Research article

Novel Sequence Types Of Extended-Spectrum and Acquired AmpC Beta-Lactamase Producing *Escherichia coli* and *Escherichia* Clade V Isolated from Wild Mammals

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ABSTRACT

The closer contact with wildlife due to the growing human population and the destruction of natural habitats emphasizes the need of gaining insight into the role of animals as source of antimicrobial resistance. Here, we aim at characterizing the antimicrobial resistance genes and phylogenetic distribution of commensal *E. coli* from 62 wild mammals. Isolates exhibiting resistance to ≥1 antibiotic were detected in 25.8% of the animals and 6.4% carried an ESBL/AmpC-producing *E. coli*. Genetic mechanisms involved in third-generation cephalosporin resistance were: i) hyperproduction of chromosomal AmpC (hedgehog), ii) production of acquired CMY-2 β-lactamase (hedgehog), iii) production of SHV-12 and CTX-M-14 ESBLs (n=2, mink and roe-deer). ESBL genes were transferable by conjugation and $bla_{\text{CMY-2}}$ was mobilized by a 95kb IncI1 plasmid. The distribution of the phylogenetic groups in the *E. coli* collection studied was B1 (44.6%), B2 (24.6%), E (15.4%), A (4.6%) and F (3.1%). Five isolates (7.7%) were cryptic *Escherichia* clades (clade IV, 4 mice; clade V, 1 mink). ESBL/AmpC-*E. coli* isolates showed different STs: ST1128/B1, ST4564/B1 (new), ST4996/B1 (new), and a non registered ST. This study contributes to better understand the *E. coli* population and antimicrobial resistance flow in wildlife and reports new AmpC-*E. coli* sequence types and a first described ESBL-producing *Escherichia* clade V isolate.

INTRODUCTION

Escherichia coli is an ubiquitous gram-negative bacillus that colonize the gastrointestinal (GI) tract of warm-blooded animals, including humans, and is widely distributed in the environment (water, soil and sediments) (Savagoau 1983). This species includes also pathogenic strains responsible for a variety of intestinal and extraintestinal diseases (neonatal meningitis, haemolytic uraemic syndrome, among others). It belongs to the Enterobacteriaceae family, whose members are becoming increasingly resistant to clinically critical antimicrobials such as fluoroquinolones and third/fourth generation cephalosporins, as shown in EARS-Net database (http://atlas.ecdc.europa.eu/public/index.aspx). Resistance to this last antimicrobial group is mainly mediated by the production of

extended-spectrum beta-lactamases (ESBL) or AmpC beta-lactamases, which are enzymes able to efficiently hydrolyze the beta-lactam ring, inactivating the antibiotic.

The extensive use, and misuse, of antibiotics in both human and veterinary medicine has led to the selection and global spread of resistant clones and gene-transfer elements (e.g. plasmids) carrying resistance determinants. These antimicrobial agents and antimicrobial resistant bacteria from hospital, farming and agricultural sources can be released from animal manure or wastewater and persist for a long time in the environment (Martínez 2009). The closer contact between wildlife and humans due to the growing human population and the destruction of natural habitats is increasing the opportunities for the transmission of antimicrobial resistance. Moreover, the high rates of antimicrobial resistant (AMR) bacteria detected in wild birds have led researchers to postulate them as sentinels, reservoirs and potential spreaders of antimicrobial resistance (Bonnedahl and Järhult 2014; Alcalá *et al.* 2016). Additionally, it is also important to remark that different natural conditions (e.g. heavy-metal rich habitats) and even antibiotic compounds produced by microbial communities can also exert a selective pressure favoring the emergence of resistance in the environment (Martínez 2009; Radhouani *et al.* 2014). Thus, there is a potential for wildlife to carry new or emerging genetic lineages associated with antimicrobial resistance.

E. coli is recognized as a reliable indicator to trace the evolution of antimicrobial resistance and is one of the most used prokaryotic model organisms in molecular biology and genetics. To understand the flow of AMR *E. coli* through different ecosystems is essential to consider the population structure of *E. coli*. The growing genomic data led to re-define the *E. coli* phylogenetic structure in seven main phylogroups (A, B1, B2, C, D, E and F) and revealed the existence of five distinct cryptic lineages (clade I to V), which include strains phenotypically indistinguishable but genetically divergent from *E. coli* (Clermont *et al.* 2011; Clermont *et al.* 2013). These cryptic *Escherichia* clades have been recovered from environmental samples (Walk *et al.* 2009; Clermont *et al.* 2011; Luo *et al.* 2011; Berthe *et al.* 2013; Vignaroli *et al.* 2014), birds and non-human mammals feces (Walk *et al.* 2009; Clermont *et al.* 2011) more frequently than from human GI tract, leading to the conclusion that they likely do not pose a risk to public health (Luo *et al.* 2011). However, data on cryptic *Escherichia* clades are still scarce.

In this paper, we aim at identifying and characterizing the antimicrobial resistant mechanisms and phylogenetic distribution of commensal *E. coli* from wild mammals. Relevant isolates, such as those producing ESBL or acquired AmpC beta-lactamases and cryptic *Escherichia* clade strains, were deeper molecular analyzed.

MATERIALS AND METHODS

Sampling and bacterial identification

Fecal samples from 62 different wild-mammals were collected between 2013-2015 in two different regions from northern Spain (Aragón and La Rioja). The animals included in this study belonged to the following species: 12 rodents (11 mice – *Apodemus sylvaticus* - and 1 rat – *Rattus rattus* -), 11 wild boars (*Sus scrofa*), 11 rabbits (*Oryctolagus cuniculus*), 8 deer (7 red deer - *Cervus elaphus* - and 1 roe deer – *Capreolus capreolus* -), 5 minks (*Mustela lutreola*), 4 hedgehogs (*Erinaceus europaeus*), 3 mouflons (*Ovis musimon*), 2 foxes (*Vulpes vulpes*), 2 martens (*Martes martes*), 2 badgers (*Meles meles)*, 1 otter (*Lutra lutra*) and 1 genet (*Genetta genetta*)**.** Fecal swabs, one per animal, were transported in Amies medium to the laboratory and conserved in refrigerated conditions until processing, within the first 48h.

Samples were streaked on Levine agar plates and MacConkey agar supplemented with cefotaxime (2 µg/ml). After an overnight incubation at 37ºC, plates were examined for suspected *E. coli* colonies. Up to two colonies per plate were randomly selected and confirmed as *E. coli* by standard biochemical tests (Gram staining, indol, triple sugar iron) and the species-specific PCR for *uidA* gene detection (Heininger *et al*. 1999).

Antimicrobial susceptibility testing

Antimicrobial susceptibility was tested by the disc diffusion method in Mueller-Hinton agar plates for the following antimicrobials: ampicillin, amoxicillin/clavulanate, ceftazidime, cefotaxime, cefoxitin, imipenem, nalidixic acid, ciprofloxacin, gentamicin, amikacin, tobramycin, chloramphenicol, trimethoprim/sulfamethoxazole and tetracycline. Interpretation of the resulting inhibition zones was done according to the Clinical Laboratory Standards Institute document (CLSI 2015). *E. coli* ATCC 25922 was used as a control strain. Additionally, double-disc synergy test using ceftazidime, cefotaxime and amoxicillin/clavulanate was performed to screen for the production of ESBLs in all the recovered cefotaxime-resistant (CTX^R) *E. coli*. When both isolates from a given plate showed the same phenotypic resistance pattern, only one isolate was selected and conserved at -80ºC for further molecular analyses.

Characterization of antimicrobial resistance genes and integrons

All the selected *E. coli* isolates were genetically characterized for the presence of specific resistance genes and mechanisms. PCR and subsequent sequencing was performed to identify the genes involved in β-lactam ($bla_{\text{CTX-M}}$, bla_{SHV}, bla_{TEM}, bla_{OXA}, bla_{PSE}), quinolone [*qnrA*, *qnrB*, *aac(6')-Ib-cr*, *qepA*], aminoglycoside [*aac(3)-I*, *aac(3)-II*, *aac(3)-III*, *aac(3)-IV*, *strA*, *strB*], tetracycline [*tet*(A), *tet*(B)], phenicol (*cmlA*, *floR*, *catB3*) and sulfonamide (*sul1*, *sul2*, *sul3*) resistances (Jouini *et al.* 2007; Alonso *et al.* 2017). The detection of acquired *amp*C genes was done by using a multiplex-PCR assay (Pérez-Pérez and Hanson 2002). Mutations in the chromosomal *amp*C promoter region were determined by PCR and sequencing. Additionally, in quinolone resistant isolates, amino acid substitutions in GyrA and ParC proteins were also screened by PCR and sequencing (Ruiz *et al.* 2012).

The presence of class 1 and class 2 integrase encoding genes (*intI1* and *intI2*, respectively), as well as the variable regions and 3'-conserved segments of the detected integrons were examined by PCR and sequencing (Sáenz *et al.* 2004; Alonso *et al.* 2017).

Conjugal transfer and plasmid characterization

Conjugation assays were carried out by filter mating to assess the transferability of ESBL and acquired AmpC encoding genes using the rifampicin-resistant *E. coli* C1520 (Lac^-) and the sodium azide-resistant *E. coli* J53 as recipient strains. A donor:recipient ratio of 1:4 was used in this approach. Transconjugants were selected on MacConkey agar plates supplemented with cefotaxime (2 µg/mL) plus rifampicin (100 µg/mL) or sodium azide (100 µg/mL). They were subjected to antibiotic susceptibility tests and PCR analysis for the detection of *bla* genes and other resistance determinants, as described above.

Plasmids were classified according to the diversity of replication proteins of major incompatibility (Inc) groups by PCR-based replicon typing (PBRT) using the genomic DNA of both original and transconjugant isolates (Carattoli *et al.* 2005). The number and size of plasmids in each transconjugant was analyzed by genomic DNA digestion with S1 nuclease followed by pulsed field gel electrophoresis (PFGE) (Schink *et al.* 2011).

Virulence genotyping of cryptic E. coli clades

Isolates belonging to *Escherichia* clade IV and V were PCR screened for the presence of the following intestinal and extraintestinal virulence factors: *eae* (encoding intim), *stx1* (shiga toxins 1), *stx2* (shiga toxins 2), *bfp* (bundle-forming pilus), *fimA* (encoding type 1 fimbriae), *hlyA* (hemolysin), *cnf1* (cytotoxic necrotizing factor), *papG* allele III (adhesion PapG class III), *papC* (P fimbriae), *aer* (aerobactin iron uptake system), *usp* (uropathogenic-specific protein), *iutA* (aerobactin receptor), *ompT* (outer membrane receptor), *malX* (pathogenicity island marker) and *sat* (secreted autotransporter toxin) (Vidal *et al.*, 2005; Alonso *et al.* 2017).

Molecular typing

The diversity of the *E. coli* phylogroups in the collection was analyzed using the multiplex PCR-based assay (Clermont *et al.* 2013). Cryptic *Escherichia* clades were confirmed and assigned to lineages I, II, III, IV or V by a previously described method based on *aes* and *chuA* allele-specific amplifications (Clermont *et al.* 2011).

Multilocus sequence typing (MLST) was carried out by amplifying and sequencing internal fragments of seven housekeeping genes (*adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA*, *recA*) for: i) All ESBL, acquired AmpC and hyperproducer chromosomal AmpC *E. coli* strains; and ii) Isolates belonging to the cryptic *Escherichia* clades. The resulting nucleotide sequences were analyzed and compared with those deposited in the Warwick MLST database (http://mlst.warwick.ac.uk/mlst/) to ascertain the corresponding sequence type (ST). A neighbor-joining dendrogram was generated with MEGA7 software to obtain a phylogenetic reconstruction based on the concatenated sequences of the seven MLST loci from our ESBL/AmpC *E. coli*, cryptic *Escherichia* clade isolates and other reference strains belonging to the genus *Escherichia* (Walk *et al.* 2009; Luo *et al.* 2011; Vignaroli *et al.* 2015).

RESULTS

Antimicrobial profiles and detection of ESBL/acquired AmpC-producing E. coli

E. coli isolates were recovered from all the 62 fecal samples analyzed. Although up to two isolates per plate were initially obtained, only in three samples >1 phenotypically distinct antimicrobial patterns were identified. Thus, a collection of 65 *E. coli* isolates were finally selected for further molecular analysis. Four of them grew in cefotaxime-supplemented MacConkey agar plates and, after the double-disk synergy test, two exhibited a positive ESBL phenotype.

E. coli isolates exhibiting resistance to at least one antimicrobial agent were detected in fecal samples of 16 out of 62 animals (25.8 %) (Table 1). The highest resistance rates were observed for ampicillin, tetracycline, trimethoprim/sulfamethoxazole and nalidixic acid. Of particular interest was the detection of five multidrug-resistant isolates (≥3 different antimicrobial classes) and four CTX^R *E. coli* strains. The genetic mechanism involved in the phenotypic resistance to third-generation cephalosporins exhibited by these isolates was: i) hyperproduction of chromosomal AmpC (n=1, hedgehog), ii) production of acquired AmpC β-lactamase (n=1, hedgehog) and, iii) production of ESBL (n=2, mink and roe deer). Interestingly, the ESBL-producing *E. coli* strain recovered from the roe deer was also resistant to tetracycline and nalidixic acid.

Molecular characterization of antimicrobial resistance genes and integrons

Molecular analysis showed the following acquired resistance determinants among the phenotypic resistant strains identified in this study [antimicrobial agent (number of resistant strains)/gene (number of strains)]: ampicillin (12)/$bla_{\text{TEM-1b}}$ (3), $bla_{\text{SHV-12}}$ (1), $bla_{\text{CTX-M-14a}}$ (1),

bla$_{CMY-2}$ (1); tetracycline (6)/*tet*(A) (4), *tet*(B) (2); sulfonamides (5)/ *sul2* (1), *sul3* (1), *sul1* + *sul2* (3). Resistance to nalidixic acid was mediated by amino acid changes in chromosomally encoded GyrA (S83L) and ParC proteins (S80R). Isolates exhibiting resistance to both nalidixic acid and ciprofloxacin demonstrated to have an additional change in GyrA (S83L + D87N) and a substitution of a serine for an isoleucine at position 80 of the ParC protein. In some strains showing resistance to ampicillin or gentamicin, the genes responsible for these phenotypes could not be identified, which suggest the involvement of other resistance determinant or mechanism not evaluated in this study. Among the CTX^{R} *E. coli* isolates (n=4; 6.1%), those identified as ESBL-producers harbored *bla*$_{SHV-12}$ (roe deer) and *bla*$_{CTXM14-a}$ (mink) genes. Of the remaining two isolates, both recovered from hedgehogs and exhibiting an AmpC phenotype, one carried the acquired *bla*$_{CMY-2}$ gene and the other presented mutations at positions -42, -18, -1 and +58 of the chromosomal *ampC* gene promoter. Class 1 integrons containing the *dfrA1-aadA1* gene cassette (GC) array were detected in 3 *E. coli* isolates (4.6%), and class 2 integrons carrying the classic *dfrA1*, *sat2* and *aadA1* GC were identified in two isolates (3.1%) (Table 1).

Plasmid content and conjugal transfer of ESBL/acquired AmpC encoding genes

In ESBL/acquired AmpC-producers, plasmid characterization demonstrated the presence of I1, F, FIB and P replicons, with sizes ranging from 20 to 95 kb (Table 2, Supplementary Figure 1). The ESBL/acquired AmpC genotype was transferable by conjugation in all the strains. The *bla*$_{CMY-2}$ gene was likely mobilized by a 95 kb IncI1 plasmid from C7389 *E. coli* to the recipient strain (Supplementary Figure 1). In ESBL-producers (C8375, C7577), the plasmids carrying *bla* genes were transferred together with other plasmids (Supplementary Figure 1). Transconjugants of MDR C7577 *E. coli* isolate only acquired the ESBL phenotype/genotype, remaining susceptible to tetracycline and nalidixic acid.

Molecular typing of ESBL, acquired AmpC and chromosomal hyperproducer AmpC E. coli isolates

The distribution in our collection of commensal *E. coli* strains from wild mammals according to the major phylogenetic groups was as follows: B1 (n=29; 44.6%), B2 (n=16; 24.6%); E (n=10; 15.4%); A (n=3; 4.6%); F (n=2; 3.1%). Five isolates were classified as cryptic *Escherichia* clades (7.7%): four recovered from mice were identified as *Escherichia* clade IV (all assigned to the lineage ST322), and one obtained from a mink as *Escherichia* clade V. This last isolate harbored the *bla*$_{CTX-M-14a}$ gene and showed a novel point mutation at position 184 (T → A) of the *icd* allele, leading to a new sequence type (not registered in the MLST database). Allelic combinations of clade IV and V isolates, together with those of some previously reported strains, are represented and compared in Table 3.

The *E. coli* strain C7577, recovered from a roe deer and carrying the *bla*$_{SHV-12}$ gene, was assigned to ST1128/B1 lineage. Molecular typing of the phenotypic AmpC isolates from hedgehogs revealed first described STs [ST/phylogroup (associated resistance mechanism)]: ST4564/B1 (production of CMY-2 enzyme) and ST4996/B1 (hyperproduction of chromosomal AmpC).

Phylogenetic reconstruction of Escherichia lineages and virulence characterization of cryptic Escherichia clades

Figure 1 shows the phylogenetic reconstruction of different *Escherichia* lineages (*E. coli*, cryptic *Escherichia* clade IV and V, *E. fergusonii*, and *E. albertii*) based on the concatenated nucleotide sequence of the 7 housekeeping loci used in MLST typing (3423 pb). Isolates typed in this study appear marked with a circle in the tree. In comparison with the *E. coli* included in the analysis, members of the cryptic clade IV and V, including those recovered in this study from one mink and mice's feces, demonstrated to be divergent from the genetically viewpoint. Virulence genotyping of the cryptic *Escherichia* clades revealed the presence of the following genes: i) C6843, C6846, C6847, C6950 (clade IV members): *fimA*; ii) C8395 (clade V member): *fimA*, *ompT*, *malX*, *aer*.

DISCUSSION

The present study, which examined the occurrence and molecular characteristics of AMR *E. coli* from wild mammals, showed a predominance of resistance against "old" antimicrobial agents (e.g. tetracycline, ampicillin, sulfonamides). This is also very common among human and livestock population (*Guerra et al.* 2003; Navajas-Benito *et al.* 2016), since these drugs have been in use for a long time both in clinical and veterinary practice. In fact, in agreement with previous studies, resistance profiles of wild mammal isolates seem to be the result of a "spill-over" from human medicine and livestock farming (Guenther *et al.* 2011). Interestingly, four of the *E. coli* isolates recovered from two hegdehogs, a roe deer and a mink exhibited resistance to third generation cephalosporins and, in most of them (n=3), this phenotype was mediated by acquired AmpC or ESBL enzymes. These mechanisms, which confer resistance to newer antibiotics used in human medicine, are still relatively unusual in isolates from wildlife. However, in the last few years, ESBL/acquired AmpC-producing isolates have been increasingly reported (Costa *et al.* 2006; Literak *et al.* 2010; Radhouani *et al.* 2012; Alcalá *et al.* 2016; Alonso *et al.* 2016). The prevalence in wild mammals vary significantly among regions and studies but can be considered as low to moderate (1.3 – 10 %), which is in accordance to our results (6.4%), in comparison with the alarming high rates described in wild birds (Simões *et al.* 2010; Hasan *et al.* 2014; Alcalá *et al.* 2016).

Focusing on these ESBL/acquired AmpC-producing isolates, different enzymes were detected in the present study. SHV-12 and CTX-M-14 (in particular, CTX-M-14a, which is considered the predominant variant worldwide) were identified as responsible for the ESBL profiles exhibited by the *E. coli* isolates from roe deer and mink origin, respectively. Although many studies reported $bla_{\text{CTX-M-1}}$ as the main ESBL gene found in wildlife (Bonnedahl *et al.* 2009; Dolejska *et al.* 2009; Literak *et al.* 2009; Poeta *et al.* 2009; Pinto *et al.* 2010; Simões *et al.* 2010; Alonso *et al.* 2016), $bla_{\text{CTX-M-14a}}$ and $bla_{\text{SHV-12}}$ have also been frequently detected, especially among wild animals from the Iberian Peninsula (Costa *et al.* 2006; Alcalá *et al.* 2016; Cristóvão *et al.* 2017). This might simply reflect the higher occurrence of SHV-12 and CTX-M-14 ESBL variants among clinical and livestock *E. coli* isolates from this geographical area. In fact, in Spain, even though CTX-M-15 is currently the predominant enzyme in human clinical specimens, SHV-12 and CTX-M-14 remain an important cause of community and healthcare-associated infections (Díaz *et al.* 2010; Merino *et al.* 2016). In the livestock production setting, these enzymes have been described as the most prevalent ESBLs identified in poultry and poultry meat (Egea *et al.* 2012; Ojer-Usoz *et al.* 2013). Moreover, both of the *bla* genes were shown to be transferable by conjugation, likely via IncI1 plasmids, which have been reported as the main group associated to the mobilization of $bla_{\text{SHV-12}}$ (Alonso *et al.* 2017) and the second more frequently found among $bla_{\text{CTX-M-14}}$-carrying *E. coli* isolates in Spain, after IncK family (Valverde *et al.* 2009). However, additional experiments would be required to confirm this point. In *E. coli* strain C7389 isolated from a hedgehog, $bla_{\text{CMY-2}}$ was identified as the gene responsible for the AmpC phenotype. This is the most commonly detected acquired AmpC β-lactamase in humans and animals worldwide (Jacoby, 2009). In our study, both $bla_{\text{CMY-2}}$ and a 95kb plasmid of the IncI1 group were transferred from the donor to the recipient strain, suggesting the location of the AmpC gene in this conjugative plasmid. IncI1 group plasmids are frequently associated with the spread of $bla_{\text{CMY-2}}$ (Hansen *et al.* 2016).

It is also interesting to note that new sequence types of *E. coli* were demonstrated to be involved in the spread of ESBL/AmpC genotype in the environment (e.g. ST4564/B1; ST4996/B1, and a non registered ST showing a new *icd* allele), which underlines the undesirable consequences of their potential entry in the community or clinical setting. The ST1128/B1 clone, found in the $bla_{\text{SHV-12}}$-carrying *E. coli*

isolate from a roe deer, was previously reported in human gut microbiota (Touchon *et al.* 2009). However, the clonal genetic relatedness among isolates from different ecosystems cannot be well established by traditional typing methods and whole-genome sequencing approaches are recommended (de Been *et al.* 2014). Remarkably, to our knowledge, this study reported for the first time the detection of an *Escherichia* clade V member carrying an ESBL gene ($bla_{\text{CTX-M-14a}}$).

Cryptic *Escherichia* clades were discovered during a research focused on the genetic diversity and population structure of *E. coli* isolated from freshwater beaches (Walk *et al.* 2007). Subsequent MLST analysis involving a larger atypical *E. coli* collection demonstrated the existence of 5 novel clades (Clade I to V), which differed from recognized *Escherichia* species by hundreds of parsimoniously informative sites (Walk *et al.* 2009). Members of clade III, IV and V were considered environmentally adapted *Escherichia* lineages that appeared to be overrepresented in habitats outside the host (water, soil and aquatic sediments). However, there are also evidences supporting the relevant presence of *Escherichia* clades in birds (7.8-28.2%) and nonhuman mammals (3.2-8.2%), most likely because they may act as a spill-over host (Clermont *et al.* 2011; Walk *et al.* 2015). Our results are consistent with these observations and demonstrated a prevalence of 7.7% of *Escherichia* clade isolates in the intestine of wild mammals (clade IV, n=4; clade V, n=1). The phylogenetic reconstruction showed in Figure 1 allow to clearly distinguish them from *E. coli* isolates, especially members of clade V which are the most divergent. It is worth noting that, as shown Table 3, identical MLST allelic numbers were shared by more than one isolate belonging to a particular clade, which demonstrate a common genetic background regardless of their sources and geographical origins. Regarding virulence and antimicrobial resistance potential, the few existing studies on cryptic *Escherichia* clades suggest a lack of many determinants involved in intestinal and extraintestinal human infections and a low level of antimicrobial resistance (except for clade I) (Ingle *et al.* 2011). With regard to the first point, among the virulence factors evaluated in this study, only *fimA* gene was identified in *Escherichia* clade IV isolates from our collection. However, the strain belonging to clade V was found to carry more of these genes (*fimA*, *malX*, *aer* and *ompT*). In fact, this last virulence factor (*ompT*) was found to be very common among clade V members (77%), as well as *aer* gene that has also been detected quite frequently (32%) (Ingle *et al.* 2011). Interestingly, a recent study indicated that strains belonging to clade V exhibit a gene repertoire and adhesion properties similar to those of intestinal pathogenic strains (Vignaroli *et al.* 2015). This observation, together with the capability of clade V to carry resistance determinants against clinically important antibiotics (as shown by the detection of our $bla_{\text{CTX-M-14a}}$-producing strain), underline the need of further research on non-*coli Escherichia* isolates to elucidate their potential role in humans as opportunistic pathogens or source of new virulence/antibiotic resistance genes.

Overall, the present study contributes to a better understanding of the *E. coli* population and antimicrobial resistance problematic in wildlife. It has been demonstrated that, although antimicrobial resistance levels among *E. coli* of wild mammals are not yet alarming, relevant mechanisms (ESBL, acquired AmpC) and genes encoding resistance against clinically critical antibiotics are also present in low selective pressure settings. Furthermore, new clones showing an AmpC phenotype and a non-*coli Escherichia* clade V isolate carrying $bla_{\text{CTX-M-14a}}$ ESBL gene have been reported for the first time. Further epidemiological studies and routine monitoring are needed to determine the role of wildlife as source of AMR bacteria or resistance genes in order to better control the global problem of antimicrobial resistance.

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REFERENCES

1. Savagoau MA. *Escherichia coli* habitats, cell types, and molecular mechanisms of gene control. *Am Nat* 1983;**122**:732-44.
2. Martínez JL. Environmental pollution by antibiotics and by antibiotic resistance determinants. *Environ Pollut* 2009;**157**:2893-902.
3. Bonnedahl J, Järhult JD. Antibiotic resistance in wild birds. *Ups J Med Sci* 2014;**119**:113-6.
4. Alcalá L, Alonso CA, Simón C *at al*. Wild birds, frequent carriers of extended-spectrum β-lactamase (ESBL) producing *Escherichia coli* of CTX-M and SHV-12 types. *Microb Ecol* 2016;**72**:861-9.
5. Radhouani H, Silva N, Poeta P *et al*. Potential impact of antimicrobial resistance in wildlife, environment and human health. *Front Microbiol* 2014;**5**:23.
6. Clermont O, Gordon DM, Brisse S *et al*. Characterization of the cryptic *Escherichia* lineages: rapid identification and prevalence. *Environ Microbiol* 2011;**13**:2468-77.
7. Clermont O, Christenson JK, Denamur E *et al*. The Clermont *Escherichia coli* phylo-typing method revisited: improvement of specificity and detection of new phylo-groups. *Environ Microbiol Rep* 2013;**5**:58-65.
8. Walk ST, Alm EW, Gordon DM *et al*. Cryptic lineages of the genus *Escherichia*. *Appl Environ Microbiol* 2009;**75**:6534-44.
9. Luo C, Walk ST, Gordon DM *et al*. Genome sequencing of environmental *Escherichia coli* expands understanding of the ecology and speciation of the model bacterial species. *Proc Natl Acad Sci USA* 2011;**108**:7200-5.
10. Berthe T, Ratajczak M, Clermont O *et al*. Evidence for coexistence of distinct *Escherichia coli* populations in various aquatic environments and their survival in estuary water. *Appl Environ Microbiol* 2013;**79**:4684-93.
11. Vignaroli C, Di Sante L, Magi G *et al*. Adhesion of marine cryptic *Escherichia isolates* to human intestinal epithelial cells. *ISME J* 2014;**9**:508-15.
12. Heininger A, Binder M, Schmidt S *et al*. PCR and blood culture for detection of *Escherichia coli* bacteremia in rats. *J Clin Microbiol* 1999;**37**:2479-82.
13. Clinical and Laboratory Standards Institute (CLSI). *Performance Standards for Antimicrobial Susceptibility Testing*. Twenty fifth informational supplement. CLSI document M100-S25. Wayne, PA: National Committee for Clinical Laboratory Standards, 2015.
14. Jouini A, Vinué L, Slama KB *et al*. Characterization of CTX-M and SHV extended-spectrum beta-lactamases and associated resistance genes in *Escherichia coli* strain of food samples in Tunisia. *J Antimicrob Chemother* 2007;**60**:1137-41.
15. Alonso CA, Gonzáles-Barrio D, Ruiz-Fons F *et al*. High frequency of B2 phylogroup among non-clonally related fecal *Escherichia coli* isolates from wild boars, including the lineage ST131. *FEMS Microbiol Ecol*. In Press.
16. Pérez-Pérez FJ, Hanson ND. Detection of plasmid-mediated AmpC beta-lactamasssse genes in clinical isolates by using multiplex PCR. *J Clin Microbiol* 2002;**40**:2153-62.
17. Ruiz E, Sáenz Y, Zarazaga M *et al*. *qnr*, *aac(6')-Ib-cr* and *qepA* genes in *Escherichia coli* and *Klebsiella* spp. Genetic environments and plasmid and chromosomal location. *J Antimicrob Chemother* 2012;**67**:886-97.
18. Sáenz Y, Briñas L, Domínguez E *et al*. Mechanisms of resistance in multiple-antibiotic-resistant *Escherichia coli* strains of human, animal and food origins. *Antimicrob Agents Chemother* 2004;**48**:3996-4001.
19. Carattoli A, Bertini A, Villa L *et al*. Identification of plasmids by PCR-based replicon typing. *J Microbiol Methods* 2005;**63**:219-28.
20. Schink AK, Kadlec K, Schwarz S. Analysis of bla_{CTX-M}-carrying plasmids from *Escherichia coli* isolates collected in the BfT-Germ Vet study. *Appl Environ Microbiol* 2011;**77**:7142-6.

21. Guerra B, Junker E, Schroeter A *et al*. Phenotypic and genotypic characterization of antimicrobial resistance in German *Escherichia coli* isolates from cattle, swine and poultry. *J Antimicrob Chemother* 2003;**52**:489-92.
22. Navajas-Benito EV, Alonso CA, Sanz S *et al*. Molecular characterization of antibiotic resistance in *Escherichia coli* strains from a dairy cattle farm and its surrounding. *J Sci Food Agric* 2016, DOI: 10.1002/jsfa.7709.
23. Guenther S, Ewers C, Wieler LH. Extended-spectrum beta-lactamases producing *E. coli* in Wildlife, yet another form of environmental pollution? *Front Microbiol* 2011;**2**:246.
24. Costa D, Poeta P, Sáenz Y *et al*. Detection of *Escherchia coli* harbouring extended-spectrum beta-lactamases of the CTX-M, TEM and SHV clases in faecal samples of wild animals in Portugal. *J Antimicrob Chermother* 2006;**58**:1311-2.
25. Literak I, Dolejska M, Radimersky T *et al*. Antimicrobial-resitant faecal Escherichia coli in wild mammals in central Europe: multiresistant *Escherichia coli* producing extended-spectrum beta-lactamases in wild boars. *J Appl Microbiol* 2010;**108**:1702-11.
26. Radhouani H, Igrejas G, Gonçalves A *et al*. Molecular characterization of extended-spectrum-beta-lactamase-producing *Escherichia coli* isolates from red foxes in Portugal. *Arch Microbiol* 2012;**195**:141-4.
27. Alonso CA, González-Barrio D, Tenorio C *et al*. Antimicrobial resistance in faecal *Escherichia coli* isolates from farmen red deer and wild small mammals. Detection of a multiresistant *E. coli* producing extended-spectrum beta-lactamase. *Comp Immunol Microbiol Infect Dis* 2016;**45**:34-9.
28. Simões RR, Poirel L, Martins Da Costa P *et al*. Seagulls and beaches as reservoirs for multidrug-resistant *Escherichia coli*. *Emerg Infect Dis* 2010;**16**:110-2.
29. Hasan B, Melhus A, Sandegren L *et al*. The gull (*Chroicocephalus brunnicepahlus*) as an environmental bioindicator and reservoir for antibiotic resistance on the coastlines of the Bay of Bengal. *Microb Drug Resist* 2014;**20**:466-71.
30. Bonnedahl J, Drobni M, Gauthier-Clerc M *et al*. Dissemination of *Escherichia coli* with CTX-M type ESBL between humans and yellow-legged gull in the south of France. *PLoS ONE* 2009;**4:**e595B.
31. Dolejska M, Bierosova B, Kohoutova L *et al*. Antibiotic-resistant *Salmonella* and *Escherichia coli* isolates with integrons and extended-spectrum bet-lactamases in surface water and sympatric black-headed gulls. *J Appl Microbiol* 2009;**106**:1941-50.
32. Literak I, Dolejska M, Radimersky T *et al*. Antimicrobial-resistant faecal *Escherichia coli* in wild mammals in central Europe: multiresistant *Escherichia coli* producing extended-spectrum beta-lactamase in wild boars. *J Appl Microbiol* 2009;**108**:1702-11.
33. Poeta P, Radhouani H, Pinto L *et al*. Wild boars as reservoirs of extended-spectrum beta-lactamase (ESBL) producing *Escherichia coli* of different phylogenetic groups. *J Basic Microbiol* 2009;**49**:584-8.
34. Pinto L, Rahouani H, Coelho C *et al*. Genetic detection of extended-spectrum beta-lactamase-containing *Escherichia coli* isolates from birds of prey from Serra da Estrela Natural Reserve in Portugal. *Appl Environ Microbiol* 2010;**76**:4118-20.
35. Cristóvão F, Alonso CA, Igrejas G *et al*. Clonal diversity of extended-spectrum beta-lactamase producing *Escherichia coli* isolates in fecal samples of wild mammals. *FEMS Microbiol Lett*. In Press.
36. Díaz MA, Hernández-Bello JR, Rodríguez-Baño J *et al*. Diversity of *Escherichia coli* stains producing extended-spectrum β-lactamase in Spain: second nationwide study. *J Clin Microbiol* 2010;**48**:2840-5.
37. Merino I, Shaw E, Horcajada JP *et al*. CTX-M-15-H30Rx-ST131 subclone is one of the main causes of healthcare-associated ESBL-producing *Escherichia coli* bacteraemia of urinary origin in Spain. *J Antimicrob Chemother* 2016;**71**:2125-30.
38. Egea P, López-Cerero L, Torres E *et al*. Increased raw poultry meat colonization by extended-spectrum β-lactamase-producing *Escherichia coli* in the south of Spain. *Int J Food Microbiol* 2012;**159**:69-73.

39. Ojer-Usoz E, González D, Vitas AI *et al*. Prevalence of extended-spectrum β-lactamase-producing Enterobacteriaceae in meat products sold in Navarra, Spain. *Meat Sci* 2013;**93**:316-21.

40. Alonso CA, Michael GB, Li J *et al*. Analysis of *bla*$_{SHV-12}$-carrying *Escherichia coli* clones and plasmids from human, animal and food sources. *J Antimicrob Chemother*. In Press.

41. Valverde A, Cantón R, Garcillán-Barcia MP *et al*. Spread of *bla*(CTX-M-14) is driven mainly by IncK plasmids disseminated among *Escherichia coli* phylogroups A, B1, and D in Spain. *Antimicrob Agents Chemother* 2009;**53**:5204-12.

42. Jacoby GA. AmpC beta-lactamases. *Clin Microbiol Rev* 2009;**22**:161-82.

43. Hansen KH, Bortolaia C, Nielsen CA *et al*. Host-Specific patterns of genetic diversity among IncI1-Iγ and IncK plasmids encoding CMY-2 β-lactamase in *Escherichia coli* isolates from humans, poultry meat, poultry and dogs in Denmark. *Appl Environ Microbiol* 2016;**82**:4705-14.

44. Touchon M, Hoede C, Tenaillon O *et al*. Organized genome dynamics in the *Escherichia coli* species results in highly diverse adaptive paths. *PLoS Genet* 2009;**5**:e10000344.

45. De Been M, Lanza BF, de Toro M *et al*. Dissemination of cephalosporin resistance genes between *Escherichia coli* strains from farm animals and humans by specific plasmid lineages. *PLoS Genet* 2014;**10**:e1004776.

46. Walk ST, Alm EW, Calhoun LM *et al*. Genetic diversity and population structure of *Escherichia coli* isolated from freshwater beaches. *Environ Microbiol* 2007;**9**:2274-88.

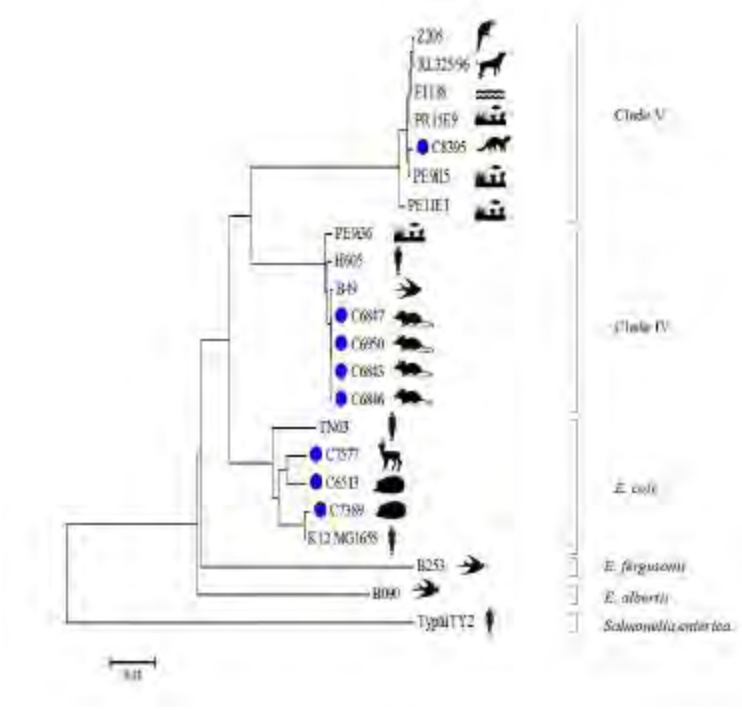

Fig. 1. Phylogenetic tree of *Escherichia* clade V, *Escherichia* clade IV, *E. coli*, *E. fergusonii*, *E. albertii* and *S. enterica* reconstructed from the concatenated sequences of the 7 MLST loci (3423 bp) using MEGA7. The evolutionary history was inferred using the Neighbor-Joining method. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. The strains noted with a black circle correspond to those identified in the present study.

Table 1. Characteristics of the *E. coli* strains showing resistance against one or more antimicrobial agents in this study.

Strain	Species	Phylogenetic group	Resistance profile[a]	Integrons		ESBL/AmpC phenotype	Mutations in QRDR		Other resistance genes outside integrons
				Class 1 (VR[b]/3'CS[c])	Class 2 (VR[b])		GyrA	ParC	
C6512	*Erinaceus europaeus*	B1	AMP	-	-				-
C6513	*Erinaceus europaeus*	B1	AMP, AMC, NAL	-	-	AmpC	S83L	S80R	-
C7388	*Erinaceus europaeus*	A	AMP, CHL, NAL, CIP, TET, SXT	-	-		S83L, D87N	S80I	*sul2*, *tet*(B), $bla_{\text{TEM-1b}}$
C7389	*Erinaceus europaeus*	B1	AMP, AMC, CAZ, CTX, FOX	-	-	AmpC			*sul3*, $bla_{\text{CMY-2}}$
C6842	*Mus musculus*	A	AMP	-	-				$bla_{\text{TEM-1b}}$
C6843	*Mus musculus*	Clade IV	GEN	-	-				-
C6895	*Mus musculus*	B1	GEN	-	-				-
C6896	*Rattus norvegicus*	E	GEN, AMK	-	-				-
C7373	*Genetta genetta*	B1	-	-	*dfrA1-sat2-aadA1*				-
C7374	*Lutra lutra*	E	AMP, TET, SXT	*dfrA1-aadA1*/*qacE*Δ*1-sul1*	-				*sul2*, *tet*(A)
C8395	*Neovison vison*	Clade V	AMP, CTX	-	-	ESBL			$bla_{\text{CTX-M-14a}}$
C7577	*Capreolus capreolus*	B1	AMP, CAZ, CTX, NAL, TET	-	*dfrA1-sat2-aadA1*	ESBL	S83L	S80R	*tet*(A), $bla_{\text{SHV-12}}$
C8415	*Ovis musimon*	B1	AMP, TET, SXT	*dfrA1-aadA1*/*qacE*Δ*1-sul1*	-				*sul2*, *tet*(A)
C8419	*Ovis musimon*	B1	AMP, TET, SXT	*dfrA1-aadA1*/*qacE*Δ*1-sul1*	-				*sul2*, *tet*(A)
C8401	*Martes foina*	B1	AMP, NAL, CIP	-	-		S83L, D87N	S80I	$bla_{\text{TEM-1b}}$
C8399	*Meles meles*	B1	AMP, TET	-	-				*tet*(B)

[a] AMP: ampicillin; AMC: amoxicillin/clavulanate; CAZ: ceftazidime; CTX: cefotaxime; FOX: cefoxitin; CHL: chloramphenicol; NAX: nalidix acid; CIP: ciprofloxacin; GEN: gentamicin; AMK: amikacin; TET: tetracycline; SXT: trimethoprim/sulfamethoxazole.

[b] VR: Variable region (gene cassettes included in the variable region).

[c] 3'CS: 3'-Conserved Region.

Table 2. Molecular typing, plasmid content and conjugative transfer of ESBL/AmpC-producing *E. coli* strains.

Strain	Species	ESBL/acquired AmpC type	Mutations in chromosomal *ampC* gene	Molecular typing		Plasmids			Conjugation and characterization of transconjugants					
				ST	Phylo group	Number	Size (kb)	Replicons	Conjugal transfer	Conjugation frequency	Resistance phenotype	Resistance genes	Plasmid replicons	Size (kb)
C8395	*N. vison*	CTX-M-14a	-	NR[a]	Clade V	3	125, 90, 65	I1, F, FIB	+	7×10^{-3}	AMP, CTX	$bla_{\text{CTX-M-14}}$	I1, F	90, 65
C7577	*C. capreolus*	SHV-12	-	ST1128	B1	2	90, 20	I1, P	+	4.3×10^{-3}	AMP, CTX, CAZ	$bla_{\text{SHV-12}}$	I1, P	90, 20
C7389	*E. europaeus*	CMY-2	-	ST4564	B1	1	95	I1	+	4.3×10^{-2}	AMP, AMC, CTX, CAZ, FOX	$bla_{\text{CMY-2}}$	I1	95
C6513	*E. europaeus*	-	-42, -18, -1, +58	ST4996	B1	ND	ND	ND	ND	ND	ND	ND	ND	ND

[a] NR: non registered.

Table 3. MLST data showing the allelic combinations of the cryptic *Escherichia* clade strains identified in this collection and others from previous studies.

Strain	Clade	Origin	*adk*	*fumC*	*gyrB*	*icd*	*mdh*	*purA*	*recA*	ST	Reference
PR15E9	V	Marine sediment	51	48	45	139	235	42	37	4105	Vignaroli *et al.* 2015
PE9i15	V	Marine sediment	51	468	220	266	235	42	37	3613	Vignaroli *et al.* 2015
PE11E1	V	Marine sediment	51	48	45	442	34	279	37	4104	Vignaroli *et al.* 2015
E1118	V	Water	51	48	45	287	34	42	37	2721	Luo *et al.* 2011
C8395	V	Mink	51	48	467	NR[a]	235	42	37	NR	This study
PE9i36	IV	Marine sediment	356	531	387	441	56	322	63	4103	Vignaroli *et al.* 2015
C6847	IV	Mouse	82	87	73	79	56	60	63	322	This study
C6950	IV	Mouse	82	87	73	79	56	60	63	322	This study
C6843	IV	Mouse	82	87	73	79	56	60	63	322	This study
C6846	IV	Mouse	82	87	73	79	56	60	63	322	This study

[a] NR: non registered.