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CASE REPORT

Pets as potential carriers of multidrug-resistant *Enterococcus faecium* of significance to public health

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SUMMARY

Enterococci are important opportunistic pathogens for humans and animals and have recently become one of the leading causes of nosocomial infections, raising concerns about their virulence and antimicrobial traits. This study describes a multidrug-resistant *Enterococcus faecium* isolated from a case of feline urinary tract infection. This strain was characterized for virulence and antimicrobial resistance markers, phylogenetic group and sensitivity to antimicrobial agents used routinely in veterinary and human practice. Other than virulence traits, the isolate harboured a variety of antimicrobial-resistance genes and chromosomal mutations, the combination of which conferred resistance to almost all of the antimicrobial compounds tested. Interestingly, this strain harboured mutations in the quinolone resistance-determining regions never been described in *E. faecium* and conferring resistance to all the quinolones tested. The combination of these resistance features, together with its virulence traits, makes this strain an example of a potentially dangerous pathogen that could easily spread in veterinary hospitals and perhaps to the environment and to humans, seriously compromising patient outcomes.

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INTRODUCTION

Enterococci are part of the normal gastrointestinal microbiota of humans and animals, usually harmless, widely distributed in several natural environments and are used as markers for antimicrobial resistance among food-producing animals. However, enterococci are also important opportunistic pathogens for humans and animals and have recently become one of the leading causes of nosocomial infections, raising concerns about their virulence traits and antimicrobial resistance properties (Shepard *et al.,* 2002). The most prevalent species of clinical importance are *Enterococcus faecium* and *Enterococcus faecalis,* which are often associated with serious human diseases, such as endocarditis, urinary tract and surgical wound infections and bacteraemia. The clinical importance of these species, especially *E. faecium*, is strictly related to their ability to colonize in patients and to develop resistance to several antimicrobial compounds, partially driven by the selective pressure produced by the use, overuse and abuse of drugs in both human and veterinary practice. As a result, the emergence of multidrug-resistant (MDR) strains among enterococcal species has become a major public health concern worldwide due to the limited therapeutic options to treat MDR enterococcal infections (Kristich *et al.,* 2014). Supporting these notions, in recent decades, hospital-acquired enterococcal infections, together with the analyses of their virulence and antimicrobial resistance traits, have been extensively reported in humans (Sharifi *et al.,* 2013). However, less attention has been given to the role of enterococci in causing clinically acquired infections in companion animals, despite their contribution to the diffusion of resistance in veterinary settings that may be amplified and spread across the environment and to humans. Only a few studies have reported the presence of MDR and pathogenic clones and investigated the genetic mechanisms of resistance in healthy and diseased companion animals hospitalized in small animal clinics and the correlation of these strains with those causing nosocomial infections in humans (de Regt *et al.,* 2012; Ghosh *et al.,* 2012). Therefore, the aim of this study was to characterize an MDR *E. faecium* isolated from a veterinary hospital-associated urinary tract infection of a cat for virulence markers, phylogenetic group and sensitivity to antimicrobial agents used routinely in veterinary and human practice.

Key words:
Enterococcus faecium, Cat, Urinary tract infection, Antimicrobial resistance, Zoonoses, Public health

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MATERIALS AND METHODS

Case Description

A 16-month-old male European intact domestic shorthair cat (4.3 kg) was referred to the Veterinary Teaching Hospital of the University of Padua (VTH-UP), Legnaro, Italy for severe traumatic injuries following a car accident. The cat suffered a double fracture of the pelvis at the level of the pubic symphysis, right hip dislocation, and fractures of the mandibular symphysis and right mandib-

ular midbody requiring surgical intervention. The patient was treated with antimicrobial therapy two days before and five days after surgery using cefazolin 25 mg/kg IV TID and cephalexin 25 mg/kg PO BID, respectively. After surgery, the patient did not urinate spontaneously. Physical examination revealed a large painful urinary bladder. An indwelling urinary catheter was placed to drain the urine. It was removed the next day, and spontaneous urination was verified. However, three days later, the patient presented for acute clinical signs of lower urinary tract infection (stranguria, pollakiuria and bacteriuria). Ultrasound-guided cystocentesis was performed to obtain a sterile urine sample for urinalysis and bacteriological investigations. The patient was returned to the referring veterinarian who reported recurrent relapses of urinary tract infections, completely resolved only after one month of repeated, high-dose antimicrobial treatments. The practitioner did not report the drugs used.

Bacterial isolation and identification

One hundred microliters of urine sample were plated onto *Columbia Agar* supplemented with *5%* sheep blood (bioMérieux), *MacConkey Agar* and *Mannitol Salt Agar* (Oxoid) and incubated in aerobiosis conditions at 37°C for 24 h. Colonies, grown in pure culture only onto *Columbia blood Agar* plate, were suspected to be *Enterococcus* spp. and were confirmed using the macro-method standard procedure (colony morphology, Gram staining, bile-aesculin hydrolysis and catalase test) for genus confirmation. The bacterial identification at the species level was definitively confirmed by multiplex-PCR assays as previously described (Kariyama *et al.,* 2000).

Table 1 - *Target genes, primer sequence and references.*

Gene	*Primer sequence 5'-3'(forward/reverse)*	*Amplicon size (bp)*	*References*
asa1	CACGTAATTCTTGCCCACCA/CAAGCATTATTGGCAGCGTT	520	Seputiene et al., 2012
gelE	AGGGATGCCCATCTTGTCAGT/CGTTCCTTTATACCGTGCTCGA	391	Seputiene et al., 2012
esp	AGCGGGAACAGGTCACAAAGC/ CCACGTCGAAAGTTCGATTTCC	419	Seputiene et al., 2012
hyl	CAGAAGAGCTGCAGGAAATG/GACTGACGTCCAAGTTTCCA	276	Seputiene et al., 2012
cylL	GTGTTGAGGAAATGGAAGCGAT/GGCTAGTTTCACTAGCCCTCT	424	Seputiene et al., 2012
acm	GGCCAGAAACGTAACCGATA/ AACCAGAAGCTGGCTTTGTC	135	Rathnayake et al., 2012
blaZ	ACTTCAACACCTGCTGCTTTC/TAGGTTCAGATTGGCCCTTAG	173	Martineau et al., 2000
pbp5	AACAAAATGACAAACGGG/TATCCTTGGTTATCAGGG	779	Jureen et al., 2003
mecA	GGGATCATAGCGTCATTATTC/AGTTCTGCAGTACCGGATTTGC	1429	Garofalo et al., 2007
tetK	TTAGGTGAAGGGTTAGGTCC/GCAAACTCATTCCAGAAGCA	718	Aarestrup et al., 2000
tetL	CATTTGGTCTTATTGGATCG/ATTACACTTCCGATTTCGG	488	Aarestrup et al., 2000
tetM	GTTAAATAGTGTTCTTGGAG/CTAAGATATGGCTCTAACAA	657	Aarestrup et al., 2000
tetO	GATGGCATACAGGCACAGAC/CAATATCACCAGAGCAGGCT	614	Aarestrup et al., 2000
tetS	TGGAACGCCAGAGAGGTATT/ACATAGACAAGCCGTTGACC	660	Aarestrup et al., 2000
vanA	GGGAAAACGACAATTGC/GTACAATGCGGCCGTTA	732	Dutka-Malen et al., 1995
vanB	ATGGGAAGCCGATAGTC/GATTTCGTTCCTCGACC	635	Dutka-Malen et al., 1995
vanC1	GGTATCAAGGAAACCTC/CTTCCGCCATCATAGCT	822	Dutka-Malen et al., 1995
vanC2	CTCCTACGATTCTCTTG/CGAGCAAGACCTTTAAG	439	Dutka-Malen et al., 1995
ermA	GAAGCGGTAAACCCCTCTG/ACCCAAAGCTCGTTGCAGAT	216	Seputiene et al., 2012
ermB	ATTGGAACAGGTAAAGGGCAT/ATCTGGAACATCTGTGGTATG	447	Seputiene et al., 2012
ermC	GAAATCGGCTCAGGAAAAGG/AGCAAACCCGTATTCCACGA	293	Seputiene et al., 2012
mrsAB	GCA AAT GGT GTA GGT AAG ACA ACT/ ATC ATG TGA TGT AAA CAA AAT	402	Fasihi et al., 2017
vatE	ACTATACCTGACGCAAATGC/ GGTTCAAATCTTGGTCCG	510	Soltani et al., 2000
aac(6')-aph(2")Ia	GAGCAATAAGGGCATACCAAAAATC/ CCGTGCATTTGTCTTAAAAAACTGG	505	Kao et al., 2011
aph(2')Ib	TATGGATCCATGGTTAACTTGGACGCTGAG/ ATTAAGCTTCCTGCTAAAATATAAACATCTCTGCT	906	Kao et al., 2011
aph(2')Ic	TGACTCAGTTCCCAGAT/AGCACTGTTCGCACCAAA	881	Kao et al., 2011
aph(2')Id	GGTGGTTTTTACAGGAATGCCATC/ CCCTCTTCATACCAATCCATATAACC	642	Kao et al., 2011
ant(6)Ia	ACTGGCTTAATCAATTTGGG/GCCTTTCCGCCACCTCACC	597	Jia et al., 2014
efrA	TTGGCTTTATGACGCCAGT/ATGCGCGTATTACCCGCAA	225	Lavilla Lerma et al., 2014
efrB	TAGTGATGATGTTCTTAATCAA/ATTGACTTGTTTAAAGCCTTCA	233	Lavilla Lerma et al., 2014
emeA	GTGACAGCCTTTGTGGCAGAT/TAGTCCGTTGATGGTTCCTTG	687	Jia et al., 2014
gyrA	GCAATGAGTGTTATCGTAGCC/CTACTGGTTCTTGTTCTGAATC	400	Oyamada et al., 2006
gyrB	CATGTTGGCTTCAAAAGCTCG/AAGCCCGTGAAGCCCGTGAA	341	Oyamada et al., 2006
parC	CGATCGTTTTGGAAGATATTCC/GCTGGTAAAACAGTGGGTTC	429	Oyamada et al., 2006
parE	AAGCCCGTGAAGCCCGTGAA/ TATCATAATTACAATCTTCAATTGA	348	Oyamada et al., 2006

Antimicrobial Susceptibility Tests

The antimicrobial susceptibility of the isolates to antimicrobials of interest in veterinary and human medicine was assessed via the agar disk diffusion method according to the recommended Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2002; CLSI, 2013). The antimicrobials were trimethoprim/sulphamethoxazole (1.25+23.75 µg), ampicillin (10 µg), penicillin (10 µg), vancomycin (30 µg), imipenem (10 µg), tetracycline (30 µg), tigecycline (15 µg), chloramphenicol (30 µg), enrofloxacin (5 µg), ciprofloxacin (5 µg), teicoplanin (30 µg), erythromycin (15 µg), quinupristin-dalfopristin (15 µg), nitrofurantoin (300 µg), linezolid (30 µg), rifampicin (5 µg), (Oxoid) and high concentrations of gentamycin (120 µg) and streptomycin (300 µg) (BD Sensi-disc, Becton-Dickinson). Breakpoints were interpreted following the CLSI susceptibility criteria except for imipenem, trimethoprim/sulphamethoxazole and tigecycline (European Committee on Antimicrobial Susceptibility Testing, 2015). *E. faecium* (ATCC 19434) was used as a quality control strain.

Multilocus Sequence Typing

Molecular typing of the isolate was determined using the *Enterococcus faecium* Multilocus Sequence Typing (MLST) scheme as described by Homan *et al.* (2002). The allelic profiles and the sequence types (STs) were obtained by submitting the allele sequences to the MLST web site (available at http://efaecium.mlst.net/).

PCR assays

PCR amplifications of virulence genes (*asa1*, *gelE*, *esp*, *hyl*, *cylL* and *acm*), the detection of genes conferring resistance to penicillins (*bla*Z, *mec*A and sequencing of C-terminal region of *pbp5* gene) tetracycline (*tet*K, *tet*L, *tet*M, *tet*O and *tet*S), glycopeptides (*van*A, *van*B, *van*C1-2), macrolides (*erm*A, *erm*B, *erm*C and *mrs*AB), streptogramin (*vat*E) and aminoglycosides (*aac*(6')Ia, *aac*(6')-*aph*(2")-Ia, *aph*(2')Ib, *aph*(2')Ic, *aph*(2')Id, *aph*(2")-Ia, *ant*(6)-Ia) and the coding of multidrug-efflux pumps (*efr*A-*efr*B and *eme*A) and the quinolone resistance-determining regions (QRDRs) were performed as previously described (Dutka-Malen *et al.,* 1995; Aarestrup *et al.,* 2000; Martineau *et al.,* 2000; Soltani *et al.,* 2000; Jureen *et al.,* 2003; Oyamada *et al.,* 2006; Kao *et al.,* 2011; Rathnayake *et al.,* 2012; Seputiene *et al.,* 2012; Jia *et al.,* 2014; Lavilla Lerma *et al.,* 2014; Fasihi *et al.,* 2017). Primer sequences and references are provided in *Table 1*. Both strands of the purified QRDR amplicons were sequenced (EMBL-EBI accession no. LN624829) and compared with the *E. faecium* DO genome (GenBank accession no. CP003583).

RESULTS

The isolate responsible of the feline UTI was an ST266, MDR *E. faecium* (EF175). This strain was resistant to ampicillin, penicillin, imipenem, trimethoprim/sulphamethoxazole, tetracycline, enrofloxacin, ciprofloxacin, erythromycin, quinupristin-dalfopristin, rifampicin and high concentrations of streptomycin, showed an intermediate sensitivity to vancomycin, nitrofurantoin, teicoplanin and chloramphenicol and was fully susceptible only to linezolid, high-level gentamycin and tigecycline (*Table 2*). PCR analyses indicated the presence of both virulence and resistance genes as well as chromosomal mutations in the QRDRs, conferring resistance to most of the antimicrobials tested (*Table 2*).

Regarding virulence traits, EF775 was positive for *acm* and *gel*E, whereas it did not carry *asa1*, *esp*, *hyl* and *cylL* genes. Although Enterococci are described as poorly sensitive to β-lactams, in this case the full resistance to the penicillins tested was ascribable to the presence of the *bla*Z gene, owing to the production of β-lactamases, and mutations in the C-terminal region of *pbp5* gene between amino-acid positions 461 and 634 (*Table 2*). Sequencing of C-terminal region of *pbp5* gene revealed mutations responsible for amino-acid changes at positions 471 H→Q, 485 M→A, 496 N→K, 499 A→T, 525 E→D, 629 E→V and a S at position 466 with respect to the reference sequence (GenBank accession no. X84860) (*Table 2*). This strain also carried different genes conferring resistance to macrolides (*erm*B and *mrs*AB), quinupristin–dalfopristin (*vat*E) and

Table 2 - *Antimicrobial resistance profile of the Enterococcus strain.*

Antimicrobial classes	*Antimicrobial resistance profile*	
	Phenotype	*Genotype*
Penicillins	Penicillin, Ampicillin	*blaZ,* mutations in the C-terminal region of *pbp*5 (S-466, H-471 to Q, M-485 to A, N-496 to K, A-499 to T, E-525 to D and E-629 to V)
Carbapenems	Imipenem	-
Glycopeptides	Vancomycin*	-
Lipoglycopeptide	Teicoplanin*	-
High level Aminoglycosides	High Level Streptomycin	*ant*(6)-Ia
Macrolides	Erythromycin	*erm*B, *mrs*AB
Ansamycins	Rifampicin	-
Tetracyclines	Tetracycline	-
Fluoroquinolones	Ciprofloxacin, Enrofloxacin	mutations in GyrA (E-88 to K) and ParC (S-82 to R)
Nitrofurantoins	Nitrofurantoin*	-
Phenicols	Chloramphenicol*	-
Streptogramins	Quinupristin-Dalfopristin	*Vat*E
Folate pathway inhibitors	Trimethoprim-Sulfamethoxazole	-

*= intermediate susceptibility

to some aminoglycosides, such as streptomycin (*ant*(6)-Ia). Finally, EF175 showed resistance to fluoroquinolones, thanks to the presence of novel nucleotide mutations and amino-acid substitutions in *Gyr*A (E-88 to K) and *Par*C (S-82 to R) (*Table 2*).

DISCUSSION

In this study, EF175 was an MDR *E. faecium* possessing both resistance genes and chromosomal mutations in the QRDR hot spot region and in the C-terminal region of *pbp*5 gene. These resistance features make this strain an example of a developing potential pandrug-resistant pathogen that could spread in veterinary hospitals and in the environmental surroundings and perhaps colonize both personnel at veterinary clinics and animal owners, compromising outcomes by the limited treatment choices that remain. EF175 showed sensitivity only to critically important antimicrobials not registered for use in animals that should not be used off-label or to topical or injectable antimicrobials which are rarely used in veterinary practice due to their route of administration and potential adverse reactions and toxicity. Interestingly, EF175 belonged to ST266, one of the most detected STs in dogs and cats, which is usually resistant to ampicillin (Damborg *et al*., 2009; de Regt *et al*., 2012). However, although evidence based on its MLST profile has proven that ST266 is not closely related to ST17 and ST78, which are currently causing the majority of human clinical infections, its recent isolation both from apparently healthy and diseased patients raises the question of whether pet animals may serve as a reservoir for resistant *E. faecium* human infections (de Regt *et al.,* 2012). Accordingly, this strain showed resistance to ampicillin and to penicillin. In this case, the resistance to β-lactams is attributable either to specific amino-acid changes already described in the C-terminal region of pbp5 at positions 466 (S insertion), 471 (H→Q), 485 (M→A), 496 (N→K), 499 (A→T), 525 (E→D) and 629 (E→V) (Jureen *et al*., 2003; Poeta *et al*., 2007, Rathnayake *et al*., 2012) and to the *bla*Z gene coding for β-lactamases. Interestingly, this latter gene has rarely been detected in clinical cases of human enterococcal infections and, except for a β-lactamase producer *E. faecium* strain from ready-to-eat raw fish, to the best of our knowledge it has never been described in *E. faecium* isolates of animal origin (Hammad *et al*., 2014). Therefore, because the majority of nosocomial invasive *E. faecium* isolates are resistant to ampicillin, and are thus considered markers for *E. faecium* isolated from hospitalized human patients in Europe, we could not exclude that this conclusion could be extended to the potentially zoonotic community and hospital-associated enterococci in veterinary medicine (Boerlin *et al.*, 2001; Simjee *et al*., 2002). Moreover, genetic analysis indicated the presence of genes other than *bla*Z, such as those conferring resistance to macrolides, quinupristin-dalfopristin and some aminoglycosides (high concentrations of streptomycin). The most studied gene-mediated antimicrobial efflux pumps, such as *efr*AB and *eme*A, which are usually found in MDR enterococci, were not detected in this strain.

Although enterococci naturally show low susceptibilities to fluoroquinolones, EF175 was fully resistant to all quinolone compounds tested. This evidence is in accordance with recent studies showing how certain hospital-adapted enterococcal strains infecting human patients may acquire high-level resistance to these compounds. Indeed, high-level ciprofloxacin resistance appears to be increasingly distributed among hospital isolates of enterococci, probably due to the increasing use of fluoroquinolones among hospitalized patients (Werner *et al*., 2010; Dalhoff *et al*., 2012). Because enterococci are common inhabitants of the human and animal gut, they reflect the antimicrobial pressure exerted in the clinical setting, serving as indicators of the diffusion of antimicrobial resistance. Similarly, the wide use of enrofloxacin, a veterinary analogue of ciprofloxacin, could explain the diffusion of quinolone-resistant strains of animal origin. However, despite the increasing number of quinolone-resistant enterococci, only a few studies exist in which ciprofloxacin resistance mutations in *E. faecium* have been investigated (Werner *et al*., 2010; Rathnayake *et al*., 2012). With the aim of filling this gap, EF175 was further examined for the presence of chromosomal mutations in QRDRs of *gyr*A, *gyr*B, *par*C and *par*E. This strain showed novel nucleotide substitutions in *gyr*A (transition from G to A at position 262) and *par*C (C to G at position 246; EMBL-EBI accession number LN624829) bringing the following amino-acid mutations: the alteration of amino-acid glutamic acid 88 to lysine in *gyr*A and the alteration of serine 82 to arginine in *par*C. Interestingly, although the amino-acid change E-88 to K in *gyr*A has never been described as a marker of fluoroquinolone resistance in *E. faecium,* Griggs *et al.* (2003) showed that this amino-acid change is involved in fluoroquinolone resistance in other Gram-positive bacteria, such as *Staphylococcus aureus* (Griggs *et al*., 2003). Conversely, to the best of our knowledge, the amino-acid change S-82 to R in *Par*C detected in this study is novel. Moving from these considerations, we assumed that resistant genes, together with the novel combinations of *gyr*A and *par*C mutations were responsible for the development of resistance to most of the compounds tested.

In addition to multidrug resistance, it is well established that virulence factors contribute to the pathogenesis of *E. faecium* and *E. faecalis*, although the traits contributing to the transition from a commensal to a hospital-adapted pathogen have not yet been identified. In accordance with other studies showing a significant prevalence of enterococci with low or high gelatinase activity isolated from dogs treated with antimicrobials in intensive care units, EF175 was positive for the *acm* and *gel*E genes. Collagen binding adhesion (Acm) plays an important role in pathogenicity of *E. faecium* while *gel*E has been reported to promote biofilm formation, and this likely confers a survival advantage, especially increasing bacterial resistance to adverse environmental conditions such as treatments with disinfectants and antimicrobial exposure (Macovei *et al*., 2009; Ghosh *et al*., 2011, Rathnayake *et al*., 2012).

In conclusion, the results of this work highlight the potential of pets as reservoirs of MDR pathogens. The combination of already described and new resistance features, together with the virulence traits, makes the strain studied an example of a potentially dangerous pathogen that could spread in veterinary hospitals and in the environment and perhaps to humans thanks to the close bond between pets, owners and veterinary personnel. This aspect of the *One Health* medicine requires adequate precautions to maximize the prevention of infections and

more surveillance efforts for the rapid identification of colonized animals and a rational drug use to prevent the spread of MDR isolates in veterinary hospitals and in the environment. These are the best options to prevent and control the spread of these MDR zoonotic pathogens.

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