

## Fluoroquinolone susceptibility in *Pseudomonas aeruginosa* isolates from dogs - comparing disk diffusion and microdilution methods

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### ABSTRACT

*Pseudomonas aeruginosa* has been determined to be the distinct cause of a number of different infections in both humans and animals. Apart from its high intrinsic resistance, which makes it difficult to treat, it also has a great capacity to acquire further resistance mechanisms. These mechanisms can be present simultaneously in one cell, conferring a multiresistant phenotype. Taking into account that the known risk factor for selection of resistant strains is excessive and inappropriate drug use, especially fluoroquinolones, the aim of this study was to determine the susceptibility of *P. aeruginosa* isolates to these antibacterial agents. The susceptibility of 90 canine isolates was determined by disk diffusion susceptibility test and the broth microdilution method. Both methods showed that isolates were significantly more sensitive to ciprofloxacin than to marbofloxacin or enrofloxacin, and more sensitive to marbofloxacin than to enrofloxacin. The results of microdilution and disk diffusion testing were in 98.9% agreement for ciprofloxacin and in 91.1% agreement for marbofloxacin, with no statistically significant difference between the two methods ( $P>0.05$ ) for these two antibiotics. For enrofloxacin, the results of microdilution and disk diffusion testing were in 76.6% agreement, with a statistically significant disagreement between the two methods ( $P<0.05$ ). In 16 cases, the disk diffusion test overestimated the number of strains susceptible to enrofloxacin compared with the microdilution test. These results suggest that for *P. aeruginosa* infections in dogs, therapy using enrofloxacin, based on the results of disk diffusion testing, may lead to ineffective treatment and could contribute to selection of resistant and multiresistant strains.

**Key words:** *Pseudomonas aeruginosa*, dog, fluoroquinolones, antimicrobial susceptibility, disk diffusion, broth microdilution

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## Introduction

*Pseudomonas aeruginosa* causes opportunistic infections in both humans and animals. In dogs, this bacterium has been recognized as the distinct cause of infections such as: otitis externa, urinary tract infections, conjunctivitis, pneumonia, endocarditis and dermatitis (MARKEY et al., 2013).

Treatment of *P. aeruginosa* infections is difficult because of its high intrinsic resistance to many commonly used antibiotics, such as penicillins, first and second generation cephalosporins, macrolides and chloramphenicol, some aminoglycosides, some fluoroquinolones, tetracyclines and sulfonamides (LIVERMORE et al., 2001). Therefore, the choice of antibiotics that can be used to treat *P. aeruginosa* infections is very narrow, especially in veterinary medicine (GIGUÈRE et al., 2013). Furthermore, apart from its high intrinsic resistance, *P. aeruginosa* also has a great capacity to acquire further resistance mechanisms, which include: efflux pump expression, decreased permeability, target site alterations and production of antibiotic-hydrolyzing enzymes. These mechanisms can be present simultaneously in one cell, conferring a multiresistant phenotype. Treatment of infections caused by such microorganisms is very challenging (STRATEVA and YORDANOV, 2009).

The known risk factors for selection of resistant and multiresistant strains are excessive drug use and inappropriate dosage regimens without previous antimicrobial susceptibility testing. This is especially true for fluoroquinolones as they are known as antimicrobials with a high tendency to select resistant bacteria if dosed inappropriately (GIGUÈRE et al., 2013).

A precise dose of a certain antimicrobial required to inhibit growth of a pathogen is determined on the basis of the minimum inhibitory concentration (MIC). According to recommendations by the Clinical and Laboratory Standards Institute (CLSI), *P. aeruginosa* isolates with enrofloxacin MIC of  $\leq 0.5$   $\mu\text{g/mL}$  are reported as susceptible, while intermediately susceptible strains have MICs between 1 and 2  $\mu\text{g/mL}$  (ANONYMOUS, 2013b). However, COLE et al. (2008) state that enrofloxacin should be dosed at 20 mg/kg once a day (high end of the enrofloxacin dose range) for the treatment of infections caused by bacteria with a MIC of 0.51-0.64  $\mu\text{g/mL}$ . Considering this, all *P. aeruginosa* isolates estimated as intermediately susceptible to enrofloxacin should be reported as resistant. Besides, without information about the MIC, clinicians cannot make an appropriate decision about the dosage regimen. In the bacteriological laboratory of the Faculty of Veterinary Medicine in Croatia disk diffusion is the method routinely used for susceptibility testing, and the question is whether this method is accurate enough to achieve appropriate therapeutic results if maximum doses of enrofloxacin are used for all *P. aeruginosa* strains estimated as susceptible.

The aim of this study was to determine the susceptibility of *P. aeruginosa* to enrofloxacin and marbofloxacin as fluoroquinolones registered for use in veterinary medicine in Croatia, and susceptibility to ciprofloxacin, which is frequently used off-label in veterinary medicine. Further, our goal was to evaluate the validity of reporting results of disk diffusion testing for chosen fluoroquinolones.

### Materials and methods

Ninety *P. aeruginosa* strains isolated from dogs in the bacteriological laboratory at the Department for Microbiology and Infectious Diseases with Clinic of the Faculty of Veterinary Medicine, University of Zagreb, in the period from December 2008 to December 2009, were used in this study. The majority of isolates were recovered from the ear canal (62.2%) and skin (13.3%). The rest were obtained from the throat (5.6%), nostrils (4.5%), conjunctival sac (4.5%), vagina (3.3%), anal sac (2.2%), foreskin (1.1%), bone fracture (1.1%), urine (1.1%) and feces (1.1%). Identification of *P. aeruginosa* was carried out according to the procedure described by MARKEY et al. (2013). Antimicrobial susceptibility to enrofloxacin, marbofloxacin and ciprofloxacin was determined by the disk diffusion and broth microdilution methods, according to the CLSI recommendations (ANONYMOUS, 2013a). Both susceptibility tests were performed from the same bacterial inoculum, adjusted to turbidity of 0.5 McFarland. The antimicrobial concentration range for microdilution testing was 0.25 to 256 µg/mL for enrofloxacin (Krka, Slovenia), 0.125 to 128 µg/mL for marbofloxacin (Vétoquinol, France) and 0.06 to 64 µg/mL for ciprofloxacin (Salutas Pharma GmbH, Germany). Disk diffusion testing was performed with 5 µg disks of enrofloxacin (Bio-Rad, USA), marbofloxacin (Bio-Rad, USA) and ciprofloxacin (bioMérieux, France).

Inhibition zone diameters and minimum inhibitory concentrations were interpreted according to the criteria recommended by the CLSI in documents VET01-S2 and M100-S24, and they are summarized in Tables 1 and 2 (ANONYMOUS, 2013b; ANONYMOUS, 2014). *Pseudomonas aeruginosa* ATCC 27853 was included in the study as a control strain.

Statistical analysis was performed using Statistica v12.5. Software (StatSoft Inc.). Basic data processing was performed by standard procedures of descriptive statistics. Significance of differences between the three fluoroquinolones was determined by the Wilcoxon matched pairs test, with statistical significance set at  $P < 0.05$ . The differences between the two methods were analyzed using separate Wilcoxon matched pairs tests for each antibiotic included in the research. According to CLSI document M37-A3, discrepancy rates were classified into three categories: very major discrepancy (false-susceptible disk diffusion test result), major discrepancy (false-resistant disk diffusion test

result) and minor discrepancy (difference in one interpretation category) (ANONYMOUS, 2008).

### Results

Disk diffusion antimicrobial susceptibility testing showed that among the tested fluoroquinolones, ciprofloxacin was the most active compound, with 95.6% sensitive strains. The least active was enrofloxacin, with 25 (27.8%) susceptible and 50 (55.6%) intermediately susceptible isolates. The results of disk diffusion testing and the criteria used for interpretation of inhibition zone diameters are summarized in Table 1. Distributions of inhibition zone diameters for each tested fluoroquinolone are summarized in Fig 1.

Table 1. Zone diameter interpretation standards and results of disk diffusion testing for 90 *Pseudomonas aeruginosa* isolates

Antimicrobial agent	Disk diffusion testing zone diameter breakpoints (mm)			Results of disk diffusion testing		
	≥S	I	≤R	Susceptible strains (%)	Intermediate strains (%)	Resistant strains (%)
Enrofloxacin	23	17-22	16	25 (27.8)	50 (55.6)	15 (16.7)
Marbofloxacin	20	15-19	14	78 (86.7)	7 (7.8)	5 (5.6)
Ciprofloxacin	21	16-20	15	86 (95.6)	2 (2.2)	2 (2.2)

S = susceptible, I = intermediately susceptible, R = resistant

Table 2. MIC Interpretive Standards and results of microdilution testing for 90 *Pseudomonas aeruginosa* isolates

Antimicrobial agent	MIC breakpoints (µg/mL)			Results of microdilution testing				
	≤S	I	≥R	Susceptible strains (%)	Intermediate strains (%)	Resistant strains (%)	MIC <sub>50</sub>	MIC <sub>90</sub>
Enrofloxacin	0.5	1-2	4	8 (8.9)	68 (75.6)	14 (15.6)	1	4
Marbofloxacin	1	2	4	73 (81.1)	9 (10.0)	8 (8.9)	1	2
Ciprofloxacin	1	2	4	85 (94.4)	3 (3.3)	2 (2.2)	0.25	0.5

MIC = minimum inhibitory concentration, MIC<sub>50</sub> = MIC that inhibited at least 50% of the isolates, MIC<sub>90</sub> = MIC that inhibited at least 90% of the isolates. S = susceptible, I = intermediately susceptible, R = resistant

From the results of disk diffusion testing, isolates were significantly more sensitive to ciprofloxacin than to marbofloxacin or enrofloxacin ( $P < 0.05$ ). Furthermore, the isolates were significantly more susceptible to marbofloxacin than to enrofloxacin ( $P < 0.05$ ).

From the results of microdilution testing, the isolates were also significantly more susceptible to ciprofloxacin than to marbofloxacin or enrofloxacin ( $P < 0.05$ ) and were significantly more susceptible to marbofloxacin than to enrofloxacin ( $P < 0.05$ ). There were 85 strains (94.4%) sensitive to ciprofloxacin with MICs between 0.06 and

1 µg/mL. Seventy-three strains (81.1%) showed susceptibility to marbofloxacin, with slightly higher MICs, mostly between 0.25 and 1 µg/mL. With eight (8.9%) susceptible strains, enrofloxacin was the least active compound. The antimicrobial activities of the three fluoroquinolones against *P. aeruginosa* strains and their MIC<sub>50</sub> and MIC<sub>90</sub> values are summarized in Table 2. Distributions of MICs for each tested fluoroquinolone are summarized in Fig. 1.

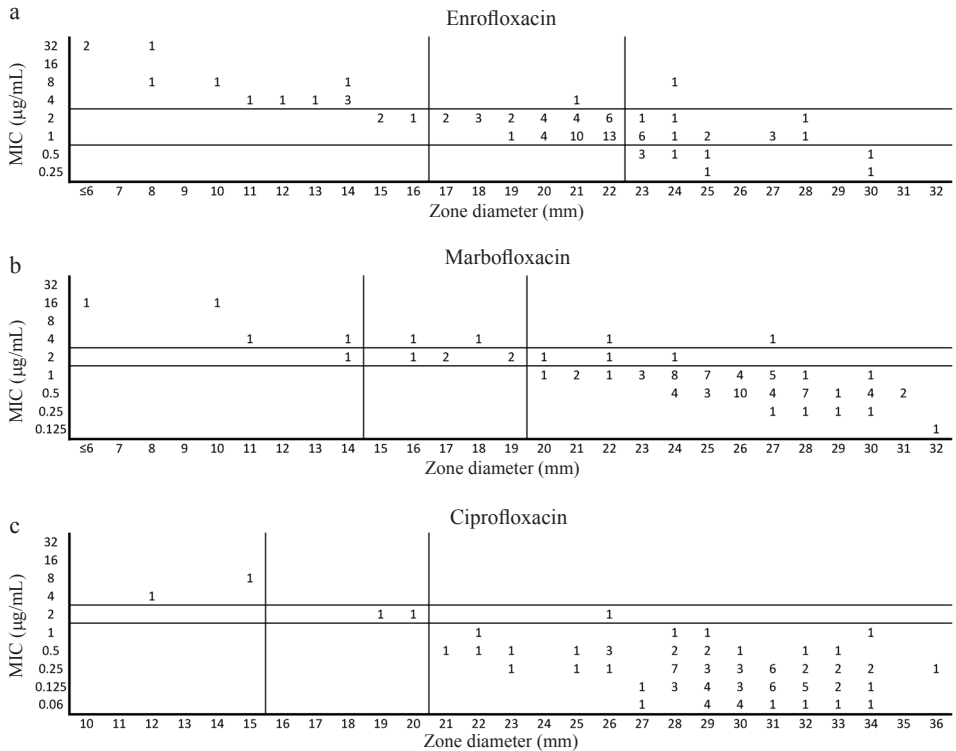


Fig. 1. Scattergrams of enrofloxacin (a), marbofloxacin (b) and ciprofloxacin (c) MICs versus disk diffusion zone diameters for 90 *Pseudomonas aeruginosa* isolates. Numbers represent the numbers of isolates. The horizontal and vertical lines represent MIC and disk diffusion breakpoints, respectively.

For ciprofloxacin, the results of microdilution and disk diffusion testing were in 98.9% agreement, with no statistically significant disagreement between the two methods ( $P>0.05$ ). There was only one minor error with one strain showing susceptibility on disk diffusion testing but intermediate susceptibility on microdilution testing.

There was no statistically significant disagreement between the results of the two methods for marbofloxacin, and the results were in 91.1% agreement. This comparison showed six minor errors and two very major errors.

For enrofloxacin, the results of microdilution and disk diffusion testing were in 76.6% agreement (69 isolates showed the same result by both methods) with a statistically significant disagreement between the two methods ( $P < 0.05$ ). Comparison identified 20 (22.2%) minor errors and one (1.1%) very major error (Fig. 1).

## Discussion

In this study we carried out a comparison of the results obtained by two methods, disk diffusion and microdilution susceptibility testing. There were no statistically significant differences between those two methods for marbofloxacin and ciprofloxacin. On the other hand, comparison showed a statistically significant disagreement between the results obtained with microdilution and disk diffusion testing for enrofloxacin. Only 76.6% of results were in agreement, and most of the disagreements were due to the disk diffusion test overestimating the number of susceptible strains compared to microdilution testing. Out of 20 minor errors, 16 errors were isolates estimated as susceptible by the disk diffusion method but intermediately susceptible by microdilution. Similar results were found by McKAY et al. (2007), where 87% agreement between microdilution and disk diffusion testing was reported for marbofloxacin, with no statistically significant disagreement between the two methods. For enrofloxacin, authors reported that the results of the two methods were in 74% agreement, with a statistically significant difference between them. In 20 instances, disk diffusion testing overestimated the number of enrofloxacin-susceptible strains compared with microdilution. On the basis of these results, the authors concluded that the use of disk diffusion testing to evaluate the enrofloxacin susceptibility of *P. aeruginosa* strains may result in inappropriate and ineffective therapy (McKAY et al., 2007). According to COLE et al. (2008), for bacteria with a minimum inhibitory concentration of 0.51-0.64 µg/mL, enrofloxacin should be dosed at 20 mg/kg (high end of the enrofloxacin dose range). On this basis, all intermediately susceptible isolates should be considered resistant, since appropriate tissue levels cannot be obtained even with a maximum dose of enrofloxacin. On the basis of the results of disk diffusion testing, it is impossible to calculate an accurate and appropriate dose of enrofloxacin for therapy since there is no information about the MIC. In addition, our study showed that most isolates interpreted as susceptible by disk diffusion showed MICs higher than 0.5 µg/mL on microdilution testing. For such isolates even a maximum dose of enrofloxacin would not be sufficient and could lead to therapeutic failure. Furthermore, the results of microdilution testing showed that only eight (8.9%) strains were susceptible to enrofloxacin, with six of them having a MIC of 0.5 µg/mL. These results suggest that

in most cases of *P. aeruginosa* infections enrofloxacin should be used at its maximum dose. In Croatia, enrofloxacin is licensed for veterinary use under several trade names, with a recommended dose of 2.5 to 5 mg/kg body weight in dogs. There is no additional data about the possibility of increasing the dose to 20 mg/kg for treating *P. aeruginosa* infections. We assume that most clinicians comply with the dosage guidelines in drug labels. This led us to the question about the validity of reporting *P. aeruginosa* strains as susceptible based on disk diffusion testing, and emphasizes the need for reviewing drug labels for enrofloxacin in Croatia, regarding dosage and duration of therapy.

Compared to this study, a lower susceptibility rate was reported in Croatia a few years ago (MEKIĆ et al., 2011) with one (1.1%) strain susceptible to enrofloxacin. In that study susceptibility testing was performed by Etest®. Besides the difference in the methods used, there is a difference between the origins of the isolates. Isolates included in the study from 2011 were all collected from the ears of dogs with otitis externa, and it is well known that otic isolates tend to show higher resistance rates to several antimicrobials, including enrofloxacin and other fluoroquinolones (PETERSEN et al., 2002; WILDERMUTH et al., 2007; HARADA et al., 2012). Nevertheless, the sensitivity rate to enrofloxacin found in this study was still lower than sensitivity rates observed in other studies, where susceptibility ranged between 16% and 51% (COLOMBINI et al., 2000; MARTIN BARRASA et al., 2000; McKAY et al., 2007; WERCKENTHIN et al., 2007).

On the other hand, the resistance to marbofloxacin found in this study is lower than the resistance rates of between 25% and 49% recorded in other studies (McKAY et al., 2007; RUBIN et al., 2008; MÜLLER and HOM, 2009). The main reason for this is probably the fact that marbofloxacin is registered in Croatia only for topical treatment of otitis externa. On the other hand, enrofloxacin is registered for systemic use, and is prescribed not only for otitis externa but also for infections of other body sites. Therefore, obviously, enrofloxacin is used more often than marbofloxacin.

Resistance to ciprofloxacin has not significantly changed since the first study was conducted in Croatia in 2002, when 93.4% of isolates were susceptible and 3.8% were resistant to ciprofloxacin (ŠEOL et al., 2002). The resistance rate reported in the Croatian study from 2011 (MEKIĆ et al., 2011) was higher (8.7%) but the main reason for that was the difference in the origin of the isolates. A similar difference in susceptibility to ciprofloxacin between isolates of different origin had already been reported by PETERSEN et al. (2002). They detected 95.2% of skin isolates and 92.9% of ear isolates that were susceptible to ciprofloxacin.

In the present study, all the isolates that were resistant to ciprofloxacin were also resistant to enrofloxacin and marbofloxacin, and there were significantly more ciprofloxacin-susceptible strains than those susceptible to enrofloxacin and/or marbofloxacin. These

findings were expected and are in agreement with the results of other studies (RIDDLE et al., 2000; TEJEDOR et al., 2003; MEKIĆ et al., 2011).

In conclusion, comparison of the two methods showed that disk diffusion testing is not a method of choice for testing the susceptibility of *P. aeruginosa* isolates to enrofloxacin. Moreover, due to the high percentage of resistant and intermediately susceptible isolates, with MICs above 0.5 µg/mL, enrofloxacin is not a good choice for treating *P. aeruginosa* infections. For such isolates even the maximum recommended dose of enrofloxacin would not be high enough to achieve appropriate tissue levels (COLE et al., 2008). On the basis of the results obtained we consider marbofloxacin and ciprofloxacin as the drugs of choice among fluoroquinolones against *P. aeruginosa*. In the light of all of the above, our results emphasize the need for the revision of drug labels for enrofloxacin in Croatia.

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**PINTARIĆ, S., K. MATANOVIĆ, B. ŠEOL MARTINEC: Osjetljivost na fluorokinolone izolata bakterije *Pseudomonas aeruginosa* izdvojenih iz pasa istražena dvjema metodama - disk-difuzijskim postupkom i mikrodilucijom. Vet. arhiv 87, 291-300, 2017.**

**SAŽETAK**

*Pseudomonas aeruginosa* značajan je uzročnik velikog broja različitih infekcija u ljudi i životinja koje je teško liječiti zbog visokog stupnja urođene rezistencije ove bakterije. Također, vrsta *P. aeruginosa* vrlo brzo razvija nove mehanizme rezistencije, a više njih se može istovremeno očitovati u samo jednom izolatu što rezultira multirezistentnim fenotipom. Uzimajući u obzir to da na selekciju rezistentnih sojeva utječe pretjerana i neodgovarajuća uporaba antibiotika, osobito fluorokinolona, cilj ovog rada bio je istražiti osjetljivost izolata bakterije *P. aeruginosa* na pojedine fluorokinolone. Disk-difuzijskim i mikrodilucijskim postupkom određena je osjetljivost 90 sojeva izdvojenih iz pasa. Na temelju rezultata obje metoda, izolati su bili značajno osjetljiviji na ciprofloksacin nego na marbofloksacin ili enrofloksacin i značajno osjetljiviji na marbofloksacin nego na enrofloksacin. Usporedbom ovih dviju metoda, uočeno je da su se rezultati dobiveni za ciprofloksacin podudarali kod 98,9% izolata, a za marbofloksacin kod 91,1% izolata. U podudarnosti interpretacijskih skupina dobivenih za ta dva antibiotika nije bilo statistički značajnih razlika. Rezultati dviju metoda za enrofloksacin bili su jednaki kod 76,6% izolata, a među metodama utvrđena je statistički značajna razlika za ovaj antibiotik ( $P < 0,05$ ). Većina „malih grešaka“ (16 od 20) rezultat je procjene testiranih izolata osjetljivim temeljem rezultata disk-difuzijske metode, a isti izolati testirani mikrodilucijom bili su umjereno osjetljivi. Takvi rezultati upućuju na zaključak da terapija enrofloksacinom temeljena samo na rezultatu disk-difuzijske metode može biti neučinkovita i kao takva može pridonijeti selekciji rezistentnih i multirezistentnih sojeva.

**Cljučne riječi:** *Pseudomonas aeruginosa*, pas, fluorokinoloni, rezistencija, disk-difuzijski postupak, mikrodilucija

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