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Prevalence and mechanisms of resistance to fluoroquinolones in *Pseudomonas aeruginosa* and *Escherichia coli* isolates recovered from dogs suffering from otitis in Greece

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ABSTRACT

The aim of this study was to investigate the prevalence and the implicated mechanisms of resistance against selected veterinary fluoroquinolones (enrofloxacin, marbofloxacin and pradofloxacin) among 101 *Pseudomonas aeruginosa* (n = 75) and *Escherichia coli* (n = 26) isolates collected from dogs suffering from otitis. Resistance ranged from 32.0% to 48.0% with differences not being considered statistically significant among the three agents or between the two bacterial species. However, individual MICs of pradofloxacin, the latest veterinary fluoroquinolone, were significantly lower than those of enrofloxacin, the oldest one, indicating an increased *in vitro* potency of the former antimicrobial. Pradofloxacin MIC_{90} was, additionally, the lowest (8 μg/ml), in *E. coli*, or among the lowest (8 μg/ml), in *P. aeruginosa* isolates. Resistance was in most cases associated with topoisomerase substitutions, with patterns GyrA:V73G in *P. aeruginosa* and GyrA:S83L + D87N/ParC:S58I + A86V in *E. coli* being reported for the first time in small animal isolates. Only 6.7% and 15.4% of *P. aeruginosa* and *E. coli* otitis isolates, respectively, carried plasmid-mediated quinolone resistance (PMQR) genes, which, moreover, contributed minimally to resistance. Efflux pump activity was additionally detected in resistant *E. coli* isolates, even those lacking topoisomerase substitutions or PMQR genes. The emergence of resistance in the canine otitis isolates seemed to be associated with previous, prolonged systemic fluoroquinolone administration. In any case, antimicrobial susceptibility testing should guide the selection of systemic FQs for the treatment of canine otitis.

1. Introduction

Otitis, an inflammation of the ear canal and related structures, is a very common clinical condition in dogs, of multifactorial etiology. Opportunistic bacterial infections are secondary causes of otitis, complicating primary disorders (e.g. allergies, autoimmune diseases), but they represent the major reason for treatment failure (Saridomichelakis et al., 2007). Apart from gram-positive (such as *Staphylococcus* spp.), gram-negative bacteria (such as *Escherichia coli*) are commonly isolated from the inflamed external ear canal and may be persistent and difficult to treat (Hariharan et al., 2006; Zamankhan Malayeri et al., 2010). Furthermore, in chronic otitis externa and/or media, *Pseudomonas aeruginosa*, either alone or in combination with other microorganisms, is the most frequent gram-negative pathogen (Colombini et al., 2000; Martín Barrasa et al., 2000).

Several approaches have been proposed for the treatment of bacterial otitis, with variable recommendations regarding the selection of topical and/or systemic antimicrobials (Morris, 2004; Rubin et al., 2008). Topical antimicrobial treatment, based on cytological and Gram's stain examination of the ear canal exudate, is the recommended initial approach in otitis externa. On the contrary, when otitis media is also present, typically in chronic or recurrent cases, selection of the

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Table 1
Distribution of MICs, MIC_{50} and MIC_{90} values (μg/ml) and resistance (%) in *P. aeruginosa* (n = 75) and *E. coli* (n = 26) isolates from cases of canine otitis.

Antimicrobial	Distribution of MICs (μg/ml)															MIC_{50} (μg/ml)	MIC_{90} (μg/ml)	Resistance (%)
	<0.03	0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32	64	128	>128			
P. aeruginosa																		
ENR	1[a]	0	3	3	3	4	9	19	**7**	9	6	5	3	3	0	**2**	**32**	**44.0**
MAR	3	4	6	6	3	10	9	10	**12**	7	3	2	0	0	0	**1**	**8**	**32.0**
PRA	3	0	1	6	4	12	13	**8**	12	11	4	0	1	0	0	**1**	**8**	**48.0**
CIP	7	6	5	7	8	11	5	8	10	3	4	0	0	1	0	**0.5**	**8**	**NA**
E. coli																		
ENR	3	3	2	1	3	0	2	2	**0**	1	2	4	1	1	1	**1**	**64**	**38.5**
MAR	3	5	1	4	0	2	1	1	**1**	4	3	0	0	1	0	**0.125**	**16**	**34.6**
PRA	5	1	4	2	1	2	0	**3**	5	1	1	1	0	0	0	**0.25**	**8**	**42.3**
CIP	7	3	1	1	1	2	1	1	1	1	4	1	1	0	1	**0.25**	**32**	**NA**

ENR: enrofloxacin; MAR: marbofloxacin; PRA: pradofloxacin; CIP: ciprofloxacin.
Clinical breakpoint values (S, I, R) for ENR (≤0.5, 1–2, ≥4 μg/ml), MAR (≤1, 2, ≥4 μg/ml) and PRA (≤0.25, 0.5–1, ≥2 μg/ml) are denoted by vertical lines across the MIC distribution rows; veterinary species-specific breakpoints for *Enterobacteriaceae* were used against *P. aeruginosa* and *E. coli* isolates (Rubin et al., 2008; CLSI, 2015). Grey areas indicate clinical resistance. Resistance rates against CIP are not reported, since no veterinary species-specific clinical breakpoints exist for this agent (NA: Not applicable).
[a]Number of isolates displaying the MIC stated in the column heading (borderline resistant isolates are in bold).

proper antimicrobial therapy becomes more complicated. Systemic administration of antimicrobials, based on culture of the exudate from the middle ear cavity and susceptibility testing is indicated in most of these cases (Morris, 2004).

Fluoroquinolones (FQs) are often used empirically or as first choice drugs to treat a range of infections, including otitis, in small animals, despite the recommendation to reserve them only when susceptibility testing indicates that there are no first-tier alternatives (Guardabassi et al., 2004). The widespread use of these agents has resulted in a continuously increasing incidence of FQ resistance in both clinical and commensal isolates from small animals (Gibson et al., 2011; Vingopoulou et al., 2014). FQ resistance in gram-negative bacteria is principally mediated by mutations in the quinolone resistance-determining region (QRDR) of genes encoding DNA gyrase (*gyrA* and *gyrB*) and topoisomerase IV (*parC* and *parE*), as well as by decreased intracellular accumulation, due to either downregulation of outer membrane proteins (porins) or overexpression of efflux pumps. Plasmid-mediated quinolone resistance (PMQR) genes have also been found to confer reduced susceptibility to FQs, mainly in *Enterobacteriaceae* (Jacoby, 2005). Only recently, have PMQR genes been described in *P. aeruginosa* isolates, mainly from humans (Poirel et al., 2012).

To the best of our knowledge, no or extremely limited information exist regarding the prevalence of FQ resistance and the implicated mechanisms, respectively, in *E. coli* isolates originated from otitis cases in small animals (Shaheen et al., 2009, 2013). Moreover, information on the molecular mechanisms of FQ resistance in *P. aeruginosa* canine otitis isolates are restricted to QRDR mutations (Tejedor et al., 2003; Rubin et al., 2008; Harada et al., 2012; Arais et al., 2016). Thus, the present study aimed at a more thorough investigation on the prevalence and the mechanisms of resistance in *P. aeruginosa* and *E. coli* isolates recovered from inflamed canine ear canals and middle ear cavities against second and third-generation veterinary FQs, namely enrofloxacin, marbofloxacin and pradofloxacin.

2. Materials and methods

2.1. Bacterial isolates

For the conduct of this study, 75 *P. aeruginosa* and 26 *E. coli* isolates, recovered in 96 ear exudate samples collected from 78 dogs suffering from with otitis externa and/or media, were studied. Dogs with unilateral or bilateral otitis externa and/or media (admitted to two university teaching animal hospitals and ten private veterinary practices between 2010 and 2014) were eligible for the study, regardless of previous topical and/or systemic FQ administration, provided that gram-negative bacilli were found on cytology smears. Samples were obtained from at least one of the four possible sites: right external auditory canal at the junction between vertical and horizontal canal, right middle ear cavity, left external auditory canal at the junction between vertical and horizontal canal, and left middle ear cavity. Sterile swabs were used to obtain ear exudate, separately from each sampling site. Thus, the number of samples per dog ranged from one to four, depending on otoscopic findings (unilateral or bilateral otitis, otitis externa or otitis externa plus otitis media) and the results of cytology. Identical isolates were excluded. Additional samples obtained upon re-examination of the same dog were included only if the recovered isolate (s) carried different resistance determinants, compared to the isolates in the original samples that had been obtained from the same sampling site. Identification of *P. aeruginosa* and *E. coli* isolates was performed by conventional biochemical tests and by use of the VITEK® 2 system (GN ID card; bioMérieux, Marcy-l'Étoile, France). All isolates were stored at −85 °C, pending further analysis.

2.2. Antimicrobial susceptibility testing

Minimum Inhibitory Concentrations (MICs) of enrofloxacin, pradofloxacin (both from Bayer AG, Leverkusen, Germany) and marbofloxacin (Vétoquinol SA, Lure, France) against all *P. aeruginosa* and *E. coli* isolates were determined by use of the broth microdilution method (Clinical and Laboratory Standards Institute (CLSI), 2013, 2017) and interpreted according to CLSI criteria (CLSI, 2015; Table 1). Although ciprofloxacin has not been authorized for use in animals, it was also included into the panel for MIC determinations (standard substance from Bayer AG), being considered as a marker of resistance, based on previous studies (Tejedor et al., 2003; Rubin et al., 2008; Harada et al., 2012; Schink et al., 2013). All experimentations were carried out in triplicate, using *P. aeruginosa* ATCC 27853 and *E. coli* ATCC 25922 as quality control strains.

Additionally, using enrofloxacin as standard, MICs against *E. coli* isolates were also calculated in the presence of 80 μg/ml of the efflux-pump inhibitor (EPI) phenylalanine-arginine-β-naphthylamide (PAβN) (Sigma-Aldrich Corp., St. Louis, USA) (Vingopoulou et al., 2014). Efflux-pump overexpression was assessed when the MIC determined in media containing EPI, was decreased by a factor of ≥4 (Shaheen et al., 2011).

2.3. PCR amplification and sequencing of PMQR genes

The presence of PMQR genes *qnrA*, *qnrB*, *qnrS*, *aac(6′)-Ib-cr*, *oqxA*,