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Antimicrobial susceptibility and mechanism of resistance to fluoroquinolones in *Staphylococcus intermedius* and *Staphylococcus schleiferi*

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The objective of the present study was to determine the antimicrobial susceptibility of 136 canine isolates of *Staphylococcus intermedius* and 10 canine isolates of *S. schleiferi* subspecies *coagulans* to 16 fluoroquinolones (FQs), and to investigate the mechanisms of resistance in the nonsusceptible isolates. Of the 136 of *S. intermedius* tested 98.5% were susceptible to all 16 FQs whereas only 40% of the 10 isolates of *S. schleiferi* subspecies *coagulans* were susceptible. Two isolates of *S. intermedius* and six isolates of *S. schleiferi*, were found to be resistant to 13 out of 16 FQs, while they retained their susceptibility to fourth generation FQs such as gatifloxacin, moxifloxacin and trovafloxacin. Sequencing of the quinolone-resistance determining regions of *gyrA* and *grlA* genes showed that in *S. intermedius*, dichotomous resistance to FQs was associated with the occurrence of one alteration in GyrA-84 and one in GrlA-80, while in *S. schleiferi* the same pattern of resistance was observed in isolates showing these changes only in *gyrA*. This study is the first to screen FQs of the second, third and fourth generation for antimicrobial resistance in clinical isolates of *S. intermedius* and *S. schleiferi* of canine origin, and to describe mutations in *gyrA* and *grlA* associated with FQ resistance in these bacterial species.

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INTRODUCTION

Bacteria in the genus of *Staphylococcus* represent part of the normal bacterial flora of the skin and mucosal surfaces of human and animals. However, many species of staphylococci are also opportunistic pathogens that can cause diseases of the skin and other body tissues and cavities, and cases of animal-to-human and human-to-animal transmission of some species of staphylococci have been documented (van Duijkeren *et al.*, 2004; Weese *et al.*, 2006). The staphylococci most frequently isolated from the skin of dogs are *S. intermedius*, a coagulase-positive staphylococcus first described as a new species in 1976 (Hajek, 1976; Hoekstra & Paulton, 2002; Sasaki *et al.*, 2005), and *S. schleiferi*, an organism with variable coagulase activity first isolated from humans in 1988 (Hoekstra & Paulton, 2002; Sasaki *et al.*, 2005; Yamashita *et al.*, 2005). Two subspecies of *S. schleiferi* have been identified: *S. schleiferi* subsp. *schleiferi* being coagulase negative and *S. schleiferi* subsp. *coagulans*, being coagulase-positive (Frank *et al.*, 2003; Kania *et al.*, 2004; May *et al.*, 2005; Yamashita *et al.*, 2005).

Staphylococcus intermedius and *S. schleiferi* are the most common etiological agents of canine pyoderma, a bacterial skin condition that usually results from a primary underlying skin disorder, such as ectoparasitism, atopy, hormonal imbalances, or immune-mediated dermatitis. Canine pyoderma may be classified by location, depth of infection, whether the bacterial infection is primary or secondary, and according to the types of invading organism. Effective treatment and management of pyoderma is achieved not only by treating the bacterial pathogens involved, but also by treating the predisposing factors when such exist (Barragry, 1994; Euzeby, 1997; Hartmann *et al.*, 2005; Sasaki *et al.*, 2005; Futagawa-Saito *et al.*, 2006). The use of fluoroquinolones (FQs) for the treatment of canine pyoderma is well documented (Heinen, 2002; Horspool *et al.*, 2004). The targets of FQ action in all gram-positive bacteria that have been studied are the bacterial enzymes DNA gyrase and DNA topoisomerase IV.

Both enzymes are composed of two pairs of subunits. In DNA gyrase these are denoted GyrA and GyrB, while in topoisomerase IV these are ParC (often also conventionally referred to as GrlA in *S. aureus*) and ParE. Resistance to FQs in gram-positive bacteria occurs as a result of changes in amino acid composition, particularly in the regions of the enzyme subunit termed the quinolone-resistance-determining region (QRDR) within GyrA and ParC. These changes make the enzyme less susceptible to FQs. Resistance mechanisms resulting in FQ degradation or modification have been found yet (Hooper, 1999; Ruiz, 2003; Jacoby, 2005; Robicsek *et al.*, 2006). In *S. aureus*, the species of gram-positive bacteria most extensively studied for FQ resistance, mutations occur predominantly at position 84 or 88 in *gyrA* and at position 80 or 84 in *grlA* (Yamagishi *et al.*, 1996; Hooper, 1999, 2002). Information regarding the gene mutations associated with FQ resistance for *S. intermedius* and *S. schleiferi* is not available. Other mechanisms of bacterial resistance to FQs include mutations that increase the expression of endogenous multidrug efflux pumps, and/or alter outer membrane diffusion channels (Hooper, 1999).

Thus, the purpose of the present study was to determine the antimicrobial susceptibility and the gene mutations conferring resistance to 16 FQs in canine clinical isolates of *S. intermedius* and *S. schleiferi*.

MATERIALS AND METHODS

Specimen

Staphylococcal isolates were obtained from clinical samples submitted to the Department of Veterinary Clinics at the University of Pisa between January 2005 and October 2006. Bacterial isolates were obtained from 367 dogs of both sexes (301 healthy dogs, 51 dogs with pyoderma and 15 dogs with otitis). None of the dogs had a history of being treated with a FQ. No more than one isolate originated from the same individual dog. A sterile swab placed in the transport medium (Transystem Film-Film/Amies; Oxoid, Milano, Italy) was used to collect specimens from the external ear or the skin of dogs. In dogs with pyoderma, the swab specimen was obtained from closed papules or pustules.

Procedures for the care and management of animals were performed in accordance with the provisions of the EC Council Directive 86/609 EEC, recognized and adopted by the Italian Government (DL 27.01.1992, no. 116).

Isolation of staphylococci

Each swab sample was inoculated within 4 h after collection onto Mannitol Salt Agar (Oxoid) and incubated aerobically for 24 h at 37 °C. Organisms obtained through bacterial culture were initially identified as staphylococci on the basis of colony characteristics on primary plating medium. One representative colony of each colony type per specimen was subcultured from the primary isolation medium to trypticase soya agar supplemented with 5% defibrinated sheep blood (blood agar plate) (Oxoid).

Molecular identification of Staphylococcus species

DNA from *Staphylococcus* cultures was extracted in guanidine lysis buffer (Sambrook *et al.*, 1989), precipitated with isopropanol and dissolved in deionized water. The 5′-end of 16S rDNA was amplified by PCR, using broad-range bacterial primers (Table 1) (Becker *et al.*, 2004). The thermal cycling conditions consisted of 30 cycles of denaturation at 94 °C for 45 sec (90 sec for the first cycle), annealing at 53 °C for 30 sec, and elongation at 72 °C for 90 sec (600 sec for the last cycle). PCR products were visualized by 1.2% agarose gel electrophoresis, and were purified using High Pure PCR Product Purification Kit in accordance with the manufacturer's instructions (Roche Applied Science, Monza, Italy). The sequencing reactions were performed in a total volume of 10 μL containing 0.5 μL of premix from the ABI prism BigDye Terminator v3.0 ready-reaction cycle-sequencing kit (Applied Biosystems, Foster City, CA, USA), 10 pmol of sequencing primer SSU-bact-519r, and 2 μL of the purified PCR product. The sequencing products were analyzed on an ABI 3730XL capillary sequencer. An alignment of 16S rDNA *Staphylococcus* sequences of the RIDOM database (Harmsen *et al.*, 2002), based on 81 type and reference strains encompassing all validly described staphylococci (GenBank accession numbers AY688029–AY688109) was performed using BIOEDIT version 7.0.1 (Hall, 1999), and was used for species identification. The pairwise distance matrix was calculated using CLUSTAL W.

Antimicrobial susceptibility tests

The bacteria identified as *S. intermedius* or *S. schleiferi* were tested for FQ susceptibility by the disc diffusion method on Muller Hinton agar (Oxoid). The following FQs were tested: ciprofloxacin, danofloxacin, difloxacin, enoxacin, enrofloxacin, flumequine, gatifloxacin, levofloxacin, lomefloxacin, marbofloxacin, moxifloxacin, norfloxacin, ofloxacin, orbifloxacin, pefloxacin and trovafloxacin (Table 2). As breakpoints for all FQs are not available from any individual organization, bacterial susceptibility to danofloxacin, difloxacin, enoxacin, gatifloxacin, lomefloxacin, norfloxacin, orbifloxacin, trovafloxacin was established in accordance with standards and criteria proposed by the Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS) (NCCLS, 1990, 2002; CLSI, 2007), while bacterial

Table 1. Primers used in this study

Primer	Sequence 5′-3′	Gene (coordinates)*
GyrAs	ATGAGYGTTATCGTKCWCGTGC	*gyrA* (79–101)
GyrAas	CCATWGARCCAAAGTTACCTTG	*gyrA* (319–340)
GrlAs	AATACRYAYGATAARAATTTCCG	*grlA* (160–182)
GrlAas	GTYGTRTCATCATAGTTTGG	*grlA* (430–449)
SSU-bact-27f †	AGAGTTTGATCMTGGCTCAG	16S rDNA (9–28)
SSU-bact-519r †	GWATTACCGCGGCKGCTG	16S rDNA (527–544)

*Coordinates are numbered from the start of *S. aureus* genes.
†Primers from Becker *et al.* (2004).

Table 2. Fluoroquinolones tested and zone breakpoints

Antimicrobials	Disk content (μg)	Interpretation of zone diameters (mm)			IC and laboratory standard source
		S	I	R	
Ciprofloxacin	5	< 19	19–21	≥ 22	CA-SFM (2006)
Danofloxacin	5	≤ 15	16–20	≥ 21	NCCLS (1990)
Difloxacin	10	≤ 17	18–20	≥ 21	NCCLS (2002)
Enoxacin	10	≤ 14	15–17	≥ 18	CLSI (2007)
Enrofloxacin	5	< 17	17–21	≥ 22	CA-SFM (2006)
Flumequine	30	< 21	21–24	≥ 25	CA-SFM (2006)
Gatifloxacin	5	≤ 19	15–17	≥ 23	CLSI (2007)
Levofloxacin	5	< 15	15–19	≥ 20	CA-SFM (2006)
Lomefloxacin	10	≤ 18	19–21	≥ 22	CLSI (2007)
Marbofloxacin	5	< 13	13–17	≥ 18	CA-SFM (2006)
Moxifloxacin	5	< 21	21–23	≥ 24	CA-SFM (2006)
Norfloxacin	10	≤ 12	13–16	≥ 17	CLSI (2007)
Ofloxacin	5	< 16	16–21	≥ 22	CA-SFM (2006)
Orbifloxacin	10	≤ 17	18–22	≥ 23	NCCLS (1990)
Pefloxacin	5	< 16	16–21	≥ 22	CA-SFM (2006)
Trovafloxacin	10	≤ 15	14–16	≥ 19	CLSI (2007)

IC, interpretative criteria; CA-SFM, Comité de l'Antibiogramme de la Société Française de Microbiologie; NCCLS (now CLSI), Clinical and Laboratory Standards Institute.

susceptibility to ciprofloxacin, enrofloxacin, flumequine, levofloxacin, marbofloxacin, moxifloxacin, ofloxacin, and pefloxacin was established in accordance with standards and criteria proposed by the Comité de l'Antibiogramme de la Société Française de Microbiologie (CASFM) (CA-SFM, 2006) (Table 2). Antimicrobial drug discs were applied (four discs on a 90-mm plate) using multidisc cartridge dispensers. All tests were read after aerobic incubation at 37 °C for 24 h.

The standardization, reliability and reproducibility of the method were assessed weekly by the use of reference strains of *Staphylococcus aureus* ATCC 25923 (Oxoid).

Amplification and sequencing of gyrA and grlA QRDRs

Consensus PCR primers were designed on sequence alignments, including available *gyrA* and *grlA* sequence data for *Staphylococcus* species (Table 1) (Sreedharan *et al.*, 1991; Fitzgibbon *et al.*, 1998; Dubin *et al.*, 1999; Linde *et al.*, 2001). PCR conditions consisted of 35 cycles of denaturation at 94 °C for 45 sec (90 sec for the first cycle), annealing at 50 °C for 30 sec, and elongation at 72 °C for 90 sec (300 sec for the last cycle). PCR products were purified and sequenced using primers grlAas and gyrAas (Table 1) as described above. Sequences determined in this study have been deposited in the GenBank database under accession numbers EF189168–EF189175.

RESULTS

Identification of Staphylococcus species

A total of 230 *Staphylococci* were isolated from samples submitted from 367 dogs. None of the dogs had a history of being treated with a FQ. Of the 230 isolates 136 (58.7%) were identified as *S. intermedius* and 10 (4.3%) were identified as *S. schleiferi* subsp. *coagulans*.

The sequences of *S. intermedius* showed 99.6–100% identity with reference sequence AY688070, while *S. schleiferi* subsp. *coagulans* showed 100% identity with reference sequence AY688091. They could be distinguished from the reference sequence of *S. schleiferi* subsp. *schleiferi* (AY688092) by single nucleotide substitution.

Of the *S. intermedius* isolates, 87 were obtained from the back skin ($n = 27$), the external auditory meatus ($n = 35$), the inner part of the thigh ($n = 13$) or the interdigital space ($n = 12$) of apparently healthy dogs. Forty-nine isolates were obtained from dogs affected by either pyoderma ($n = 39$) or otitis externa ($n = 10$).

Of the *S. schleiferi* isolates, four strains were from the back skin ($n = 1$) or the external auditory meatus ($n = 3$) of healthy dogs, while six other strains were isolated from dogs affected by pyoderma ($n = 3$) or otitis externa ($n = 3$).

Antimicrobial susceptibility tests

A total of 134 out of the 136 *S. intermedius* isolates (98.5%) and four out of the 10 *S. schleiferi* isolates (40%) were susceptible to all FQs (Table 3). The resistance pattern observed in two skin isolates of *S. intermedius* (one from a diseased dog and one from a healthy one) and in six isolates of *S. schleiferi* (one from the external auditory meatus of a healthy dog, two from the back skin and three from the external auditory meatus of diseased dogs), all obtained from unrelated cases, included the majority of FQs (13 out of 16) while gatifloxacin, moxifloxacin and trovafloxacin retained their *in vitro* activity (Table 3).

Table 3. Antimicrobial susceptibility of *Staphylococcus intermedius* ($n = 136$) and *S. schleiferi* ($n = 10$)

Antibiotic	*S. intermedius*		*S. schleiferi*	
	Susceptible (*n*)	Susceptibility rate (%)	Susceptible (*n*)	Susceptibility rate (%)
Ciprofloxacin	134	98.5	4	40
Danofloxacin	134	98.5	4	40
Difloxacin	134	98.5	4	40
Enoxacin	134	98.5	4	40
Enrofloxacin	134	98.5	4	40
Flumequine	134	98.5	4	40
Gatifloxacin	136	100	10	100
Levofloxacin	134	98.5	4	40
Lomefloxacin	134	98.5	4	40
Marbofloxacin	134	98.5	4	40
Moxifloxacin	136	100	10	100
Norfloxacin	134	98.5	4	40
Ofloxacin	134	98.5	4	40
Orbifloxacin	134	98.5	4	40
Pefloxacin	134	98.5	4	40
Trovafloxacin	136	100	10	100

Table 4. Alterations in GrlA subunit of topoisomerase IV and GyrA subunit of DNA gyrase of *Staphylococcus intermedius* ($n = 2$) and *S. schleiferi* subsp. *coagulans* ($n = 6$) associated with quinolone resistance

	GyrA			GrlA			
Species	Position	Susceptible	Resistant	Position	Susceptible	Resistant	Frequency
S. intermedius	84	Ser	Leu	80	Ser	Arg	100% (2/2)
	88	Glu	Glu	84	Asp	Asp	
S. schleiferi	84	Ser	Ser	80	Ser	Arg	33% (2/6)
subsp. *coagulans*	88	Glu	Gly	84	Asp	Asp	
	84	Ser	Ser	80	Ser	Ser	66% (4/6)
	88	Glu	Gly	84	Asp	Asp	

Sequencing of gyrA and grlA QRDRs

Quinolone-resistance-determining region sequencing results of *gyrA* codons 84 and 88 and *grlA* codons 80 and 84 are summarized in Table 4. All FQ-susceptible strains of both *S. intermedius* and *S. schleiferi* subsp. *coagulans* had a Ser and a Glu residue at position 84 and 88 of *gyrA*, respectively, and had a Ser and an Asp residue at position 80 and 84 of *grlA*, respectively. The FQ-resistant strains of *S. intermedius* presented an alteration in both *gyrA* (Ser-84 to Leu) and *grlA* (Ser-80 to Arg). In resistant *S. schleiferi* subsp. *coagulans*, two strains had an alteration in both *gyrA* (Glu-88 to Gly) and *grlA* (Ser-80 to Arg) (genotype 1), while the majority of them (four out of six) presented an amino acid change only in *gyrA* (Glu-88 to Gly) (genotype 2).

DISCUSSION

This study is the first to screen several FQs of the second, third, and fourth generation for antimicrobial resistance in clinical isolates of *S. intermedius* and *S. schleiferi* of canine origin, and to describe mutations in *gyrA* and *grlA* associated with FQ resistance in these bacterial species in Italy.

Most of the isolates of the present study were identified as *S. intermedius*. The frequency of isolation of S. *intermedius* observed in the present study (58.7%) is similar to that observed by other authors who reported *S. intermedius* as the predominant inhabitants in the skin and the external auditory meatus of healthy and diseased dogs (Biberstein *et al.*, 1984; Hoekstra & Paulton, 2002; Futagawa-Saito *et al.*, 2006). *Staphylococcus schleiferi* subsp. *coagulans* was isolated 10 times (4.3%). While the number of infections caused by *S. schleiferi* reported in the literature is low, the frequency of *S. schleiferi* infection may be under-reported because of its possible misidentification in routine laboratory practice as *S. intermedius* or even as *S. aureus* (Yamashita *et al.*, 2005). In this study, partial 16S rDNA sequencing allowed a rapid, precise identification of the organism and made it possible to distinguish *S. schleiferi* subsp. *coagulans* from *S. schleiferi* subsp. *schleiferi*.

Staphylococcus intermedius and *S. schleiferi* isolates of canine origin have been reported to be resistant to more than one antibiotic. Resistance to penicillin and tetracycline has been frequently seen in isolates of *S. intermedius* of different geographic origins, whereas resistance to other antimicrobial agents such as FQs is comparatively low (Lloyd *et al.*, 1999; Werckenthin *et al.*, 2001; Hoekstra & Paulton, 2002; Kania *et al.*, 2004). Only a limited number of studies have investigated the antimicrobial susceptibility of *S. schleiferi*, which have frequently been found to be resistant to methicillin, while a few isolates were also resistant to FQs (Frank *et al.*, 2003; Kania *et al.*, 2004; May *et al.*, 2005; Yamashita *et al.*, 2005). A recent retrospective study from the USA documented resistance rates to methicillin of 17% in *S. intermedius* and of 40% in *S. schleiferi* (Morris *et al.*, 2006). Methicillin-resistant strains were also resistant to many other antimicrobials, including FQs, with resistance rates to enrofloxacin and marbofloxacin of 35% and 33% respectively, in *S. intermedius*, and of 55% and 29%, respectively, in *S. schleiferi* (Morris *et al.*, 2006). The present data show that the prevalence of resistance to FQs among *S. intermedius* is quite low in dogs (1.5%). On the contrary, a high prevalence of FQ-resistant *S. schleiferi* (60%) was observed. Resistant strains showed a pattern of dichotomous resistance: they became resistant to second and third generation FQs (13 out of 16 of the molecules investigated) but maintained full susceptibility to fourth generation FQs such as gatifloxacin, moxifloxacin and trovafloxacin. The pattern of dichotomous resistance to FQs as observed in our study, has already been observed previously in *S. aureus*, as well as in coagulase-negative staphylococci that were found to be ciprofloxacin-resistant, but trovafloxacin-susceptible (Fitzgibbon *et al.*, 1998; Dubin *et al.*, 1999; Sanders, 2001). It has been proposed that the susceptibility of bacteria to FQs is determined primarily by which one of the two target enzymes is more sensitive. In older generation FQs, DNA gyrase tends to be the primary target in gram-negative organisms, whereas topoisomerase IV is the primary target in gram-positive bacteria (Hooper, 2001; Sanders, 2001; Hooper, 2002; Blondeau, 2004). On the contrary, the newer generation FQs, such as gatifloxacin, moxifloxacin and trovafloxacin, have a dual-binding mechanism of action, inhibiting both DNA gyrase and topoisomerase IV in gram-positive species (Lu *et al.*, 1999; Peterson, 2001; Robicsek *et al.*, 2006). Furthermore, it has been proposed that the primary target among FQs may vary depending upon the specific bacteria: i.e. DNA gyrase is the primary target of gatifloxacin and moxifloxacin in pneumococci, while they target both enzymes in *S. aureus*. As a consequence, a pattern of dichotomous resistance to quinolones, especially between the older and newer generation FQs, has emerged (Ouabdesselam *et al.*, 1995; Fitzgibbon *et al.*, 1998; Pan & Fisher, 1998; Dubin *et al.*, 1999; Pan & Fisher, 1999; Nagai *et al.*, 2000; Blondeau, 2004). In the present study, *grlA* sequencing of FQ-susceptible *S. intermedius*

and *S. schleiferi* subsp. *coagulans* showed an Asp residue in position 84, instead of the Glu residue present in *S. aureus grlA*. The same residue had previously been reported in this position for other staphylococci such as *S. haemolyticus, S. simulans, S. capitis, S. hominis, S. epidermidis, S. saprophyticus, S. lugdunensis* and *S. warneri* (Hooper, 1999). Our QRDR sequencing results showed that resistance in *S. intermedius* is correlated with a double alteration in *gyrA*-84 and *grlA*-80. Similar findings have been observed in previous studies which reported that in *S. aureus* resistance to ciprofloxacin, levofloxacin, sparfloxacin and ofloxacin is generally associated with one change in *gyrA*-84 or 88 and one in *grlA*-80 or 84, while resistance to trovafloxacin is associated with a second change in *grlA*-80 or 84, depending on the position of the first mutation (Fitzgibbon *et al.*, 1998; Dubin *et al.*, 1999; Hooper, 2001; Linde *et al.*, 2001). On the basis of these considerations, it may be speculated that the mutant strains of *S. intermedius* presumably maintained their susceptibility to gatifloxacin, moxifloxacin and trovafloxacin, because of the requirement of an additional alteration in *grlA*-84 which was not observed in our study. The Ser-84 to Leu change in *S. intermedius* GyrA observed in this study is identical to that seen in *S. aureus*, while the Ser-to-Arg change at *grlA*-80, found in resistant *S. schleiferi* subsp. *coagulans*, has not been reported before for staphylococci. In other studies, FQ-resistant staphylococci with a change in position 80 in *grlA* present a Ser to Phe, Tyr, or Leu alteration here (Yamagishi *et al.*, 1996; Fitzgibbon *et al.*, 1998; Dubin *et al.*, 1999; Hooper, 1999; Linde *et al.*, 2001; Ba *et al.*, 2006). Although mutations in both *gyrA* and *grlA* seem to be necessary for FQ resistance in most staphylococci, previous studies have shown that exceptions may occur: quinolone-resistant strains of *S. pneumoniae* with a single mutation in *gyrA* have been found (Nagai *et al.*, 2000). In our study, the sequencing of *gyrA* and *grlA* QRDRs of resistant *S. schleiferi* confirms that a single Glu-88 to Gly alteration in *gyrA* is sufficient to confer FQ resistance.

In conclusion, the present data indicate that the prevalence of resistance to FQs in *S. intermedius* isolated from dogs does not appear to be significant (1.5%), whereas a high level (60%) of FQ resistance was found among *S. schleiferi* isolates. In *S. intermedius*, dichotomous resistance to FQs was associated with the occurrence of two alterations in *gyrA*-84 and *grlA*-80. The same pattern of resistance was also observed in *S. schleiferi* showing a change in *gyrA* only.

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