	0.02–0.04 (200–400)
	MIC_{50}	0.03 (281)	0.015 (146)	0.03 (275)	0.02 (200)
	MIC_{90}	0.03 (281)	0.03 (292)	0.03 (275)	0.04 (400)
Pseudomonas aeruginosa[a] (n = 20)	MIC range	0.06–0.125 (563–1173)	0.06–0.125 (585–1120)	0.06–0.25 (550–2292)	0.04–0.09 (400–800)
	MIC_{50}	0.125 (1173)	0.06 (585)	0.06 (550)	0.09 (800)
	MIC_{90}	0.125 (1173)	0.125 (1120)	0.25 (2292)	0.09 (800)
Proteus mirabilis (n = 20)	MIC range	0.03 (281)	0.015–0.03 (146–292)	0.03–0.06 (275–550)	0.02 (200)
	MIC_{50}	0.03 (281)	0.015 (146)	0.03 (275)	0.02 (200)
	MIC_{90}	0.03 (281)	0.03 (292)	0.03 (275)	0.02 (200)
Malassezia pachydermatis (n = 20)	MIC range	0.06 (563)	0.06 (585)	0.125 (1146)	0.04–0.09 (400–800)
	MIC_{50}	0.06 (563)	0.06 (585)	0.125 (1146)	0.09 (800)
	MIC_{90}	0.06 (563)	0.06 (585)	0.125 (1146)	0.09 (800)

[†]Values converted into μg/mL based on the density values of each tested samples; MSSP meticillin-susceptible *S. pseudintermedius*, MRSP meticillin-resistant *S. pseudintermedius*; a includes antimicrobial-sensitive *P. aeruginosa* (n = 10) and *P. aeruginosa* resistant to one or two antimicrobials (n = 10).

Antifungal susceptibility testing

The antifungal susceptibility testing of *M. pachydermatis* isolates was performed using a previous method.[23] Briefly, isolates were cultured onto SDA supplemented with 1% Tween 80 (Sigma-Aldrich) and incubated for 48–72 h at 35 ± 1°C. A two-fold serial dilution of the antimicrobial stock solution was prepared in 1% DMSO at ×100 concentration from wells 12 to 3 (from 0.2 to 100%). Aliquots (2 μL) of each concentration were then added to each well in the challenge plate using an electronic multichannel pipette and each dilution was then further diluted to 1:100 in Sabouraud's dextrose broth (SDB) (Oxoid™; Hampshire, UK) supplemented with 1% Tween 80 in 96-well microtitre plates. The final concentration of tested antimicrobial agent (essential oils and their major phenolic compounds) and control antimicrobial agent (amphotericin B) in each plate was 0.002–1% (18–9,760 μg/mL) and 0.008–4 μg/mL, respectively. Preliminary testing found that 1% DMSO in the final concentration did not exhibit any antifungal activity (data not shown). *Malassezia pachydermatis* isolates were suspended in SDB supplemented with 1% Tween 80 to obtain a uniform fungal suspension with optical density (OD) 0.1 (equivalent to ≈ 1–5 × 10^6 cfu/mL) at 530 nm wavelength (Eppendorf BioPhotometer plus). Subsequently, 20 μL of a 1:100 dilution of the fungal suspension in saline was added to 180 μL of SDB in each well to create a final inoculum concentration of approximately 1-5 × 10^3 cfu/mL. After incubation of the plates at 35 ± 1°C for up to 72 h, the minimum fungicidal concentration (MFC) end-point was determined using both visual end-point reading of 50% growth inhibition compared with the positive growth control and spectrophotometric end-point reading of OD 530 nm. The MFC, MFC_{50} and MFC_{90} were calculated as reported above for bacteria.

Statistical analysis

The antimicrobial activity of essential oils and their main chemical compounds were compared based on unpaired Student's *t*-test using GraphPad Prism v8.0 (San Diego, CA, USA).

Results

Antimicrobial activity of essential oils and their main phenolic compounds against isolates from dogs with otitis externa

Oregano oil, carvacrol, thyme oil and thymol demonstrated *in vitro* antimicrobial activity against bacterial isolates from dogs with otitis externa with MIC_{90} values ranging from 0.02% (200 μg/mL) to 0.25% (2,292 μg/mL) (Tables 1, S1–4). The drug-resistant isolates, such as MRSP and *P. aeruginosa* showed similar low MICs to the tested essential oils and their main phenolic compounds, with MIC_{90} values ranging from 0.03% (275 μg/mL) to 0.25% (2,292 μg/mL).

For *S. pseudintermedius* isolates, carvacrol was active at lower concentrations compared to the other compounds (MIC_{90} = 0.015%; 146 μg/mL). In addition, MRSP isolates exhibited susceptibility to essential oils and their phenolic compounds over similar MIC ranges compared to MSSP isolates. Oregano oil and thyme oil showed antimicrobial activity against β-haemolytic *Streptococcus* isolates at MIC_{90} values of 0.03% (275–281 μg/mL) and were similar to those obtained for *Staphylococcal* isolates (Table 1). However, a two-fold increase in MIC_{90} values were observed for carvacrol and thymol (MIC_{90} = 0.03–0.04%; 292–400 μg/mL) when compared to those for the staphylococcal isolates (MIC_{90} = 0.015–0.02%; 146–200 μg/mL).

Essential oils and their phenolic compounds showed similar antimicrobial activities against *P. aeruginosa* isolates. The antimicrobial activities for *P. aeruginosa* isolates (MIC_{90} values ranged from 0.09 to 0.25%; 800 to 2,292 μg/mL) were higher compared to those for Gram-positive isolates (MIC_{90} values ranged from 0.015 to 0.04%; 146–400 μg/mL). *Pseudomonas aeruginosa* isolates were most susceptible to thymol (MIC_{90} = 0.09%; 800 μg/mL) compared to other tested compounds.

Oregano oil, carvacrol, thyme oil and thymol showed similar antimicrobial activity against *P. mirabilis* (MIC_{90} values ranged from 0.02–0.03%; 200–292 μg/mL) and was comparable to that against Gram-positive isolates. However, the selected oils and phenolic compounds were more effective in inhibiting *P. mirabilis* isolates than *P. aeruginosa* isolates (MIC_{90} values ranged from 0.09 to 0.25%; 800 to 2,292 μg/mL).

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Table 2. Minimum inhibitory concentration (MIC) values (μg/mL) for ampicillin, gentamicin and amphotericin B against quality-control bacterial strains obtained in the present study and acceptable range according to CLSI standards.[21]

	Acceptable range of MIC (μg/mL)			MIC obtained (μg/mL)		
Bacterial control strain	AMP	GEN	AMB	AMP	GEN	AMB
Staphylococcus aureus ATCC 29213	0.5–2	0.12–1	–	0.5–1	0.25–0.5	–
Escherichia coli ATCC 25922	2–8	0.25–1	–	2–4	0.5–1	–
Pseudomonas aeruginosa ATCC 27853	–	0.5–2	–	–	1–2	–
Candida albicans ATCC 90028	–	–	0.5–2	–	–	0.5–1
Candida krusei ATCC 6258	–	–	0.25–2	–	–	0.25–1

AMB amphotericin B, AMP ampicillin, GEN gentamicin.

Table 3. Minimum bactericidal concentrations (% values) (μg/mL) and minimum fungicidal concentrations of oregano oil, carvacrol, thyme oil and thymol against 100 isolates from dogs with otitis externa

		Concentration % values (μg/mL†)			
Organism	Value	Oregano	Carvacrol	Thyme	Thymol
Staphylococcus pseudintermedius [n = 20; MSSP(10), MRSP (10)]	MBC range	0.015–0.03 (140–281)	0.03–0.06 (292–585)	0.015–0.03 (137–275)	0.02–0.04 (200–400)
	MBC_{50}	0.015 (140)	0.03 (292)	0.03 (275)	0.04 (400)
	MBC_{90}	0.03 (281)	0.03 (292)	0.03 (275)	0.04 (400)
β-haemolytic *Streptococcus* spp. (n = 20)	MBC range	0.03 (281)	0.015–0.06 (146–585)	0.03 (275)	0.02–0.09 (200–800)
	MBC_{50}	0.03 (281)	0.03 (292)	0.03 (275)	0.04 (400)
	MBC_{90}	0.03 (281)	0.06 (585)	0.03 (275)	0.09 (800)
Pseudomonas aeruginosa‡ (n = 20)	MBC range	0.06–0.125 (563–1173)	0.06–0.125 (585–1120)	0.06–0.25 (550–2292)	0.04–0.09 (400–800)
	MBC_{50}	0.125 (1173)	0.06 (585)	0.06 (550)	0.09 (800)
	MBC_{90}	0.125 (1173)	0.125 (1120)	0.25 (2292)	0.09 (800)
Proteus mirabilis (n = 20)	MBC range	0.03 (281)	0.015–0.03 (146–292)	0.03–0.06 (275–550)	0.02 (200)
	MBC_{50}	0.03 (281)	0.015 (146)	0.03 (275)	0.02 (200)
	MBC_{90}	0.03 (281)	0.03 (292)	0.03 (275)	0.02 (200)
Malassezia pachydermatis (n = 20)	MFC range	0.06 (563)	0.06 (585)	0.125 (1146)	0.04–0.09 (400–800)
	MFC_{50}	0.06 (563)	0.06 (585)	0.125 (1146)	0.09 (800)
	MFC_{90}	0.06 (563)	0.06 (585)	0.125 (1146)	0.09 (800)

†Values converted into μg/mL based on the density values of each tested samples; MSSP meticillin-susceptible *S. pseudintermedius*, MRSP meticillin-resistant *S. pseudintermedius*.
‡Includes antimicrobial-sensitive *P. aeruginosa* (n = 10) and *P. aeruginosa* resistant to one or two antimicrobials (n = 10).

The MIC values of quality control strains against ampicillin and gentamicin were within the CLSI range (Table 2).

Antifungal activity of essential oils and phenolic compounds against *M. pachydermatis*

Oregano oil, carvacrol, thyme oil and thymol showed antifungal activity against *M. pachydermatis* isolates; they were more sensitive to oregano oil and carvacrol (MIC_{90} = 0.06%; 563–585 μg/mL), followed by thymol (MIC_{90} = 0.09%; 800 μg/mL) and thyme oil (MIC_{90} = 0.125%; 1,146 μg/mL) (Table 1). Additionally, *M. pachydermatis* was reported to have the same MFC values as their MIC values (Table 3). The MIC values of quality control strains against amphotericin B were within the CLSI range (Tables 2, S5).

MBC, MFC values and time-dependent killing assays

Minimum bactericidal concentrations and MFC values for oregano oil, carvacrol, thyme oil and thymol against all of the isolates tested are shown in Table 3. The MBC values for all compounds were equal to the MIC concentration for Gram-negative isolates; however, the MBC values for carvacrol and thymol were two dilutions higher than the MIC values for Gram-positive isolates (Tables S6–10). The MFC values for all compounds were equal to the MIC concentrations for *M. pachydermatis* isolates (Table 3).

For the kill kinetics assays, it was found that oregano oil and carvacrol took 30 m to eliminate *S. pseudintermedius* below the level of detection at 2× MIC, whereas for thyme oil and thymol, kill time was 4 h at 2× MIC, indicating a bactericidal effect in all compounds (Figure 1 a). By comparison with ampicillin kill kinetics in which *S. pseudintermedius* was eliminated only after 24 h, the tested compounds were more effective in killing *S. pseudintermedius*. For *P. aeruginosa*, all compounds, including gentamicin, took 1 h to clear the population at 2× MIC (Figure 1 c). A similar trend was observed for kill kinetics assays when luminescent strains were performed (Figure 1 b and d).

Comparison of the antimicrobial activity of essential oils with their main phenolic compounds against canine otitis externa isolates

Carvacrol exhibited lower MIC values when compared to oregano oil against β-haemolytic *Streptococcus* spp. (190 ± 67 versus 282 ± 0 μg/mL, $P < 0.001$), *P. aeruginosa* (585 ± 0 versus 1,051 ± 250 μg/mL, $P < 0.0001$) and *P. mirabilis* (161 ± 44 versus 281 ± 0 μg/mL, $P < 0.0001$) (Figure 2 a). No differences were observed in *S. pseudintermedius* and *M. pachydermatis* when the MIC values were compared for carvacrol versus oregano oil. Thymol showed lower MIC values when compared to thyme oil against *S. pseudintermedius* (180 ± 41 versus

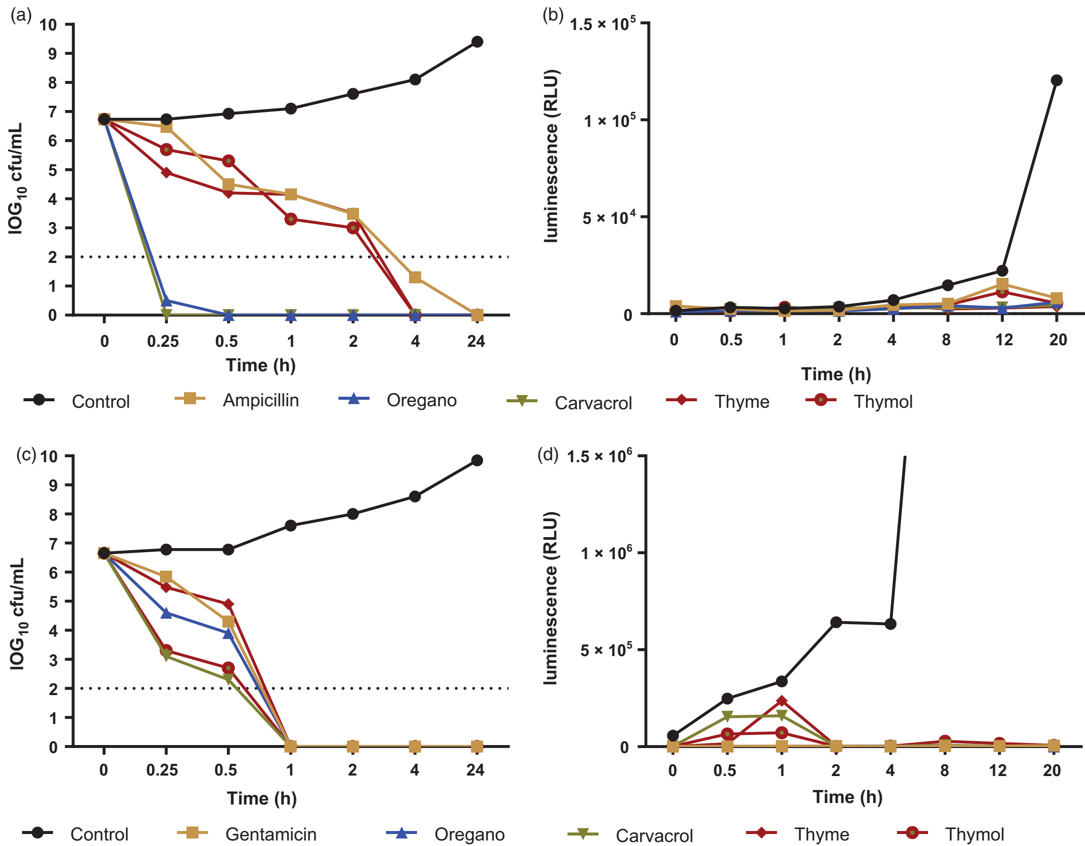

Figure 1. Time-dependent kill kinetic assays of *Staphylococcus pseudintermedius* and *Pseudomonas aeruginosa*.
[(a) *S. pseudintermedius*, (b) *Staphylococcus aureus* ATCC 12600 (Xen29), (c) *P. aeruginosa* and (d) *P. aeruginosa* ATCC PAO1 (Xen 41)] following exposure to oregano oil, thyme oil, carvacrol and thymol at 2× MIC compared to a positive control and growth control.

261 ± 42 μg/mL, $P < 0.01$), β-haemolytic *Streptococcus* spp. (220 ± 60 versus 275 ± 0 μg/mL, $P < 0.05$), *P. mirabilis* (195 ± 15 versus 275 ± 0 μg/mL, $P < 0.0001$) and *M. pachydermatis* (680 ± 188 versus 1,146 ± 0 μg/mL, $P < 0.0001$) (Figure 2 b).

Discussion

The antimicrobial activity of essential oils previously has been attributed to their phenolic component contents.[24] To the best of the authors' knowledge, this is the first study that evaluates the potential of essential oils (oregano, thyme) and their phenolic components (carvacrol, thymol) as novel antimicrobial agents against a comprehensive range of canine otitis externa isolates. In this study, both oregano oil and thyme oil showed good antimicrobial activity against Gram-negative and Gram-positive bacteria as well as *M. pachydermatis* with MIC_{90} values ranging from 0.02 to 0.25% (200–2,292 μg/mL). These results are in agreement with previous studies including oregano oil and thyme oil as potential antimicrobial agents against a broad range of bacteria;[14,17] however, these studies mostly used the disk diffusion technique and presented the MIC values. In the present study, the antimicrobial activities of the essential oils and their phenolic components are reported as MIC range, MIC_{50} and MIC_{90} values. These values allowed determination of the effectiveness of these oils or components against a large panel of isolates of bacterial and fungal species, 20 isolates each in the present study.

All tested compounds also showed bactericidal activity against *S. pseudintermedius*, β-haemolytic streptococci, *P. mirabilis* and *P. aeruginosa* isolates at their MIC values or two-fold MIC values. The kill kinetic assays revealed that the bacteria treated with the essential oils and their phenolic compounds were inactivated over a relatively short time at 2× MIC compared to the growth control, confirming the bactericidal activity of the tested compounds. The present results were consistent with previous studies that reported both essential oils exhibiting similar bactericidal activity with their respective phenolic compounds.[25,26] In addition, the MBC values of oregano oil, thyme oil, carvacrol and thymol against *S. epidermis*, *P. mirabilis* and *P. aeruginosa* reported in previous studies[25,26] ranged from 1× to 2× of their MIC values, in agreement with the present study.

The results herein showed that *P. aeruginosa* isolates are susceptible to the tested essential oils and their

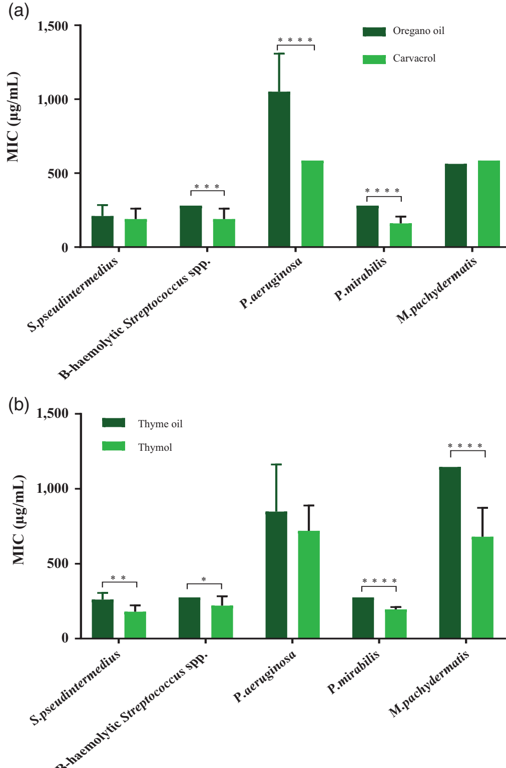

Figure 2. Comparison between the antimicrobial activity of (a) oregano oil and carvacrol, (b) thyme oil and thymol for isolates of *Staphylococcus pseudintermedius*, β-haemolytic *Streptococcus* spp., *Pseudomonas aeruginosa*, *Proteus mirabilis* and *Malassezia pachydermatis*.
Analysis was determined using unpaired Student's *t*-test and presented as mean ± SD, n = 20.
$*P < 0.05$; $**P < 0.01$; $***P < 0.001$; $****P < 0.0001$.

phenolic components; however, this is in disagreement with a previous study,[17] which showed no antibacterial activity against oregano oil. This contradiction may be due to the different techniques and concentrations used in the previous and present studies. In the previous study, disk diffusion testing and a low concentration of essential oils (30 µg) were applied rather than the CLSI broth microdilution method and a range of essential oil concentrations as used in the present study. In addition, both carvacrol and thymol showed very similar antimicrobial activities against *P. aeruginosa*, further confirming the antimicrobial activities of these phenolic components as reported previously.[16,27] Overall, these favourable responses are driving further interest in phenolic components as novel antimicrobial agents for topical treatment of otitis externa in dogs.

The emergence and rapid spread of MDR pathogens have become a global challenge to current antimicrobial therapy. In the present study, the antimicrobial activity of essential oils and their phenolic compounds were evaluated against MRSP and drug-resistant *P. aeruginosa*. The results herein are in agreement with previous studies, reporting the antimicrobial activity of similar essential oils against meticillin-resistant *S. aureus*[28] and antimicrobial-resistant *P. aeruginosa*.[29] Similar to the results herein, a previous study also showed carvacrol and thymol possessing antimicrobial activities against antimicrobial-resistant *S. aureus* and *E. coli*.[30] The proposed mechanism includes the hydrophobic nature of carvacrol and thymol in their distribution into membranes, and the presence of free hydroxyl groups of these compounds disrupting the ion gradients of bacterial cells.[31] This suggests that phenolic compounds could act differently on bacteria compared to antimicrobials, emphasizing the potential of phenolic compounds in eradicating resistant pathogens.

The present findings also showed that the main phenolic components of essential oils exhibited stronger antimicrobial activities compared to their respective essential oils. The MIC values of both carvacrol and thymol were significantly lower compared to oregano oil and thyme oil against β-haemolytic *Streptococcus* spp., *P. aeruginosa* and *P. mirabilis*. These results support previous studies where the inhibitory concentration of carvacrol was four times lower than oregano essential oil in inhibiting the growth of bacteria.[32,33] Therefore, phenolic components such as carvacrol and thymol could be used as novel treatments for canine otitis externa, in particular if caused by antimicrobial-resistant pathogens.

Furthermore, these phenolic compounds herein showed potential antifungal activity against *M. pachydermatis*. This is in agreement with the antifungal activity of essential oils against *Malassezia* species and *Candida albicans* reported in previous studies.[13,14,34] The ratio of $MFC_{90} : MIC_{90} = 1$ demonstrated that both of the tested essential oils and their phenolic compounds in the present study exhibited fungicidal activity.

Our findings show that oregano oil, thyme oil, carvacrol and thymol could be developed as novel treatments for sensitive and resistant bacteria and fungal organisms involved in canine otitis externa. The use of phenolic compounds is suggested for the development of otitis externa treatment due to the high antimicrobial activity and the variance of phenolic composition in essential oils originating from different geographical locations.

Acknowledgements

We thank Amanda Ruggero, Lora Bowes and Anh Hong Nguyen at the University of South Australia for their assistance with technical assistance.

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Supporting Information

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

Tables S1–S5. MIC values for essential oils and their major components against *Staphylococcus* pseudintermedius, β-haemolytic *Streptococcus* spp., *Pseudomonas aeruginosa, Proteus mirabilis* and *Malassezia pachydermatis*, isolated from dogs with otitis externa.

Tables S6–10. MBC and MFC values for essential oils and their major components against *Staphylococcus* pseudintermedius, β-haemolytic *Streptococcus* spp., *Pseudomonas aeruginosa, Proteus mirabilis* and *Malassezia pachydermatis*, isolated from dogs with otitis externa.

Résumé

Contexte – Les pathogènes multirésistants représentent un défi majeur pour la thérapeutique antimicrobienne et compliquent fréquemment les otites externes canines.

Hypothèses/Objectifs – Nous avons étudiés l'efficacité *in vitro* de l'huile d'origan, de l'huile de thym et de leurs principaux composés phénoliques, contre les souches bactériennes et fongiques associées aux otites externes canines. Il est supposé que les composés phénoliques principaux auraient une meilleure activité antimicrobienne en comparaison avec les huiles essentielles.

Matériels et méthodes – La sensibilité antimicrobienne a été testée par microdilution sur plaque pour déterminer la concentration minimale inhibitrice et les concentrations bactéricides/fongicides (MICs, MBCs et MFCs). Un test cinétique temps dépendant a été réalisé pour confirmer l'activité antifongique et

bactéricide des huiles et de leurs constituants phénoliques. Cent souches bactériennes et fongiques ont été utilisées, comprenant *Staphylococcus pseudintermedius* sensible à la méticiline (n = 10), *S. pseudintermedius* résistante à la méticiline (n = 10), *Streptococcus spp.* β-hémolytique (n = 20), *Pseudomonas aeruginosa* (n = 20; comprenant 10 souches résistantes à un ou deux antimicrobiens), *Proteus mirabilis* (n = 20) et *Malassezia pachydermatis* (n = 20) de chiens avec otite externe.
Résultats – L'huile d'origan, l'huile de thym, le carvacrol et le thymol ont montré une activité antibactérienne contre toutes les souches bactériennes et fongiques testées. Les valeurs de MIC90 allant de 0.015 à 0.03% (146–292 μg/mL) pour la bactérie Gram-positive et *P. mirabilis*. Pour *P. aeruginosa* et *M. pachydermatis*, les valeurs de MIC90 allaient de 0.09 à 0.25% (800–2,292 μg/mL).
Conclusions et importance clinique – L'huile d'origan, de thym, le carvacrol et le thymol ont montré une bonne activité bactéricide et fongicide contre 100 souches de chiens avec otite externe, comprenant des souches hautement résistantes. Ces huiles essentielles et leurs constituants phénoliques principaux ont le potentiel d'être étudiés *in vivo* pour le traitement des otites externes canines.

RESUMEN

Introducción – los patógenos resistentes a múltiples fármacos presentan un reto global importante en la terapia antimicrobiana y con frecuencia complican la otitis externa en perros.
Hipótesis/Objetivos – Se investigó la eficacia *in vitro* del aceite de orégano, el aceite de tomillo y sus principales componentes fenólicos contra los aislados bacterianos y fúngicos asociados con la otitis externa canina. Se planteó la hipótesis de que los principales componentes fenólicos tendrían una mayor actividad antimicrobiana en comparación con el aceite esencial relativo.
Métodos y materiales – las pruebas de susceptibilidad a los antimicrobianos se realizaron mediante microdilución en caldo con técnica de cultivo puntual para determinar las concentraciones mínimas inhibitorias y bactericidas/fungicidas (MIC, MBC y MFC). Se realizó un ensayo de cinética de tiempo de destrucción para confirmar la actividad bactericida y fungicida de los aceites y sus componentes fenólicos. Cien aislados bacterianos y fúngicos, incluyendo *Staphylococcus pseudintermedius* susceptible a meticilina (n = 10), *S. pseudintermedius* resistente a meticilina (n = 10), *Streptococcus sp.* β-hemolítico (n = 20), *Pseudomonas aeruginosa* (n = 20; incluidos 10 aislamientos resistentes a uno o dos antimicrobianos), *Proteus mirabilis* (n = 20) y *Malassezia pachydermatis* (n = 20) de perros con otitis externa.
Resultados – el aceite de orégano, el aceite de tomillo, el carvacrol y el timol exhibieron actividad antibacteriana contra todos los aislamientos bacterianos y fúngicos probados. Los valores de MIC_{90} variaron de 0,015 a 0,03% (146–292 μg/mL) para las bacterias Gram-positivas y *P. mirabilis*. Para *P. aeruginosa* y *M. pachydermatis*, los valores de MIC_{90} variaron de 0,09 a 0,25% (800–2,292 μg/mL).
Conclusiones e importancia clínica – el aceite de orégano, el aceite de tomillo, el carvacrol y el timol mostraron una buena actividad bactericida y fungicida *in vitro* frente a 100 aislados de perros con otitis externa, incluidos algunos aislados altamente resistentes a los fármacos. Estos aceites esenciales y sus principales componentes fenólicos tienen el potencial de ser investigados *in vivo* para el tratamiento de la otitis externa canina.

Zusammenfassung

Hintergrund – Multiresistente Pathogene stellen eine große globale Herausforderung bei der antimikrobiellen Therapie dar und verkomplizieren häufig eine Otitis externa von Hunden.
Hypothese/Ziele – Die *in vitro* Wirksamkeit von Oreganoöl, Thymianöl und ihrer hauptsächlichen phenolischen Bestandteile gegen bakterielle und mykologische Isolate, die mit einer Otitis externa im Zusammenhang stehen, wurden untersucht. Es wurde die Hypothese aufgestellt, dass die hauptsächlichen Phenol-Komponenten eine größere antimikrobielle Aktivität im Vergleich zu den relativen essentiellen Ölen haben.
Methoden und Materialien – Es wurden Hemmstoffnachweise mittels Mikrodilutionsbouillon mit einer Spot- Plating Technik durchgeführt, um eine minimale Hemmstoffkonzentration und bakterielle/fungizide Konzentrationen (MICs, MBCs und MFCs) zu bestimmen. Ein Time-Kill Kinetik Assay wurde durchgeführt, um bakterizide und fungizide Aktivitäten der Öle und ihrer phenolischen Bestandteile zu bestätigen. Es wurden einhundert bakterielle und mykologische Isolate, inklusive Methicillin-empfindlichem *Staphylococcus pseudintermedius* (n = 10), Methicillin-resistentem *S. pseudintermedius* (n = 10), β-*hämolytischem Streptococcus spp.* (n = 20), *Pseudomonas aeruginosa* (n = 20; inklusive 10 Isolaten, die resistent zu einem oder zwei antimikrobiellen Wirkstoffen waren), *Proteus mirabilis* (n = 20) und *Malassezia pachydermatis* (n = 20) von Hunden mit einer Otitis externa verwendet.
Ergebnisse – Oreganoöl, Thymianöl, Carvacrol und Thymol zeigten eine antibakterielle Aktivität gegenüber allen getesteten bakteriellen und mykologischen Isolaten. MIC_{90} Werte reichten von 0,015 bis 0,03% (146-292 μg/mL) für Gram-positive Bakterien und *P. mirabilis*. Für *P. aeruginosa* und *M. pachydermatis* lagen die MIC_{90} Werte zwischen 0,09 und 0,25% (800-2,292 μg/mL).
Schlussfolgerungen und klinische Bedeutung – Oreganoöl, Thymianöl, Carvacrol und Thymol zeigten eine gute *in vitro* bakterizide und fungizide Aktivität gegenüber 100 Isolaten von Hunden mit Otitis externa, wobei einige hochresistente Isolate dabei waren. Diese essentiellen Öle und ihre hauptsächlichen

phenolischen Bestandteile haben das Potential *in vivo* für die Therapie einer Otitis externa des Hundes weiter untersucht zu werden.

要約

背景 – 多剤耐性病原体は、抗菌薬治療における世界的な主要課題であり、犬外耳炎をしばしば複雑化する。

仮説/目的 – 本研究の目的は、犬の外耳炎に関連する細菌および真菌分離株に対するオレガノ油、タイム油、およびそれらの主要フェノール成分のin vitroにおける有効性を調査することであった。主要なフェノール成分は、関連精油と比較してより高い抗菌活性を持つと仮定した。

材料と方法 – 最小発育阻止濃度および殺菌/殺真菌濃度(MIC、MBC、MFC)決定に対し、spot-plating技術による微量液体希釈法を用いて薬剤感受性試験を実施した。オイルおよびそのフェノール成分の殺菌および殺真菌活性の証明に、time-kill kinetics assayを実施した。外耳炎を有する犬から採材したメチシリン感受性Staphylococcus pseudintermedius(n = 10)、メチシリン耐性S. pseudintermedius(n = 10)、b溶血性連鎖球菌(n = 20)、緑膿菌(n = 20; 1つまたは2つの抗菌薬に耐性を持つ10の分離株を含む)、Proteus mirabilis(n = 20)、およびMalassezia pachydermatis(n = 20)を含む100の細菌および真菌分離株を本研究に供した。

結果 – オレガノ油、タイム油、カルバクロールおよびチモールは、試験したすべての細菌および真菌分離株に対し抗菌活性を示した。 MIC_{90}値は、グラム陽性菌およびP. mirabilisの0.015〜0.03%(146〜2921g/ mL)の範囲にわたった。 緑膿菌およびM. pachydermatisの場合、MIC_{90}値は0.09〜0.25%(800〜2,2921g/ mL)の範囲に及んだ。

結論と臨床的重要性 – オレガノ油、タイム油、カルバクロールおよびチモールは、外耳炎を有する犬から採材された100の分離株(いくつかの高度な薬剤耐性分離株を含む)に対し、in vitroにおいて良好な殺菌および殺菌活性を示した。これらの精油とその主要なフェノール成分は、犬外耳炎治療のに対し、in vivoでさらに調査される可能性を持っている。

摘要

背景 — 多重耐药菌的抗菌治疗是一项全球均需面临的重要挑战,并且其经常使犬外耳炎复杂化。

假设/目的 — 研究牛至油、百里香油及其主要酚类成分,对细菌和真菌引起的犬外耳炎的体外疗效。与精油相比,推测主要酚类成分具有更高的抗菌活性。

方法和材料 — 使用肉汤微量稀释和点镀技术进行药敏试验,以确定最小抑菌、杀菌和杀真菌浓度(MIC、MBC和MFCs)。采用时间-杀菌动力试验以确定精油及其酚类成分的杀菌和杀真菌活性。共选取从犬外耳炎中分离的100株细菌和真菌菌株,包括甲氧西林敏感(n = 10)和甲氧西林耐药(n = 10)的假中间型葡萄球菌、b-溶血性链球菌(n = 20)、铜绿假单胞菌(n = 20;包括对一种或两种抗生素耐药的10个菌株)、奇异变形杆菌(n = 20)和厚皮马拉色菌(p = 20)。

结果 — 牛至油、百里香油、香芹酚和百里香酚对所有检测的细菌和真菌菌株均有抗菌活性。对于革兰氏阳性菌和奇异变形杆菌,MIC_{90}值范围为0.015-0.03%(146-292μg/ mL);对于铜绿假单胞菌和厚皮马拉色菌,MIC_{90}的数值范围为0.09-0.25%(800-2292μg/ mL)。

结论和临床意义 — 牛至油、百里香油、香芹酚和百里香酚对100株犬外耳炎菌株(其中还包括一些高度耐药的菌株),均有良好的体外杀菌和杀真菌活性。精油及其主要酚类成分治疗犬外耳炎的效果,需要进行更多的体内试验来获得。

Resumo

Contexto – Patógenos multirresistentes representam um grande desafio global na terapia antimicrobiana e frequentemente complicam otites externas em cães.

Hipótese/Objetivos – Investigou-se a eficácia *in vitro* do óleo de orégano, óleo de tomilho e seus principais componentes fenólicos contra isolados bacterianos e fúngicos associados com otite externa canina. A hipótese foi de que os principais componentes fenólicos teriam maior atividade antimicrobiana quando comparados com o óleo essencial relativo.

Métodos e materiais – Realizou-se teste de suscetibilidade a antimicrobianos utilizando a microdiluição em caldo com técnica de plaqueamento pontual para determinar a concentração inibitória mínima e as concentrações bactericidas/fungicidas (MICs, MBCs e MFCs). Um ensaio de cinética de tempo de morte foi realizado para confirmar a atividade bactericida e fungicida dos óleos e seus componentes fenólicos. Foram utilizados cem isolados bacterianos e fúngicos de cães com otite externa, incluindo *Staphylococcus pseudintermedius* suscetível à meticilina (n = 10), *S. pseudintermedius* resistente à meticilina (n = 10), *Streptococcus spp* β-hemolítico (n = 20), *Pseudomonas aeruginosa* (n = 20, incluindo 10 isolados resistentes a um ou dois antimicrobianos), *Proteus mirabilis* (n = 20) e *Malassezia pachudermatis* (n = 20).

Resultados – O óleo de orégano, óleo de tomilho, carvacrol e timol exibiram atividade antimicrobiana contra todos os isolados bacterianos e fúngicos testados. Os valores de MIC_{90} variaram entre 0,015 e 0,03% (146–292 μg/mL) para as bactérias Gram-positivas e *P. mirabilis*. Para *P. aeruginosa* e *M. pachydermatis*, os valores de MIC90 variaram de 0,09 a 0,25% (800–2,292 μg/mL).

Conclusões e significância clínica – O óleo de orégano, óleo de tomilho, carvacrol e timol demonstraram boa atividade bactericida e fungicida *in vitro* contra os 100 isolados de cães com otite externa, incluindo isolados altamente multirresistentes. Estes óleos essenciais e seus constituintes fenólicos possuem potencial no tratamento da otite externa canina e devem ser investigados *in vivo*.

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AUSTRALIAN VETERINARY JOURNAL
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SMALL ANIMALS

Prevalence of positive reactions in intradermal and IgE serological allergy tests in dogs from South Australia, and the subsequent outcome of allergen-specific immunotherapy

C Han, WY Chan and PB Hill*

Objective To determine the prevalence of positive allergen reactions in intradermal and IgE serological tests in dogs presenting to a dermatology referral centre in South Australia and the clinical efficacy of subsequent allergen-specific immunotherapy.

Design Retrospective study.

Methods Results from 108 intradermal allergy tests, 25 IgE serological assays and immunotherapy outcomes in 37 dogs were retrospectively analysed. Immunotherapy outcomes were determined as excellent, good, modest or failure using a global assessment of efficacy matrix which incorporated pruritus scores, lesion severity, medication requirements, and owner and clinician opinion.

Results The most common positive reactions in intradermal allergy tests were Red clover (59%), *Dermatophagoides farinae* (29%), *Tyrophagus putrescentiae* (28%), Yellow dock (25%) and *Malassezia pachydermatis* (24%). In the IgE serological tests, Yorkshire fog grass (40%), Yellow dock (36%), Kentucky bluegrass (36%) and *T. putrescentiae* (36%) were the most commonly reported positive results. The outcome of allergen-specific immunotherapy was judged to be excellent in 20% of dogs, good in 15%, modest in 18% and a failure in 47%.

Conclusion As has been reported in other geographical areas, environmental mites and plant pollens frequently gave positive reactions in allergy tests in South Australia. However, the prevalence of individual allergen reactions differed between intradermal and IgE serological tests, with *M. pachydermatis* being identified as a common cause of hypersensitivity in intradermal tests but not in IgE serological assays. Immunotherapy was judged to be a beneficial treatment in 35% of dogs but was essentially unsuccessful in 65%.

Keywords allergy; dog; IgE; immunotherapy; intradermal; South Australia

Aust Vet J 2019 doi: 10.1111/avj.12892

Canine atopic dermatitis is a genetically predisposed inflammatory and pruritic allergic skin disease, with characteristic clinical features that is associated most commonly with IgE antibodies to environmental allergens.[1] A tentative diagnosis of atopic dermatitis can be made based on the presence of typical historical and clinical features, and after other similar pruritic dermatoses have been ruled out.[2] Allergy tests can be used to demonstrate IgE-mediated hypersensitivity and are used to identify offending allergens for allergen-specific immunotherapy.[2,3]

Intradermal allergy testing and IgE serological testing are the two most common ways of detecting allergen sensitivity in dogs. Many clinicians prefer intradermal testing, as it has been used extensively for many decades and directly assesses the skin, the target organ of the disease, and its physiological response to allergens.[4] Intradermal testing is optimised by using allergens specific to the regional location of the patient and more accurate results are obtained when individual allergen extracts are used.[5] IgE serological assays involve ELISA based technologies to detect circulating IgE.[6] Despite some disagreement between intradermal and IgE serological tests,[6] studies have found no significant difference in the results of allergen-specific immunotherapy prescribed based on intradermal tests or IgE serological assays.[7]

Allergen-specific immunotherapy has been used to treat allergic dogs since the first reported successful treatment in 1941,[8] with varying degrees of success described in the literature. Currently, it is indicated in dogs with a definitive diagnosis of atopic dermatitis in which allergen sensitivity has been defined by intradermal or IgE serological tests.[9] It is currently the only therapy that can prevent further development of the disease, offer long-term remission and low ongoing treatment frequency to patients.[9]

The purpose of this study was to determine the prevalence of positive allergen reactions in dogs presenting to the Companion Animal Health Centre at the University of Adelaide's School of Animal and Veterinary Sciences in South Australia, from both intradermal and IgE serological tests. The clinical efficacy of subsequent allergen-specific immunotherapy was also evaluated.

Materials and methods

Data collection and study design

In this retrospective study, data were obtained from the Cornerstone practice management software used by the Companion Animal Health Centre at the University of Adelaide (South Australia). Records between 2011 and mid-2017 from dogs that underwent intradermal or IgE serological tests, and those that had been prescribed immunotherapy, were selected for analysis.

Intradermal allergy test

Intradermal allergy tests were performed in 108 dogs (60 males and 48 females) at various ages ranging from 7 months to 9 years (median of 2). The intradermal allergy test comprised 60 allergens, made up of 3 mites, 5 insects, 18 trees, 15 grasses, 13 weeds,

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Table 1. Allergens included in the intradermal and IgE serological tests, and the prevalence of positive reactions

Allergen	Intradermal test prevalence (%) (n = 108)	IgE serological test prevalence (%) (n = 25)
Mites		
Dermatophagoides farinae	29	24
Dermatophagoides pteronyssinus	21	20
Tyrophagus putrescentiae	28	36
Insects		
Ctenocephalides felis	6	8
Insect mix (house fly, moth, cockroach, mosquito)	4	
Cockroach mix	6	8
Mosquito	6	
Ant	13	16
Trees		
Wattle	6	24
Casuarina	7	28
Eucalyptus	6	8
Melaleuca	12	20
Privet	6	16
Birch	5	8
Chinese elm	7	12
Pine mix	4	16
Red oak	4	16
Olive	5	20
Palm	7	
Peppercorn	10	
Black willow	10	8
Plane tree	5	12
Maple	16	24
Silver poplar	7	16
Liquidamber	5	12
Cypress	5	4
Grasses		
Cocksfoot grass	17	
Couch	13	32
Kentucky bluegrass	12	36
Johnson grass	12	32
Perennial ryegrass	13	28
Paspalum	8	28
Canary grass	14	32
Yorkshire fog	8	40
Brome grass	17	32
Sweet vernal	19	28
Timothy grass	18	28
Bent grass	10	28
Wheat	7	
Oat	8	
Alfalfa	5	
Weeds		
Kochia	15	
Lamb's tongue	10	16
Fat hen	15	20
Ragweed	10	24
Rough pigweed	16	28
Sheep sorrel	12	28
Yellow dock	25	36
Red clover	59	
Dandelion	13	20
Daisy	13	12
Mugwort	6	12
Tobacco	4	
Dog fennel	8	
Mustard weed		20
Moulds		
Alternaria tenuis	4	4
Aspergillus mix	5	0
Cladosporidium herbarum	5	0
Penicillium mix	5	0
Other		
Mixed feathers	2	
Malassezia pachydermatis	24	0

4 moulds, 1 feather and 1 yeast (Table 1). Allergens were imported from Greer (Lenoir, NC, USA) and purchased from Dermcare (Slacks Creek, Queensland, Australia). Histamine was used as a positive control at 0.0275 mg/mL and saline as a negative control. All allergens were tested at a concentration of 1000 PNU/mL except for the mites (1:2000 w/v); flea, ant and tobacco (1:1000 w/v); insect mix (400 PNU/mL); and cypress (1:400 w/v). Appropriate drug withdrawal times were applied before testing. Reactions were subjectively graded from 0 to 5 by comparing the size and erythema of the wheal to histamine, which was arbitrarily assigned a score of 4. Only immediate reactions (within 25 min of allergen injection) were recorded. Any reaction of 2 or greater was considered to be a positive reaction.

IgE serological test

IgE serological tests were performed in 25 dogs (14 males and 11 females) at various ages ranging from 8 months to 9 years (median of 2). Sixteen of these dogs had also undergone intradermal allergy testing. The remaining nine dogs only had the IgE serological test. IgE serological testing was undertaken in preference to intradermal testing

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for several reasons, including owner preference, an inability to return to the clinic for the skin test, an aversion to clipping of the hair coat, concerns over sedation, or active skin disease over the testing site. IgE serological testing was performed using the Heska Allercept test (Heska, Loveland, CO, USA) and performed by Gribbles Veterinary Pathology (Glenside, South Australia, Australia). The test comprised 48 allergens made up of 6 mites/insects, 16 trees, 11 grasses, 10 weeds and 5 moulds/yeasts (Table 1). From 2011 until 2014, the reactions were graded using ELISA Absorbance units in which a result of 150 or greater was considered positive. From 2015 onwards, the test was measured using Heska IgE Receptor Binding Units and a result of 10 or greater is considered positive.

Allergen-specific immunotherapy

Allergen-specific immunotherapy was prescribed to 37 dogs (17 males and 20 females) based on the results of the allergy tests. All of these dogs had undergone an intradermal test, but four dogs had also had an IgE serological test. No dogs were prescribed immunotherapy on the basis of an IgE test alone. Reasons for immunotherapy not being pursued included a negative allergy test, the typical slow rate of onset, the reported success rate, the cost of the treatment, the owner not wishing to inject their own dog, or a preference to try alternative therapies. Immunotherapy vials were formulated by Dermcare (Slacks Creek, Queensland, Australia) at a concentration of 15,000 PNU/mL. If 15 or fewer positive reactions were observed, all allergens were included in the treatment set. If more than 15 positive reactions were observed, 15 allergens were selected based on a subjective assessment of likely exposure, perceived relevance and potential cross reactivity between allergen groups. Immunotherapy was administered by subcutaneous injection by the owners following a standard schedule. Briefly, dogs were given gradually increasing doses of the allergen extract over a 22 day period, followed by administration of a full 1 mL dose every week for 3 doses, every 2 weeks for 3 doses and then every 3 weeks on a continuing basis. Dogs were evaluated after 3 and 9 months of treatment, and then at 8–12 month intervals (see A1 for dosage schedule).

Assessment of treatment outcomes

The outcome of immunotherapy was determined using a global assessment of efficacy matrix (Table 2). This incorporated a collective assessment of multiple parameters, including pruritus score, lesion severity, requirement for additional medications and owner's and clinician's opinions. Due to case-to-case variation in the various parameters, the column descriptions were not applied rigorously, but were used as a guide to aid classification of a patient into one of four treatment responses (excellent, good, modest, none). In other words, a case had to meet the majority, but not all, of the criteria within a particular row to be classified as one of the treatment outcomes. However, ongoing requirement for anti-pruritic medications prevented a dog from being classified as a good or excellent response. This method of assessment was deemed most appropriate for a retrospective study of this type of treatment in which data have to be extracted from medical records and other modalities are typically used concurrently. Categorisation of each dog into one of the four treatment outcomes was performed independently by two of the authors (C. Han and P.B. Hill). In the case of any disagreements, the cases were further discussed until agreement was reached. Cases needed to have been on immunotherapy for at least 9 months to be judged as a success or a failure, unless the treatment had been stopped by the owner due to perceived lack of efficacy or the occurrence of adverse reactions prior to this. Analysis of the final outcome was undertaken in three different ways in relation to these withdrawals – as treatment failures, as potential treatment successes, or with withdrawn cases removed from the analysis altogether.

Statistical analysis

Descriptive and analytical statistics were computed using Microsoft Excel and GraphPad prism. Prevalences of positive reactions were reported for allergy test results, and Fisher's exact test was used to compare age, sex and desexing status of dogs that received immunotherapy. A Kruskal Wallis test was used to compare the number of allergens used to treat dogs with different treatment outcomes.

Table 2. Global assessment of efficacy matrix: Outcomes of immunotherapy were classified into excellent, good, modest or failure based on pruritus level, pruritus score, skin lesions, requirement for concurrent medications and the owner and clinician's opinions of the dog's response to treatment

Treatment outcome	Subjective pruritus level	Pruritus score	Skin lesions	Concurrent medications	Owner's opinion	Clinician's opinion
Excellent	None	0–2	None or very mild	No other medications	Very satisfied	Excellent response
Good	Mild	2–4	Mild, but may have had some flares	Reduced but some still needed	Satisfied	Good response
Modest	Only slight reduction	4–6	Moderate lesions	Ongoing for control of signs	Unsure of benefit	Minimal response but may be worth continuing
Failure	Unchanged	Unchanged	Unchanged	Unchanged	No benefit	Poor response and should stop treatment

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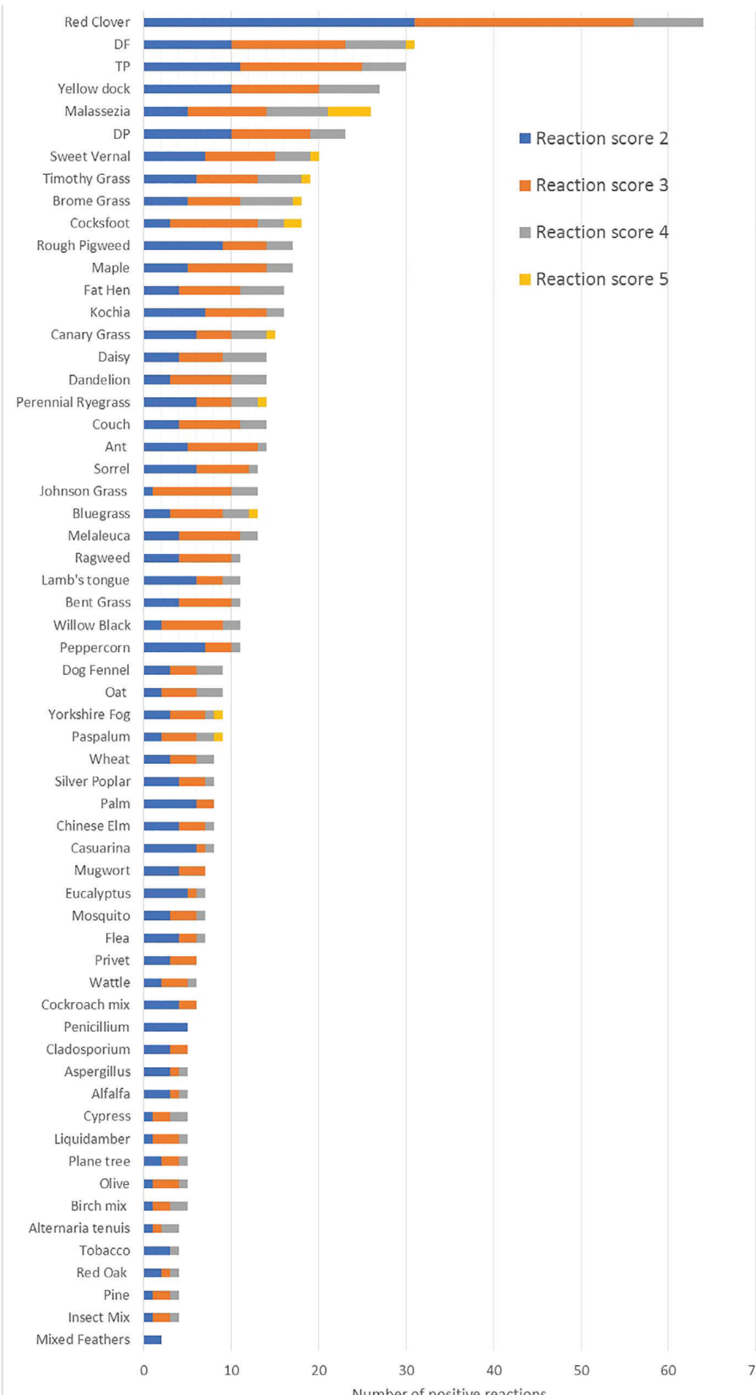

Figure 1. The number of positive reactions for each allergen in the 108 intradermal tests. The reactions were scored from 0–5 subjectively by comparison to the histamine reaction, which was arbitrarily assigned a score of 4. Blue represents a score of 2, orange a score of 3, grey a score of 4 and yellow a score of 5. DF, *Dermatophagoides farinae*; TP, *Tyrophagus putrescentiae*; DP, *Dermatophagoides pteronyssinus*.

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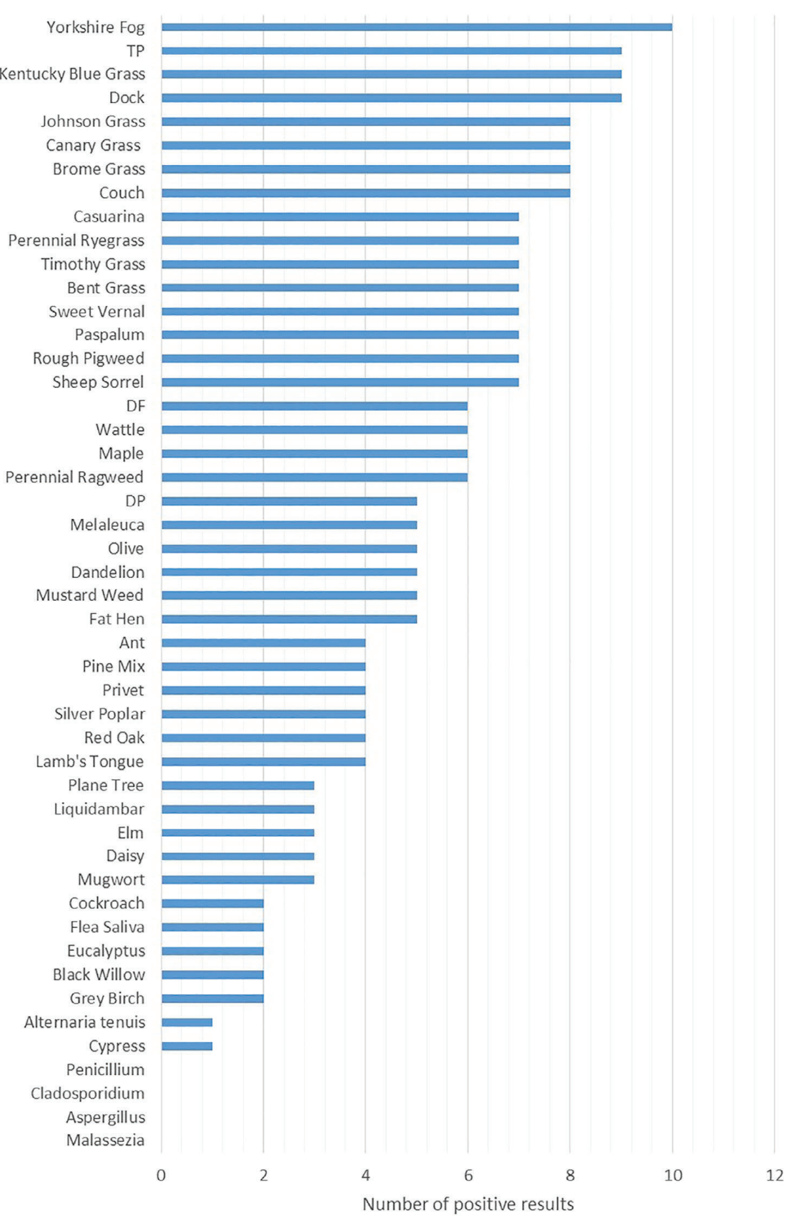

Figure 2. **The number of positive reactions for each allergen in the 25 IgE serological tests. DF, *Dermatophagoides farinae*; TP, *Tyrophagus putrescentiae*; DP, *Dermatophagoides pteronyssinus*.**

Results

Of the 108 intradermal tests performed, 22 were negative and 86 were positive to at least one allergen (80%). Of the 25 IgE serological tests performed, 7 were negative and 18 were positive to at least one allergen (68%). The number of positive reactions seen for each allergen in the 86 positive intradermal tests and the 18 positive IgE serological tests is shown in Figures 1 and 2, respectively. To allow direct comparison, the prevalence of positive reactions in the two tests is shown in Table 1. In the intradermal tests, the five most commonly reacting allergens were Red clover (positive in 64 tests, prevalence of 59%), *Dermatophagoides farinae* (n = 31, 29%), *Tyrophagus putrescentiae* (n = 30, 28%), Yellow dock (n = 27, 25%) and *Malassezia pachydermatis* (n = 26, 24%). Other allergens with a prevalence of positive reactions over 15% included *Dermatophagoides pteronyssinus*, sweet vernal grass, timothy grass, cocksfoot grass, brome grass, maple and rough pigweed. At least one

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Table 3. Outcome of allergen-specific immunotherapy in 34 dogs

Dog	Age	Sex	Breed	Pruritus score	Skin lesions	Concurrent medications	Clinician's opinion	Owner's opinion	Comment
1	2	FS	Great Dane	3	Very mild	None	Excellent	Very satisfied	No requirement for prednisolone and skin appeared virtually normal
2	5	FS	EBT	2	Very mild	None	Excellent	Very satisfied	No requirement for Apoquel
3	1	FS	SBT	2	Very mild	None	Excellent	Very satisfied	Skin appeared virtually normal, and no requirement for ongoing Apoquel
4	1	FS	Rottweiler	0	None	None	Excellent	Very satisfied	Dog appeared normal
5	2	MN	Border Terrier	0	Very mild	None	Excellent	Very satisfied	Dog appeared normal
6	1	MN	GSD	3	Very mild	None	Excellent	Very satisfied	Dramatic improvement in pruritus scores and overall skin condition
7	4	FS	Shih Tzu	0	None	None	Excellent	Very satisfied	Owner considers the dog to be essentially normal
8	1	MN	Brittany Spaniel	2–3	None	Occasional antihistamine	Good	Satisfied	Skin appeared virtually normal, and no requirement for ongoing prednisolone
9	3	MN	SBT	3	Mild	Mometasone cream	Good	Satisfied	Owner happy with pruritus level, but still some active dermatitis
10	1	FS	Lab	2–4	None	Apoquel every 2–3 days	Good	Satisfied	Overall improvement but still needs Apoquel every 2–3 days
11	2	MN	Bull Mastiff	3	Mild	Occasional antibiotics	Good	Satisfied	AD controlled but still suffered intermittent folliculitis
12	4	FS	Border Terrier	2	Mild	Occasional ear drops	Good	Satisfied	Occasional flare
13	4	M	American Bulldog	1–5	Moderate	Mometasone cream/ antibiotics	Modest	Unsure of benefit	Waxing and waning with intermittent flares and recurrent furunculosis
14	5	MN	Dalmation	4	Mild	Apoquel	Modest	Unsure of benefit	Owner thought there had been an improvement but still required daily Apoquel
15	3	FS	Golden Retriever	2–4	Moderate	Prednisolone	Modest	Unsure of benefit	Response was variable with multiple flares
16	6	FS	Boxer	3–6	Moderate	Prednisolone/antibiotics	Modest	Unsure of benefit	Multiple flares requiring prednisolone
17	1	FS	BT	N/R	Moderate	Mometasone/prednisolone	Modest	Unsure of benefit	Owner considered good improvement but still active skin disease and recurrent Malassezia dermatitis
18	6	FS	Kelpie	4–6	Moderate	Cortavance	Modest	Unsure of benefit	Owner considered skin disease better than before immunotherapy, but pruritus scores still high
19	4	MN	GSD	5	Mild	Prednisolone	None	No benefit	No change in prednisolone requirement
20	1	MN	Dobermann	5–6	Moderate	Prednisolone/Mometasone	None	No benefit	Still required daily prednisolone
21	2	FS	Mastiff	3–6	Mild	Apoquel	None	No benefit	Still required daily Apoquel
22	2	FS	SBT	2–3	Moderate	Apoquel	None	No benefit	Dog was never very pruritic but continued to get recurrent pyoderma. Eventually controlled with Apoquel
23	2	FS	Boxer	5–7	Mild	Prednisolone/Apoquel	None	No benefit	Relapsed when not on prednisolone or Apoquel
24	1	FS	Boxer	3–6	Moderate	Apoquel	None	No benefit	Relapsed when not on Apoquel
25	1	MN	Irish Wolfhound	3–5	Moderate	Apoquel	None	No benefit	Relapsed when not on Apoquel
26	7	MN	Rhodesian Ridgeback	5	Moderate	Prednisolone	None	No benefit	Owner discontinued immunotherapy with no follow up
27	3	MN	Rottweiler	6.5	Moderate	Prednisolone	None	No benefit	Relapsed when not on daily prednisolone
28	2	FS	SBT	3.5	Mild	Unknown	None	No benefit	Owner discontinued immunotherapy with no follow up
29	4	MN	Poodle	2–3	Mild	Apoquel	None	No benefit	Relapsed when not on prednisolone or Apoquel
30	1	FS	Labrador	4–8	Moderate	Prednisolone/Apoquel	None	No benefit	Still required daily prednisolone or Apoquel
31	4	FS	Lab	6	None	Prednisolone	None	No benefit	Discontinued due to high pruritus score and prednisolone requirement
32	2	MN	Beagle	5–6	Mild	None	None	No benefit	Owner discontinued treatment due to high pruritus scores
33	6	FS	SBT	N/R	Moderate	Prednisolone	None	No benefit	Immunotherapy stopped due to swelling of neck after first few injections, with no follow up
34	6	MN	Lab	N/R	Moderate	Prednisolone	None	No benefit	Owner discontinued immunotherapy with no follow up

The cases were assessed using the global assessment of efficacy matrix shown in Table 2. AD, atopic dermatitis; BT, bull terrier; EBT, english bull terrier; FS, female spayed; GSD, German shepherd dog; MN, male neutered; N/R, not recorded; SBT, staffordshire Bull terrier.

positive reaction was observed for all 60 allergens, albeit in some cases in a very small number of tests. The number of positive reactions in an individual dog ranged from 1 to 47 with a median of 5.

In the IgE serological tests, the four most commonly reacting allergens were Yorkshire fog grass (positive in 10 tests, 40%), *T. putrescentiae* (n = 9, 36%), Kentucky bluegrass (n = 9, 36%) and Yellow dock (n = 9,

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36%). Twenty-eight other allergens had a prevalence of positive reactions over 15%. *M. pachydermatis*, Penicillium, Aspergillus and Cladosporidium had no positive results in any dogs tested. The number of positive reactions in an individual dog ranged from 1 to 40, with a median of 9.

Of the 37 dogs that were prescribed immunotherapy, three could not have their treatment efficacy assessed because they had not yet shown a beneficial response but had only been on treatment for 3 months at the time of the study. Four dogs failed to return for their first scheduled evaluation and the immunotherapy was discontinued. One owner discontinued the treatment due to an adverse reaction (swelling of the neck after the first few injections). For the data set of 34 cases, if the five withdrawals were classified as treatment failures, the outcome of immunotherapy was judged to be excellent in 20% of dogs, good in 15%, modest in 18% and a failure in 47% (Figure 3). If the five withdrawals had continued with the treatment, and their final outcomes were eventually good or excellent, the failure rate would have been 32% and the success rate would have been 50%. If these dogs were left out of the analysis altogether, there would have been a 38% failure rate and a 41% chance of a good or excellent outcome.

The number of allergens included in an immunotherapy vaccine had no significant effect on the outcome (Kruskal Wallis, P = 0.7). 81% of dogs had their immunotherapy started at 4 years of age or younger, but there was no significant difference in efficacy between these dogs and those that were started later (P = 0.37). Likewise, there were no significant differences in efficacy between males and females (P > 0.99), and between entire and desexed dogs (P = 0.47). However, these results should be interpreted with caution because the low numbers of patients may not have yielded sufficient power to demonstrate statistically significant differences.

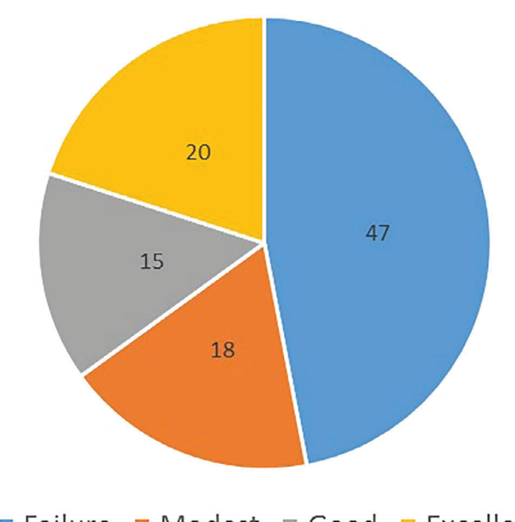

Figure 3. **The percentage of 34 dogs with outcomes of excellent, good, modest or failure in response to treatment with immunotherapy. Outcomes of immunotherapy were categorised based on a global assessment of efficacy matrix (Tables 2 and 3).**

Discussion

In this study, household environmental mites, certain grass and weed pollens, and *M. pachydermatis* were shown to be the most relevant allergens in dogs with atopic dermatitis in South Australia. When comparing the two different testing methodologies (intradermal allergy testing and IgE serological assay), the prevalence of positive reactions to the environmental mites (*D. farinae*, *D. pteronyssinus* and *Tyrophagus putresceantiae*) were very similar in the two tests and in the region of 20%–30%. On a worldwide basis, dust mites and food storage mites are reported as very common allergens in canine atopic dermatitis with prevalence rates for positive reactions ranging from 14%–34% (South Eastern Australia),[10] 38% (Norway),[11] 42%–49% (UK),[12] 55% (France),[13] 54%–64% (Japan),[14] 61%–63% (Korea),[15,16] 53%–70% (Greece),[17] 53%–74% (Thailand),[18] and 71% in the USA.[19] The lower prevalence rates reported in this and the other Australian study may relate to the more rural lifestyle observed by dogs in some parts of the country.

The prevalence of positive reactions to grass, tree and weed pollen was generally higher in the IgE serological test compared to the intradermal test. In the intradermal test, tree pollens were typically positive in about 5%–10% of dogs, and grass and weed pollens were positive in about 10%–20% of dogs. In the IgE test, tree pollens were typically positive in 10%–25% of dogs with grasses and weeds yielding positive reactions in about 25%–30% of cases. The reason for this discrepancy is not known but it is accepted that intradermal testing and IgE serology do not correlate precisely.[20–22] However, whether the intradermal test is under-recognising pollen allergies (low sensitivity leading to false negative reactions) or the IgE assay is over-reporting pollen allergies (poor specificity leading to false positive reactions) is not known. One possibility for the lower rate of positive pollen reactions in the intradermal test could relate to the concentration of allergen used. Intradermal allergy testing is not a standardised procedure and different concentrations of allergens are reported by different authors. We used a concentration of 1000 PNU/mL for pollen allergens as recommended by the manufacturer, but other authors have reported concentrations as low as 500 PNU/mL.[10] Studies to assess irritant thresholds of allergens in normal dogs have reported tolerable concentrations of up to 8000 PNU/mL for certain allergens in one study[23] but the validity of this methodology was questioned in a subsequent publication.[24] However, It is possible that use of a higher allergen concentration may have yielded more positive pollen reactions. Yellow dock was the only pollen allergen that appeared in the top four reactions in both the intradermal test (fourth most prevalent reaction) and the IgE assay (second most prevalent reaction), confirming its importance as a major allergen in South Australia.

A major exception to these general findings was the high prevalence of positive reactions to Red clover in this study. 59% of dogs had a positive reaction to this allergen in the intradermal test, the highest in the set of 60. This allergen has not been widely reported in previous studies so direct comparison is not possible, but 10% of dogs

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reacted to it in London and 5% in Edinburgh.[12] A number of possible explanations could account for this high rate of positivity. First, it could represent genuinely high rates of hypersensitivity to this allergen in South Australian dogs. Clovers are common in lawns and pastures[25] so there is ample opportunity for exposure. Also, Red clover is known not to cross-react with other weed allergens so mono-sensitisation would be more common than with other weeds.[26] Second, it could represent a high rate of false positive reactions in which an irritant component of the extract is able to elicit a wheal reaction in non-allergic dogs. This appears unlikely as nearly 40% of dogs did not show a reaction, and the phenomenon occurred over multiple batches of the allergen. A third possibility is that these are clinically irrelevant reactions that are still mediated by an IgE reaction but do not necessarily account for the dog's clinical signs. Unfortunately, this uncertainty cannot be resolved due to the lack of comparative data, and the absence of Red clover from the IgE serological test.

An important finding in this study that was not seen in most other studies of this type is the high prevalence of positive reactions to the commensal yeast *M. pachydermatis*. The immunological relevance of this yeast has only been recognised over the last 20 years[27,28] and it has only been available as a commercially available allergen for part of that time. Malassezia was not included as an allergen in most of the reported studies because they pre-dated its availability.[10,19] Despite this, it clearly represents a major allergen in dogs with atopic dermatitis, being positive in 24% of intradermal tests in this study. It also resulted in some of the strongest positive reactions, with many tests being scored as a 5 (meaning that the wheal size exceeded that of the positive control). The lack of positive reactions in the IgE serological assay is surprising, but this could simply be a quirk of the lower numbers tested. Other studies have reported positive serological reactions to the yeast in atopic dogs,[15] and immunotherapy against the organism has now been reported.[29]

The outcomes of allergen-specific immunotherapy reported in this study appear slightly less favourable than those previously reported. Only 35% of dogs were judged to have had a good or excellent response and 47% were considered failures. However, we did include 5 dogs in the failure group that had immunotherapy discontinued by their owners without veterinary input. It is possible that some of these might have improved if the treatment had been continued for longer. Our rationale for counting these dogs as failures was to allow a realistic overall impression of this type of treatment to be established. Despite initial counselling and guidance, some owners may discontinue this type of therapy because they perceive it is not working, is causing adverse effects, or is simply not appropriate for their needs. These owners are likely to consider the treatment a failure. In previous studies, success rates of 60%–75% are typically reported.[30,31] However, the lower success rate in our study can likely be explained by the group of cases referred to as 'modest' responses. The 18% of dogs in this category did appear to gain some degree of benefit from the treatment according to the owners, but it was not sufficient to permit other forms of treatment to be significantly reduced. Some of the apparent benefit could have been due to placebo effects because the only placebo controlled study to investigate the efficacy of immunotherapy reported that 20% of dogs receiving placebo injections improved by more than 50%.[30] In terms of outlining realistic expectations for an owner, the authors do not believe that dogs in this group could be reasonably described as a meaningful success. Using this interpretation, we concluded that 65% of dogs did not gain great benefit from the treatment. However, other authors have interpreted these sub-optimal responses differently. For example, in one study,[31] the 20% of dogs that showed less than a 50% improvement were classified as having a 'moderate' improvement and were still judged a success, even though some of these dogs may have improved by only 10%. In another study, this group of dogs, representing 40% of the total, were classified as 'partial' responders (defined as being controlled but only with the use of concurrent systemic agents).[32] A further study defined a 'moderate' response as a minor improvement but the level of medication could not be decreased. Again, 20% of the dogs fell into this group. In each of these studies, a group of dogs with potentially very marginal improvements were being classified as having a moderate outcome, and hence a treatment success. Our results are most comparable to the study by Colombo,[33] which reported an excellent response in 22% of dogs, a measurable improvement in 22% of dogs, but no improvement in 56% of dogs.

In this study, we chose an end point at 9 months because this is when dogs came for re-evaluation after finishing their induction and initial course of maintenance vaccines. Many dermatologists assess the efficacy of immunotherapy after a 12 month period, but there is no scientific evidence as to why this should be the case. To the authors' knowledge, there is no reliable evidence that documents a cohort of dogs who improved dramatically between 9–12 months of treatment. There is also no sound immunological theory as to why an improvement would take 9 months to commence. As such, we do not believe that the outcomes of immunotherapy would have been significantly improved if the study had continued for 12 months. However, this cannot be proven one way or the other with the data generated in this study.

In conclusion, this study has revealed Red clover, environmental mites, Yellow dock, Yorkshire fog, Kentucky bluegrass and *M. pachydermatis* to be the most prevalent reactions in allergy tests in dogs in South Australia. Immunotherapy proved to be a successful treatment in 35% of dogs in which it was used.

Acknowledgment

W.Y. Chan is supported by the Ministry of Higher Education of Malaysia and Universiti Putra Malaysia.

Conflict of interest and sources of funding

The authors declare no conflicts of interest. This study was funded by The University of Adelaide clinical research project fund.

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APPENDIX

Table A1 **Immunotherapy dosage schedule**

	Volume (mL)	Day
Vial 1 1500 PNU/mL	0.1	0
	0.2	2
	0.4	4
	0.6	6
	0.8	8
	1.0	10
Vial 2 15,000 PNU/mL	0.1	12
	0.2	14
	0.4	16
	0.6	18
	0.8	20
	1.0	22
	1.0	+1 week × 3
	1.0	+2 weeks × 3
	1.0	+3 weeks

(Accepted for publication 13 October 2019)

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