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Characterization of Antibiotic Resistance Genes and Virulence Factors in Faecal Enterococci of Wild Animals in Portugal

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With 4 tables

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Summary

Antibiotic susceptibility was tested in 140 non-selected enterococci (73 *Enterococcus faecalis*, 45 *E. faecium* and 22 of other species) recovered from faecal samples of 77 wild animals in Portugal. Susceptibility testing for 11 antibiotics (vancomycin, teicoplanin, ampicillin, streptomycin, gentamicin, kanamycin, chloramphenicol, tetracycline, erythromycin, quinupristin–dalfopristin and ciprofloxacin) was determined by disk diffusion and agar dilution methods. Forty-four isolates (31.4%) showed susceptibility to all the antibiotics tested (5.5% of *E. faecalis*; 62.2% of *E. faecium*; and 78.6% of *E. hirae*). Neither ampicillin-resistance nor acquired-vancomycin-resistance was detected and 1.4% of the isolates showed high-level-resistance for gentamicin or streptomycin. Tetracycline and erythromycin resistances were shown in 28.6% and 20.1% of the isolates, respectively. Antibiotic resistance genes were studied by polymerase chain reaction (PCR) and sequencing and *tet*(M) + *tet*(L), *erm*(B) or *aac*(6′)-*aph*(2″) genes were detected in most of tetracycline-, erythromycin- or gentamicin-resistant enterococci respectively. Genes encoding virulence factors were studied by PCR and a wide variety of virulence genes were detected in most of *E. faecalis* isolates but were rarely found in *E. faecium* and not detected in the other species. The prevalence of genes encoding virulence factors in *E. faecalis* was as follows: *cpd* (98.6%), *gel*E (75.3%), *agg* (30.1%), *fsr* (17.8%), *ace* (9.6%) and *esp* (4.1%). Low percentages of antibiotic resistance was found in the faecal enterococci of wild animals but a wide variety of virulence genes were detected among *E. faecalis* isolates although were rare in the other species.

Introduction

Enterococci are Gram-positive bacteria, which inhabit the gastrointestinal tract of humans and most animals, and can also be frequently found in soil, plants, water and food products (Tannock, 1995; Aarestrup et al., 2000; Aarestrup, 2004). They have intrinsic resistance to the semi-synthetic penicillins (e.g. oxacillin), aminoglycosides (low level), vancomycin (low level for some species as *Enterococcus gallinarum*, E. *casseliflavus* and *E. flavescens*), lincosamides (most of them), polymyxines, streptogramin A (*E. faecalis*) and monobactams. Enterococci are also considered an important human pathogen because of their ability to acquire virulence traits or antibiotic resistance genes and they are important causes of nosocomial infections worldwide (Murray, 1998; Ellerbroek et al., 2004). Antibiotic resistance has been previously studied in faecal enterococci from farm and pet animals (Aarestrup et al., 2000; Butaye et al., 2001), but this type of studies are very scarce in wild animals (Livermore et al., 2001; Butaye et al., 2002), and the mechanisms implicated in resistance were not analysed in any of them; there are only some few reports exclusively focused on detecting faecal colonization by vancomycin-resistant enterococci in wild animals (Mallon et al., 2002; Rice et al., 2003; Poeta et al., 2005). Different virulence factors have been reported in enterococci (Mundy et al., 2000; Eaton and Gasson, 2001), although the molecular mechanism of virulence is still not completely understood in this genus. Virulence factors have been mainly detected in bacteria of the *E. faecalis* species, being *E. faecium* generally free of these determinants (Eaton and Gasson, 2001). Very few studies do exist in which virulence factors have been studied in enterococci of food and animal origin (Eaton and Gasson, 2001; Mannu et al., 2003; Ben Omar et al., 2004) and none of them has been focused on isolates of wild animals. The objective of this work was to analyse the prevalence of antibiotic resistance and the mechanisms implicated, as well as the presence of genes encoding virulence determinants in non-selected enterococci recovered from faecal samples of wild animals (birds, mammals and others) in Portugal.

Materials and Methods

Samples and bacteria

Enterococci were recovered from faecal samples of 77 wild animals (14 birds of prey, 10 owls, seven foxes, six wild rabbits, five European genets, four forest wildcats, four salamanders, three storks, three magpies, three deer, three vipers, three otters, two wolves, two mouflon, two badgers, one partridge, one hedgehog, one pigeon, one ferret, one quails and one wild boar). During 2003–2004, faecal samples were obtained from animals of different Natural Parks of the north and center of Portugal in collaboration in most of the cases with CRATAS (Center of Collecting, Welcome and Handling of Wild Animals). This center is located in the Trás-os-Montes e Alto Douro University and receives injured animals found in its natural environment. It is necessary to

refer that the number of animals and species is determined by availability of animals at CRATAS during the studied 2 years. None of the animals had been previously fed by humans or had received antibiotics. Faecal samples were diluted, sampled in Slanetz–Bartley agar plates (Merck; 5289, Merck KGa A, Darmstadt, Germany), without antibiotic supplementation, and incubated 48 h at 35°C. Colonies with typical enterococcal morphology were identified by cultural characteristics and by biochemical tests using the API ID20 Strep system (BioMérieux, La Balme Les Grottes, France). Species identification was confirmed by the use of the polymerase chain reaction (PCR) using primers and conditions for the different enterococcal species (Dutka-Malen et al., 1995; Robredo et al., 1999). Two colonies per animal were studied and kept for further studies.

Antibiotic susceptibility testing

Antibiotic susceptibility was tested for 11 antibiotics of interest in animal and human medicine (vancomycin, 30 μg; teicoplanin, 30 μg; ampicillin, 10 μg; streptomycin, 300 μg; gentamicin, 120 μg; kanamycin, 120 μg; chloramphenicol, 30 μg; tetracycline, 30 μg; erythromycin, 15 μg; quinupristin–dalfopristin, 15 μg; and ciprofloxacin, 5 μg), by the disk diffusion method recommended by the National Committee for Clinical Laboratory Standards (NCCLS, 2003b); antibiotic disks were obtained from Oxoid (Oxoid Ltd, Basingstoke, UK), with the exception of aminoglycoside disks that were prepared in the laboratory. Minimal inhibitory concentrations (MICs) of vancomycin (Eli Lilly, Indianapolis, IN, USA), teicoplanin (Hoeschst Marion Roussell, Paris, France) and quinupristin–dalfopristin (Rhône-Poulene Rorer, Vitry sur Seine, France) were determined by the agar dilution method according to the National Committee for Clinical Laboratory Standards (NCCLS, 2003a). Serial 2-fold dilutions were tested for antibiotic MIC determinations (from 0.25 to 64 μg/ml). The breakpoints for resistance were the following ones: vancomycin or teicoplanin, ≥32 μg/ml (a MIC of 8–16 μg/ml of vancomycin or 16 μg/ml of teicoplanin was considered as intermediate susceptibility); quinupristin–dalfopristin, ≥4 μg/ml. Only high-level resistance to aminoglycosides was considered in the susceptibility of our enterococci. *E. faecalis* strain ATCC 29212 and *Staphylococcus aureus* strain ATCC 25923 were used for quality control.

Antibiotic resistance genes

They were studied by PCR in all resistant enterococci and the following genes were tested: *tet*(M), *tet*(L), *erm*(A), *erm*(B), *erm*(C), *aph*(3′)-IIIa, *ant*(6)-Ia, *aac*(6′)-Ie-*aph*(2″)-*Ia*, *vat*(D), *vat*(E), *van*A, *van*B, *van*C-1 and *van*C-2/3 (Van de Klundert and Vliegenthart, 1993; Dutka-Malen et al., 1995; Miele et al., 1995; Sutcliffe et al., 1996; Aarestrup et al., 2000; Del Campo et al., 2000; Robredo et al., 1999). Primers used in PCR reactions are included in Table 1. Positive and negative controls were included and all of them were obtained from our collection of the University of La Rioja (Spain). Some of the amplicons were sequenced in order to confirm their identity, using the ABI Prism 366 system (Applied Biosystems, Foster City, CA, USA), and the obtained sequences were compared with those included in the EMBL database.

Detection of virulence factors

The presence of genes encoding different virulence factors were tested by PCR in all enterococcal isolates recovered in this study, using primers and conditions previously described (Eaton and Gasson, 2001; Pillai et al., 2002; Mannu et al., 2003). Primers used in PCR reactions are included in Table 1. The genes tested were the following ones (virulence factor encoded): *cpd* (pheromone determinant), *gel*E (gelatinase), *fsr* (regulator of the expression of *gel*E), *ace* (accessory colonization factor), *agg* (aggregation substance) and *esp* (extracellular surface protein). Positive and negative controls were included in all analyses and bacteria were part of the collection of the University of Rioja (Spain). Some of the obtained amplicons were confirmed by sequencing.

Results

A total of 140 enterococci were recovered from the 77 faecal samples analysed. The species detected were the following ones: *E. faecium* (45 isolates, 32.1%), *E. faecalis* (73 isolates, 52.1%), *E. hirae* (14 isolates, 10%), *E. casseliflavus* (four isolates, 2.8%), *E. gallinarum* (two isolates, 1.4%) and *Enterococcus* spp. (two isolates, 1.4%). Enterococci were recovered from all tested faecal samples with the exception of those coming from cold blood animals (four salamanders and three vipers) from which no enterococci growth was obtained.

Antibiotic susceptibility

The percentage of antibiotic resistance detected in our enterococcal isolates is shown in Table 2. Forty-four of the 140 isolates (31.4%) showed susceptibility to all the antibiotics tested (5.5% of *E. faecalis*; 62.2% of *E. faecium*; 78.6% of *E. hirae* and one of the two *Enterococcus* spp.). In general, higher percentages of resistance were obtained in the series of *E. faecalis* with respect to *E. faecium* (with the exception of ciprofloxacin), or to the other enterococcal species. It is especially remarkable the low resistance rates detected in the non-*E. faecalis* and non-*E. faecium* isolates in our series. No ampicillin-resistant isolates were found in this study and only two *E. faecalis* isolates, recovered from an owl, showed high-level-gentamicin resistance (Table 3). No vancomycin-resistance was demonstrated in our series, with the exception of the four *E. casseliflavus* and the two *E. gallinarum* isolates (recovered from a mouflon, a deer and a fox), which showed a vancomycin MIC of 8 μg/ml (Table 3). Quinupristin–dalfopristin resistance was not detected among *E. faecium* isolates but, as expected, it was found in most of our *E. faecalis* isolates. This type of resistance was shown in two additional isolates (one *E. hirae* and one *Enterococcus* sp.) recovered from two birds of prey (both of them *Acciper gentillis*).

Mechanisms of antibiotic resistance

Table 3 shows the antibiotic resistance genes detected in our series of enterococci in relation with their specific antibiotic resistance phenotype. The *tet*(M) gene was found in all tetracycline-resistant isolates, independently of the enterococcal species or the animal origin, in most of the cases associated with *tet*(L) gene. All erythromycin-resistant

Table 1. Primers used in PCR reactions for the detection of genes implicated in antibiotic resistance and virulence in enterococci

Gene detected	Sequence of the primer (5′ to 3′)	Amplicon (bp)	Reference
aac(6′)-*Ie-aph*(2″)-*Ia*	F: CCAAGAGCAATAAGGGCATA R: CACTATCATACCACTACCG	220	Van de Klundert and Vliegenthart, 1993
aph(3′)-*IIIa*	F: GCCGATGTGGATTGCGAAAA R: GCTTGATCCCCAGTAAGTCA	292	Van de Klundert and Vliegenthart, 1993
ant(6)-*Ia*	F: ACTGGCTTAATCAATTTGGG R: GCCTTTCCGCCACCTCACCG	577	Del Campo et al., 2000
erm(B)	F: GAAAAGRTACTCAACCAAATA R: AGTAACGGTACTTAAATTGTTTAC	639	Sutcliffe et al., 1996
erm(A)	F: TCTAAAAAGCATGTAAAAGAA R: CTTCGATAGTTTATTAATATTAGT	645	Sutcliffe et al., 1996
erm(C)	F: TCAAAACATAATATAGATAAA R: GCTAATATTGTTTAAATCGTCAAT	642	Sutcliffe et al., 1996
tet(M)	F: GTTAAATAGTGTTCTTGGAG R: CTAAGATATGGCTCTAACAA	576	Aarestrup et al., 2000
tet(L)	F: CATTTGGTCTTATTGGATCG R: CAATATCACCAGAGCAGGCT	456	Aarestrup et al., 2000
vanA	F: GGGAAAACGACAATTGC R: GTACAATGCGGCCGTTA	732	Miele et al., 1995
vanB	F: ATGGGAAGCCGATAGTC R: GATTTCGTTCCTCGACC	635	Dutka-Malen et al., 1995
vanC-1	F: GGTATCAAGGAAATC R: CTTCCGCCATCATCT	822	Dutka-Malen et al., 1995
vanC-2/3	F: CTCCTACGATTCTCTTG R: CGAGCAAGACCTTTAAG	439	Dutka-Malen et al., 1995
vat(D)	F: CCGAATCCTATGAAAATGTATCC R: GCAGCTACTATTGCACCATCCC	413	Robredo et al., 2000
vat(E)	F: ACGTTACCCATCACTATG R: GCTCCGATAATGGCACCGAC	282	Robredo et al., 2000
ace	F: AAAGTAGAATTAGATCCACAC R: TCTATCACATTCGGTTGCG	320	Mannu et al., 2003
gelE	F: AGTTCATGTCTATTTTCTTCAC R: CTTCATTATTTACACGTTTG	403	Eaton and Gasson, 2001
agg	F: AAGAAAAAGAAGTAGACCAAC R: AAACGGCAAGACAAGTAAATA	1553	Eaton and Gasson, 2001
esp	F: AAACGGCAAGACAAGTAAATA R: GCGTCAACACTTGCATTGCCGAA	955	Eaton and Gasson, 2001
fsr	F: AACCAGAATCGACCAATGAAT R: GCCCCTCATAACTCAATACC	3268	Pillai et al., 2002
cpd	F: TGGTGGGTTATTTTTCAATTC R: TACGGCTCTGGCTTACTA	782	Eaton and Gasson, 2001

Table 2. Percentages of antibiotic resistance[a] detected in 140 enterococci of different species recovered from wild animals

Antibiotic	*Enterococcus faecalis* ($n = 73$)	*Enterococcus faecium* ($n = 45$)	*Enterococcus hirae* ($n = 14$)	Other species[b] ($n = 8$)	All enterococci ($n = 140$)
Ampicillin	0	0	0	0	0
Gentamicin[c]	2.7	0	0	0	1.4
Streptomycin[c]	2.7	0	0	0	1.4
Kanamycin[c]	4.1	2.2	0	0	2.1
Tetracycline	34.2	26.7	14.3	12.5	28.6
Erythromycin	37	4.3	0	0	20.7
Chloramphenicol	0	0	0	0	0
Ciprofloxacin	4.1	20.0	0	0	8.6
Quinupristin–dalfopristin	94.5	0	7.1	12.5	50.7
Vancomycin	0	0	0	75[b]	4.3
Teicoplanin	0	0	0	0	0

[a]The disk diffusion method was used, with the exception of vancomycin and teicoplanin in which the MIC determined by agar dilution method is considered.
[b]Species included: *E. casseliflavus* (four isolates), *E. gallinarum* (two isolates), *Enterococcus* spp. (two isolates).
[c]High-level resistance is considered.

enterococci harboured the *erm*(B) gene. The *vat*(D) or *vat*(E) genes (formerly named as *sat*A or *sat*G, respectively), encoding streptogramin A acetyltransferases, were not detected in the two quinupristin–dalfopristin-resistant non-*E. faecalis* isolates (*E. hirae* and *Enterococcus* sp.) of our study, which showed quinupristin–dalfopristin MIC values of 4 and 8 μg/ml respectively. The *aac*(6′)-Ie-*aph*(2″)-Ia gene was observed in the two high-level gentamicin-resistant *E. faecalis* isolates recovered from an owl in this study; these two isolates showed a multi-resistant phenotype that included, in

Table 3. Antibiotic resistance phenotypes and genes detected in the series of 140 enterococci recovered from wild animals

Antibiotic resistance phenotype[a]	Enterococcal isolates		Genes detected by PCR	
	Number and species	Animal origin	Genes	Number of isolates
STR-KAN-GEN-TET-ERY-QD	2 *E. faecalis*	Owl	*ant*(6)-*Ia* + *aph*(3′)-*IIIa* + *aac*(6′)-Ie-*aph*(2″)-Ia + *tet*(M) + *tet*(L) + *erm*(B)	2
KAN-TET-ERY-QD	1 *E. faecalis*	Wild rabbit	*aph*(3′)-*IIIa* + *tet*(M) + *tet*(L) + *erm*(B)	1
KAN-TET-ERY	1 *E. faecium*	Badger	*aph*(3′)-*IIIa* + *tet*(M) + *tet*(L) + *erm*(B)	1
TET-ERY-CIP-QD	2 *E. faecalis*	Bird of prey	*tet*(M) + *tet*(L) + *erm*(B)	2
TET-ERY-QD	8 *E. faecalis*	Owls, European genet and magpie	*tet*(M) + *tet*(L) + *erm*(B)	8
TET-CIP-QD	1 *E. faecalis*	Owl	*tet*(M) + *tet*(L)	1
TET-CIP	5 *E. faecium*	Pigeon, quail, stork and bird of prey	*tet*(M) + *tet*(L) *tet*(M)	4 1
TET-QD	11 *E. faecalis* and 1 *Enterococcus* spp.	Birds of prey, fox, magpie and owls	*tet*(M) + *tet*(L)	12
TET	6 *E. faecium*, 2 *E. hirae*	Birds of prey, European genet, forest wildcat, stork and quails	*tet*(M) + *tet*(L) *tet*(M)	6 2
ERY-QD	14 *E. faecalis*	Wolf, European genet, wild rabbit, magpie, bird of prey and fox	*erm*(B)	14
ERY	1 *E. faecium*	Wolf	*erm*(B)	1
CIP	4 *E. faecium*	Bird of prey, forest wildcat, wild rabbit and fox	ND	4
QD	30 *E. faecalis*, 1 *E. hirae*	Birds of prey, fox, European genet, forest wildcat, wild rabbit, magpie, partridge, stork, owls and otter	ND	31
VAN[b]	4 *E. casseliflavus*	Mouflon and deer	*vanC-2/3*	4
	2 *E. gallinarum*	Fox	*vanC-1*	2
Susceptible	28 *E. faecium*, 11 *E. hirae*, 4 *E. faecalis* and 1 *Enterococcus* sp	A wide variety of animals	–	44

[a]Antibiotics: STR, streptomycin; KAN, kanamycin; GEN, gentamicin; TET, tetracycline; ERY, erythromycin; CIP, ciprofloxacin; VAN, vancomycin; QD, quinupristin–dalfopristin. Susceptible: isolates that showed susceptibility to all antibiotics tested.
[b]Intermediate susceptibility (vancomycin MIC of 8 μg/ml).
E., *Enterococcus*; ND, no resistance genes were detected.

addition to resistance to all the tested aminoglycosides, resistance to tetracycline, erythromycin and quinupristin–dalfopristin. Only three of the wild animals studied harboured high-level kanamycin-resistant enterococci, in which *aph*(3′)-IIIa gene was shown.

Detection of virulence factors in enterococci from wild animals

Genes encoding virulence factors were studied in our series of 73 *E. faecalis* isolates and results are shown in Table 4. The *cpd* gene (a sex pheromone determinant) was detected in all but one of the *E. faecalis* isolates, and 28 of them showed at least three of the six genes encoding virulence factors tested. Eleven *E. faecalis* isolates harboured four virulence genes, and they were recovered from three birds of prey, five owls, one forest wildcat, one otter and one European genet. The prevalence of the different genes encoding virulence factors in our series of *E. faecalis* was as follows: *cpd* (98.6%), *gel*E (75.3%), *agg* (30.1%), *fsr* (17.8%), *ace* (9.6%) and *esp* (4.1%). One *E. faecalis* isolate, recovered from a wild rabbit, was free of the virulence factors analysed in this study. Virulence determinants were also studied by PCR in all the 45 *E. faecium* and in the remaining 22 *Enterococcus* spp. of our study. All results were negative with the exception of two *E. faecium* isolates, recovered form a bird of prey, in which the *gel*E gene was detected.

Discussion

Enterococci were detected in most of the faecal samples of the wild animals tested with the exception of the seven cold blood animals, in which no enterococcal isolates were recovered. This fact could be associated with the different intestinal microbiota of cold blood animals in relation with hot blood animals. The low percentages of antibiotic resistance detected in our faecal enterococci from wild animals in relation with percentages referred by other authors for faecal enterococci of humans or food animals (Aarestrup et al., 2000; Butaye et al., 2001; Del Campo et al., 2003; Mutnick et al., 2003), could be associated with differences in antibiotic use in the different groups. In this sense, our results corroborate the wide use of antibiotics in human medicine and veterinary, and the significant differences found in an antibiotic-free ecosystem such as that of wild animals, in which low percentages of antibiotic resistance were detected.

In our study, no ampicillin-resistant enterococci were detected, even among the *E. faecium* isolates. Nowadays, it is quite frequent the detection of clinical *E. faecium* strains with an ampicillin-resistant phenotype because of modifications in their penicillin-binding proteins (PBP5), although this resistance phenotype has been more frequently detected in isolates of human origin than in those of animal origin (Mannu et al., 2003). In addition, no acquired vancomycin resistance has

Table 4. Detection of genes encoding virulence factors in 73 *Enterococcus faecalis* isolates from wild animals of this study

Type of genes encoding virulence factors detected by PCR	Number of isolates	Animal from which enterococci were recovered
cpd, *gel*E, *ace*, *esp*	1	Bird of prey
cpd, *gel*E, *ace*, *agg*	4	Owl, forest wildcat, otter and bird of prey
cpd, *gel*E, *agg*, *fsr*	6	Owl, bird of prey and European genet
cpd, *gel*E, *agg*	10	Owl, bird of prey, fox, wild rabbit, otter and European genet
cpd, *gel*E, *fsr*	4	Owl and forest wildcat
cpd, *gel*E, *ace*	1	Magpie
cpd, *gel*E	29	Stork, forest wildcat, wild rabbit, partridge, fox, owl and bird of prey, European genet, wolf and magpie
cpd, *agg*, *fsr*	1	Stork
cpd, *agg*, *esp*	1	Owl
cpd, *esp*	1	Owl
cpd, *fsr*	2	Owl and magpie
cpd, *ace*	1	Magpie
cpd	11	European genet, fox, magpie, owl and bird of prey
ND	1	Wild rabbit

ND, no resistance genes were detected; PCR, polymerase chain reaction.

been detected among our enterococci of wild animals, but this type of resistance was previously demonstrated in faecal enterococci of food animals, pets and humans in Portugal when vancomycin-supplemented media was used (Poeta et al., 2005). The majority of the tetracycline-resistant enterococcal isolates of this study harboured the *tet*(M) gene, associated or not with *tet*(L). The *tet*(M) gene is implicated in ribosomal protection and *tet*(L) codifies an efflux pump; both of them have been referred by other authors as important mechanisms of tetracycline resistance, and have been described, as in our study, frequently associated together in the same strain (Aarestrup et al., 2000; Del Campo et al., 2003). The *erm*(B) gene, detected in most of our erythromycin-resistant isolates, encodes an *erm* methylase that modifies the 23S rRNA, and has been previously found as the most common macrolide resistance gene (Del Campo et al., 2003). Only two of our enterococci showed quinupristin–dalfopristin resistance (with the exception of the *E. faecalis* isolates) and none of them harboured the *vat*(D) or *vat*(E) resistance genes. These resistance genes were also not detected in a series of quinupristin–dalfopristin-resistant *E. faecium* faecal isolates of healthy human volunteers in a previous study and were found only in 25% of those obtained from food-handlers (Del Campo et al., 2003). Nevertheless, the *vat*(E) gene was frequently found among *van*A-containing *E. faecium* strains recovered from poultry and humans in other study (Robredo et al., 2000). However, most of our *E. faecalis* isolates showed quinupristin–dalfopristin resistance, being this type of resistance intrinsic in this enterococcal species; an efflux pump encoded by the *lsa* gene may play a role in this type of resistance (Singh and Murray, 2005).

It is of interest to underline that most of the *E. faecalis* isolates of this study harboured at least one of the genes encoding virulence factors and 38% of them harboured three virulence genes. The more frequently detected genes were *cpd*, *gel*E and *agg* (98.6–30.1%), being *esp* gene the less frequently detected one ($<5\%$). The aggregation substance (the product of the *agg* virulence determinant) is a pheromone-inducible surface protein of *E. faecalis*, which promotes mating aggregate formation during bacterial conjugation (Mundy et al., 2000). As an important component of the bacterial pheromone-responsive genetic exchange system, this aggregation substance mediates adhesion to a variety of eukaryotic cell surfaces, and promotes internalization by cultured human intestinal cells (Mundy et al., 2000). In our study, the *agg* virulence determinant was always associated with the presence of the *cpd* pheromone determinant. This association has been previously reported by others (Eaton and Gasson, 2001). Sex pheromones are secreted by potential recipient strains and induce genes leading to the production of the aggregation substance in potential donor cells. This process enhances the transfer frequency of the sex pheromone plasmid into plasmid-free recipient cells because of donor and recipient cell aggregation.

Gelatinase, encoded by the *gel*E gene, is an extracellular zinc endopeptidase that hydrolyses collagen, gelatin, hemoglobin and other bioactive compounds, and it has been shown to exacerbate endocarditis in an animal model although this activity is not requirement for pathogenesis (Jones and Deshpande, 2003). In our study, a high proportion of the *E. faecalis* isolates showed this virulence determinant (75.3%). However, the *fsr* locus of *E. faecalis* confers virulence in animal models (Pillai et al., 2002) and seems to be much more frequently detected in human clinical isolates (100% in isolates implicated in endocarditis) than in stool isolates (53%), (Pillai et al., 2002). In our case, only 17.8% of the *E. faecalis* isolates of wild animals harboured this virulence factor. This fact could indicate a lower virulence capacity of strains of wild animals respect to those of other origins.

The *ace* gene (accessory colonization factor), found in almost 10% of our *E. faecalis* isolates, codes for a putative protein with similar characteristics to a collagen-binding protein (Eaton and Gasson, 2001; Mannu et al., 2003). The *esp* gene, that encodes an enterococcal surface protein associated with the ability to form a biofilm onto abiotic surfaces, was found in only three *E. faecalis* isolates recovered from one owl and one bird of prey (Table 4). Other authors have found this virulence factor in *E. faecalis* isolates of foods and human clinical origin, but in a higher proportion than that found in this study (Eaton and Gasson, 2001). In addition, the *esp* gene has been more frequently found in antibiotic-resistant *E. faecium* isolates than in susceptible ones in a previous report (Coque et al.,

2002). Resistance to four or more of the tested antibiotics were shown in five of our 73 *E. faecalis* isolates (6.8%), and three of them were the only ones that harboured the *esp* gene; in our series, it seems also that the *esp* gene is associated with the most resistant enterococcal strains.

Enterococcus faecium is a species generally free of known virulence factors (Mannu et al., 2003), although there are some references reporting virulence determinants in occasional isolates of this species (Jett et al., 1994; Eaton and Gasson, 2001; Coque et al., 2002; Semedo et al., 2003). The *gel*E and *agg* factors have been previously found in *E. faecium*, *E. hirae*, *E. casseliflavus*, *E. gallinarum* and other species in a previous study (Semedo et al., 2003), suggesting that virulence determinants are a common trait in the genus *Enterococcus*; these authors also found the *esp* gene in strains of *E. faecium* and *E. durans* but not in *E. casseliflavus* or *E. gallinarum* isolates.

In conclusion, relatively low levels of antibiotic resistance were detected in enterococcal isolates recovered from wild animals in Portugal, and the mechanisms of resistance implicated in resistant isolates are similar to those detected in enterococcal isolates of human origin. Nevertheless, these resistance levels are much lower than those detected in enterococci of farm animals, humans, or in pets (Aarestrup et al., 2000; Butaye et al., 2001; Del Campo et al., 2003). Most of the studied animals are carnivorous, omnivorous and opportunistic and this fact could explain the detection, in some of the isolates, of antimicrobial resistance genes, even when no known antibiotic pressure is applied. It is also of interest to underline the wide dissemination of genes encoding virulence factors observed in our series of *E. faecalis* from wild animals, but not in the other enterococcal species. More studies should be carried out in the future to evaluate the evolution of enterococci harbouring antibiotic resistance genes and virulence determinants in different ecosystems.

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