Short Communication

Wild boars as reservoirs of extended-spectrum beta-lactamase (ESBL) producing *Escherichia coli* of different phylogenetic groups

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ESBL-producing *E. coli* isolates have been isolated from eight of seventy seven faecal samples (10.4%) of wild boars in Portugal. The ESBL types identified by PCR and sequencing were $bla_{\text{CTX-M-1}}$ (6 isolates) and $bla_{\text{CTX-M-1}}$ + $bla_{\text{TEM1-b}}$ (2 isolates). Further resistance genes detected included *tet*(A) or *tet*(B) (in three tetracycline-resistant isolates), *aad*A (in three streptomycin-resistant isolates), *cml*A (in one chloramphenicol-resistant isolate), *sul*1 and/or *sul*2 and/or *sul*3 (in all sulfonamide-resistant isolates). The *intI*1 gene encoding class 1 integrase was detected in all ESBL-producing *E. coli* isolates. One isolate also carried the *intI*2 gene, encoding class 2 integrase. The ESBL-producing *E. coli* isolates could be assigned to phylogenetic groups B1 (3 isolates), B2 (3 isolates) or A (2 isolates). Amino acid change in GyrA protein (Ser83Leu or Asp87Tyr) was detected in three nalidixic acid-resistant and ciprofloxacin-susceptible isolates. Two amino acid changes in GyrA (Ser83Leu + Asp87Asn) and one in ParC (Ser80Ile) were identified in two nalidixic acid- and ciprofloxacin-resistant isolates. As evidenced by this study wild boars could be a reservoir of antimicrobial resistance genes.

Keywords: Antibiotic resistance / Wild boars / ESBL-producing *E. coli* / β-lactamases / Phylogenetic groups

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Introduction

β-lactams are among the most clinically important antimicrobial agents in both human and veterinary medicine. However, resistance to these antibiotics has been increasingly observed in bacteria, including those of animal origin. The mechanisms of β-lactam resistance include inaccessibility of the drugs to their target, target alterations and/or inactivation of the drugs by β-lactamases. The genes encoding these enzymes often coexist with other antimicrobial resistance determinants and can also be associated with transposons/integrons, increasing the potential enrichment of multidrug resistant bacteria by multiple antimicrobial agents as well as dissemination of the resistance determinants among bacterial species. Characterization of β-lactam-resistant animal-derived bacteria warrants further investigation of the type and distribution of β-lactamases in bacteria of animal origin and their potential impact on human medicine [1].

Extended-spectrum beta-lactamases (ESBLs) derived from the TEM-1 beta-lactamase were first identified in the USA in outbreak strains of *Klebsiella pneumoniae* in

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the middle to late 1980s, together with the SHV-5 ESBL [2]. The CTX-M family of beta-lactamases has recently emerged and has been associated with *Escherichia coli* strains of human and animal origin in Europe. These strains have been isolated from farm animals, food, pets and environmental samples in different European countries [3] and have caused a main apprehension in diverse countries being frequently implicated in human infections [4]. Antibiotic resistance of ESBL-positive faecal *Escherichia coli* isolates of wild animals has been studied by our research group in Portugal [5, 6] allowing the scientific community for a better understanding of the problem of antibiotic resistance in the wildlife and the consequences of this aspect in different ecosystems.

The objective of our study was to detect ESBL-containing *E. coli* isolates in faecal samples of wild boars in Portugal, to characterize the phylogenetic groups of these isolates and the type of ESBL encoding genes they harbor.

Material and methods

Faecal samples and isolation of bacteria

Seventy seven faecal samples obtained from wild boars were collected during two consecutively years (from December of 2005 to February of 2006 and from November 2006 to February of 2007) in North of Portugal during hunts of wild boars organized by different corporations of hunters. This kind of hunting is organized all of the years during a short period of time having like main objective the ecological control of the animal population increase and is supervised by the forest brigade of the General Corporation of Forestry Resources, and this programme is under the Agriculture and Fishery Ministry of Portugal. Faecal samples were streaked on Levine agar (Oxoid Limited, Basingstoke, Hampshire, United Kingdom) supplemented with cefotaxime (2 mg ml^{-1}). After 24 h of incubation at 37 °C colonies typical for *E. coli* were selected, purified and identified by standard bacteriological methods (i.e. Gram staining, catalase, oxidase, indol, methyl-red/Voges-Proskauer, citrate and urease), and by using the API 20E biochemical identification system (bioMérieux, La Balme Les Grottes, France). One *E. coli* isolate per faecal sample was retained for further studies.

Antimicrobial susceptibility testing

All recovered *E. coli* isolates were tested by the agar disc-diffusion method [7]. Antimicrobials tested: ampicillin (10 μg), amoxicillin-clavulanic acid (AMC) (20 μg + 10 μg), cefoxitin (30 μg), ceftazidime (CAZ) (30 μg), cefotaxime (CTX) (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) (1.25 μg + 23.75 μg), tetracycline (30 μg) and chloramphenicol (30 μg). *E. coli* ATCC 25922 was used as a quality control strain. Broad-spectrum cephalosporin-resistant isolates were selected for further studies. Agar plate-based screening for ESBL production was carried out by the double-disc diffusion test [using CTX (30 μg), CAZ (30 μg) and AMC (20 μg + 10 μg)] [7].

Characterization of antimicrobial resistance genes

DNA of *E. coli* isolates was extracted by the boiling method. The presence of genes encoding TEM, SHV, OXA and CTX-M type β-lactamases was verified by specific PCRs [6] and sequencing. DNA sequences were compared with those included in the GenBank database as well as with those deposited at the website http://www.lahey.org/Studies/, in order to determine the specific type of β-lactamase gene. The following resistance genes were also studied by PCR [6]: *tet*(A) and *tet*(B) in tetracycline-resistant isolates, *aad*A in streptomycin-resistant isolates, *cml*A in chloramphenicol-resistant isolates, and *sul*1, *sul*2 and *sul*3 in SXT-resistant isolates. The presence of the *intI*1 and *intI*2 genes, encoding class 1 and 2 integrases, respectively was studied by PCR. Positive and negative controls were from the strain collection of the University of Trás-os-Montes and Alto Douro, Portugal.

Characterization of the mechanisms of quinolone resistance

The quinolone resistance-determining region (QRDR) of the *gyr*A gene, as well as the analogous region of the *par*C gene, was amplified by PCR in all quinolone-resistant *E. coli* isolates [8]. Amplified fragments were purified (Qiagen), and both strands were sequenced in an Applied Biosystem 3730 automatic sequencer (Genome Express, France), using the same primer set as for PCR. Sequences obtained were compared with those previously reported for *gyr*A (GenBank accession number X06373) and *par*C genes (M58408 with the modification included in L22025).

Detection of phylogenetic groups: The ESBL-positive isolates were assigned to one of the four main phylogenetic groups A, B1, B2 and D, following the PCR strategy previously published based in the presence or absence of *chu*A, *yja*A or *tsp*E4.C2 genes [9].

Results and discussion

From 77 faecal samples, 8 (10.4%) tested positive for *E. coli* on Levine-CTX agar. The obtained *E. coli* isolates (n = 8) showed intermediate or full resistance to CTX and exhibited a positive screening test for ESBL production, being three of them also resistant to aztreonam. Eight, 5, 4, 3 and one of the ESBL-positive isolates showed resistance to SXT, nalidixic acid, tetracycline, streptomycin and chloramphenicol, respectively (Table 1). The beta-lactamase genes detected in these isolates were the following ones: $bla_{\text{CTX-M-1}}$ in 6 isolates and $bla_{\text{CTX-M-1}}$ + $bla_{\text{TEM-1}}$ in two isolates. The bla_{SHV} and bla_{OXA} genes were not found in any isolate. A variety of resistance genes were observed among our ESBL-positive strains: *tet*(A) and *tet*(A) + *tet*(B) (in two and one tetracycline-resistant strains, respectively), and *aad*A (in three streptomycin-resistant strains), *cml*A (in one chloramphenicol isolate). Two SXT-resistant isolates harboured simultaneously *sul*1 and *sul*2 genes; one isolate carried both *sul*2 and *sul*3 genes; four isolates harboured only the *sul*3 gene and two isolates only the *sul*2 gene. The presence of class 1 integrons was demonstrated in all of the cephalosporin resistant strains, and one of them also carried the *intI*2 gene (Table 1).

The antimicrobial susceptibility patterns of the wild boar *E. coli* strains indicated the presence of ESBLs responsible for resistance to broad-spectrum cephalosporins and in some cases also to aztreonam. Significantly, the isolates were more resistant to cefotaxime and aztreonam than to ceftazidime, suggesting that they were CTX-M producers. So far, more than 65 CTX-M-type β-lactamases have been identified in clinical isolates but mostly in enterobacterial species such as *E. coli*. In some countries, CTX-M-type enzymes are the ESBLs most frequently isolated from *E. coli* strains. They have been involved in several outbreaks in long-term care facilities and are also becoming a problem in the community [10]. The emergence of resistance is the result of the resistance determinants and resistant bacteria that can spread between different ecosystems and selected by widespread use of the same antibiotics in animals and humans. An in vivo experiment was performed to analyze the effects of veterinary β-lactam drugs on the selection and persistence of ESBL-producing *E. coli* in the intestinal flora of pigs and the study provides evidence that the cephalosporins used in pig production select for CTX-M-producing *E. coli* strains [11].

In our study, all ESBL-containing *E. coli* isolates carried the $bla_{\text{CTX-M-1}}$ gene. The $bla_{\text{CTX-M-1}}$ gene has been previously reported in *E. coli* isolates of human and animal origins in different countries [3]. This gene was also referred by our research group from healthy pets and wild animals [5, 6, 12]. In Spain, the presence of ESBL and plasmidic class C beta-lactamase-producing *Enterobacteriaceae* were studied in animals and the majority of the strains (73%) from pig farms had CTX-M-1 β-lactamases [13]. Additionally there seems to be a certain association between the CTX-M variant detected in *E. coli* strains and the animal species of origin. CTX-M-14 is mainly found in birds, CTX-M-1 in pigs and CTX-M-2 in cattle [14]. On the other hand, this study revealed the presence of the $bla_{\text{TEM-1b}}$ gene in two isolates that also carried the $bla_{\text{CTX-M-1}}$ gene. This association is frequent and has already been described in the literature [15].

Table 1. Characteristics of beta-lactamase-producing *Escherichia coli* from wild boars.

Isolate	Antibiotic resistance to beta-lactams[a]	Antibiotic resistance to other antibiotics[b]	Beta-lactamases	Resistance genes	Amino acid changes in:		Genetic elements[c]	Phylogenetic group
					GyrA	ParC		
J27	AMP-ATM-CTX	NAL-TET-STR-SXT	CTX-M-1 + TEM1-b	*tet*A + *aad*A + *sul*2	Ser83Leu	wild	*intI*1 + *intI*2	A
J31	AMP-CTX	STR-SXT	CTX-M-1	*aad*A + *sul*2 + *sul*3	–	–	*IntI*1	B2
J33	AMP-CTX	NAL-STR-CHL-SXT	CTX-M-1	*aad*A + *cml*A + *sul*1 + *sul*2	Asp87Asn	wild	*IntI*1	B1
J42	AMP-ATM-CTX	SXT	CTX-M-1	*sul*3	–	–	*IntI*1	B1
J51	AMP-CTX	NAL-SXT	CTX-M-1	*sul*3	Ser83Leu	wild	*IntI*1	B2
J64	AMP-ATM-CTX	NAL-CIP-TET- SXT	CTX-M-1 + TEM1-b	*tet*A + *sul*2	Ser83Leu + Asp87Asn	Sr80Ile	*IntI*1	B1
J69	AMP-CTX	TET-SXT	CTX-M-1	*sul*3	–	–	*IntI*1	A
J71	AMP-CTX	NAL-CIP-TET-SXT	CTX-M-1	*tet*A + *tet*B + *sul*3	Ser83Leu + Asp87Asn	Sr80Ile	*IntI*1	B2

[a] AMP, ampicillin; ATM, aztreonam; CTX, cefotaxime
[b] CIP, ciprofloxacin; NAL, nalidixic acid; TET, tetracycline; STR, streptomycin; CHL, chloramphenicol; SXT, sulfamethoxazole/trimethoprim
[c] Integron genes detected

The *gyr*A and *par*C genes were amplified and sequenced in all five quinolone-resistant isolates and the deduced amino acid changes detected in GyrA and ParC proteins are shown in Table 1. Two amino acid changes in GyrA (Ser83Leu + Asp87Asn) and one in ParC (Ser80Ile) were identified in the two nalidixic acid- and ciprofloxacin-resistant isolates found in this study and only one amino acid change in GyrA (Ser83Leu or Asp87Tyr) was found in the three nalidixic acid-resistant and ciprofloxacin-susceptible isolates. It has been observed a correlation in the type and number of amino acid changes in GyrA and ParC proteins with the level of resistance to nalidixic acid and ciprofloxacin. This observation has been previously detected both in human and animal *E. coli* isolates [8].

ESBL-positive *E. coli* isolates corresponded to phylogenetic groups B1 (3 isolates), B2 (3 isolates) or A (2 isolates). Phylogenetic studies have shown that *E. coli* strains fall into four main phylogenetic groups A, B1, B2, and D and this analysis provide a useful tool to investigate the evolutionary origins of pathogenic *E. coli* strains [9]. To our knowledge, there is only one report that analyzes *E. coli* clones in wild boars and in this study although the occurrence of certain virulence genes and phylogenetic groups, all clones were susceptible to all antimicrobial agents tested [16]. The presence of three CTX-M-1 containing *E. coli* isolates of the B2 phylogenetic group is of interest because this phylogroup has been associated in previous reports with more virulent isolates [9].

Concluding remarks

It is of interest to underline the high prevalence of ESBL-producing *E. coli* of the CTX-M-1 class detected in this study in wild animals such as wild boars. As previously indicated there seems to be a certain association between the CTX-M variant detected in *E. coli* strains and the animal species of origin being the CTX-M-1 class frequently found in pigs. This beta-lactamase was also detected in *E. coli* strains classified in three different phylogroups being the B2 associated with human extraintestinal virulence factors. On the other hand wild boars are omnivorous and will travel large distances for searching food and territory. As they inhabit near human and other animal populations, the possibility that these animals eat rests of food of humans can not be excluded at all. It seems that ESBLs are widely distributed in bacteria of different ecosystems. Monitoring ESBL-producing *E. coli* strains in wild animals and specifically in wild boars, should be continued in order to investigate their evolution and to analyze the factors that contribute to their selection and spread.

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