



Detection of antibiotic resistant enterococci and *Escherichia coli* in free range Iberian Lynx (*Lynx pardinus*)

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HIGHLIGHTS

- Antimicrobial resistance in *Enterococcus* spp. and *E. coli* from wild Iberian lynx
- High percentages of resistance to tetracycline, erythromycin and streptomycin
- Half of *E. coli* isolates were ascribed to phylogenetic groups B2 and D.
- High prevalence of resistance genes and virulence determinants in commensal *E. coli*

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ABSTRACT

Thirty fecal samples from wild specimens of Iberian lynx were collected and analyzed for *Enterococcus* spp. (27 isolates) and *Escherichia coli* (18 isolates) recovery. The 45 isolates obtained were tested for antimicrobial resistance, molecular mechanisms of resistance, and presence of virulence genes. Among the enterococci, *Enterococcus faecium* and *Enterococcus hirae* were the most prevalent species (11 isolates each), followed by *Enterococcus faecalis* (5 isolates). High percentages of resistance to tetracycline and erythromycin (33% and 30%, respectively) were detected among enterococcal isolates. The *tet(M)* and/or *tet(L)*, *erm(B)*, *aac(6′)-Ie-aph(2′)-Ia*, *ant(6)-Ia*, or *aph(3′)-IIIa* genes were detected among resistant enterococci. Virulence genes were detected in one *E. faecalis* isolate (*cpd*, *cytB*, and *cytL*) and one *E. hirae* isolate (*cytL*). High percentages of resistance were detected in *E. coli* isolates to tetracycline (33%), streptomycin (28%), nalidixic acid (28%), and sulfamethoxazole–trimethoprim (SXT, 22%). Additionally, the *bla_{TEM}*, *tet(A)*, *aadA*, *cmlA*, and different combinations of *sul* genes were detected among most ampicillin, tetracycline, streptomycin, chloramphenicol and SXT-resistant isolates, respectively. Two isolates contained a class 1 integron with the gene cassette arrays *dfrA1* + *aadA1* and *dfrA12* + *aadA2*. The *E. coli* isolates were ascribed to phylo-groups A (n = 5); B1 (n = 4); B2 (n = 6), and D (n = 3), with the virulence gene *fimA* present in all *E. coli* isolates. This study found resistance genes in wild specimens of Iberian lynx. Thus, it is important to notice that multiresistant bacteria have reached species as rare and completely non-synanthropic as the Iberian lynx. Furthermore, the susceptibility of this endangered species to bacterial infection may be affected by the presence of these virulence and resistance genes.

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1. Introduction

The intensive use of antimicrobial agents in human and animals has caused a selective pressure that combined with several mechanisms for bacterial genetic transfer, resulted in the selection and dissemination of resistant bacteria (Schwarz et al., 2001). Commensal bacteria can constitute a reservoir of resistance genes that might be transmitted

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to other commensal or pathogenic bacteria (Gonçalves et al., 2012; Silva et al., 2010). *Enterococcus* spp. and *Escherichia coli* are common inhabitants of the gastrointestinal tracts of human and animals that can easily acquire and transfer resistance genes (Sørum and Sunde, 2001). Consequently, these commensal bacteria can be used as indicators of changes in antimicrobial resistance (Silva et al., 2010). Additionally, these bacteria are associated with both community- and hospital-acquired infections (Paterson and Bonomo, 2005; Ruiz et al., 2002).

The problem of antimicrobial resistant enterococci and *E. coli* is not only restricted to the clinical setting but also to other environments such as the intestinal tract of healthy humans, food-producing animals and wild animals (Aarestrup et al., 2000; Radhouani et al., 2012; Sáenz et al., 2004). Recent studies in wild animals confirm that these animals might act as reservoirs of genetic elements containing antimicrobial resistance genes that could be spread across the environment. Nevertheless, the flow of resistant microorganisms and resistant genes from the environment and humans to wildlife, or vice-versa, remains poorly comprehended (Silva et al., 2010).

The Iberian lynx (*Lynx pardinus*) is a critically endangered species (IUCN, 2012). Total population for this species is estimated to a maximum of 150 adults, surviving in two breeding population on southern Spain (Doñana and Sierra Morena). The Iberian lynx is a rigorous food and habitat specialist. The habitat dependence (Mediterranean scrublands) is directly correlated with its specialization on European rabbit (*Oryctolagus cuniculus*), composing 80–99% of their diet (Ferrerías et al., 2010). The major threats to the Iberian lynx population are the decline in rabbit populations, habitat destruction, and road kills. Associated to these threats additionally, there are reports of demographic bottlenecks and loss of genetic diversity (Ferrerías et al., 2010). Lastly, their predatory and traveling habits might expose this species to food remains or fecal material from farm animals or even from humans that carry antimicrobial-resistant bacteria.

The purpose of this study was to determine the prevalence of antimicrobial resistance, the mechanisms of resistance, and the detection of virulence genes in fecal *Enterococcus* spp. and *E. coli* isolates from wild specimens of Iberian lynx. Furthermore, studying the antimicrobial resistance in intestinal enterococci and *E. coli* among Iberian lynx, an endangered species, is of capital importance, since the susceptibility of this endangered species to bacterial infection may be affected by the presence of virulence and resistance genes.

2. Material and methods

2.1. Fecal samples and bacterial isolates

Antimicrobial resistance was studied in enterococci and *E. coli* isolates recovered from 30 fresh fecal samples obtained from wild specimens of Iberian lynx (*L. pardinus*) between 2008 and 2010. Each fecal sample analyzed belonged to a different animal. Sample collection was obtained in Doñana National Park and in Sierra Morena, South Spain, and took place during procedures and took place during procedures or handling by conservation program personnel, e.g. placement of radio tracking systems.

For enterococci recovery, samples were seeded in Slanetz–Bartley agar (Oxoid Limited, Basingstoke, UK) plates. One colony 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 *Enterococcus faecalis*, *Enterococcus faecium*, *Enterococcus casseliflavus* (Dutka-Malen et al., 1995a, 1995b), *Enterococcus gallinarum* (Miele et al., 1995), *Enterococcus hirae*, and *Enterococcus durans* species (Arias et al., 2006).

For *E. coli* isolation, samples were seeded in Levine agar (Oxoid) 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).

2.2. Antimicrobial susceptibility testing

Antimicrobial susceptibility was performed by the disk diffusion method (CLSI, 2011). The enterococci susceptibility to 11 antimicrobial agents (vancomycin; teicoplanin; ampicillin; chloramphenicol; tetracycline; erythromycin; quinupristin–dalfopristin; ciprofloxacin; streptomycin; gentamicin; and kanamycin) was tested. High-level resistance (HLR) was evaluated for aminoglycosides. *E. faecalis* ATCC 29212 and *Staphylococcus aureus* ATCC 25923 strains were used for quality control.

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

2.3. Assay of gelatinase and beta-hemolytic activities in enterococci

Gelatinase is an extracellular metalloendopeptidase that can hydrolyse gelatin, collagen, and other bioactive peptides which suggests that it may take part in inflammatory processes. Similarly, hemolysin, commonly referred to as cytolysin, has been demonstrated to contribute to the severity of enterococcal disease (Archimbaud et al., 2002).

Evaluation of gelatinase and hemolysin production was performed in enterococci as previously reported (López et al., 2009).

2.4. Characterization of antimicrobial resistance mechanisms and detection of virulence genes

The presence of the resistance genes [*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* and the Tn916- and Tn5397-specific sequences] were analyzed by PCR in resistant enterococci using previously reported primers and conditions (Table 1). The presence of genes encoding different virulence factors (*gelE*, *agg*, *ace*, *cpd*, *fsr*, *esp*, *hyl* and *cyl* LLSABM) was also studied by PCR (Eaton and Gasson, 2001; Klare et al., 2005; Poeta et al., 2005).

Likewise, the presence of resistance genes [*bla*_{TEM}, *bla*_{SHV}, *tet*(A), *tet*(B), *aadA*, *strA*–*strB*, *aac*(3)-II, *aac*(3)-IV, *sulI*, *sul2*, *sul3*, *cmlA* and *floR*] were studied by PCR in the resistant *E. coli* isolates (Table 1). Additionally, the *intl1* and *intl2* genes, encoding classes 1 and 2 integrases, respectively, and their variable regions were also analyzed by PCR and sequencing among our *E. coli* isolates (Sáenz et al., 2004).

Lastly, the phylogenetic groups and virulence determinants often found in pathogenic *E. coli* (*stx1*–*stx2*, *fimA*, *papG* allele III, *cnf1*, *papC* and *aer*) were investigated (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.

3. Results and discussion

3.1. Fecal samples and bacterial isolates

Enterococci were recovered from 27 fecal samples and *E. coli* in 18 of the 30 tested fecal samples. In our study, *E. faecium* and *E. hirae* were the most prevalent species (11 isolates each), followed by *E. faecalis* (5 isolates). These results are similar to results obtained in wild boars in Portugal (Poeta et al., 2007), but different from isolates recovered

Table 1
Genetic elements and resistance genes investigated.

Resistance genes/ Genetic elements	Mechanism of resistance/ Mobile elements	Reference
<i>erm(A)</i> , <i>erm(B)</i>	Target site modification	Sutcliffe et al., 1996
<i>tet(M)</i>	Ribosomal protection	Aarestrup et al., 2000
<i>tet(L)</i> , <i>tet(K)</i>	Efflux	Aarestrup et al., 2000
<i>aph(3')-IIIa</i> , <i>aac(6')-Ie-aph(2'')-Ia</i>	Aminoglycoside-modifying enzymes	van de Klundert and Vliegthart, 1993
<i>ant(6)-Ia</i>	Aminoglycoside-modifying enzyme	del Campo et al., 2000
<i>catA</i>	Chloramphenicol acetyltransferase	Aarestrup et al., 2000
<i>vatD</i> , <i>vatE</i>	Virginiamycin acetyltransferases	Robredo et al., 2000
<i>tn916-</i> , <i>tn5397-</i>	Conjugative transposons	Agero et al., 2006
<i>bla_{TEM}</i>	Beta-lactamase enzyme	Belaouaj et al., 1994
<i>bla_{SHV}</i>	Beta-lactamase enzyme	Pitout et al., 1998
<i>tet(A)</i> , <i>tet(B)</i>	Efflux	Guardabassi et al., 2000
<i>aadA</i>	Target site modification	Madsen et al., 2000
<i>strA-strB</i>	Target site modification	Madsen et al., 2000
<i>aac(3)-II</i> , <i>aac(3)-IV</i>	Gentamicin acetyltransferases	van de Klundert and Vliegthart, 1993
<i>sul1</i> , <i>sul2</i> , <i>sul3</i>	Target site modification	Sáenz et al., 2004
<i>cmlA</i>	Efflux	Sáenz et al., 2004
<i>floR</i>	Efflux	Ng et al., 1999
<i>int11</i> , <i>int12</i>	Integrases	Mazel et al., 2000

from other wild animals where *E. faecium* and *E. faecalis* are usually the prevalent enterococcal species (Poeta et al., 2005; Radhouani et al., 2011; Silva et al., 2010).

3.2. Antimicrobial susceptibility, resistance mechanisms and virulence genes in enterococci

Sixteen of the 27 enterococci isolates (59%) showed susceptibility to all the antimicrobial agents tested (8 *E. hirae*, 6 *E. faecium*, and 2 *E. faecalis*). No ampicillin-, chloramphenicol, vancomycin- or teicoplanin-resistant enterococcal isolates were found in this study, and quinupristin–

dalfopristin resistance was detected only in one *E. faecalis* isolate. On the other hand, high frequency of resistance was identified for tetracycline (33%) and erythromycin (30%) among our isolates, and low frequency for ciprofloxacin (7%), streptomycin (7%), kanamycin (7%), and gentamicin (4%). Similar results were previously described in a study performed with wild rabbits in Northern Portugal (Silva et al., 2010).

Table 2 shows the antimicrobial resistance genes detected by PCR in the enterococci and *E. coli* isolates. The *tet(M)* and *tet(L)* genes, encoding a ribosomal protection mechanism and an efflux-mediated mechanism for tetracycline resistance, respectively, are frequently reported in enterococcal isolates (Radhouani et al., 2012; Silva et al., 2010). In our study, most of the tetracycline-resistant isolates contained both genes. Additionally, 4 of our 7 *tet(M)*-positive enterococci carried specific genes of Tn916/Tn1545 or Tn5397 transposons. Similarly, 6 of the 7 isolates that showed resistance to erythromycin presented the *erm(B)* gene. The *aac(6')-aph(2'')* (1 isolate), *ant(6)-Ia* (1 isolate) and *aph(3')-IIIa* (2 isolates) genes detected in our HLR-aminoglycoside enterococci have been previously detected in wild animals (Poeta et al., 2005, 2007; Radhouani et al., 2012; Silva et al., 2010).

It is of interest to underline the low prevalence of virulence determinants among our enterococci. Two isolates harbored *cyl* genes (*cylI*, *cylB* and *cylL*); however, beta-hemolytic activity was not observed among our isolates, as they did not contain the whole *cyl* operon. Also, none of our isolates showed gelatinase activity. One *E. faecalis* isolate harbored a sex pheromone determinant (*cpd*).

3.3. Antimicrobial susceptibility, resistance mechanisms and virulence genes in *E. coli* isolates

Half of the *E. coli* isolates were susceptible to all tested antimicrobial agents. No resistances were detected to ceftioxin, cefotaxime, ceftazidime, aztreonam, imipenem, and gentamicin. High percentages of resistance were observed to tetracycline (33%), streptomycin (28%), nalidixic acid (28%), and sulfamethoxazole–trimethoprim (22%).

Table 2
Resistance genes detected in antimicrobial resistant enterococci and *Escherichia coli* isolates obtained from fecal samples of Iberian lynx.

Bacteria	Antimicrobial agent	Number of resistant isolates	Genes detected by PCR	
			Resistance genes and genetic elements	Number of isolates
Enterococci (n = 27)				
<i>Enterococcus faecium</i> (n = 11)	Tetracycline (30 µg)	3	<i>tet(L)</i>	1
			<i>tet(M)</i> + <i>tet(L)</i>	1
	Erythromycin (15 µg)	4	<i>tet(M)</i> + <i>tet(L)</i> + <i>tn916</i>	1
			<i>erm(B)</i>	4
<i>Enterococcus hirae</i> (n = 11)	Tetracycline (30 µg)	3	–	–
			<i>tet(M)</i> + <i>tet(L)</i>	1
			<i>tet(M)</i> + <i>tet(L)</i> + <i>tn916</i>	1
<i>Enterococcus faecalis</i> (n = 5)	Tetracycline (30 µg)	3	<i>tet(M)</i> + <i>tet(L)</i> + <i>tn5397</i>	1
			<i>tet(L)</i>	1
			<i>tet(M)</i> + <i>tet(L)</i>	1
	Erythromycin (15 µg)	3	<i>tet(M)</i> + <i>tet(L)</i> + <i>tn916</i>	1
			<i>erm(B)</i>	2
			–	–
			<i>ant(6)-Ia</i>	1
<i>E. coli</i> (n = 18)	Ciprofloxacin (5 µg)	1	<i>aac(6')-Ie-aph(2'')-Ia</i>	1
	Streptomycin (300 µg)	2	<i>aph(3')-IIIa</i>	2
	Gentamicin (120 µg)	1	<i>aph(3')-IIIa</i>	1
	Kanamycin (120 µg)	2	<i>bla_{TEM}</i>	1
	Ampicillin (10 µg)	1	<i>tet(A)</i>	2
	Tetracycline (30 µg)	6	–	–
	Amikacin (30 µg)	1	–	–
	Tobramycin (10 µg)	1	–	–
	Streptomycin (10 µg)	5	<i>aadA</i>	3
	Nalidixic acid (30 µg)	5	–	–
Ciprofloxacin (5 µg)	4	–	–	
Sulfamethoxazole · Trimethoprim (25 µg)	4	<i>sul1</i>	1	
		<i>sul3</i>	2	
		<i>sul1</i> + <i>sul3</i>	1	
Chloramphenicol (30 µg)	2	<i>cmlA</i>	2	

Similar results were detected in other studies performed with fecal *E. coli* isolates from wild animals (Costa et al., 2008; Radhouani et al., 2009). Still, higher levels of resistance have been previously detected in *E. coli* isolates from wild birds in Portugal (Radhouani et al., 2012).

The classical TEM enzymes are the predominant plasmid-mediated β -lactamases of Gram-negative bacteria and have been previously found among ampicillin-resistant *E. coli* isolates from different origins (Briñas et al., 2002). In our study the *bla*_{TEM} gene was detected in the ampicillin-resistant *E. coli* isolate. The mechanism of tetracycline resistance through active efflux encoded by the *tet(A)* gene was detected in 2 tetracycline-resistant *E. coli* isolates and is the most frequent one reported among isolates from different origins (Radhouani et al., 2009; Sáenz et al., 2004). Moreover, the *cmlA* gene was identified in the 2 chloramphenicol-resistant isolates. The presence of the *aadA* gene was shown in 3 of 5 streptomycin-resistant *E. coli* isolates. Different combinations of *sul1*, and/or *sul3* genes were identified in the sulfamethoxazole–trimethoprim-resistant *E. coli* isolates (Table 2).

The presence of class 1 integrons was detected in 2 isolates containing the gene cassette arrays *dfrA1* + *aadA1* and *dfrA12* + *aadA2*. The presence of integrons among our commensal *E. coli* isolates is a cause for concern. This genetic structure might enhance the dissemination of resistance genes to other bacteria, by mobile elements such as plasmids and transposons. Similar structures have been reported in *E. coli* isolates from other wild animals in Portugal (Radhouani et al., 2009).

All *E. coli* isolates harbored at least one virulence determinant. The *fimA* gene was detected in 15 isolates, the *papC*, *papGIII* and *fimA* genes in 2 isolates, the *aer*, *cnf1* and *fimA* genes in 1 isolate. The predominance of *fimA* (type 1 fimbriae) virulence gene is in accordance with a previous work conducted with *E. coli* isolates from wild birds (*Buteo buteo*) in Portugal (Radhouani et al., 2012). Previous studies have shown that the presence of virulence genes in commensal isolates might be part of a survival mechanism that ensures greater genetic diversity, increasing their survival capability in the host animal, and not as a process of active virulence gene acquisition (Chapman et al., 2006). The distribution of the phylogenetic groups was: 5 isolates ascribed to the phylogenetic group A, 4 isolates to B1, 6 isolates to B2 and 3 isolates into the phylogroup D. A similar ratio has been previously reported among intestinal *E. coli* clones from wild boars in Germany (Schierack et al., 2009). Nonetheless, this phylogenetic distribution was unexpected, as former studies performed in Portugal within fecal *E. coli* isolates from wild animals detected a higher prevalence of strains belonging to phylogroups A and B1 (Radhouani et al., 2009, 2012).

The Iberian lynx population lives in an ecosystem where contact with humans is infrequent (dense scrubland for shelter and open pasture for hunting rabbits, with rare or none human dwellings and occasional livestock contact) and without apparent antimicrobial resistance selection pressures. Nonetheless, the extensive ranges of these animals [male ranges: 9.3 ± 1.2 km²; female ranges: 7.8 ± 2.0 km²; (Ferrerías et al., 2010)] could expose this species to food remains or fecal material from farm animals or even from humans that carry resistant strains (Aarestrup et al., 2008). Additionally, there are several examples of transfer of resistant bacteria between animals, and from animals to man via the food chain (Aarestrup et al., 2008; Teale, 2002). Therefore, Iberian lynx might be contaminated through the food chain, as the presence of resistant *E. coli* strains has been previously detected in their predominant prey (Silva et al., 2010). The (re)introduction of captive animals carrying resistant bacteria, either due to contamination via food chain or through the selection pressure caused by the use of antimicrobial agents in management protocols, could also explain the obtained results.

4. Conclusion

This study shows wild specimens of Iberian lynx carrying antimicrobial resistance bacteria. Thus, it is important to notice that

multiresistant bacteria have reached species as rare and completely non-synanthropic as the Iberian lynx. The same genes found in bacteria from environment and human origins encode the antimicrobial resistance mechanisms found in this study, indicating the possible circulation of bacteria and resistance genes between animal, environment and human ecosystems. On the other hand, the Iberian lynx is a critically endangered species; the virulence encoding genes found in these *E. coli* strains are genome located, thus, it is unlikely that they would be individually transmitted to other pathogenic strains (Hacker and Kaper, 2000). Nonetheless, the B2 strains are closely related with extra-intestinal virulence and the presence of virulence determinants in these strains is a cause of concern. In case of infection the virulence genes can be expressed, representing an increased health risk. Increased risk of infection and therapeutic failure due to virulence/resistance genes can consequently represent a serious setback for the species conservation program.

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