Letters in Applied Microbiology

Letters in Applied Microbiology ISSN 0266-8254

ORIGINAL ARTICLE

Antimicrobial resistance in faecal enterococci and *Escherichia coli* isolates recovered from Iberian wolf

A. Gonçalves[1,2,3,4], G. Igrejas[1,2], H. Radhouani[1,2,3,4], S. Correia[1,2,3,4], R. Pacheco[1,2,3,4], T. Santos[1,2,3,4], R. Monteiro[1,2,3,4], A. Guerra[5], F. Petrucci-Fonseca[5], F. Brito[3,4], C. Torres[6] and P. Poeta[3,4]

1 Institute for Biotechnology and Bioengineering/Center of Genomics and Biotechnology, University of Trás-os-Montes and Alto Douro, Vila Real, Portugal
2 Department of Genetics and Biotechnology, University of Trás-os-Montes and Alto Douro, Vila Real, Portugal
3 Center for Animal Science and Veterinary, Vila Real, Portugal
4 Department of Veterinary Science, University of Trás-os-Montes and Alto Douro, Vila Real, Portugal
5 Grupo Lobo, Lisbon Faculty of Science, Lisbon, Portugal
6 Área de Bioquímica y Biología Molecular, Universidad de La Rioja, Logroño, Spain

Significance and Impact of the Study: This study shows antimicrobial resistance in commensal bacteria from the free-range, Portuguese, Iberian wolf population. The results indicate that the Iberian wolf could contribute to the spread of resistant bacteria throughout the environment. Additionally, in case of infection, an increased risk of therapeutic failure due to the presence of multiresistant bacteria may represent a health problem for this endangered species. Future studies must be performed to analyse the possible contamination of these animals through the environment and/or the food chain.

Keywords
Antimicrobial resistance, *E. coli*, *Enterococcus*, Iberian wolf.

Correspondence
Patrícia Poeta, Department of Veterinary Sciences, University of Trás-os-Montes and Alto Douro, Vila Real, Portugal.
E-mail: ppoeta@utad.pt

2012/1267: received 13 July 2012, revised 10 January 2013 and accepted 12 January 2013

doi:10.1111/lam.12044

Abstract

The aim of this study was to report the antimicrobial resistance, the molecular mechanisms associated and the detection of virulence determinants within faecal *Enterococcus* spp. and *Escherichia coli* isolates of Iberian wolf. Enterococci (n = 227) and *E. coli* (n = 195) isolates were obtained from faecal samples of Iberian wolf (*Canis lupus signatus*). High rates of resistance were detected for tetracycline and erythromycin among the enterococci isolates, and most of resistant isolates harboured the *tet*(M) and/or *tet*(L) and *erm*(B) genes, respectively. The bla_{TEM}, *tet*(A) and/or *tet*(B), and *aadA* or *strA-strB* genes were detected among most ampicillin-, tetracycline- or streptomycin-resistant *E. coli* isolates, respectively. *E. coli* isolates were ascribed to phylogroups A (n = 56), B1 (91), B2 (13) and D (35). The occurrence of resistant enterococci and *E. coli* isolates in the faecal flora of Iberian wolf, including the presence of resistant genes in integrons, and virulence determinants was showed in this study. Iberian wolf might act as reservoir of certain resistance genes that could be spread throughout the environment.

Introduction

Enterococcus spp. and *Escherichia coli* are common inhabitants of the intestinal tract of animals and humans and are also commonly found in food products, plants, water and soil (Silva *et al.* 2010). These bacteria are associated with both community- and hospital-acquired infections because they sometimes carry virulence determinants often found in pathogenic strains and can also easily acquire and transfer resistance genes (Ruiz *et al.* 2002; Paterson and Bonomo 2005; Aarestrup *et al.* 2008). Consequently, the level of antimicrobial resistance in these micro-organisms is considered to be a good indicator that might be used to track the evolution of antimicrobial resistance in different ecosystems (Sørum and Sunde 2001; Silva *et al.* 2010). Studies concerning the incidence of resistant enterococci and *E. coli* isolates in wild animals are available in the literature (Caprioli *et al.* 1991; Lilleheaug *et al.* 2005; Poeta *et al.* 2005; Costa *et al.* 2008; Schierack *et al.* 2009; Silva *et al.* 2010; Allen *et al.* 2011;

Radhouani *et al.* 2012). Still, the flow of resistant microorganisms and resistant genes from livestock and humans to wildlife, or vice-versa, remains poorly understood despite the fact that wild animals may act as reservoirs of resistant genetic elements that could be spread across the environment (Sørum and Sunde 2001; Aarestrup *et al.* 2008; Radhouani *et al.* 2010b).

The Portuguese population of Iberian wolf (*Canis lupus signatus*) is endangered (Cabral *et al.* 2008). The Wolf Group, a Portuguese, independent, nonprofit organization that works for the wolf conservation and its ecosystem in Portugal, identifies the Portuguese wolf population with approximately 300 individuals, 90% of which inhabit the area north of Douro River and are in continuity with the Spanish wolf population (Álvares 2004). This species typically hunts deer and wild boars. However, due to the decline of prey population along with habitat loss, livestock has become an important portion of their diet (Álvares 2004). Their predatory and travelling habits might expose this species to food remains or faecal material from farm animals or even from humans that carry resistant strains.

Previous studies carried out by our group were focused to analyse the faecal colonization by vancomycin-resistant enterococci and *E. coli* isolates producing extended-spectrum β-lactamases (ESBL) in the free-range, Portuguese, Iberian wolf population (Gonçalves *et al.* 2011, 2012). In the referred studies, faecal samples were seeded on Slanetz–Bartley supplemented with vancomycin and Levine agar plates supplemented with cefotaxime to recover the vancomycin-resistant enterococci isolates and the cefotaxime-resistant *E. coli* isolates, respectively. The present study has been focused to investigate the antimicrobial resistance, the molecular mechanisms associated and the detection of virulence determinants within nonselected faecal *Enterococcus* spp. and *E. coli* isolates of Iberian wolf, to obtain a deeper knowledge about the distribution of antimicrobial resistance genes in this wild ecosystem.

Results and discussion

Enterococci were recovered from 227 of 237 faecal samples (96%) and *E. coli* in 195 of tested faecal samples (82%). *Enterococcus faecium* and *Ent. faecalis* are usually the prevalent enterococcal species among isolates recovered from faecal samples of wild animals (Silva *et al.* 2010). Accordingly, *Ent. faecium* was the prevalent isolated species (117 isolates), being *Ent. hirae* (63 isolates) the second most frequent species, followed by *Ent. faecalis* (27 isolates) and *Ent. durans* (20 isolates). Interestingly, similar results were obtained in a study performed with wild boars in Northern Portugal (Poeta *et al.* 2007).

None of our enterococci, recovered in nonselective plates, showed vancomycin resistance. Nevertheless, this type of resistance was previously detected in this species when enterococci were recovered using vancomycin-supplemented agar plates (Gonçalves *et al.* 2011). This fact suggests that VRE might be present within the faecal enterococcal population of wild wolves, but in a lower proportion when compared with the vancomycin susceptible ones, and could not be detected when nonsupplemented plates were used for bacterial isolation.

The enterococcal isolates showed high frequency of resistance to tetracycline (55%), erythromycin (22%) and ciprofloxacin (15%) and lower values for quinupristin–dalfopristin (11%), kanamycin (7%), ampicillin (4%), streptomycin (3%), chloramphenicol (2%) and gentamicin (1%). With the exception of tetracycline resistance, similar results were previously described in a study performed with wild rabbits in Northern Portugal (Silva *et al.* 2010). In a study performed with wild boars, similar values of resistance to tetracycline, ciprofloxacin, kanamycin, ampicillin, streptomycin and chloramphenicol were also found. It is noteworthy that only 34% of the *Enterococcus* isolates were susceptible to all tested antimicrobial agents.

Table 1 shows the antimicrobial resistance genes detected by PCR. Most of tetracycline-resistant isolates harboured both *tet*(M) and *tet*(L) genes. Additionally, 39% and 8% of our *tet*(M)-positive enterococci carried specific sequences of Tn*916*/Tn*1545* and Tn*5397* transposons, respectively. The *tet*(M) and *tet*(L) genes, encoding for tetracycline resistance, are commonly reported among enterococci (Silva *et al.* 2010). The *erm*(B) and *erm*(A) genes, responsible for acquired erythromycin resistance, were detected in 22 and one of the 50 erythromycin-resistant isolates, respectively. Among our high-level-aminoglycoside-resistant enterococci, the *aac*(6′)-*aph*(2″) gene was found in one gentamicin-resistant isolate; the *ant*(6)-Ia gene, also in one streptomycin-resistant isolate; and the *aph*(3′)-IIIa gene, in 15 kanamycin-resistant isolates. These genes have also been found in previous reports among high-level-aminoglycoside-resistant enterococci from wild animals in Portugal (Poeta *et al.* 2005; Silva *et al.* 2010). None of the *Enterococcus* isolates recovered in this study presented one of the tested genes encoding virulence factors. Likewise, none showed beta-haemolytic or gelatinase activity. These results are in agreement with other authors, who showed that generally, virulence factors appear more commonly associated with clinical enterococcal isolates and most frequently associated with *Ent. faecalis* (López *et al.* 2009).

Concerning the *E. coli* isolates, 57% showed susceptibility to all antimicrobial agents tested. None of the *E. coli* isolates exhibited resistance to cefotaxime, ceftazidime,

Table 1 Resistance genes detected in antimicrobial-resistant enterococci and *E. coli* isolates obtained from faecal samples of Iberian wolf

Bacteria	Antimicrobial agent (disc charge)	Number of resistant isolates	Genes detected by PCR	
			Resistance genes and genetic elements	Number of isolates
Enterococci (*n* = 227)				
Enterococcus faecium (*n* = 117)	Tetracycline (30 μg)	75	*tet*(M)	4
			tet(L)	3
			tet(M) + *tet*(L)	34
			tet(M) + Tn*916*	18
			tet(M) + Tn*5397*	2
			tet(M) + Tn*916* + Tn*5397*	1
			tet(M) + *tet*(L) + Tn*916*	11
			tet(M) + *tet*(L) + Tn*5397*	2
	Erythromycin (15 μg)	38	*erm*(A)	1
			erm(B)	13
	Ampicillin (10 μg)	9	–	–
	Ciprofloxacin (5 μg)	27	–	–
	Quinupristin–dalfopristin (15 μg)	1	*vatD* or *vatE*	0
	Streptomycin (300 μg)	5	*ant*(6)-Ia	1
	Gentamicin (120 μg)	2	*aac*(6′)-Ie-*aph*(2″)-Ia	1
	Kanamycin (120 μg)	10	*aph*(3′)-IIIa	9
Enterococcus hirae (*n* = 63)	Tetracycline (30 μg)	20	*tet*(M)	2
			tet(M) + *tet*(L)	8
			tet(M) + Tn*916*	2
			tet(M) + Tn*5397*	2
			tet(M) + *tet*(L) + Tn*916*	5
			tet(M) + *tet*(L) + Tn*5397*	1
	Ampicillin (10 μg)	1	–	–
	Ciprofloxacin (5 μg)	3	–	–
	Quinupristin-dalfopristin (15 μg)	2	*vatD* or *vatE*	0
Enterococcus faecalis (*n* = 27)	Chloramphenicol (30 μg)	4	*catA*	0
	Tetracycline (30 μg)	19	*tet*(L)	3
			tet(M)	2
			tet(M) + *tet*(L)	8
			tet(M) + *tn*916	1
			tet(M) + *tet*(L) + Tn*916*	5
	Erythromycin (15 μg)	12	*erm*(B)	9
	Ciprofloxacin (5 μg)	3	–	–
	Streptomycin (300 μg)	2	*ant*(6)-Ia	0
	Gentamicin (120 μg)	1	*aac*(6′)-Ie-*aph*(2″)-Ia	0
	Kanamycin (120 μg)	6	*aph*(3′)-IIIa	6
Enterococcus durans (*n* = 20)	Tetracycline (30 μg)	10	*tet*(M)	1
			tet(M) + *tet*(L)	5
			tet(M) + *tn*916	1
			tet(M) + *tet*(L) + *tn*916	2
			tet(M) + *tet*(L) + *tn*5397	1
Escherichia coli (*n* = 195)	Ampicillin (10 μg)	49	bla_{TEM}	32
	Tetracycline (30 μg)	58	*tet*(A)	23
			tet(B)	14
	Gentamicin (10 μg)	1	*aac*(3)-II	1
	Amikacin (30 μg)	2	–	–
	Tobramycin (10 μg)	4	–	–
	Streptomycin (10 μg)	48	*aadA*	26
			strA-strB	22
	Nalidixic acid (30 μg)	20	–	–
	Ciprofloxacin (5 μg)	6	–	–
	Sulfamethoxazole–trimethoprim (25 μg)	24	*sul1*	4
			sul2	6
			sul1 + *sul2*	13
			sul1 + *sul2* + *sul3*	1
	Chloramphenicol (30 μg)	10	*cmlA*	2

aztreonam and imipenem. Additionally, resistance to amoxicillin plus clavulanic acid, cefoxitin, gentamicin and amikacin was $\leq 1\%$. Higher levels of resistance were detected for tetracycline (30%), ampicillin (25%), streptomycin (25%) and sulfamethoxazole–trimethoprim (12%). Lower levels of resistance were shown for nalidixic acid (10%), chloramphenicol (5%), ciprofloxacin (3%) and tobramycin (2%). The results obtained in our study are similar with those previously reported among wild animals in Portugal (Costa *et al.* 2008; Radhouani *et al.* 2009).

As indicated in Table 1, 32 of the 49 ampicillin-resistant *E. coli* isolates harboured the bla_{TEM} gene. This result was expected, as classical TEM enzymes are the predominant mechanism of ampicillin resistance in *E. coli* (Silva *et al.* 2010). The *tet*(A) and *tet*(B) genes, encoding for tetracycline resistance, were detected in 23 and 14 isolates, respectively, and had been the most frequent genes reported among isolates from different origins (Sáenz *et al.* 2004). The *cmlA* gene, encoding a chloramphenicol-specific exporter, was identified in 2 of our 10 chloramphenicol-resistant isolates. The *aadA* or *strA-strB* genes were detected among the streptomycin-resistant isolates. These genes, encoding enzymes for streptomycin resistance, were previously found and are widely disseminated in streptomycin-resistant *E. coli* isolates from animals, humans and plants (Sunde and Norstrom 2005). The gene encoding for gentamicin acetyltransferase AAC(3)-II was detected in one isolate. Different combinations of *sul1*, *sul2* and *sul3* genes were identified in all the sulfamethoxazole–trimethoprim-resistant *E. coli* isolates. The presence of class 1 and class 2 integrons was confirmed in 13 and one isolates, respectively, all containing the *dfrA1* + *aadA1* cassette array. Similar structures have been reported in *E. coli* isolates from different sources (Sáenz *et al.* 2004; Radhouani *et al.* 2009). Concerning the phylogenetic groups, 56 isolates were ascribed to the phylogenetic group A; 91 isolates, to B1; 13 isolates, to B2; and 35 isolates, into the phylogroup D. The faecal origin of our isolates might explain the higher prevalence of A and B1 phylogroups, because these groups are comprised mostly by commensal isolates (Radhouani *et al.* 2010a). The prevalence of virulence determinants was as follows: the *fimA* gene was detected in 142 isolates; the *aer* and *fimA* genes, in 27 isolates; the *papC* and *fimA* genes, in six isolates; the *papC* and *cnf1*, in one isolate; and the *aer*, *papC* and *fimA* genes, in three isolates. The predominance of *fimA* (type 1 fimbriae) is in agreement with a previous work performed in wild animals (Radhouani *et al.* 2012). The *fimA* gene, encoding the major subunit of the *E. coli* type 1 fimbriae, was the most frequently found virulence factor-encoding gene detected in *E. coli* isolates responsible for urinary tract infection in humans (Ruiz *et al.* 2002). Still, the presence of virulence genes in our commensal isolates should not be interpreted as a process of active virulence gene acquisition, but as a part of a survival mechanism that ensures greater genetic diversity, increasing their survival capability in the host animal (Chapman *et al.* 2006).

It has been observed an association between the level of antimicrobial resistance in faecal bacteria from wildlife populations and the level of contact of these animals with people. For instance, while a high prevalence of antibiotic resistance in faecal Enterobacteriaceae from wild rodents that had not been exposed to antibiotics was detected in England, the absence of resistance among Enterobacteriaceae of wild moose, deer and voles was reported in Finland (Gilliver *et al.* 1999; Osterblad *et al.* 2001). The Portuguese Iberian wolf population live in ecosystems other than those closely related to humans or containing obvious antimicrobial resistance selection pressures. Still, travelling large distances could expose this species to food remains or faecal material from farm animals or even from humans that carry resistant strains (Aarestrup *et al.* 2008). The acquisition of antimicrobial-resistant enterococci and *E. coli* isolates by this species could also be explained through the predatory behaviour of these animals. There are several examples of transfer of resistant bacteria between animals and from animals to man via the food chain (Teale 2002; Aarestrup *et al.* 2008). Consequently, Iberian wolf might be contaminated through the food chain, as the presence of resistant *E. coli* strains has been previously detected in its preys (Caprioli *et al.* 1991; Lillehaug *et al.* 2005; Schierack *et al.* 2009; Silva *et al.* 2010).

Concluding, the Iberian wolf might act as reservoir of resistant bacteria. The results obtained in this study indicate that Iberian wolf harbours multiresistant enterococci and *E. coli* isolates and, through their predatory and travelling habits, could spread these resistant bacteria, and/or their resistant genes, throughout the environment.

Materials and methods

Two hundred and thirty-seven faecal samples were obtained from free-ranging Iberian wolf (*Canis lupus signatus*) during 2008 and 2009. Faecal samples were collected from the soil in the north-east of Portugal in five locations/mountainous regions (Falperra, Alvão, Minhéu, Padrela and Candedo). These locations are in the north side of the Douro River and are remote from urban centres. Still, some small villages are inside the habitat range of the Iberian wolf. Sample gathering was carried out during surveillance studies performed by the Wolf Group. For enterococci recovery, samples were seeded in Slanetz–Bartley agar plates. One colony per sample with typical

enterococcal morphology was 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 polymerase chain reaction (PCR) using specific primers and conditions for *Ent. faecalis*, *Ent. faecium*, *Ent. casseliflavus* (Dutka-Malen *et al.* 1995), *Ent. gallinarum* (Miele *et al.* 1995), *Ent. hirae* and *Ent. durans* (Arias *et al.* 2006). For *E. coli* isolation, samples were seeded in Levine agar plates. One colony per sample with typical *E. coli* morphology was selected and identified by classical biochemical methods (Gram staining, catalase, oxidase, indol, Methyl-Red-Voges-Proskauer, citrate and urease) and by the API 20E system (BioMérieux, La Balme Les Grottes, France).

Antimicrobial susceptibility was performed by the disc diffusion method according to the criteria of CLSI (CLSI 2011). Susceptibility to 11 antimicrobial agents (Oxoid Limited, Basingstoke, UK) [vancomycin (30 μg); teicoplanin (30 μg); ampicillin (10 μg); chloramphenicol (30 μg); tetracycline (30 μg); erythromycin (15 μg); quinupristin–dalfopristin (QD) (15 μg); ciprofloxacin (5 μg); streptomycin (300 μg); gentamicin (120 μg); and kanamycin (120 μg)] was tested for enterococci. High-level resistance (HLR) was evaluated for aminoglycosides. *Enterococcus faecalis* ATCC 29212 and *Staphylococcus aureus* ATCC 25923 strains were used as quality control strains. Evaluation of gelatinase and haemolysin production in enterococci was performed as previously reported (López *et al.* 2009).

Additionally, susceptibility of the *E. coli* isolates was determined for 16 antimicrobial agents (Oxoid) [ampicillin (10 μg), amoxicillin plus clavulanic acid (30 μg), cefoxitin (30 μg), cefotaxime (30 μg), ceftazidime (30 μg), aztreonam (30 μg), imipenem (10 μg), gentamicin (10 μg), amikacin (30 μg), tobramycin (10 μg), streptomycin (10 μg), nalidixic acid (30 μg), ciprofloxacin (5 μg), sulfamethoxazole–trimethoprim (SXT) (25 μg), tetracycline (30 μg) and chloramphenicol (30 μg)] by the disc diffusion method (CLSI 2011). *E. coli* ATCC 25922 was used as a quality control strain. Also, ESBL phenotypic detection was carried out by double-disc diffusion test (CLSI 2011).

Deoxyribonucleic acid of the enterococci isolates was extracted using the InstaGene Matrix DNA extraction kit (Bio-Rad, Hercules, CA, USA) and following the manufacturer's instructions. For the *E. coli* isolates, DNA was extracted by the boiling method (Solberg *et al.* 2006). The DNA purity and concentration were determined using the NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The presence of the following resistance genes was analysed by PCR among the antimicrobial-resistant enterococci: *erm*(A), *erm*(B), *tet*(M), *tet*(L), *tet*(K), *aph*(3′)-IIIa, *ant*(6)-Ia, *aac*(6′)-Ie-*aph*(2″)-Ia, *catA*, *vatD*, *vatE,* as well as of Tn*916*- and Tn*5397*-specific sequences (De Leener *et al.* 2004; Poeta *et al.* 2005). The presence of different virulence genes (*gel*E, *agg*, *ace*, *cpd*, *fsr*, *esp*, *hyl* and *cyl*LLLSABM) was also studied by PCR in enterococcal isolates (Eaton and Gasson 2001; Pillai *et al.* 2002; Creti *et al.* 2004; Klare *et al.* 2005). In the resistant *E. coli* isolates, the following antimicrobial resistance genes were studied by PCR: *tet* (A), *tet*(B), *aadA, strA-strB*, *aac(3)-II, aac(3)-IV*, *sul1*, *sul2*, *sul3*, *cmlA* and *floR*. Additionally, the presence of the *intl1* and *intI2* genes, encoding class 1 and 2 integrases, respectively, and their variable region were analysed by PCR and sequencing (Sáenz *et al.* 2004). Lastly, the phylogenetic groups and virulence determinants frequently identified in pathogenic *E. coli* isolates (*stx1-stx2*, *fimA*, *papG* allele III, *cnf1*, *papC* and *aer*) were studied (Clermont *et al.* 2000; Ruiz *et al.* 2002). Positive and negative controls from the collection of strains of the University of Trás-os-Montes and Alto Douro (Portugal) were included in all PCR assays.

Acknowledgements

Alexandre Gonçalves has a PhD fellowship granted by FCT-Fundação para a Ciência e a Tecnologia and ESF-European Social Fund (SFRH/BD/47833/2008). Hajer Radhouani has a PhD fellowship granted by FCT-Fundação para a Ciência e a Tecnologia and ESF-European Social Fund (SFRH/BD/60846/2009). Susana Correia has a PhD fellowship granted by FCT-Fundação para a Ciência e a Tecnologia and ESF-European Social Fund (SFRH/BD/75160/2010).

Conflict of interest

None of the authors of this article has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the article.

References

Aarestrup, F.M., Wegener, H.C. and Collignon, P. (2008) Resistance in bacteria of the food chain: epidemiology and control strategies. *Expert Rev Anti Infect Ther* **6**, 733–750.

Allen, S.E., Boerlin, P., Janecko, N., Lumsden, J.S., Barker, I.K., Pearl, D.L., Reid-Smith, R.J. and Jardine, C. (2011) Antimicrobial resistance in generic *Escherichia coli* isolates from wild small mammals living in swine farm, residential, landfill, and natural environments in southern Ontario, Canada. *App Environ Microbiol* **77**, 882–888.

Álvares, F. (2004) *Status and Conservation of the Iberian Wolf in Portugal. In WolfPrint*. UK: The UK Wolf Conservation Trust.

Arias, C.A., Robredo, B., Singh, K.V., Torres, C., Panesso, D. and Murray, B.E. (2006) Rapid identification of *Enterococcus hirae* and *Enterococcus durans* by PCR and detection of a homologue of the *E. hirae mur-2* Gene in *E. durans*. *J Clin Microbiol* **44**, 1567–1570.

Cabral, M.J., Almeida, P.R., Almeida, J., Almeida, N.F., Dellinger, T., Oliveira, M.E., Palmeirim, J., Queiroz, A.I. *et al.* (2008) *Livro Vermelho dos Vertebrados de Portugal*. Lisboa: Instituto da Conservação da Natureza e Biodiversidade.

Caprioli, A., Donelli, G., Falbo, V., Passi, C., Pagano, A. and Mantovani, A. (1991) Antimicrobial resistance and production of toxins in *Escherichia coli* strains from wild ruminants and the alpine marmot. *J Wildlife Dis* **27**, 324–327.

Chapman, T.A., Wu, X.Y., Barchia, I., Bettelheim, K.A., Driesen, S., Trott, D., Wilson, M. and Chin, J.J. (2006) Comparison of virulence gene profiles of *Escherichia coli* strains isolated from healthy and diarrheic swine. *App Environ Microbiol* **72**, 4782–4795.

Clermont, O., Bonacorsi, S. and Bingen, E. (2000) Rapid and simple determination of the *Escherichia coli* phylogenetic group. *Appl Environ Microbiol* **66**, 4555–4558.

CLSI (2011) *Performance Standards for Antimicrobial Susceptibility Testing; Twenty-First Edition: Informational Supplement. CLSI Document M100-S21*. Wayne, PA, USA: Clinical and Laboratory Standards Institute.

Costa, D., Poeta, P., Sáenz, Y., Vinué, L., Coelho, A.C., Matos, M., Rojo-Bezares, B., Rodrigues, J. *et al.* (2008) Mechanisms of antibiotic resistance in *Escherichia coli* isolates recovered from wild animals. *Microb Drug Resist* **14**, 71–77.

Creti, R., Imperi, M., Bertuccini, L., Fabretti, F., Orefici, G., Di Rosa, R. and Baldassarri, L. (2004) Survey for virulence determinants among *Enterococcus faecalis* isolated from different sources. *J Med Microbiol* **53**, 13–20.

De Leener, E., Martel, A., Decostere, A. and Haesebrouck, F. (2004) Distribution of the *erm*(B) gene, tetracycline resistance genes, and Tn*1545*-like transposons in macrolide- and lincosamide-resistant enterococci from pigs and humans. *Microb Drug Resist* **10**, 341–345.

Dutka-Malen, S., Evers, S. and Courvalin, P. (1995) Detection of glycopeptide resistance genotypes and identification to the species level of clinically relevant enterococci by PCR. *J Clin Microbiol* **33**, 24–27.

Eaton, T.J. and Gasson, M.J. (2001) Molecular screening of *Enterococcus* virulence determinants and potential for genetic exchange between food and medical isolates. *Appl Environ Microbiol* **67**, 1628–1635.

Gilliver, M.A., Bennett, M., Begon, M., Hazel, S.M. and Hart, C.A. (1999) Antibiotic resistance found in wild rodents. *Nature* **401**, 233–234.

Gonçalves, A., Igrejas, G., Radhouani, H., Lopez, M., Guerra, A., Petrucci-Fonseca, F., Alcaide, E., Zorrilla, I. *et al.* (2011) Detection of vancomycin-resistant enterococci from faecal samples of Iberian wolf and Iberian lynx, including *Enterococcus faecium* strains of CC17 and the new singleton ST573. *Sci Total Environ* **410**, 266–268.

Gonçalves, A., Igrejas, G., Radhouani, H., Estepa, V., Pacheco, R., Monteiro, R., Brito, F., Guerra, A. *et al.* (2012) Iberian wolf as a reservoir of extended-spectrum beta-lactamase-producing *Escherichia coli* of the TEM, SHV, and CTX-M groups. *Microb Drug Resist* **18**, 215–219.

Klare, I., Konstabel, C., Mueller-Bertling, S., Werner, G., Strommenger, B., Kettlitz, C., Borgmann, S., Schulte, B. *et al.* (2005) Spread of ampicillin/vancomycin-resistant *Enterococcus faecium* of the epidemic-virulent clonal complex-17 carrying the genes *esp* and *hyl* in German hospitals. *Eur J Clin Microbiol Infect Dis* **24**, 815–825.

Lillehaug, A., Bergsjo, B., Schau, J., Bruheim, T., Vikoren, T. and Handeland, K. (2005) *Campylobacter* spp., *Salmonella* spp., verocytotoxic *Escherichia coli*, and antibiotic resistance in indicator organisms in wild cervids. *Acta Vet Scand* **46**, 23–32.

López, M., Sáenz, Y., Rojo-Bezares, B., Martinez, S., del Campo, R., Ruiz-Larrea, F., Zarazaga, M. and Torres, C. (2009) Detection of *van*A and *van*B2-containing enterococci from food samples in Spain, including *Enterococcus faecium* strains of CC17 and the new singleton ST425. *Int J Food Microbiol* **133**, 172–178.

Miele, A., Bandera, M. and Goldstein, B.P. (1995) Use of primers selective for vancomycin resistance genes to determine *van* genotype in enterococci and to study gene organization in *vanA* isolates. *Antimicrob Agents Chemother* **39**, 1772–1778.

Osterblad, M., Norrdahl, K., Korpimaki, E. and Huovinen, P. (2001) Antibiotic resistance. How wild are wild mammals? *Nature* **409**, 37–38.

Paterson, D.L. and Bonomo, R.A. (2005) Extended-spectrum beta-lactamases: a clinical update. *Clin Microbiol Rev* **18**, 657–686.

Pillai, S.K., Sakoulas, G., Gold, H.S., Wennersten, C., Eliopoulos, G.M., Moellering, R.C. Jr and Inouye, R.T. (2002) Prevalence of the fsr locus in *Enterococcus faecalis* infections. *J Clin Microbiol* **40**, 2651–2652.

Poeta, P., Costa, D., Sáenz, Y., Klibi, N., Ruiz-Larrea, F., Rodrigues, J. and Torres, C. (2005) Characterization of antibiotic resistance genes and virulence factors in faecal enterococci of wild animals in Portugal. *J Vet Med B Infect Dis Vet Public Health* **52**, 396–402.

Poeta, P., Costa, D., Igrejas, G., Rodrigues, J. and Torres, C. (2007) Phenotypic and genotypic characterization of antimicrobial resistance in faecal enterococci from wild boars (*Sus scrofa*). *Vet Microbiol* **125**, 368–374.

Radhouani, H., Poeta, P., Igrejas, G., Gonçalves, A., Vinué, L. and Torres, C. (2009) Antimicrobial resistance and phylogenetic groups in isolates of *Escherichia coli* from

seagulls at the Berlengas nature reserve. *Vet Rec* **165**, 138–142.

Radhouani, H., Pinto, L., Coelho, C., Gonçalves, A., Sargo, R., Torres, C., Igrejas, G. and Poeta, P. (2010a) Detection of *Escherichia coli* harbouring extended-spectrum {beta}-lactamases of the CTX-M classes in faecal samples of common buzzards (*Buteo buteo*). *J Antimicrob Chemother* **65**, 171–173.

Radhouani, H., Poeta, P., Pinto, L., Miranda, J., Coelho, C., Carvalho, C., Rodrigues, J., Lopez, M. *et al.* (2010b) Proteomic characterization of *van*A-containing *Enterococcus* recovered from seagulls at the Berlengas Natural Reserve, W Portugal. *Proteome Sci* **8**, 48.

Radhouani, H., Poeta, P., Gonçalves, A., Pacheco, R., Sargo, R. and Igrejas, G. (2012) Wild birds as biological indicators of environmental pollution: antimicrobial resistance patterns of *Escherichia coli* and enterococci isolated from common buzzards (*Buteo buteo*). *J Med Microbiol* **61**, 837–843.

Ruiz, J., Simon, K., Horcajada, J.P., Velasco, M., Barranco, M., Roig, G., Moreno-Martinez, A., Martinez, J.A. *et al.* (2002) Differences in virulence factors among clinical isolates of *Escherichia coli* causing cystitis and pyelonephritis in women and prostatitis in men. *J Clin Microbiol* **40**, 4445–4449.

Sáenz, Y., Briñas, L., Dominguez, E., Ruiz, J., Zarazaga, M., Vila, J. and Torres, C. (2004) Mechanisms of resistance in multiple-antibiotic-resistant *Escherichia coli* strains of human, animal, and food origins. *Antimicrob Agents Chemother* **48**, 3996–4001.

Schierack, P., Romer, A., Jores, J., Kaspar, H., Guenther, S., Filter, M., Eichberg, J. and Wieler, L.H. (2009) Isolation and characterization of intestinal *Escherichia coli* clones from wild boars in Germany. *App Environ Microbiol* **75**, 695–702.

Silva, N., Igrejas, G., Figueiredo, N., Gonçalves, A., Radhouani, H., Rodrigues, J. and Poeta, P. (2010) Molecular characterization of antimicrobial resistance in enterococci and *Escherichia coli* isolates from European wild rabbit (*Oryctolagus cuniculus*). *Sci Total Environ* **408**, 4871–4876.

Solberg, O.D., Ajiboye, R.M. and Riley, L.W. (2006) Origin of class 1 and 2 integrons and gene cassettes in a population-based sample of uropathogenic *Escherichia coli*. *J Clin Microbiol* **44**, 1347–1351.

Sørum, H. and Sunde, M. (2001) Resistance to antibiotics in the normal flora of animals. *Vet Res* **32**, 227–241.

Sunde, M. and Norstrom, M. (2005) The genetic background for streptomycin resistance in *Escherichia coli* influences the distribution of MICs. *J Antimicrob Chemother* **56**, 87–90.

Teale, C.J. (2002) Antimicrobial resistance and the food chain. *J App Microbiol* **92** (Suppl.), 85S–89S.