Veterinary Microbiology 181 (2015) 261–270

Contents lists available at ScienceDirect

Veterinary Microbiology

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

Is transmission electron microscopy (TEM) a promising approach for qualitative and quantitative investigations of polymyxin B and miconazole interactions with cellular and subcellular structures of *Staphylococcus pseudintermedius*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Malassezia pachydermatis*?

Michael Voget[a,*], Dorothea Lorenz[b], Elisabeth Lieber-Tenorio[c], Ruediger Hauck[d], Michael Meyer[e], Michael Cieslicki[f]

[a] *ECON GmbH, Lübeck, Germany*
[b] *Leibnitz Institut für molekulare Pharmakologie, Berlin, Germany*
[c] *Friedrich-Loeffler Institut, Jena, Germany*
[d] *Freie Universität Berlin, Fachbereich Veterinärmedizin, Germany*
[e] *MBC-Statistik, Falkensee, Germany*
[f] *Elanco Animal Health, Germany*

ARTICLE INFO

Article history:
Received 15 January 2015
Received in revised form 29 September 2015
Accepted 2 October 2015

Keywords:
Otitis
Therapy
Polypeptide antibiotic
Imidazole antifungal
Synergy
In vitro

ABSTRACT

Antimicrobial therapy using a combination of polymyxin B and miconazole is effective against the main bacterial pathogens associated with otitis externa in dogs, and a synergistic effect of both drugs has been shown previously. The objective of the present investigation was to visualize ultrastructural changes after exposure of *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus pseudintermedius* and *Malassezia pachydermatis* to *polymyxin B and miconazole* by transmission electron microscopic (TEM). For this, cultures of *E. coli*, *P. aeruginosa*, *S. pseudintermedius* and *M. pachydermatis* were exposed to polymyxin B and miconazole, alone or in combination for 24 h. Ultrastructural changes were observed most frequently in the cell envelope of the four microorganisms. Exposure to polymyxin B seemed to cause more damage than miconazole within the range of concentrations applied. Treatment resulted in changes of the cell size: in *E. coli*, cell size increased significantly after treatment with either compound alone; in *P. aeruginosa*, cell size decreased significantly after treatment with polymyxin B and with miconazole; exposure of *S. pseudintermedius* to miconazole caused a decrease in cell size; in *M. pachydermatis*, cell size increased significantly after treatment with polymyxin B.; in *E.coli*, *S. pseudintermedius* and *M. pachydermatis*, cell size changed highly significant, in *P. aeruginosa* significantly after exposure to the combination of both compounds. In conclusion, by using a different approach than previous investigations, this study confirmed a clear combinatory effect of polymyxin B and miconazole against the tested microorganisms involved in canine otitis externa. It is the first time that visualization technologies were applied to compare the effect of single drugs to their combinatory effects on cellular and subcellular entities of selected bacterial and yeast species.

1. Introduction

Inflammation of the external ear is a common problem in dogs. Canine otitis externa can be acute or chronic and is caused by several different factors (Anon., 2014; Griffin, 2014a; Miller et al., 2013). Various microorganisms are commonly isolated from inflamed external ear canals and considered important for pathogenesis (Rosser, 2004). The most common are the yeast *Malassezia pachydermatis* and the various bacteria including

Abbreviations: TEM, Transmission electron microscopy; MIC, Minimal inhibitory concentration; FICI, Fractional inhibitory concentration index.

* Corresponding author.
E-mail address: michael.voget@econ-ag.de (M. Voget).

http://dx.doi.org/10.1016/j.vetmic.2015.10.002
0378-1135/

Staphylococcus spp., *Pseudomonas aeruginosa*, *Proteus mirabilis* and *Escherichia coli* (Bugden, 2013; Oliveira et al., 2008; Zamankhan Malayeri et al., 2010). Consequently, ear cleaning and topical application of antimicrobials are important for the successful therapy of otitis externa (Bond, 2012; Griffin, 2014b).

Antimicrobial therapy using a combination of polymyxin B and miconazole was shown to be effective against the main bacterial pathogens associated with otitis externa in several clinical studies (Engelen et al., 2010; Engelen and Anthonissens, 2000; Rougier et al., 2005; Studdert and Hughes, 1991).

Polymyxin B is a polypeptide antibiotic, which mainly interacts with Gram-negative bacteria by binding to the lipid A component of their lipopolysaccharides within the outer membrane of the cellular envelope (Kadar et al., 2013; Velkov et al., 2010). Polymyxin B becomes completely integrated into the outer membrane, causing disorganization and neutralizing the charge on the cell surface. In consequence the envelope loses its barrier function (Kadar et al., 2013; Velkov et al., 2010). Polymyxin B also shows some activity against *Staphylococcus* spp. (Boyen et al., 2012) and has antifungal properties (Schwartz et al., 1972; Zhai et al., 2010). In the last few years polymyxin B, which was discovered already in the 1940s, has received new attention as a last line antimicrobial against multiresistant bacteria (Velkov et al., 2013).

Miconazole is a fungicidal imidazole derivate. Its main mode of action is interacting with the cytochrome P450 complex in fungal cell membranes inhibiting the biosynthesis of ergosterol, which is essential for the stability of the fungal cell membrane. Furthermore, it causes toxic levels of methylated sterol and interferes with the activities of oxidative and peroxidative enzymes leading to toxic concentrations of hydrogen peroxide (Barasch and Griffin, 2008; Piérard et al., 2012). Besides a broad spectrum of activity against fungi, including *M. pachydermatis*, it is also active against Gram-positive bacteria (Piérard et al., 2012).

The efficacy of treating canine otitis externa with a combination of polymyxin B and miconazole was underlined by showing a synergistic effect of both drugs against type strains of *P. aeruginosa*, *E. coli* and *M. pachydermatis* (Pietschmann et al., 2009). However, in the same study no synergistic effect against *Staphylococcus intermedius* was detected. Another study investigating clinical isolates of bacteria from canine otitis externa from different European countries demonstrated synergy of both compounds against *E. coli* and *P. aeruginosa* but not against *Staphylococcus pseudintermedius* and *P. mirabilis* (Chiavassa, 2013; Pietschmann et al., 2013). Both studies were conducted by determining minimal inhibitory concentrations (MIC), minimal bactericidal concentrations and the fractional inhibitory concentration index (FICI) according to Odds (2003). Pietschmann et al. (2009) also used fluorescence microscopy and flow cytometry to demonstrate the antibiotic effects and their synergy by visualizing the proportion of living and killed bacterial cells.

The work presented here is one of a series aligning with the previous studies mentioned above. The first study examined effects on strains provided by the DSZM German collection of Microorganisms and Cell Cultures (Pietschmann et al., 2009) and reported an eightfold reduction of MIC for the combination of both drugs in *S. intermedius.* The corresponding FICi was 0.63. In *E. coli, P. aeruginosa* and *M. pachydermatis* the combination resulted in a reduction of the MIC between fourfold and hundredfold with FICi's between 0.06 and 0.5. Independent research work (Chiavassa, 2013) with *M. pachydermatis* isolates from different regions in Italy reported reductions of MIC's for miconazole between eightfold and more than thirty fold and for Polymyxin B more than hundredfold when exposed to the combination of both. The FICI was calculated to be <0.5, a clear indicator for synergism. Another study (Pietschmann et al., 2013) showed synergistic effects of both drugs against strains of *E. coli* and *P. aeruginosa* isolated from dogs in Italy, France and Germany, which were diagnosed to suffer from *otits externa* (FICi = 0.25 and 0.50, respectively). No interaction was seen against *S. pseudintermedius* strains (FICi = 1.25).

The objective of the present investigation was to visualize and to analyze quantitatively morphological changes occurring at cellular and sub cellular levels after exposure of *E. coli*, *P. aeruginosa*, *S. pseudintermedius* and *M. pachydermatis* to polymyxin B and miconazole, alone or in combination, by transmission electron microscopy (TEM).

Concerning the synergism of both compounds, we relied on the findings reported earlier (Pietschmann et al., 2009, 2013; Chiavassa, 2013). TEM was not understood as alternative approach to examine synergism of drug combinations, it was intended to give a deeper insight into the impact of the combinatory drug effects reported earlier. Furthermore, TEM additionally provided quantitative data such as diameters. These data enabled a statistical modelling of the effect of miconazole, polymyxin B and the combination on cell size.

2. Methods

2.1. Bacterial and fungal strains

Type strains of *E. coli* (DSM 1103/ATCC 25922), *P. aeruginosa* (DSM 1117/ATCC 27853), *S. pseudintermedius* (DSM 21284) *M. pachydermatis* (DSM 6172/CIP 649-86, CDC 16334) were investigated in this study.

2.2. Antimicrobials

For all experiments freshly prepared antimicrobial solutions were used. Polymyxin B sulfate (Genaxxon Bioscience, Ulm, Germany) was dissolved in de-ionized water. Miconazole nitrate salt (TCI Deutschland, Eschborn, Germany) was dissolved in ethanol as co-solvent while heating for 2 h at 60 °C. Both preparations were filter sterilized (pore size 0.2 μm) prior use.

Table 1
OD_{600} of *S. pseudintermedius* cultures after addition of different concentrations of polymyxin B, miconazole and their combination.

Time after addition of compounds (h)	Control 1 (+25 μl/ml ethanol)	Control 2	Polymyxin (5 μg/ml)	Polymyxin (15 μg/ml)	Miconazole (5 μg/ml)	Miconazole + polymyxin (0.5 μg/ml*)	Miconazole + polymyxin (2.5 μg/ml*)	Miconazole + polymyxin (5 μg/ml*)
0	0.443	0.443	0.423	0.423	0.443	0.443	0.443	0.423
0.5	0.692	0.798	0.668	0.348	0.346	0.686	0.742	0.6
1	0.95	1.075	0.91	0.54	0.305	0.915	0.875	0.78
1.5	1.67	1.61	1.14	0.59	0.24	1.17	1.23	0.87
2	2.15	2.21	1.71	0.68	0.22	1.81	1.51	1.26
2.5	2.5	2.62	2.35	0.88	0.24	2.38	2.34	1.77
3	3.7	4.4	2.86	0.94	0.23	2.98	2.26	2.08

*Of each compound.