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## Short Communication

## Revised Version

**Phenotypic and genotypic characterization of antimicrobial resistance in faecal enterococci from wild boars (*Sus scrofa*).**

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## Abstract

The objective was to study the prevalence of antimicrobial resistance and the mechanisms implicated in faecal enterococci of wild boars in Portugal. One-hundred and thirty-four enterococci (67 *E. faecium*, 54 *E. hirae*, 2 *E. faecalis*, 2 *E. durans* and 9 *Enterococcus* spp.) were recovered from 67 wild boars (two isolates/sample), and were further analysed. High percentages of resistance were detected for erythromycin, tetracycline, and ciprofloxacin (48.5%, 44.8% and 17.9%, respectively), and lower values were observed for high-level-kanamycin, -streptomycin, chloramphenicol, and ampicillin resistance (9%, 6.7%, 4.5%, and 3.7%, respectively). No isolates showed vancomycin or high-level-gentamicin resistance. The *erm*(B), *tet*(M), *aph*(3')-IIIa, and *ant*(6)-I genes were demonstrated in all erythromycin, tetracycline, kanamycin, and streptomycin resistant isolates, respectively. Specific genes of Tn916/Tn1545 and Tn5397 transposons were detected in 78% and 47% of our *tet*(M)-positive enterococci, respectively. The *tet*(S) and *tet*(K) genes were detected in one isolate of *E. faecium* and *E. hirae*, respectively. Three *E. faecium* isolates showed quinupristin-dalfopristin resistance and the *vat*(E) gene was found in all of them showing the *erm*(B)-*vat*(E) linkage. Four *E. faecium* isolates showed ampicillin-resistance and all of them presented seven amino acid substitutions in PBP5 protein (461Q→K, 470H→Q, 485→A, 496N→K, 499A→T, 525E→D and 629E→V), in relation with the reference one; a serine insertion at 466'position was found in three of the isolates. Faecal enterococci from wild boars harbour a variety of antimicrobial resistance mechanisms and could be a reservoir of antimicrobial resistance genes and resistant bacteria that could eventually be transmitted to other animals or even to humans.

## 1. Introduction

Enterococci are saprophytic bacteria of the gastrointestinal tract of humans and most animals, but they have also emerged as important causes of nosocomial and community acquired infections. They are intrinsically resistant to many antimicrobial agents, but its ability to acquire resistance to other agents is well known (Murray, 1998).

The problem of antimicrobial resistance in enterococci is not only restricted to the clinical setting but also to other environments such as the intestinal tract of healthy humans and also of food-producing animals and pets (Devriese et al., 1996; Aarestrup et al., 2000; Torres, et al., 2003; Guardabassi et al., 2004; Da Costa et al., 2007); these resistant enterococci might act as reservoir of antimicrobial resistance genes that could be transmitted to other pathogenic bacteria, and for this reason might represent a problem in public health. Studies of antimicrobial resistance of intestinal enterococci in wild animals are scarce and generally restricted to the analysis of vancomycin-resistant enterococci (Lauková, 1999; Mallon et al., 2002; Poeta et al., 2005b). In a study performed in the past by our group, it was shown that acquired antimicrobial resistance is also detected in the intestinal enterococcal microbiota of wild animals (Poeta et al., 2005a); only one wild boar was included in the referred study, although this type of animals are important because they could enter, in some occasions, into the food chain. The objective of the present study was to analyse the prevalence of antimicrobial resistance and the mechanisms implicated in faecal enterococci of wild boars in Portugal.

## 2. Material and Methods

### 2.1 Samples and bacteria

Enterococcal isolates were recovered from 67 faecal samples obtained of wild boars (two isolates per animal). Samples were collected from December 2005 to

February 2006 in North of Portugal during hunts of wild boars organized by different corporations of hunters. The animals lived in freedom and, to our knowledge, they had not received any feed or antimicrobials from humans during their lifetime.

Faecal samples were diluted and transferred into enrichment medium Azide Dextrose Broth, incubated 24h at 37°C, and then spread onto Slanetz-Bartley agar plates, being incubated 48h at 37°C. Colonies with typical enterococcal morphology were identified to the genus and species level by gram staining, catalase test, bile-aesculin reaction and by biochemical tests using the API ID20 Strep system (BioMérieux, La Balme Les Grottes, France). Species identification was confirmed by PCR using primers and conditions for the different enterococcal species (Dutka-Malen et al., 1995; Torres et al., 2003; Arias et al., 2006).

## 2.2 Antimicrobial susceptibility testing

Susceptibility for 11 antimicrobial agents (vancomycin, teicoplanin, ampicillin, streptomycin, gentamicin, kanamycin, chloramphenicol, tetracycline, erythromycin, quinupristin-dalfopristin, and ciprofloxacin) was tested by the disk diffusion CLSI method (Clinical Laboratory Standards Institute, 2005). The agar dilution CLSI method was also used for ampicillin susceptibility testing. High-level resistance was considered for aminoglycosides. *E. faecalis* strain ATCC 29212 and *Staphylococcus aureus* strain ATCC 25923 were used for quality control.

## 2.3 Antimicrobial resistance genes

Macrolide [*erm*(A), *erm*(B), *erm*(C)], streptogramin [*vat*(D), and *vat*(E)], tetracycline [*tet*(M), *tet*(K), *tet*(L), *tet*(S), *tet*(O)], aminoglycoside [*aph*(3')-IIIa, *aac*(6')-*aph*(2''), *ant*(6)-Ia], vancomycin [*vanA*, *vanB*, *vanC*-1, *vanC*-2/3], and chloramphenicol [*catA*] resistance genes were tested by PCR in all enterococcal isolates

which showed resistance for these antimicrobials, using primers and conditions previously reported (Aarestrup et al., 2000; De Leener et al., 2004; Soltani et al., 2000; Sutcliffe et al., 1996; Torres et al., 2003). To determine whether the *erm*(B) gene was linked with the *vat*(E) gene, a primer-pair [linkage *erm*(B)-*vat*(E)] was used (De Leener et al., 2005). Specific PCR assays for detection of *tdnX* and *int* genes were also used in *tet*(M)-positive isolates, to demonstrate the presence of the Tn5397-like and Tn916/Tn1545-like transposons, respectively (Agerso et al., 2006; De Leener et al., 2004). Positive and negative controls were used in all PCRs, belonging to the strain collection of the University of La Rioja (Spain). DNA sequencing was used to verify the identity of the gene products of at least one randomly selected isolate for each gene.

#### 2.4 Analysis of beta-lactamase activity

The production of beta-lactamase was tested in ampicillin resistant *E. faecium* isolates using nitrocefin discs (Cefinase, Becton-Dickinson, Cokeyesville, MD, USA).

#### 2.5 PCR amplification of *pbp5* gene and DNA sequence analysis.

The total DNA of *E. faecium* isolates was obtained by the InstaGene Matrix (Bio-Rad; CA, USA). The C-terminal region of *pbp5* gene was amplified by PCR in all ampicillin-resistant *E. faecium* isolates, using PBP5F-1 (5'-AACAAAATGACAAACGGG-3') and PBP5R (5'-TATCCTTGGTTATCAGGG-3') primers (Jureen et al., 2003). The purified PCR products (QiaQuick Gel Extraction kit, Qiagen Inc.) were sequenced on both strands (Applied Biosystem 3730 DNA sequencer) using PBP5F-2 (5'-GACAAACGGGATCTCACAAG-3') and PBP5R primers. Duplicated sequences were obtained from independent PCR reactions and were compared with that of *pbp5* gene included in GenBank with the accession no. X84860.

### 3. Results

A total of 134 enterococcal isolates were recovered from the 67 faecal samples of wild boars analysed in this study. Table 1 shows the distribution of enterococcal species found and the percentage of antimicrobial resistance detected. *E. faecium* was the most prevalent detected species (50%), followed by *E. hirae* (40.3%). It is interesting to underline the relatively low prevalence of *E. faecalis* and *E. durans* species (1.5%, each one).

High percentages of resistance were detected for erythromycin, tetracycline, and ciprofloxacin (48.5%, 44.8% and 17.9%, respectively) among our enterococci, being lower the values detected for kanamycin (9%), streptomycin (6.7%), chloramphenicol (4.5%), and ampicillin (3%). No gentamicin- or vancomycin-resistant enterococci were detected in this study (Table 1). In general, higher percentages of antimicrobial resistance were detected in *E. faecium* than in the other enterococcal species tested. Three of our *E. faecium* isolates showed quinupristin-dalfopristin resistance (4.5%), and, as expected, the two *E. faecalis* isolates presented also quinupristin-dalfopristin resistance (intrinsic resistance).

Table 2 shows the presence of antimicrobial resistance genes in enterococci of the different species in relation with its specific phenotype of resistance. All 65 erythromycin-resistant enterococci harboured the *erm*(B) gene. Three of these erythromycin-resistant enterococci (all of them of the *E. faecium* species) also showed quinupristin-dalfopristin resistance and harboured the *vat*(E) gene, linked to *erm*(B) gene.

Sixty enterococci showed tetracycline-resistance and the following genes were detected: *tet*(M) (8 isolates); *tet*(M)+ *tet*(L) (50 isolates); *tet*(M)+ *tet*(L)+ *tet*(S) (1



isolate) and *tet*(M)+ *tet*(L)+ *tet*(K) (1 isolate). The *int* gene of transposon Tn916/Tn1545 was identified in 47 of the tetracycline-resistant isolates, and the *tdnX* gene of Tn5397 in 27 *E. faecium* and one *E. hirae* isolates. Fifteen *E. faecium* isolates harboured both the *tdnX* gene of Tn5397 and the *int* gene of Tn916/1545 (Table 2).

All high-level kanamycin and streptomycin resistant enterococci and most of the chloramphenicol-resistant isolates harboured the *aph*(3')-IIIa, *ant*(6)-I and *catA* gene, respectively (Table 2). Four *E. faecium* isolates showed ampicillin resistance (MIC range 128-256 µg/ml) and none of them produced the β-lactamase enzyme. Table 3 shows the alleles of the C-terminal region of *pbp5* gene of these resistant isolates. Seven amino acid substitutions (461Q→K, 470H→Q, 485→A, 496N→K, 499A→T, 525E→D and 629E→V) were identified in PBP5 of all ampicillin-resistant isolates, and an additional one (586V→L) was identified in *E. faecium* PG JN5. The three isolates with the highest ampicillin MIC (256 µg/ml) showed a serine insertion at position 466'.

#### 4. Discussion

The detection of *E. faecium* and *E. hirae* as the predominant enterococcal species in the faeces of wild boars in this study shows strong similarities with data previously reported for faecal enterococci of pigs (Kuhn et al., 2003). Nevertheless, *E. faecium* and *E. faecalis* have been referred as the predominant species in the faecal content in other animals (Aarestrup et al., 2002; Butaye et al., 2001; Kuhn et al., 2003; De Leener, 2005). Wild boars and pigs are close related animal species and this situation could explain the similarities observed in the distribution of enterococcal species in their intestinal microbiota.

In general, and with few exceptions, *E. faecium* was the enterococcal species that showed higher percentages of antimicrobial resistance, mainly associated with

erythromycin, tetracycline, and ciprofloxacin, and to a lower level with kanamycin, streptomycin, ampicillin, and quinupristin-dalfopristin. It is interesting to underline that most of our *E. faecalis*, *E. durans* and *Enterococcus* spp isolates presented a susceptible phenotype for the tested antimicrobial agents. Isolates of the *E. hirae* species showed percentages of antimicrobial resistance lower than *E. faecium* but higher than the other species.

None of our enterococci showed glycopeptide resistance. Nevertheless, *vanA* enterococci were recovered from faecal samples of wild boars in a previous study carried out by our group using vancomycin-supplemented agar plates (4 µg/ml) for enterococci isolation (Poeta et al., 2007a). This fact could indicate that vancomycin-resistant enterococci might be present within the faecal enterococcal population of wild animals but in a low proportion respect to the vancomycin-susceptible ones, and could not be detected when non-supplemented-plates are used for bacterial isolation.

Three ampicillin-resistant *E. faecium* isolates presented a serine insertion at position 466' in PBP5 sequence in addition to other important amino acid changes at positions 485, 496, 499, 525 and 629, and the nucleotidic sequence was similar to *pbp5* allele F already reported in a previous work in high-level ampicillin resistant *E. faecium* isolates of poultry (Poeta et al., 2007b). Insertions of aspartic acid or serine at this 466' position had also been previously detected in strains of human origin with an increased level of ampicillin resistance (Rybkin et al., 1998; Jureen et al., 2003). The serine insertion seems to be an essential determinant in beta-lactam resistance, affecting the antibiotic recognition (Rybkin et al., 1998; Jureen et al., 2003). The amino acid change 586V→L, detected in one of our ampicillin-resistant *E. faecium* isolates, could be associated with the increased ampicillin MIC in this strain (128 µg/ml). Some authors

(Ligozzi et al., 1996) suggested that the region from positions 558 to 586 might play an important role in the  $\beta$ -lactam binding site of PBP5.

The *tet(M)* gene is frequently implicated in tetracycline resistance in enterococci (Aarestrup et al., 2000; Del Campo et al., 2003; Kuhn et al., 2003), and in our study was found in all our tetracycline-resistant isolates. *tet(M)* gene has been previously found associated with conjugative transposons related to the Tn916/Tn1545 family (De Leener et al., 2004; Agerso et al., 2006). Recently, another conjugative transposon Tn5397, originally found in *Clostridium difficile*, was found in *E. faecium* isolates from broilers (Agerso et al., 2006). In this study 78.% and 47% of our *tet(M)*-positive enterococci carried specific genes of Tn916/Tn1545 and Tn5397 transposons, respectively, although the association of *tet(M)* gene with these transposons was not analysed.

In conclusion, enterococci of the intestinal tract of wild boars can constitute a reservoir of antimicrobial resistant genes and potentially these genes or the resistant bacteria could be transmitted to other animals or even to humans. The genes detected in enterococci of wild animals are similar to those found in enterococci of human origin indicating the possible circulation of bacteria and resistance genes between the animal and human ecosystems. Surveillance studies should be continued in the future to follow up the prevalence of antimicrobial resistance in saprophytic bacteria of different origins in order to detect the emergence and dissemination of new or already-known antimicrobial resistance genes in different ecosystems.

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**Table 1.** Antimicrobial resistance in 134 enterococci of different species isolated from faecal samples of wild boars (*Sus scrofa*).

Antimicrobial agent	Number (percentage) of enterococci of different species showing resistance to different antimicrobial agents:				
	<i>E. faecium</i> (N=67)	<i>E. hirae</i> (N=54)	<i>E. faecalis</i> (N=2)	<i>E. durans</i> and <i>Enterococcus</i> spp. (N=11)	Total (N=134)
Ampicillin	4 (6)	0	0	0	4 (3)
Gentamicin	0	0	0	0	0
Streptomycin	7 (10.4)	2 (3.7)	0	0	9 (6.7)
Kanamycin	9 (13.4)	3 (5.6)	0	0	12 (9)
Tetracycline	41 (61.2)	17 (31.5)	2 (100)	0	60 (44.8)
Erythromycin	46 (68.7)	17 (31.5)	2 (100)	0	65 (48.5)
Chloramphenicol	4 (6)	2 (3.7)	0	0	6 (4.5)
Ciprofloxacin	20 (29.9)	4 (7.4)	0	0	24 (17.9)
Quinupristin-Dalfopristin	3 (4.5)	0	2 (100)	0	5 (3.7)
Vancomycin	0	0	0	0	0
Teicoplanin	0	0	0	0	0



**Table 2.** Antimicrobial resistance genes and presence of genetic mobile elements in *Enterococcus* spp. isolates recovered from wild boars (*Sus scrofa*)

Detection of resistance genes and mobile elements in different enterococcal species with specific resistant phenotypes <sup>b</sup>									
Resistance to the antimicrobial agents <sup>a</sup>	<i>E. faecium</i>				<i>E. hirae</i>			<i>E. faecalis</i>	
	No. of resistant isolates	Genes and genetic elements detected		No. of resistant isolates	Genes and genetic elements detected		No. of resistant isolates	Genes and genetic elements detected	
		Genes and genetic elements	No. of isolates		Genes and genetic elements	No. of isolates		Genes and genetic elements	No. of isolates
ERY	46	<i>erm</i> (B)	46	17	<i>erm</i> (B)	17	2	<i>erm</i> (B)	2
ERY-QD	3	Linkage <i>erm</i> (B)- <i>vat</i> (E)	3	0			0		
TET	41	<i>tet</i> (M)+ Tn916/Tn1545	1	17	<i>tet</i> (M) + Tn916/Tn1545	7	2	<i>tet</i> (M) + <i>tet</i> (L) + Tn916/Tn1545	2
		<i>tet</i> (M) + <i>tet</i> (L) + Tn916/Tn1545	13		<i>tet</i> (M) + <i>tet</i> (L) + Tn916/Tn1545	8			
		<i>tet</i> (M) + <i>tet</i> (L) + Tn5397	12		<i>tet</i> (M) + <i>tet</i> (L) + Tn5397	1			
		<i>tet</i> (M) + <i>tet</i> (L) + Tn916/Tn1545 + Tn5397	14		<i>tet</i> (M)+ <i>tet</i> (L)+ <i>tet</i> (K) + Tn916/Tn1545	1			
		<i>tet</i> (M) + <i>tet</i> (L) + <i>tet</i> (S) + Tn916/Tn1545 + Tn5397	1						
CHL	4	<i>cat</i> (A)	3	2	<i>cat</i> (A)	2	0		
		No genes detected	1						
KAN	9	<i>aph</i> (3')-IIIa	9	3	<i>aph</i> (3')-IIIa	3	0		
STR	7	<i>ant</i> (6)-I	7	2	<i>ant</i> (6)-I	2	0		

<sup>a</sup>ERY, erythromycin; QD, quinupristin-dalfopristin; TET, tetracycline; CHL, chloramphenicol; KAN, kanamycin; STR, streptomycin.

<sup>b</sup>*E. durans* (n=2) and *Enterococcus* spp. (n=9) did not show antibiotic resistance.

**Table 3.** Polymorphism in the C-terminal region of *pbp5* gene of the 4 ampicillin-resistant *E. faecium* isolates recovered from wild boars (*Sus scrofa*)

<i>E. faecium</i> isolate	Amino acid at positions: <sup>a</sup>																	Ampicillin MIC (µg/ml)
	461	466	470	485	496	497	499	524	525	558	582	586	593	607	629	631	634	
X84860	Q	—	H	M	N	F	A	E	E	A	G	V	L	L	E	G	N	—
PG J3	<b>K</b>	<b>S</b>	<b>Q</b>	<b>A</b>	<b>K</b>	F	<b>T</b>	E	<b>D</b>	A	G	V	L	L	<b>V</b>	G	N	256
PG J4	<b>K</b>	<b>S</b>	<b>Q</b>	<b>A</b>	<b>K</b>	F	<b>T</b>	E	<b>D</b>	A	G	V	L	L	<b>V</b>	G	N	256
PG J38	<b>K</b>	<b>S</b>	<b>Q</b>	<b>A</b>	<b>K</b>	F	<b>T</b>	E	<b>D</b>	A	G	V	L	L	<b>V</b>	G	N	256
PG JN5	<b>K</b>	—	<b>Q</b>	<b>A</b>	<b>K</b>	F	<b>T</b>	E	<b>D</b>	A	G	<b>L</b>	L	L	<b>V</b>	G	N	128

<sup>a</sup>In bold letters are indicated the amino acids that are changed with respect to the reference one (GenBank accession no. X84860).