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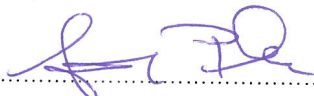
**Development of advanced  
antimicrobial combinations for the  
treatment of canine otitis externa**

PhD Thesis

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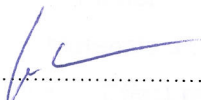
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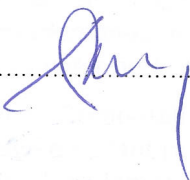
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## 1. Introduction and objective

One aim of this study was to determine antimicrobial susceptibility of *P. aeruginosa* strains to marbofloxacin and gentamicin and investigate the possible synergistic, additive, indifferent or antagonistic effect between the two antibacterial agents with special emphasis on those strains that were resistant to each antibiotic monotherapy.

We were also interested in determining the permeation of gentamicin across a monolayer of the porcine intestinal epithelial cell line, IPEC-J2 in the presence and absence of 1% DMSO using HPLC with fluorescence detection.

Another goal of this examination was to investigate the risk and process of resistance development of certain antibacterials. We investigated the development of resistance after 8-day serial passages in sublethal concentrations of marbofloxacin and the marbofloxacin–gentamicin (1:1) combination in *Staphylococcus*

*pseudintermedius* and *Pseudomonas aeruginosa* strains isolated from external otitis cases in dogs.

Furthermore, we determined Minimum inhibitory concentration (MIC) values of marbofloxacin and ketoconazole in selected *Staphylococcus pseudintermedius*, *Staphylococcus aureus*, *Streptococcus canis*, *Pseudomonas aeruginosa* and *Malassezia pachydermatis* strains isolated from dogs (from skin and the external ear canal). The first examinations were performed in 2010 and we repeated it with the same way in 2017–2018 to check the rate of resistance developed among the mentioned microbes in 2010 and 7–8 years later.

A further objective was to study Minimum inhibitory concentration (MIC) values of ketoconazole in selected *Malassezia pachydermatis* strains isolated from dogs and grown on biofilms and as planktonic forms.

## 2. Materials and methods

### 2.1. Effect of the combination of marbofloxacin and gentamicin on the antimicrobial susceptibility of *Pseudomonas aeruginosa* strains

A total of 68 *P. aeruginosa* strains isolated from dogs showing the clinical signs of otitis externa were used in this study. The origin of the strains were Hungarian and were collected from different territories. The broth microdilution method was performed in accordance with CLSI M7-A9. 2012 with a two-fold dilution in 96-well sterile microtiter plates.

In order to describe interactions between marbofloxacin and gentamicin, we determined the fractional inhibitory concentration (FIC) index.

## 2.2. Penetration of gentamicin and gentamicin supplemented with 1% DMSO across biological monolayer membranes *in vitro*

The IPEC-J2 cell line were used in this study. Cells form a differentiated monolayer and are attached to each other via tight junctions apically. The high transepithelial electrical resistance (TEER) of IPEC-J2 monolayers grown on Transwell polyester filters coated with rat tail collagen shows the functional integrity of a continuous, uninterrupted cell layer.

Only monolayers with a TEER value of at least 8000 Ohm/cm<sup>2</sup> were used as a model system to study the cellular barrier permeability

For the statistical evaluation unpaired, two-sample Student's t-probe were used.



### 2.3. Effect of 8-day serial passage on the development of resistance against marbofloxacin–gentamicin (1:1) combination in selected *Pseudomonas aeruginosa* and *Staphylococcus pseudintermedius* strains

Sixteen non-duplicate clinical isolates of *P. aeruginosa* isolated from dogs and cats and were used in the present study. Preparation of bacterial suspension and MIC determination were performed according to CLSI M07-A9.

Following the determination of the MICs, bacteria were inoculated on the following day in a different microplate containing the same concentrations of marbofloxacin or the marbofloxacin–gentamicin (1:1) combination. For each strain, the highest concentration of the antibacterial where bacterial growth occurred was selected and from this well, bacteria were passaged to another plate with all of the concentrations present.

For the detection of mutations in the genes of QRDRs as well as the efflux regulatory genes that are associated with fluoroquinolone or aminoglycoside resistance in *P. aeruginosa*, PCR amplification and sequencing were performed.

Carbonyl cyanide m-chlorophenyl hydrazone is a proton conductor that causes uncoupling of the proton gradient that is maintained by the activity of electron carriers in the electron transport chain. CCCP acts as an efflux pump inhibitor in *P. aeruginosa* thereby increasing sensitivity to fluoroquinolones and other antimicrobials.

Besides *P. aeruginosa* examination, we also carried out thirty two clinical isolates of *S. pseudintermedius* strains isolated from dogs and cats.

Trends of MIC values were modelled with power functions of the days elapsed.

## 2.4. Comparative susceptibility examination between *Staphylococcus* spp., *Streptococcus canis* and *Pseudomonas aeruginosa* strains collected in 2010 and 2017-2018

One hundred and twenty three (123) strains of staphylococci, thirty seven (37) strains of *S. canis* (Lancefield group G), fifty two (52) strains of *P. aeruginosa* and forty (40) strains of *M. pachydermatis* isolated from dogs and cats in 2010 were investigated in this experiment.

Eighty nine (89) strains of *Staphylococcus pseudintermedius* and *S. aureus*, fifty five (55) strains of *P. aeruginosa* and eighty (80) strains of *M. pachydermatis* strains isolated from dogs in 2017-2018 were investigated in comparison with the MICs of the strains isolated in 2010.

## 2.5. Activity of ketoconazole against biofilm producing and planktonic *Malassezia pachydermatis* strains

Eight strains of *Malassezia pachydermatis* strains were involved in this experiment. All of the strains were isolated from dogs suffering from clinical signs of malassezia infections of the skin or the ear.

*M. pachydermatis* strains multiplied in Sabouraud broth for 48 hours were centrifuged at 3000 g for 10 minutes. The fungi were washed with sterile physiological saline, centrifuged at 3000 g for 10 minutes and resuspended in physiological saline. A suspension of approximately  $10^6$  CFU/ml was prepared with a 10-fold dilution. The germ-count of the suspensions was tested with inoculation to Sabouraud agar plates and counting the number of CFU.

For forming biofilms we used in this study a sterile, disposable Foley catheter was used.

Biofilm production: 25  $\mu$ l of a standardized ( $10^6$  CFU/ml) yeast suspension was dropped on the surface of the catheter segments and were put in a thermostat at 37 °C for 1 hour (attachment phase) to help adhering of the yeasts to the catheter surface. After 1 hour these segments (along with the yeasts on the surface) were submerged in 1 ml of Sabouraud broth including different concentrations of ketoconazole. Fungal suspensions were placed in a thermostat at 37 °C for 72 hours.

Planktonic forms of yeasts: in these cases no attachment phase was utilised, the fungi were directly inoculated into Sabouraud broth containing different concentrations of ketoconazole. Thus, the organisms remained in planktonic forms without any attachment. Fungal suspensions were place in a thermostat at 37 °C for 72 hours.

Determination of MIC-values of ketoconazole with broth microdilution in *M. pachydermatis* strains

### 3. Results and conclusions

#### 3.1. Effect of the combination of marbofloxacin and gentamicin on the antimicrobial susceptibility of *Pseudomonas aeruginosa* strains

The results of our study demonstrate full synergy has been found between marbofloxacin and gentamicin in 48.5% of the *P. aeruginosa* strains investigated, with a mean FIC index of 0.546 indicating only partial synergy for all of the strains. During the examination no antagonistic effect was observed in any of the strains, therefore, according to our results the justified and targeted usage of the marbofloxacin/gentamicin combination in infections caused by *P. aeruginosa* in the veterinary field may yield beneficial results, especially in topical products where the toxic effects of gentamicin might be negated if the tympanic membrane is intact.

### 3.2. Penetration of gentamicin and gentamicin supplemented with 1% DMSO across biological monolayer membranes *in vitro*

Although our studies were conducted on monolayer IPEC-J2 cells our results suggest that gentamicin alone or supplemented with 1% DMSO cannot penetrate through the intact tympanic membrane into the inner ear. The tight barrier due to IPEC-J2 cells grown in a monolayer and linked by tight junction proteins is less complex than the multi-layered tympanic membrane therefore our approach is over-predictive. Furthermore, our data show that the addition of the organic solvent DMSO did not alter the transport of gentamicin through an intestinal epithelial monolayer, hence it might not enhance the oral absorption of gentamicin if ingested accidentally. Whether our *in vitro* results can be extrapolated to the *in vivo* situation remains to be investigated.

### 3.3. Effect of 8-day serial passage on the development of resistance against marbofloxacin–gentamicin (1:1) combination in selected *Pseudomonas aeruginosa* and *Staphylococcus pseudintermedius* strains

Results of the present study revealed that the ratio of resistant *P. aeruginosa* strains was significantly lower when passaged for 8 days in sublethal concentrations of gentamicin and marbofloxacin solution as compared to sublethal concentrations of marbofloxacin solution alone. No strain reached higher MIC value than 32 µg/ml.

We carried out the examination with *S. pseudintermedius*. Average multiplication of the MICs on day 8 passaged in marbofloxacin alone and the marbofloxacin–gentamicin (1:1) combination were 11.67 compared to 3.00, respectively. In addition it has to be stated that the original (day 1) results of the combination were usually lower, thus the multiplication factor indicates a much lower



absolute increase. All of our results showed highly significant differences ( $p < 0.0001$ ). Considering these results, it can be pronounced that the marbofloxacin–gentamicin (1:1) combination can significantly hinder the development of fluoroquinolone resistance in *S. pseudintermedius* bacteria.

### 3.4. Comparative susceptibility examination between *Staphylococcus* spp., *Streptococcus canis* and *Pseudomonas aeruginosa* strains collected in 2010 and 2017-2018

The comparative susceptibility test shows MIC values of different *Staphylococcus* spp., *Streptococcus canis*, *P. aeruginosa* and *Malassezia pachydermatis* strains isolated from dog skin concerned by dermatitis in 2010 in comparison with MICs gained from the same sources in 2017-2018. The ratio of sensitive microbial population is given in percent.

*Staphylococcus* spp., *S. canin*, *P. aeruginosa* and *M. pachydermatis* show definite susceptibility to marbofloxacin and ketoconazole.

Besides that on significant difference were observed in MICs between 2010 and 2017–2018. Based on these results it can be stated, that marbofloxacin and ketoconazole are still been valuable and useful antibiotic against the most important bacteria and *M. pachydermatis*.

### 3.5. Activity of ketoconazole against biofilm producing and planktonic *Malassezia pachydermatis* strains

According to our results it can be stated, that *M. pachydermatis* strains showed very high susceptibility to ketoconazole in their planktonic (suspended) forms. However, when planted on surface catheters to produce biofilms their susceptibility was changed significantly. It is highlighted in our study that biofilm producing forms

of a yeast strain can show 25–3125 times decrease in susceptibility (25–3125 times increase in MIC) compared to the planktonic forms of the respective strain.

These data can be of utmost importance when evaluating the *in vivo* efficacy of an antifungal. *In vitro* susceptibility data can be essentially changed in living systems. Thus, the effect of ketoconazole can be much less pronounced against these strains and forms of yeasts. According to these results, a much higher concentration of ketoconazole is recommended in dermatological products than the average MIC<sub>90</sub> values, if *M. pachydermatis* infection is suspected. This can inhibit and even kill biofilm producing strains, thereby decreasing the development of resistance in these fungi.

## 4. New scientific results

Synergic effect between marbofloxacin and gentamicin against *Pseudomonas aeruginosa* were discovered.

It was proven that addition of 1% DMSO to gentamicin did not disrupt paracellular integrity of IPEC-J2 monolayer and it did not alter the transport of gentamicin.

Examination revealed that the ratio of resistant *Pseudomonas aeruginosa* strains was significantly lower when passaged for 8 days in sublethal concentrations of gentamicin and marbofloxacin combination solution as compared to sublethal concentrations of marbofloxacin solution alone.

Experiment revealed that the ratio of resistant *Staphylococcus pseudintermedius* strains was significantly lower when passaged for 8 days in sublethal concentrations of gentamicin and

marbofloxacin combination solution as compared to sublethal concentrations of marbofloxacin solution alone.

In our examination we did not find significant difference in MIC values of staphylococci, *Streptococcus canis*, *Pseudomonas aeruginosa* against marbofloxacin and *Malassezia pachydermatis* against ketoconazole between 2010 and 2017–2018.

According to the results described it can be stated, that *Malassezia pachydermatis* strains collected from Hungarian dogs suffering from clinical signs of malassezia infections of the skin or the ear showed 25–3125 times decrease in susceptibility in biofilm (25–3125 times increase in MIC) compared to the planktonic forms of the respective strain.

## 5. Relating author's publications

### 5.1. Publications related to the topic of the present dissertation

#### 5.1.1. Full Text papers in peer-reviewed journal

Gyetvai B., Jerzsele Á., Pászti-Gere E., Nagy G., Gálfi P.: Gentamicin sulphate permeation through porcine intestinal epithelial cell monolayer. *Acta Veterinaria Hungarica* 63 (1), pp. 60–68 2015.

El Zowalaty ME., Gyetvai B.: Effectiveness of Antipseudomonal Antibiotics and Mechanisms of Multidrug Resistance in *Pseudomonas aeruginosa* *Polish Journal of Microbiology* 65:(1) 23-32. 2016.

Jerzsele Á., Albrecht V., Palócz O., Gálfi P., Gyetvai B.: A biofimképzés hatása antibiotikumokkal szembeni *in vitro* érzékenységre kutyából izolált *Pseudomonas*

*aeruginosa* törzseknél. Magyar Állatorvosok Lapja 137:(1) 45-52. 2013.

Jerzsele Á., Balázs B., Kálmánfi E., Lajos Z., Gálfi P., Gyetvai B.: Kutyából és macskából izolált *Malassezia pachydermatis* törzsek *in vitro* érzékenységi vizsgálata. Magyar Állatorvosok Lapja 135. 351-356. 2013.

Jerzsele Á., Balázs B., Lajos Z., Gálfi P., Gyetvai B.: Külsőhallójárat-gyulladásban szenvedő kutyákból izolált *Pseudomonas aeruginosa* törzsek *in vitro* érzékenységi vizsgálata. Magyar Állatorvosok Lapja 135:(12) 746-750. 2013.

Jerzsele Á., Gyetvai B., Csere I., Gálfi P.: Biofilm formation in *Malassezia pachydermatis* strains isolated from dogs decreases susceptibility to ketoconazole and itraconazole. Acta Veterinaria Hungarica 62 (4), 473–480 2014.

### 5.1.2. Oral presentations on Hungarian national conferences

Gyetvai B., Csere I., Szaniszló F., Lang Zs., Gálfi P., Csikó Gy.: Marbofloxacin és ketokonazol hatóanyag tartalmú készítmények hatékonyságának vizsgálata pozitív kontrollkészítménnyel szemben. Akadémiai Beszámolók 2013.

Gyetvai B., Jerzsele Á., Lang Zs., Gálfi P.: *Staphylococcus* spp. és *Pseudomonas aeruginosa* törzsek 8-napos sorozatpasszálásának hatása szubletálisan alkalmazott marbofloxacinnal, illetve marbofloxacin-gentamicin kombinációval szembeni rezisztencia kialakulására. Akadémiai Beszámolók 2014.



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