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

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