The joint *in vitro* action of polymyxin B and miconazole against pathogens associated with canine otitis externa from three European countries

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Background – Canine otitis externa, an inflammation of the external ear canal, can be maintained and worsened by bacterial or fungal infections. For topical treatment, combinations of anti-inflammatory and antimicrobial ingredients are mainly used.

Hypothesis/Objectives – This study was conducted to elucidate the *in vitro* activity of polymyxin B and miconazole against clinical bacterial isolates from three European countries, to investigate possible differences in sensitivity and to assess drug interactions.

Animals – Seventeen strains of *Escherichia coli*, 24 strains of *Pseudomonas aeruginosa*, 24 strains of *Proteus mirabilis* and 25 strains of *Staphylococcus pseudintermedius* from dogs with diagnosed otitis externa had been isolated in Germany, France and Italy.

Methods – Drug activities were evaluated by minimal inhibitory concentration (MIC) and minimal bactericidal concentration. The potentiation of polymyxin B plus miconazole was calculated using the fractional inhibitory concentration index (FICI). An FICI ≤ 0.5 defined synergy. Furthermore, geographical variations in the FICI and MIC were assessed by statistical analysis.

Results – Bacterial susceptibilities were comparable in different European countries, because there were no significant MIC and FICI variations ($P > 0.05$). As a single agent, polymyxin B had bactericidal activity against most *E. coli* and *P. aeruginosa* strains and, in higher concentrations, against *S. pseudintermedius* strains. Miconazole was bactericidal against all *Staphylococcus* strains. Synergy was demonstrated against strains of *E. coli* and *P. aeruginosa* (FICI = 0.25 and 0.50, respectively), whereas overall there was no interaction against *S. pseudintermedius* strains (FICI = 1.25). *Proteus mirabilis* strains were not inhibited by each of the drugs individually or by their combination.

Conclusions and clinical importance – *In vitro* synergy of polymyxin B and miconazole against *E. coli* and *P. aeruginosa* isolates indicates a rationale for applying both agents in combination to treat otitis externa when infected with these types of bacteria.

Introduction

Canine otitis externa, an acute or chronic inflammation of the external ear canal epithelium, is a common presentation in small animal practice. Although not life-threatening, the therapeutic intervention can be challenging and frustrating because several perpetuating factors frequently prevent healing. Micro-organisms are common secondary factors that can maintain and worsen the disease process. *Staphylococcus* spp. are among the most common bacterial pathogens isolated from dogs with otitis externa,[1] other significant bacteria are *Pseudomonas aeruginosa*, *Escherichia coli* and *Proteus mirabilis*.

Antimicrobial therapy using polymyxin B and miconazole has been proved to be effective against the main bacterial pathogens associated with otitis externa in clinical studies.[2–5] While miconazole kills fungi and some Gram-positive bacteria,[6] polymyxin B has antifungal properties[7] and antibacterial activity against a wide variety of Gram-negative and, to a lesser extent, Gram-positive microbes.[8] Most importantly, this agent is effective against various strains of antibiotic-resistant bacteria.[9]

The polymyxins are cationic polypeptides that target and disrupt the bacterial cell membrane. This causes an

increase in the permeability of the cell envelope, leakage of cell contents and, subsequently, cell death. When combined with various drugs, polymyxins have a potential for enhanced activity, which is related to their ability to increase the penetration of other agents into the cell.[10]

In the past, several studies demonstrated synergistic antimicrobial *in vitro* activity when polymyxins are combined with different antimicrobial agents.[11,12] By combining polymyxin B with miconazole, synergism was reported against *Candida albicans*,[13] against strains of *Staphylococcus aureus* and *E. coli*[14] and against type strains of *E. coli*, *P. aeruginosa* and *Malassezia pachydermatis*.[14,15]

So far, the incidence of resistance to miconazole has been low in clinical isolates[16] or in laboratory experiments.[17] Resistance to polymyxin B, largely due to lipopolysaccharide modifications,[11,18] was either low[19] or could not be detected at all in canine and feline bacterial strains.[20] In human isolates of *P. aeruginosa*, resistance to polymyxins was reported to be <5% for specific subpopulations within a species.[11,21] Inconsistent with these reports, in distinct geographical regions of the world higher prevalence of resistance has been reported.[22] Recent data from the SENTRY Antimicrobial Surveillance Program 2006–2009 described excellent *in vitro* activity of polymyxins against a worldwide collection of Gram-negative pathogens, with a trend towards greater resistance in Asia-Pacific and Latin-America regions.[23]

The objective of the present study was an analysis of synergism of a combination of polymyxin B and miconazole *in vitro* not only on type strains[15] but also on clinical strains of *Staphylococcus pseudintermedius*, *P. aeruginosa*, *E. coli* and *P. mirabilis* associated with canine otitis externa. Equal molar concentrations of both drugs were combined to approach the mode of action. A second objective was a survey of the susceptibility of these bacterial species in different European countries.

Materials and methods

Bacterial strains

For broad and representative sampling, bacterial strains were taken from different regions of Germany, France and Italy. The bacteria were sampled in 2009 and 2010 by different laboratories from cases of acute canine otitis externa using regular submissions by veterinary practices, clinics or veterinary faculties for identification of bacterial genus and species. The veterinary practices and faculties made the diagnosis of acute otitis externa (abrupt onset of signs and symptoms). They collected samples from one ear of each dog with sterile cotton swabs (various brands), which were sent daily to resident laboratories (Laboklin GmbH & Co. KG, Bad Kissingen, Germany; Vébiotel, Arcueil, Cedex, France; Department of Animal Production, Epidemiology and Ecology, School of Veterinary Medicine of Turin, Italy).

For analysis of susceptibility and synergy testing, bacterial samples were transferred to a single laboratory. In this study, we included isolates of *E. coli* (haemolytic and nonhaemolytic) originating from Germany and France (*n* = 17); samples from Italy did not contain *E. coli*. Isolates of *P. aeruginosa* (*n* = 24), *P. mirabilis* (*n* = 24) and *S. pseudintermedius* (formerly *S. intermedius*;[24] *n* = 25) were investigated from Germany, France and Italy. Regardless of regional aspects, meticillin-resistant *S. pseudintermedius* (MRSP) strains from The Netherlands (*n* = 5) were included in this study to compare their susceptibility to polymyxin B and miconazole with that of strains from Germany, France and Italy. The strains were sampled from dogs suffering from acute otitis externa.

For the bacterial species investigated in this study, type strains ATCC 25922, ATCC 27853, ATCC 29663 and ATCC 29906 served as quality controls (QC strain).

For certainty, we repeated bacterial characterization. We plated the isolates on trypticase soy agar and cetrimide agar (heipha GmbH, Eppelheim, Germany). Purified isolates were identified from their appearance on solid medium, cell morphology, odour, pigment production, Gram properties, haemolysis and catalase and oxidase reaction. Additional biochemical species identification was achieved by applying API Staph ID 32 for *Staphylococcus* spp., API 20 NE for nonenteric Gram-negative rods and API 20 E for enteric bacteria (BioMérieux, Nürtingen, Germany).

Antimicrobial agents

Polymyxin B sulfate (Sigma-Aldrich, Taufkirchen, Germany) was dissolved in deionized water. Miconazole nitrate salt (Sigma-Aldrich) was dissolved in a solution containing 1.88 mol/L polyethyleneglycol 400 and 5.43 mol/L ethanol. Both antibiotic solutions were filter sterilized (Minisart nylon filter, pore size 0.2 µm; Sartorius, Göttingen, Germany) prior to use.

For quality control of the antimicrobial agents, aliquots of the antibiotic stock solutions of polymyxin B sulfate and miconazole nitrate were retained and analysed for content of active substance by high-pressure liquid chromatography (HPLC) using the Merck/Hitachi LaChrom 2 HPLC-System with UV-Detector and Software Merck/Hitachi D-7000 HSM HPLC System Manager Software on the first day and 6 weeks after preparation. In the polymyxin B sulfate solution, the content decreased by 14.41%, and in the miconazole nitrate solution there was a decrease of 9.26% after 6 weeks.

Additionally, the sterile filtering process was controlled for potential losses of antimicrobial agents. Solutions of polymyxin B sulfate and miconazole nitrate were filtered using either Minisart Plus syringe filter (cellulose acetate membrane with GF prefilter; Sartorius Stedim Biotech GmbH, Göttingen, Germany) or Minisart NML syringe filter [surfactant-free cellulose acetate (SFCA) membrane; Sartorius Stedim Biotech GmbH]. There was no detectable loss of polymyxin B or miconazole following sterile filtration with Minisart Plus or Minisart NML filters. We concluded that the antibiotics did not bind to the surface of the filters.

The procedures and the detailed results of these parallel experiments are reported in ECON report numbers 1022-08 and 1022-09 (M. Voget, M. Armbruster, unpublished results).

Susceptibility tests

Susceptibility to antimicrobial agents was assessed using the broth microdilution method according to recommendations of the Clinical and Laboratory Standards Institute, protocol M7-A8.[25] The minimal inhibitory concentration (MIC) end-points were evaluated visually, and the results were verified photometrically at an optical density (OD) of 490 nm. The MIC was read as the lowest concentration of antimicrobial substance which inhibited visible growth. Following MIC determination, subcultures onto Müller-Hinton agar plates (Sifin, Berlin, Germany), free of antibacterial substances, were made from wells that failed to show macroscopic growth and reincubated for an additional 18–24 h to determine the minimal bactericidal concentration (MBC). The MBC was read as the lowest concentration of antimicrobial substance which reduced bacterial counts by 99.9% (3-$\log_{10}$ reduction in colony-forming units). Bactericidal activity was interpreted as a ratio of MBC to MIC ≤ 4.

Synergy trials were performed as chequerboard interactions in microtitre plates. Bacteria were added to a twofold serial dilution of a single antibiotic agent or in combination with an identical dilution of the other tested antibiotic agent. Concentrations tested in combination consisted of equal molar concentrations of polymyxin B and miconazole. They varied from 275.0 mg/L polymyxin B combined with 93.75 mg/L miconazole (corresponding to 2.25×10^{-4} mol/L of

each drug) to 1.68×10^{-2} mg/L polymyxin B combined with 5.72×10^{-3} mg/L miconazole (corresponding to 1.35×10^{-8} mol/mL of each drug). One negative (no bacteria) and two positive controls (no antibiotic, and no antibiotic but solvent of miconazole solution) were used on each plate.

Statistical analysis

The fractional inhibitory concentration index (FICI) was calculated according to the equation FICI = MIC(A_B)/MIC(A) + MIC(B_A)/MIC(B) where MIC(A) and MIC(B) denote the MIC of drug A and the MIC of drug B alone, MIC(A_B) and MIC(B_A) are corresponding MICs of drug A in the presence of drug B and *vice versa*. An FICI ≤ 0.5 was interpreted as synergy, and a FICI $> 0.5-4$ was interpreted as no interaction of both drugs.[26]

The MICs and calculated FICIs were summarized descriptively by the sample size *n*, the mode, the median, the interquartile range (IQR) and the minimal and maximal values. The median enables a description of the central tendency largely unaffected by outliers, whereas the mode facilitates the recognition of samples having more than one maximum. The Kruskal–Wallis rank sum test was used to study whether FICI calculated from samples taken in the different countries originate from the same distribution. Subsequently, one-sided Wilcoxon signed-rank tests were performed to test whether the FICIs were smaller than 1.0 and 0.5, respectively. In all hypothesis tests, $P < 0.05$ was considered significant. The *P*-values are labelled p_c for the test of grouping by country, and $p_{<1.0}$ and $p_{<0.5}$ for the test of upper limits of the FICI. All calculations were carried out with the R-package for statistical computing (R Foundation for Statistical Computing, Vienna, Austria).[27]

Results

Geographical variations

The geographical variation of MIC and FICI was studied in samples taken in Germany, France and Italy. For polymyxin B, there was no evidence of a geographical variation of MIC in *E. coli* (p_c, *P*-value for test of MIC grouping by country = 0.314), *P. aeruginosa* ($p_c = 0.420$) and *S. pseudintermedius* ($p_c = 0.496$). Likewise, geographical region was not a relevant factor for the MIC of miconazole in *S. pseudintermedius* ($p_c = 0.775$). Furthermore, we found no significant geographical variation of FICI for all the aforementioned strains (Tables 1–3). Hence, MIC and FICI data were pooled, and the results of the combined data set are presented below.

Minimal inhibitory concentrations

Against *E. coli* strains from Germany and France, MICs of polymyxin B ranged from 0.13 to 4.30 μg/mL; the mode and median MIC of the sample pooled for statistics were 0.27 μg/mL (Table 1). There was no inhibition of bacterial growth when miconazole was applied alone.

Against *P. aeruginosa* strains from Germany, France and Italy, MICs of polymyxin B ranged from 0.27 to 1.07 μg/mL. In two strains of *P. aeruginosa* from Germany, there was no inhibition by polymyxin B at the maximal experimentally accessible concentration of 275 μg/mL (Table 2). The mode and median MIC of polymyxin B was 0.54 μg/mL (Table 2). Miconazole when given alone did not inhibit bacterial growth.

Against *S. pseudintermedius* strains from Germany, France and Italy, MICs of polymyxin B ranged from 4.30 to 34.38 μg/mL. The mode and median MIC of polymyxin B were 8.59 and 17.19 μg/mL, respectively. Three strains from Italy were not inhibited by the maximal experimen-

Table 1. *In vitro* activity of polymyxin B and miconazole against *Escherichia coli* strains

	QC strain	Germany	France	Total
n		12	5	17
MIC for polymyxin B (μg/mL)				
Mode	0.27	0.27	—	0.27
Median		0.27	0.54	0.27
IQR		0.27–0.27	1.07–0.27	0.54–0.27
Minimum		0.13	0.13	0.13
Maximum		1.07	4.30	4.30
FICI for polymyxin B and miconazole				
Mode	0.25	0.25	0.25, 0.50	0.25
Median		0.25	0.25	0.25
IQR		0.50–0.25	0.50–0.25	0.50–0.25
Minimum		0.06	0.06	0.06
Maximum		0.50	0.50	0.50
P-values				
p_c				0.955
$p_{<1.0}$				1.3×10^{-4}
$p_{<0.5}$				0.001

Abbreviations: FICI, fractional inhibitory concentration index; IQR, interquartile range; MIC, minimal inhibitory concentration; *n*, number of isolates; p_c, *P*-value for test of FICI grouping by country; $p_{<1.0}$ and $p_{<0.5}$, *P*-values for tests of FICI < 1.0 and FICI < 0.5; and QC, quality control.

tally accessible concentration of 275 μg/mL polymyxin B (Table 3). The finding that three strains from Italy were not inhibited at the highest concentration is reflected by the bimodal MIC with most frequent observations of 4.30 and > 275 μg/mL. All MRSP strains were inhibited by polymyxin B at concentrations of 8.59–17.19 μg/mL (Table 4).

The MICs of miconazole against the *Staphylococcus* strains from all countries were in the range of 0.73–11.72 μg/mL, while the MRSP strains ranged from 1.47 to 5.86 μg/mL. In the pooled *S. pseudintermedius* samples, the mode and median MIC values of miconazole were 1.47 μg/mL. In our MRSP samples, we found the most frequent inhibition at 5.86 μg/mL, whereas the median inhibition of the samples was

Table 2. *In vitro* activity of polymyxin B and miconazole against *Pseudomonas aeruginosa* strains

	QC strain	Germany	France	Italy	Total
MIC for polymyxin B (μg/mL)					
n		8	8	8	24
Mode	0.27	0.27	0.54	0.54	0.54
Median		0.54	0.54	0.54	0.54
IQR		1.07–0.27	0.54–0.27	1.07–0.54	1.07–0.27
Minimum		0.27	0.27	0.27	0.27
Maximum		>275	1.07	1.07	>275
FICI for polymyxin B and miconazole					
n		6	8	8	22
Mode	0.50	0.50	0.25	0.50	0.50
Median		0.50	0.25	0.50	0.50
IQR		0.50–0.50	0.50–0.25	0.63–0.44	0.50–0.25
Minimum		0.13	0.13	0.25	0.13
Maximum		0.50	1.00	1.00	1.00
P-values					
p_c					0.200
$p_{<1.0}$					4.7×10^{-5}
$p_{<0.5}$					0.348

Abbreviations are as for Table 1.

Table 3. *In vitro* activity of polymyxin B and miconazole against *Staphylococcus pseudintermedius* strains

	QC strain	Germany	France	Italy	Total
n		8	8	9	25
MIC for polymyxin B (μg/mL)					
Mode	8.59	17.19	8.59	4.30, >275	8.59
Median		17.19	8.59	17.19	8.59
IQR		17.19–8.59	8.59–4.30	>275–4.30	17.19–8.59
Minimum		8.59	4.30	4.30	4.30
Maximum		34.38	34.38	>275	>275
MIC for miconazole (μg/mL)					
Mode	2.93	1.47	1.47	1.47	1.47
Median		1.47	1.47	1.47	1.47
IQR		2.93–1.47	1.47–1.47	2.93 -1.47	2.93–1.47
Minimum		1.47	0.73	0.73	0.73
Maximum		5.86	5.86	11.72	11.72
FICI for polymyxin B and miconazole					
Mode	1.00	0.50	1.00	0.75, 1.25	1.25
Median		0.59	1.00	1.00	1.00
IQR		1.06–0.55	1.47–1.31	1.25–0.75	1.25–0.56
Minimum		0.50	0.56	0.83	0.83
Maximum		1.50	1.50	2.06	2.06
P-values					
p_c					0.288
$p_{<1.0}$					0.348
$p_{<0.5}$					1.000

Abbreviations are as for Table 1.

Table 4. *In vitro* activity of polymyxin B and miconazole against meticillin-resistant *Staphylococcus pseudintermedius* (MRSP) strains

	MRSP
n	5
MIC for polymyxin B (μg/mL)	
Mode	17.19
Median	17.19
IQR	17.19–17.19
Minimum	8.59
Maximum	17.19
MIC for miconazole (μg/mL)	
Mode	5.86
Median	2.93
IQR	5.86–2.93
Minimum	1.47
Maximum	5.86
FICI for polymyxin B and miconazole	
Mode	0.75
Median	0.75
IQR	0.75–0.75
Minimum	0.50
Maximum	1.25

Abbreviations are as for Table 1.

2.93 μg/mL. None of the strains of *P. mirabilis* was inhibited by polymyxin B or by miconazole at the concentrations tested.

Minimal bactericidal concentrations

Susceptibility to polymyxin B and miconazole was evaluated by MBC determination and calculation of the MBC/MIC ratio (Fig. S1 in Supplementary material). For *E. coli*, 100% of the clinical strains from Germany and France had an MBC/MIC ratio of 1. For 46% of the clinical *P. aeruginosa* strains from three countries, the MBC/MIC ratio was also 1. Another 42% of these strains had a MBC/MIC ratio of 2, 4% of the strains had a MBC/MIC ratio of 8, and 8% of the strains were not inhibited by polymyxin B, hence the MBC/MIC ratio was not calculable. The MICs for the Gram-negative QC strains conformed to published values.[28] For polymyxin B, 67% of the clinical strains of *S. pseudintermedius* and all MRSP strains showed an MBC/MIC ratio of 1. Furthermore, 13, 7, and 3% of these strains showed MBC/MIC ratios of 2, 4 and 8, respectively, while 10% were not inhibited by polymyxin B. Miconazole had bactericidal activity against all isolates of *Staphylococcus* too. The MBC/MIC ratio was 1 for 50% of the clinical *S. pseudintermedius* strains, and for the MRSP strains, 2 for 40% and 4 for 10% of the strains (Fig. S1 in Supplementary material). The combination of both drugs had no impact on the MBC/MIC ratios. The results from MIC and MBC testing indicated that polymyxin B exhibited bactericidal activity against most strains tested, while miconazole was bactericidal solely against the Gram-positive isolates.

Synergism

For the *E. coli* samples from Germany and France pooled for statistical analysis, the FICI was determined to be 0.25. The minimal and maximal values were 0.06 and 0.50. The FICI was significantly smaller than 0.5 ($p_{<0.5}$ = 0.001). Consequently, the criteria for synergy were met.

The minimal and maximal FICI values for *P. aeruginosa* were 0.13 and 1.00, respectively. There was evidence for FICI < 1 ($p_{<1.0}$ = 4.7 × 10^{-5}). Although it could not be shown that FICI < 0.5 ($p_{<0.5}$ = 0.348), the mode and median FICI were exactly 0.5, which indicates a synergistic interaction of both drugs.

For *S. pseudintermedius*, the FICI mode of 1.25 is somewhat higher than the median of 1.00. The minimal and maximal FICI were 0.38 and 2.06, respectively. Due to the variation of the data, there was not sufficient evidence to prove that FICI < 1 ($p_{<1.0}$ = 0.348) for the strains from Germany, France and Italy. Thus, interaction

between both drugs if applied against *S. pseudintermedius* isolates could not be demonstrated. For the MRSP strains, both location parameters, the mode and median were 0.75.

Discussion

This study aimed to investigate whether a synergistic effect of polymyxin B with miconazole was exerted on clinical strains from three different countries in Europe. To produce objective data for the prudent application of polymyxin B and miconazole, the present study evaluated the efficacy of these drugs alone and in combination against clinical isolates of *E. coli*, *P. aeruginosa*, *S. pseudintermedius* and *P. mirabilis* using MIC, MBC and FICI determination.

Our data revealed no evidence of different sensitivity to polymyxin B and miconazole of clinical strains from cases of canine otitis externa in the European countries France, Germany and Italy in terms of MIC and FICI. Thus, the results were presented in a joint statistical analysis. In addition, our investigation confirms a synergistic activity of polymyxin B combined with miconazole against strains of *E. coli* and *P. aeruginosa*. Although the *S. pseudintermedius* strains on average did not fulfil the rigorous criteria for synergism, there was a substantial reduction in MIC if both antibiotic agents were acting together.

Clinical isolates of *E. coli* and *P. aeruginosa* showed a high level of susceptibility to polymyxin B, with MICs being consistently low. In general, *Staphylococcus* spp. are regarded as poor targets for polymyxins, with high MIC values ranging from 8 to 64 μg/mL.[9,15,29] Our results confirm these data, in that the MIC values for the *S. pseudintermedius* strains from all countries were in the range of 4.3–34.4 μg/mL and for the MRSP strains in the range of 8.6–17.19 μg/mL. In contrast to these data, in a recent study[30] MIC values for MRSP strains were remarkably low, ranging from 0.25 to 4 μg/mL, while for MRSA strains the MIC values were significantly higher and ranged from 8 to 64 μg/mL.

It is noteworthy that miconazole was able to kill the *S. pseudintermedius* isolates, and the MICs from 0.73 to 11.72 μg/mL are extensively consistent with data from previous *in vitro* studies.[15] Both polymyxin B and miconazole alone and in combination could inhibit the MRSP strains and exerted strong bactericidal activity as well. For the majority of strains tested, the MBC did not exceed four times the MIC, thus bactericidal activity was confirmed. This conforms to the known mode of action of polymyxins and anticipates a rapid killing of the target pathogens.

The bactericidal activity of polymyxin B and miconazole is of clinical importance. While many infections respond equally well to bacteriostatic agents as to bactericidal ones,[31] in theory the killing of bacteria should produce a more rapid resolution of infection along with an improved clinical outcome,[32] and the faster elimination of bacterial pathogens should also minimize the likelihood of the emergence of resistance and spread of infection. However, *in vitro* testing methods that are used to categorize antibacterial agents as bactericidal may not duplicate the

conditions found *in vivo*. For instance, polymyxin B is known to be inactivated in purulent exudates.[33] Thus, clinicians must consider drug concentrations at the site of infection or local factors that impair drug activity, such as low pH, the presence of pus or high protein concentration, to determine the optimal treatment.

None of the strains except *Proteus* exhibited resistance to miconazole, but polymyxin B was not active against two clinical isolates of *P. aeruginosa* from Germany and three *Staphylococcus* strains from Italy. This might indicate waning susceptibility to polymyxins in different European countries. A progressive increase in MICs of polymyxins was assigned to prolonged treatment with polymyxins;[34,35] thus, data from microbiological field studies may point to the usage of polymyxins in certain regions.

The clinical isolates of *P. mirabilis* revealed resistance to both drugs if given alone. Unexpectedly, resistance is maintained if both drugs are given in combination. Intrinsic resistance of *Proteus* species to polymyxins is established and based on changes in lipid A. The isolates from our study that remained unaffected by polymyxin may provide a contribution to our understanding of how both antibacterial agents co-operate to damage bacteria. In Gram-negative microbes, the synergistic action of polymyxin and miconazole is supposed to originate from the ability of polymyxin to stimulate the uptake of the hydrophobic miconazole to the intracellular space,[36] where it increases the level of reactive oxygen species.[37] In bacteria resistant to polymyxin B, the drug has no access to the cell membrane and cannot disrupt it; thus, miconazole fails to penetrate into the cells and leaves those bacteria unaffected. In Gram-positive bacteria, the cell membrane is not exposed, hence polymyxin B has little activity against them. In these microbes, the synergistic interaction of polymyxin B and miconazole may result predominantly from miconazole that impairs the cell wall and alters its permeability, which then allows polymyxin to gain access through the cell wall to disrupt the cytoplasmic membrane.

To explore the mechanism of action and potential targets for the antimicrobial agents, our study presents systematically increasing concentrations of polymyxin B and miconazole either alone or combined in equal molarity. Results from this setting were compared with data from previous studies where identical masses of the antibiotic substances were combined.[15] Both approaches yielded matching results, because the antibacterial agents acted synergistically against the Gram-negative *E. coli* and *P. aeruginosa* and showed on average no interaction against *S. pseudintermedius* strains. Thus, the impact of both agents on the bacterial cell is a monomolar reaction, as one mole of polymyxin B combined with one mole of miconazole is sufficient to produce these results.

Synergism was addressed by means of FICI. The mode was 0.25, 0.50, 1.25 and 0.75 for *E. coli*, *P. aeruginosa*, *S. pseudintermedius* and the MRSP strains, respectively. These results agree with the median except for the *Staphylococcus* strains from Germany, France and Italy (1.00). In general, there is a close correspondence between the median, separating the higher and the lower half of the sample, and the mode, the most frequent

value. Both are used to describe the central tendency of FICI from a different perspective. Accordingly, in a common interpretation, 'no interaction' corresponds to FICI = 1, synergism corresponds to FICI < 1, and for antagonism FICI is >1. However, due to inherent inaccuracies of the experimental method, a more rigorous limit of FICI $\leq$ 0.5 for synergism and FICI > 4 for antagonism is required by the editorial policies of many journals.[26,38] More recently, symmetrical limits of 0.5 and 2 have been proposed for 'no interaction'.[39] Further minor deviations of the FICI from 1 do not appear to be relevant practically. Thus, we tested whether the experimentally determined FICIs are <1.0 and <0.5. For *E. coli*, there is high evidence of synergism, in that the FICI = 0.25 is significantly smaller than the rigorous limit of 0.5. For *P. aeruginosa*, the FICI determined from pooled data is clearly smaller than 1, but not lower than the rigorous limit. Instead, the FICI estimated from samples from three countries is located exactly at the upper limit of 0.5 for synergism. For *S. pseudintermedius* strains, the median of FICI is 1.0, and the IQR of 0.56–1.25 suggests no interaction. This also holds true for the MRSP strains, although the median FICI of 0.75 is lower. While most strains were rendered more susceptible when both drugs were used in combination, a definite synergistic effect for all strains was missing. With this bacterial species, *in vitro* synergy may be strain dependent, because for some *S. pseudintermedius* isolates a boosted effect was obvious while for others there was no interaction.

The combination of both drugs has the potential for synergistic action not only *in vitro*, but it may be also of clinical relevance. Polymyxins produce concentration-dependent killing, with an initial kill followed by regrowth.[11] In the light of the routine occurrence of regrowth, combination therapy may prove to be more efficacious, because this strategy suppresses bacterial regrowth at subinhibitory concentration and/or avoids the appearance of heteroresistant strains. From *in vitro* susceptibility testing of polymyxin B combined with miconazole, a synergistic effect against the Gram-negative bacterial isolates was evident. If we consider topical remedies for combating veterinary otic bacterial pathogens, they provide antimicrobial drugs in excess. With preparations for local administration, up to 1000 times the MIC values for the bacteria tested are present at the site of application, and persistence of miconazole in the external ear canal for 10 days was shown at concentrations exceeding the MIC values that inhibit 90% of bacterial isolates.[40] These high local concentrations exert a strong killing potential and minimize the risk of microbial resistance.

Overall, our results revealed that clinical strains from three European countries show a similar *in vitro* susceptibility to polymyxin B and miconazole. Antibiotic synergism of polymyxin B and miconazole against the Gram-negative *E. coli* and *P. aeruginosa* strains was demonstrated, and both agents, when applied in combination, showed a substantial bactericidal activity against almost all of the strains tested. In search of effective chemotherapeutic approaches for treating otitis externa, combination therapy using polymyxin B and miconazole can be of potent therapeutic value against the pathogens com-

monly associated with this disease. The *in vitro* synergy of polymyxin B and miconazole may result in better treatment results for otitis externa associated with Gram-negative bacterial infections.

Acknowledgements

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Supporting Information

Additional Supporting Information may be found in the online version of this article.
Figure S1. MBC/MIC ratios of polymyxin B or miconazole for bacterial isolates from all countries.

Résumé

Contexte – L'otite externe canine, une inflammation du conduit auriculaire externe, peut être entretenue et aggravée par les infections bactériennes ou fongiques. Pour le traitement topique, les associations d'anti-inflammatoires et d'antimicrobiens sont principalement utilisées.

Hypothèses/Objectifs – Cette étude a été menée pour déterminer l'activité *in vitro* de la polymyxine B et du miconazole contre les souches bactériennes cliniques isolées dans trois pays européens, d'étudier les différences possibles de sensibilité et de déterminer les interactions médicamenteuses.

Sujets – Dix-sept souches d'*Escherichia coli*, 24 souches de *Pseudomonas aeruginosa*, 24 souches de *Proteus mirabilis* et 25 souches de *Staphylococcus pseudintermedius* ont été isolées de chiens atteints d'otite externe en Allemagne, France et Italie.

Résultats – L'activité des molécules a été évaluée par la concentration minimale inhibitrice (CMI) et la concentration minimale bactéricide. La potentialisation de la polymyxine B et du miconazole a été calculée par l'indice de concentration inhibitrice fractionnaire (FICI). Un FICI ≤ 0.5 définissait la synergie. En outre, les variations géographiques dans le FICI et la CMI étaient évaluées par analyses statistiques.

Résultats – Les sensibilités bactériennes étaient comparables dans les différents pays européens parce qu'aucune différence significative n'a été mise en évidence entre les variations de CMI et de FICI ($P >$ 0.05). La polymyxine B en tant que seul agent avait une activité bactéricide contre la plupart des souches de *E. coli* et *P. aeruginosa*, et, à plus forte concentration, contre les souches de *S. pseudintermedius*. Le miconazole était bactéricide contre toutes les souches de *S. pseudintermedius*. Une synergie a été mise en évidence contre les souches de *E. coli* et *P. aeruginosa* (FICI = 0.25 et 0.50, respectivement), alors

qu'aucune interaction n'a été mise en évidence contre les souches de *S. pseudintermedius* (FICI = 1.25). Les souches de *Proteus mirabilis* n'ont été inhibées par aucune des molécules, individuellement ou en association.

Conclusions et importance clinique – La synergie *in vitro* de la polymyxine B et du miconazole contre les souches d'*E. coli* et de *P. aeruginosa* justifie l'application de la combinaison des deux agents dans le traitement de l'otite externe lors d'infection par ces bactéries.

Resumen

Introducción – la otitis externa canina, inflamación del canal auditivo externo, puede perpetuarse y empeorar debido a la presencia de infecciones bacterianas o fúngicas. Para el tratamiento tópico se utilizan fundamentalmente combinaciones de ingredientes antiinflamatorios y antimicrobianos.

Hipótesis/objetivos – este estudio se condujo para elucidar la actividad *in vitro* de polimixina B y miconazol frente a aislados clínicos bacterianos de tres países europeos, investigar posibles diferencias en sensibilidad y analizar interacciones de fármacos.

Animales – diecisiete cepas de *Escherichia coli*, 24 cepas de *Pseudomonas aeruginosa*, 24 cepas de *Proteus mirabilis* y 25 cepas de *Staphylococcus pseudintermedius* de perros diagnosticados con otitis externa asilados de Alemania, Francia e Italia.

Métodos – se evaluó la actividad de los fármacos mediante la concentración inhibitoria minima (MIC) y la concentración bactericida minima. La potenciación de polimixina B y miconazol se calculó usando el índice de concentración fraccional inhibitoria (FICI). Un FICI $\leq$ 0,5 definía sinergismo. Además se analizaron estadísticamente las variaciones en la FICI y MIC dependiendo de la región de origen.

Resultados – la susceptibilidad bacteriana fue comparable en los diferentes países europeos ya que no hubo diferencias significativas en MIC y FICI (P > 0,05). Como agente único la polimixina B tuvo actividad antimicrobiana frente a la mayoría de cepas de *E. coli* y *P. aeruginosa,* y a mayores concentraciones frente a cepas de *S. pseudintermedius*. El miconazol fue bactericida frente a todas las cepas de *Staphylococcus*. Se observó sinergismo frente a cepas de *E. coli* y *P. aeruginosa* (FICI = 0.25 y 0,50, respectivamente), mientras en general no hubo sinergismo frente a las cepas de *S. pseudintermedius* (FICI = 1.25). Cepas de *Proteus mirabilis* no fueron inhibidas por los fármacos individualmente ni en combinación.

Conclusiones e importancia clínica – el sinergismo *in vitro* de la polimixina B y el miconazol frente a aislados de *E. coli* y *P. aeruginosa* indica un motivo para utilizar ambos agentes en combinación para tratar casos de otitis externa producidos por infecciones con estas bacterias.

Zusammenfassung

Hintergrund – Die canine Otitis externa, eine Entzündung des äußeren Ohrkanals, kann durch eine bakterielle Infektion oder durch eine Infektion mit Hefepilzen aufrechterhalten bzw. verschlimmert werden. Zur topischen Behandlung werden hauptsächlich Kombinationen aus entzündungshemmenden und antimikrobiellen Wirkstoffen verwendet.

Hypothese/Ziele – Diese Studie wurde durchgeführt, um die *in vitro* Aktivität von Polymyxin B und Mikonazol gegenüber klinischen Bakterienisolaten aus drei europäischen Ländern zu beleuchten und um mögliche Unterschiede in der Sensibilität zu untersuchen und um Interaktionen von Medikamenten zu beurteilen.

Tiere – Siebzehn Stämme von *Escherichia coli*, 24 Stämme von *Pseudomonas aeruginosa*, 24 Stämme von *Proteus mirabilis* und 25 Stämme von *Staphylokokkus pseudintermedius* von Hunden mit einer diagnostizierten Otitis externa waren in Deutschland, Frankreich und Italien isoliert worden.

Methoden – Die Wirkstoffaktivitäten wurden mittels minimaler inhibitorischer Konzentration (MIC) und minimaler bakterizider Konzentration evaluiert. Die Potenzierung von Polymyxin B plus Mikonazol wurde mittels „Fractional Inhibitory Concentration Index" (FICI) kalkuliert. Ein FICI $\leq$ 0,5 definierte eine Synergie. Weiters wurden geographische Variationen des FICI und der MIC mittels statistischer Analyse beurteilt.

Ergebnisse – Die bakteriellen Empfindlichkeiten waren in den verschiedenen europäischen Ländern vergleichbar, da keine signifikanten Unterschiede bei MIC und FICI bestanden (P > 0,05). Als alleiniger Wirkstoff zeigte Polymyxin B eine bakterizide Wirkung gegenüber den meisten *E. coli* und *P. aeruginosa* Stämmen und, in höheren Konzentrationen, gegenüber *S. pseudintermedius* Stämmen. Eine Synergie wurde gegen *E. coli* und *P. aeruginosa* Stämme (FICI = 0.25 bzw. 0,50) demonstriert, während insgesamt keine Interaktion gegen *S. pseudintermedius* Stämme (FICI = 1.25) bestand. *Proteus mirabilis* Stämme wurden von keinem dieser Wirkstoffe, weder individuell noch in Kombination, inhibiert.

Schlussfolgerungen und klinische Bedeutung – Eine *in vitro* Synergie von Polymyxin B und Mikonazol gegenüber *E. coli* und *P. aeruginosa* Isolaten bekräftigt die Argumentation dafür, beide Wirkstoffe in Kombination zu verwenden, um eine Otitis externa, bei der diese Bakterien vorkommen, zu behandeln.

要約

背景 – 犬の外耳炎は外耳道の炎症で、細菌や真菌感染によって持続ならびに悪化する可能性がある。外用療法には、抗炎症性、および抗菌性物質の組み合わせが主に使用されている。

仮説/目的 – この研究はヨーロッパ3国で臨床的に分離した細菌に対するポリミキシンBならびにミコナゾールの in vitro の活性を解明するため、感受性の違いを調査するため、および薬剤の相互作用を評価するために実施した。

供与動物 – ドイツ、フランスならびにイタリアで外耳炎と診断した犬から分離した Escherichia coli 17菌株、Pseudomonas aeruginosa 24菌株、Proteus mirabilis 24菌株、Staphylococcus pseudintermedius 25菌株。

方法 – 薬剤活性は最小阻止濃度 (MIC) および最小殺菌濃度により評価した。ポリミキシンBとミコナゾールの相乗作用は fractional inhibitory concentration index (FICI) を用いて計算した。FICI ≤ 0.5 を相乗効果と決めた。さらに、FICI ならびに MIC の地理的な差を統計学的な解析により評価した。

結果 – 細菌の感受性は MIC および FICI の変動に有意差が見られなかったため、それぞれのヨーロッパの国で同等で有った (P > 0.05)。単剤として、ポリミキシンBはほとんどの E.coli ならびに P. aeruginosa 菌株に、高濃度では S. pseudintermedius 菌株に対しても抗菌活性が認められた。ミコナゾールはすべての Staphylococcus 菌株に対して殺菌活性を示した。相乗作用は E. coli ならびに P. aeruginosa 菌株に対して認められた (それぞれの FICI = 0.25 および 0.50) 一方、全体的に S. pseudintermedius 菌株に対して相互作用が見られなかった (FICI = 1.25)。Proteus mirabili 菌株はそれぞれの薬剤単独あるいはそれらの組み合わせによって阻止されなかった。

結論および臨床的な重要性 – 分離された E. coli および P. aeruginosa に対するポリミキシンBとミコナゾールの in vitro の相乗作用はこれらの細菌の感染の際、両方の薬剤を組み合わせて外耳炎の治療として適用できる根拠を示した。

摘要

背景 – 犬外耳炎——外耳道炎症的持续发生和恶化，都是细菌或真菌感染所引起。局部治疗，主要使用抗炎和抗微生物药物组合。

假设/目的 – 本研究针对来自3个欧洲国家的临床菌株，评估多粘菌素B和咪康唑的体外抗菌活性，调查可能存在的敏感性差异，并评估药物间的相互作用。

动物 – 在德国、法国和意大利，从诊断为外耳炎的犬耳道内，分离出的17个大肠杆菌菌株、24个绿脓杆菌菌株、24个奇异变形杆菌菌株和25个假中间型葡萄球菌菌株。

方法 – 药物活性通过最低抑菌浓度（MIC）和最低杀菌浓度评估。多粘菌素B加咪康唑的增效作用通过部分抑菌浓度指数（FICI）评估。FICI ≤ 0.5 定义为协同作用。此外FICI和MIC的地理学变化通过统计学分析评估。

结果 – 不同的欧洲国家细菌敏感性相似，因为MIC和FICI没有显著变化（P > 0.05）。作为单个产品，多粘菌素B对大部分大肠杆菌和绿脓杆菌有杀菌活性，高浓度时可抗假中间型葡萄球菌。咪康唑对所有葡萄球菌有杀菌活性。对大肠杆菌和绿脓杆菌（FICI = 0.25和0.50）有协同作用，对假中间型葡萄球菌全部没有相互作用（FICI = 1.25）。每种单独的药物或联合用药对奇异变形杆菌没有抑制作用。

结论和临床价值 – 多粘菌素B和咪康唑的体外协同作用显示，大肠杆菌和绿脓杆菌感染的外耳炎可联合应用这两种药物治疗。