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EVALUATING SYNERGY BETWEEN MARBOFLOXACIN AND GENTAMICIN IN *PSEUDOMONAS AERUGINOSA* STRAINS ISOLATED FROM DOGS WITH OTITIS EXTERNA

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The aim of this study was to determine antimicrobial susceptibility of *Pseudomonas aeruginosa* strains to marbofloxacin and gentamicin, and investigate the possible synergistic, additive, indifferent or antagonistic effects between the two agents. *P. aeruginosa* strains can develop resistance quickly against certain antibiotics if used alone, thus the need emerges to find synergistic combinations. A total of 68 *P. aeruginosa* strains isolated from dogs were examined. In order to describe interactions between marbofloxacin and gentamicin the checkerboard microdilution method was utilized. The MICs (minimum inhibitory concentrations) for marbofloxacin and gentamicin were in the range 0.25–64 mg/L and 0.25–32 mg/L, respectively. The combination of marbofloxacin and gentamicin was more effective with a MIC range of 0.031–8 mg/L and a MIC_{90} of 1 mg/L, compared to 16 mg/L for marbofloxacin alone and 8 mg/L for gentamicin alone. The FIC (fractional inhibitory concentration) indices ranged from 0.0945 (pronounced synergy) to 1.0625 (indifference). Synergy between marbofloxacin and gentamicin was found in 33 isolates. The mean FIC index is 0.546, which represents a partial synergistic/additive effect close to the full synergy threshold. *In vitro* results indicate that marbofloxacin and gentamicin as partially synergistic agents may prove clinically useful in combination therapy against *P. aeruginosa* infections. Although marbofloxacin is not used in the human practice, the interactions between fluoroquinolones and aminoglycosides may have importance outside the veterinary field.

Keywords: *Pseudomonas aeruginosa*, marbofloxacin, gentamicin, combination, otitis, synergy

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Introduction

Pseudomonas aeruginosa is a frequently isolated Gram-negative bacterium in veterinary practice, associated mainly with external otitis, deep pyoderma, keratoconjunctivitis, respiratory and urinary tract infections [1, 2]. This bacterium can develop resistance rapidly against several antibiotics, even during the course of antimicrobial treatment [3, 4]. Pandrug-resistant strains not susceptible to any of these substances have also been reported in the human medicine [5]. Accordingly, synergistic combinations of antibiotics are used frequently to eliminate this pathogen in human and veterinary practice alike. It was presented that synergism exists between enrofloxacin and gentamicin in 5 *P. aeruginosa* strains [6].

Marbofloxacin is a second generation fluoroquinolone with marked efficacy against *P. aeruginosa*. It has better activity [2, 7, 8] and a longer postantibiotic effect against the pathogen compared to enrofloxacin [9]. 91.3% incidence of sensitivity to marbofloxacin was reported in *Pseudomonas* spp. isolated from dogs was proven [10]. 183 *P. aeruginosa* strains isolated from the outer ear canal of dogs were investigated and they found that 93.4% were sensitive to marbofloxacin [11]. 26.1% resistance ratio was reported [1] in *P. aeruginosa* strains isolated from otitis cases in dogs and cats. Our previous study indicated 23% resistance and 16% moderate susceptibility ratio among 56 isolates [12]. These data show an increasing tendency of marbofloxacin resistance among *P. aeruginosa* strains in companion animals.

Gentamicin is an aminoglycoside frequently used in veterinary medicine. It is applied as a topical medication in dermatology or given parenterally for respiratory, urinary or systemic *P. aeruginosa* infections. 65.2% incidence of susceptibility [10] was reported to gentamicin in *Pseudomonas* spp. isolated from chronic otitis externa cases in dogs. 183 *P. aeruginosa* strains isolated from the outer ear canal of dogs [11] were investigated; 83.1% of the strains were susceptible to gentamicin. Similar results [13] were reported, 84.6% of the investigated 39 *P. aeruginosa* strains were sensitive to gentamicin.

The aim of our 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.

Materials and Methods

Bacteria involved in the study

A total of 68 *P. aeruginosa* strains isolated from dogs showing the clinical signs of otitis externa were used in this study. Acquired *P. aeruginosa* strains were grown on MacConkey agar and were identified by Gram staining, microscopic examination, growth in selective cetrimide agar (Sharlau Chemie, Sentmenat, Spain), colony morphology, pigment production and oxidase positivity. The strains were collected and stored at –80 °C in Mueller–Hinton broth (Biolab Ltd., Budapest, Hungary) supplemented with 20% sterile glycerol. Isolates were subcultured on 5% sheep blood agar plates directly before the investigation. A reference strain of *P. aeruginosa* (ATCC No. 27853) was used as quality control in consonance with Committee of Laboratory Standards Institute (CLSI) guidelines [14].

In vitro *susceptibility tests*

The broth microdilution method was performed in accordance with CLSI guideline M7-A7 with a twofold dilution in 96-well sterile microtiter trays. For the determination of MICs (minimum inhibitory concentrations) for the single antibiotics, final concentrations of marbofloxacin or gentamicin in the wells were set to 128, 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.125 and 0.063 mg/L in Mueller–Hinton broth.

Bacteria grown for 24 hours in Mueller–Hinton broth were centrifuged at 3000 *g* for 10 min. The bacteria were washed and resuspended in physiological saline. The optical density of the suspension at 600 nm was set to 0.1 with the appropriate amount of physiological saline, that corresponded to 10^8 colony forming units (CFU) per mL. *P. aeruginosa* strains were diluted and distributed into the microtiter wells to achieve approximately a 10^5 CFU/mL bacterial density. The inoculated trays were incubated for a period of 24 h at 37 °C and evaluated with the unaided eye. Data were reported as MIC ranges and MIC at which 90% of the strains were inhibited (MIC_{90}). Resistance threshold was determined according to CLSI and EUCAST (European Committee of Antimicrobial Susceptibility Testing) breakpoints. Isolates having MIC values of 4 mg/L or higher for marbofloxacin, and 8 mg/L or higher for gentamicin, were considered resistant. When evaluating the combination, the lower threshold was used.

Investigating antibiotic synergy

In order to describe interactions between marbofloxacin and gentamicin, the checkerboard microdilution method was utilized [15, 16] for determining the lowest fractional inhibitory concentration (FIC) index [17]. Similar to the single antibiotic susceptibility tests, *P. aeruginosa* strains were distributed in the microtiter wells to achieve a 10^5 CFU/mL bacterial density. The inoculated trays were incubated for 24 h at 37 °C. The combined effects of the antibiotics were evaluated as synergy, addition, indifference, and antagonism. To evaluate these interactions, the values for marbofloxacin (FIC_M) and gentamicin (FIC_G) were determined at each dilution.

$$FIC_M = MIC_{M\ combination} / MIC_{M\ alone}$$

$$FIC_G = MIC_{G\ combination} / MIC_{G\ alone}$$

The FIC indices were calculated for each strain according to the method of Elioponlos and Moellering [15] as $FIC_{index} = FIC_M + FIC_G$ and the results were interpreted as follows: synergy (<0.5), partial synergy/addition (0.5–1.0), indifference (1.0–4.0), and antagonism (>4.0). Mean FIC_{index} was calculated to analyse the interactions for all investigated bacteria (n = 68) as

$$\text{Mean } FIC_{index} = \sum FIC_{index} / 68.$$

In addition, data was also illustrated on isobolograms to confirm and analyse interactions between marbofloxacin and gentamicin. The isobologram, a graph of equally effective dose pairs, is a commonly used method to describe drug synergism. In these graphs, the MIC values of the single drugs and the different ratio combinations are plotted as axial points in a Cartesian plot. The straight line connecting $MIC_{marbofloxacin}$ and $MIC_{gentamicin}$ are those dose pairs that will produce an additive effect [18]. Effective dose pairs found below the additive line are considered synergistic combinations, when confirmed with regression analysis.

Results

The sensitivity of the investigated *P. aeruginosa* strains to the two investigated antimicrobials applied as a single treatment varied within broad limits (Table I). The MICs for marbofloxacin and gentamicin ranged from 0.25–64 mg/L and 0.25–32 mg/L, respectively. Three strains were resistant to both antimicrobials alone, but two of these isolates were susceptible to the combination. The reference strain (ATCC No. 27853) showed MICs of 2 mg/L for marbofloxa-

Table I. Distribution of minimum inhibitory concentrations (MICs), MIC_{90} values and percentage of resistant strains in case of marbofloxacin, gentamicin and their 1:1 combination against *P. aeruginosa* isolated from dogs (n = 68)

	No. of isolates with MIC (mg/l)												MIC_{90} (mg/l)	% of resistant strains*	Mean FIC index
	128	64	32	16	8	4	2	1	0.5	0.25	0.125	0.063			
Marbofloxacin	0	4	0	7	8	6	23	11	8	1	0	0	16	36.8%	
Gentamicin	0	0	4	0	3	17	27	14	2	1	0	0	8	10.3	
Marbofloxacin:gentamicin (1:1) combination	0	0	0	0	1	0	4	10	33	17	1	2	1	1.5	0.546

* according to CLSI breakpoints
FIC = fractional inhibitory concentration

Table II. Minimum inhibitory concentrations of gentamicin resistant strains to marbofloxacin, gentamicin and the 1:1 ratio combination. Synergism between the antibacterials is described as partial (1 > FIC > 0.5) or full (FIC < 0.5) synergy

Strain No.	$MIC_{marbofloxacin}$	$MIC_{gentamicin}$	$MIC_{combination}$	FIC	Synergy
1	2	8	1	0.625	Partial
2	0.5	8	0.5	1.0625	None
3	1	32	0.5	0.515625	Partial
4	2	8	0.5	0.3125	Full
5	4	32	0.5	0.140625	Full
6	16	32	8	0.75	Partial
7	4	32	2	0.5625	Partial

Gray cells represent resistance according to CLSI breakpoints
MIC = minimum inhibitory concentration
FIC = fractional inhibitory concentration

cin as well as gentamicin. With respect to CLSI and EUCAST breakpoints, the ratio of resistant strains was 36.8% and 10.3% for marbofloxacin and gentamicin, respectively.

Four gentamicin resistant strains were sensitive and 3 strains were resistant to marbofloxacin, of which 2 proved to be susceptible to the combination (Table II).

Regarding the combination, the FIC indices ranged from 0.0945 (pronounced synergy) to 1.0625 (indifference). Additive and indifferent interactions were observed in 31 and 4 strains, respectively. Synergy between marbofloxacin and gentamicin was found in 33 isolates, while no antagonistic effect was observed in any of the strains. Taking into account the FIC indices acquired with the checkerboard method and isobologram analysis, the 1:1 ratio of the antimicrobials proved to be the most effective. The feature of this fixed ratio combination is its much better activity against *P. aeruginosa* with a MIC range of 0.031–8 mg/L and a MIC_{90} of 1 mg/L compared to the 16 and 8 mg/L values for marbofloxacin and gentamicin alone, respectively. Only 1 strain (1.5%) was resistant to the combination. The mean FIC index for the investigated *P. aeruginosa* strains was 0.546, which represents a partial synergistic/additive effect close to the full synergy threshold. The isobolograms acquired were convex without any exception, showing no antagonistic effect between the two antimicrobials in any of the isolates (Fig. 1).

Discussion

Multidrug resistant and extensively drug-resistant *P. aeruginosa* strains became abundant in the last decades in Europe and cause frequent therapeutic failure in veterinary and human practice [5, 19]. Taking into account the low permeability of the cell wall and the presence of efflux pumps [20], the broad range of β-lactamases produced, biofilm production [21], and the high genetic versatility of this species, only a very limited number of antimicrobials are at the clinicians disposal. Enrofloxacin and marbofloxacin are licensed only for veterinary use, the latter being more active against *P. aeruginosa* [2, 8]. A total of 349 *P. aeruginosa* strains isolated from dogs and cats were investigated [8], 23% of these proved to be resistant to marbofloxacin, a lower ratio compared to our results (36.8%). Regarding gentamicin, Martin Barrasa et al. [10] reported 65.2% incidence of susceptibility to the aminoglycoside among *Pseudomonas* spp. isolated from chronic otitis externa cases in dogs. The 83.1% of 183 *P. aeruginosa* strains were found susceptible to gentamicin [11]. In our study 89.7% of the isolates were susceptible to gentamicin. There were 3 gentamicin resistant strains that were also resistant to marbofloxacin. Two of these strains proved to be sus-

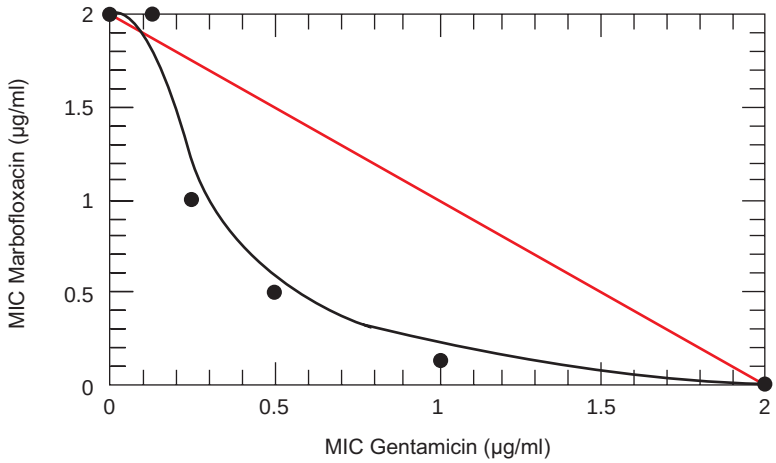

Figure 1. Sample isobologram of marbofloxacin and gentamicin in one *P. aeruginosa* strain. Convex curves (points below the additive line) indicate synergy, concave curves (points above the additive line) show antagonistic effect between two antimicrobials in a certain strain

ceptible to the marbofloxacin: gentamicin combination and the MIC of the third strain was also decreased. Thus, those multiresistant strains resistant to the single antimicrobial therapy can be eliminated by the application of marbofloxacin and gentamicin combination.

Resistance in *Pseudomonas aeruginosa* can develop even during therapy with most of the antimicrobials, including fluoroquinolones [22, 23]. Multiresistant, extensively drug-resistant and panresistant strains of *P. aeruginosa* have already been reported, and a number of workers found that there is no sole antimicrobial that has 100% activity against this bacterium [5, 24, 25]. An incidence of 39–42% multiresistant *P. aeruginosa* isolated from domestic animals between 1989–1997 was reported [26]. In order to kill these multiresistant organisms and delay the development of resistance, a synergistic combination of two bactericidal antibiotics is often required as these might be able to break up resistance in the microorganism [27]. The combination of gentamicin and ciprofloxacin improved survival rate in postsurgical septicaemia in humans [28]. The *in vitro* interactions between enrofloxacin and gentamicin in 7 *P. aeruginosa* strains were investigated [6]. The fluoroquinolone and the aminoglycoside showed synergy in 5 and additive effect in 2 isolates.

The results of our study demonstrate synergy between marbofloxacin and gentamicin in 33 (48.5%) isolates, addition and indifference in 31 (45.5%) and 4 (6%) isolates, respectively. Analysing the FIC values in all of the isolates, the mean FIC index proved to be 0.546 that describes a partially synergistic effect of the combination. In the study of [6] the mean FIC value of 0.442 indicated

full synergy between enrofloxacin and gentamicin. The reason of this difference compared to our results may be the lower activity of enrofloxacin against *P. aeruginosa* that could be enhanced significantly by the addition of gentamicin, or just the small number of isolates examined.

The underlying mechanism of synergy between fluoroquinolones and aminoglycosides has not yet been elucidated. Fluoroquinolone resistance is attributed to increased efflux pump activity and/or chromosomal mutations in the gyrA or parC genes encoding DNA gyrase or topoisomerase IV [29]. Either of these changes can lead to low or high level resistance, and are frequently observed together in resistant strains. As aminoglycosides cause misreading of the genetic code resulting in synthesis of mistranslated proteins, this phenomenon might influence efflux pump activity and expression, leading to decreased survival in the presence of the antimicrobials. Although it might be plasmid encoded [30], fluoroquinolone resistance is primarily chromosomal, while the genes encoding aminoglycoside modifying enzymes are usually found on plasmids [31]. Thus, plasmid and chromosomal resistance mechanisms are usually required jointly in a microorganism to achieve high level resistance to the combination of marbofloxacin and gentamicin.

The usage of fluoroquinolones in veterinary dermatology is recommended in those cases when susceptibility testing indicates that the pathogen is resistant to other classes of antimicrobials [32, 33]. Some authors would recommend systemic usage of these antimicrobials instead of topical administration for treatment of external otitis or pyoderma [34], however, a much higher concentration can be achieved with the latter method. As mentioned earlier, selection of resistant organisms is much less likely when fluoroquinolones are used in combination, as several mutation mechanisms should occur at the same time to achieve a high level resistance in *P. aeruginosa* that can lead to therapeutic failure. However, non-prudent use of fluoroquinolones and consequent development of resistance may lead to public health risk as bacteria can be reservoirs of antibiotic-resistant bacteria [33, 35, 36].

In conclusion, 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. The threshold of full synergy is at 0.5 FIC, and 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.

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Conflict of Interest

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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