Research Article

HIGH FREQUENCY OF B2 PHYLOGROUP AMONG NON-CLONALLY RELATED FAECAL *Escherichia coli* ISOLATES FROM WILD BOARS, INCLUDING THE LINEAGE ST131

Carla Andrea Alonso [1], David González-Barrio [2], Francisco Ruiz-Fons [2], Laura Ruiz-Ripa [1], Carmen Torres [1,*]

[1] *Área Bioquímica y Biología Molecular, Universidad de La Rioja, Logroño, Spain.*

[2] *Health & Biotechnology (SaBio) Group, Spanish Wildlife Research Institute IREC (CSIC-UCLM-JCCM), Ciudad Real, Spain.*

*Corresponding author. +34 941 299750

E-mail address: carmen.torres@unirioja.es (C. Torres)

Full postal address: Madre de Dios 51, 26006 Logroño, Spain. Área Bioquímica y Biología Molecular. Universidad de La Rioja.

Keywords: wild boars, antimicrobial resistance, *Escherichia coli*, virulence genes, B2 phylogroup, ST131

Running title: AMR and phylogenetic distribution of *E. coli* from wild boars.

Conflicts of interest: Do not exist in relation with this manuscript.

ABSTRACT

Wild boars are worldwide distributed mammals which population is increasing in many regions, like the Iberian Peninsula, leading to an increased exposition to humans. They are considered reservoirs of different zoonotic pathogens and have been postulated as potential vectors of antimicrobial-resistant bacteria (AMR). This study aimed to determine the prevalence of antimicrobial resistance and phylogenetic distribution of *Escherichia coli* from wild boar faeces. Antimicrobial resistance and integron content was genetically characterized and *E. coli* of B2 phylogroup were further analyzed by molecular typing and virulence genotyping. The prevalence of antimicrobial-resistant *E. coli* was low, with only 7.5% of isolates being resistant against at least one antimicrobial, mainly ampicillin, tetracycline and/or sulfonamide. An unexpected elevated rate of B2 phylogroup (47.5%) was identified, most of them showing unrelated PFGE patterns. ST131/B2 (*fimH* 22 sublineage), ST28/B2, ST1170/B2, ST681/B2 and ST625/B2 clones, previously described in extraintestinal infections in humans, were detected in B2 isolates, and carried one or more genes associated with extraintestinal pathogenic *E. coli* (ExPEC). This study demonstrated a low prevalence of antimicrobial resistance in *E. coli* from wild boars, although they are not exempt of AMR bacteria, and a predominance of genetically diverse B2 phylogroup, including isolates carrying ExPEC which may contribute to the spread of virulence determinants among different ecosystems.

INTRODUCTION

Wild boars (*Sus scrofa*) are among the widest worldwide distributed ungulates which, in many regions, are experiencing an important demographic explosion. These animals are considered reservoirs and/or vectors of several zoonotic pathogens, including different viruses like hepatitis B virus, Japanese encephalitis virus, Influenza A virus or Nipah virus, and bacteria such as *Leptospira* spp., *Campylobacter* spp., *Salmonella* spp. or shiga-toxin producing *Escherichia coli* (STEC) (Ruiz-Fons, 2015). Moreover, some studies have also suggested wild boars to be potential carriers of antimicrobial-resistant bacteria (AMR) and, worryingly, extended-spectrum beta-lactamase (ESBL) producing strains (Poeta *et al.,* 2009; Literak *et al.,* 2010).

Considering that many factors, such as the frequent use of shared habitat resources by wild and livestock species, the increasing human settlements in natural areas and the consumption of wildlife meat, may lead to a closer contact between species from different settings, it is important to gain insight into the characteristics of commensal bacteria from free-living animals in order to understand the flow of virulence and antibiotic-resistant genes and bacterial clones. *E. coli* is usually considered a good indicator in surveillance studies due to its capability to colonize the gut of many animal species and to act both as commensal and pathogen bacteria. Moreover, its intraspecific genetic structure has been deeply studied and, currently, *E. coli* population is classified in seven phylogenetic groups designated as A, B1, B2, C, D, E and F (Clermont *et al.*, 2013). These phylogroups seem to differ in different aspects regarding their metabolic activity, ecological niche and their virulence or antimicrobial-resistance profiles (Carlos *et al.*, 2010). In fact, strains belonging to B2 and, in a lesser extent, D phylogroups have been associated with extraintestinal infections in humans (Picard *et al.*, 1999).

In this work, we aimed to determine the prevalence of AMR *E. coli*, phenotypically characterize and genetically analyze their antimicrobial resistance profiles and integron content, and to study the distribution of phylogroups in commensal *E. coli* from faeces of wild boars hunted in central Spain. Strains belonging to B2 phylogroup, due to its clinical relevance, were further characterized by determining the presence/absence of several virulence determinants and the main associated genetic lineages.

MATERIALS AND METHODS

Sample collection and processing

Intestine segments with faecal content were collected from 79 wild boars (*Sus scrofa*) hunted during the regular hunting season (October to February 2014-2015) in two provinces of central Spain (Ciudad Real and Toledo). Samples were sent to the laboratory in refrigerated conditions for processing.

In total, 0.5 g of faeces were diluted in sterile saline solution (0.85%, 1 mL). Once diluted, 20 µL were directly cultured on Levine agar plates and incubated overnight at 37ºC. Additionally, in order to discriminate possible cefotaxime (CTX) resistant isolates, 100 µL of the faecal solution were inoculated in 5 mL of brain heart infusion broth supplemented with CTX (2 µg/mL) and, after overnight incubation, 10 µL were seeded onto MacConkey agar plates containing CTX (2 µg/mL). Strains compatible with *E. coli* were randomly selected (up to two per sample) and confirmed by standard biochemical tests (gram-staining, triple sugar iron, indol) and species-specific PCR (Heininger *et al.*, 1999).

Antimicrobial susceptibility testing

Susceptibility to 15 antimicrobials (ampicillin, amoxicillin/clavulanate, ceftazidime, cefotaxime, cefoxitin, imipenem, nalidixic acid, ciprofloxacin, gentamicin, amikacin, tobramycin, chloramphenicol, sulfonamide, trimethoprim/sulfamethoxazole and tetracycline) was tested by the disk diffusion method in accordance with Clinical and Laboratory Standards Institute document (CLSI, 2015). *E. coli* ATCC 25922 was used as a control strain. When both isolates from a given sample exhibited identical phenotypic resistance profile, only one isolate was selected for further molecular characterization.

Characterization of antimicrobial resistance genes and integrons

On the basis of phenotypic resistance patterns, the presence of genes encoding beta-lactam (*bla*$_{\text{TEM}}$, *bla*$_{\text{SHV}}$, *bla*$_{\text{OXA}}$), quinolone (*qnrA*, *qnrB*, *qnrS*, *aac(6´)-Ib-cr*, *qepA*), tetracycline (*tet*(A), *tet*(B)) and sulphonamide (*sul1*, *sul2*, *sul3*) resistance was tested by PCR and subsequent sequencing (Belaaouaj *et al.*, 1994; Pitout *et al.*, 1998; Guardabassi *et al.*, 2000; Mazel *et al.*,

2000; Steward *et al*., 2001; Maynard *et al*., 2003; Perreten *et al.*, 2003; Park *et al*., 2006; Yamane *et al*., 2007; Cattoir *et al*., 2007) (Table 1). In addition, amino acid changes in ParC and GyrA proteins were studied by PCR and sequencing of the corresponding genes in quinolone-resistant isolates (Oram and Fisher, 1991; Vila *et al.*, 1996).

The presence of class 1 or class 2 integrons was examined by PCR amplification of the integrase genes (*intI1* or *intI2*, respectively) (Mazel *et al.*, 2000). The composition and organization of the variable region was determined by the primer-walking strategy (Vinué *et al.*, 2008).

Virulence genotyping

All *E. coli* isolates were tested for the presence of genes encoding intim (*eae*) and shiga toxins 1 (*stx1*) and 2 (*stx2*) by using a previously described PCR protocol (Vidal *et al.*, 2005) (Table 1).

In those isolates belonging to B2 phylogroup, additional PCRs were performed for the detection of the following virulence determinants: *fimA* (encoding type 1 fimbriae), *hlyA* (hemolysin), *cnf1* (cytotoxic necrotizing factor), *papG* allele III (adhesion PapG class III), *papC* (P fimbriae), *bfp* (bundle-forming pilus) and *aer* (aerobactin iron uptake system) (Yamamoto *et al.*, 1995; Velasco *et al*., 2001; Ruiz *et al.*, 2002) (Table 1).

Additionally, for the ST131 clone, some of the most commonly associated virulence factors were tested by specific PCRs: *usp* (uropathogenic-specific protein), *iutA* (aerobactin receptor), *ompT* (outer membrane receptor), *malX* (pathogenicity island marker) and *sat* (secreted autotransporter toxin) (Bauer e*t al.*, 2002; Johnson *et al.*, 2003; Rodríguez-Siek *et al.*, 2005; Jakobsen *et al.*, 2008) (Table 1).

Molecular typing

E. coli isolates were classified in the seven main phylogenetic groups (A, B1, B2, C, D, E and F) using the Clermont multiplex PCR method, as previously described (Clermont *et al.*, 2013).

Genetic diversity of strains assigned to B2 phylogroup was further studied by pulsed-field-gel-electrophoresis (PFGE) after *Xba*I digestion of the genomic DNA (Gautom *et al.*, 1997). Resulting profiles were analyzed by BioNumerics software 2.0 (Applied Maths, Belgium) using the UPGMA algorithm and Dice similarity coefficient.

Multilocus sequence typing (MLST) was carried out for: A) All the isolates showing resistance to antimicrobials; B) In strains assigned to B2 phylogroup, those isolates belonging to the most frequent PFGE patterns and other selected ones representing different virulence profiles (including all the ones carrying ≥2 virulence genes). Internal fragments of seven standards housekeeping genes (*adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA* and *recA*) were amplified and sequenced. Nucleotide sequences were compared with those deposited in the MLST Warwick website (http://mlst.warwick.ac.uk/mlst) in order to obtain the specific allelic combination and sequence type (ST).

Additionally, a previously described primer combination was used to know if the isolate assigned to ST131 belonged to O25 serogroup (Clermont *et al.*, 2007). This strain was further characterized by *fimH*-based subtyping (Weissman *et al.*, 2012).

RESULTS AND DISCUSSION

E. coli isolates were detected in Levine agar plates (non-supplemented with antimicrobials) in all the 79 samples analyzed. Two *E. coli* isolates per sample were initially obtained (158 isolates). After antimicrobial susceptibility testing, in most of the cases (all but one), both isolates recovered from the same sample showed an identical antimicrobial resistance profile, from which only one was finally selected for further genetic characterization. Nevertheless, two phenotypically distinct *E. coli* isolates were identified in one of the samples and both of them were included in the study. For this reason, a collection of 80 *E. coli* isolates obtained in Levine agar plates were further genetically characterized in this study. No *E. coli* growth was detected in cefotaxime-supplemented MacConkey agar plates.

Only 6 of the 80 *E. coli* isolates (7.5%) showed resistance to at least one of the 15 antimicrobial agents tested, although none of them exhibited resistance to third-generation cephalosporins (cefotaxime and ceftazidime). This frequency is in agreement with the available literature, which shows lower antimicrobial resistance rates among *E. coli* from wild-boars (Literak *et al.*, 2010; Navarro-Gonzalez *et al.*, 2013) or other wild mammals (Alonso *et al.*, 2016), compared to domestics and livestock animals. However, unlike previous studies (Poeta *et al.*, 2009, Literak

169 *et al.*, 2010), no ESBL-producing strains were detected. The highest resistance rates were
170 recorded for tetracycline (n=6), sulfonamides (n=5) and ampicillin (n=3) (Table 2). Tetracycline
171 resistance, encoded by *tet*(A) and/or *tet*(B) genes, was detected in all the AMR *E. coli*. This
172 antibiotic has been in use for many decades both in human and veterinary medicine and
173 different factors such as faecal contamination of water or manure spreading may probably lead
174 to the selection of resistant bacteria in the enteric microbiota of wild species surroundings
175 human influenced areas. Considering resistance to sulfonamides, in 3 out of 5 cases it was
176 shown to be related to the presence of classic class 1 integrons containing the conserved
177 *qacEΔ1-sul1* region. In two of them, *aadA1* gene was detected within the variable region of the
178 integron structure and, in one isolate, the array *dfrA1-aadA1* was identified. The presence of
179 class 1 integrons containing streptomycin (*aad*) resistance encoding gene, alone or in
180 combination with the trimethoprim (*dfr*) resistance determinant, has been frequently reported in
181 bacteria from different origins, including animals (Guerra *et al.*, 2003), humans (Vinué *et al.*,
182 2008) and environmental samples (Ben Said *et al.*, 2016, Navajas-Benito *et al.*, 2016). Although
183 some studies have reported significant rates of class 2 integrons in wild boars commensal
184 bacteria (Literak *et al.*, 2010), we did not detect this class of integrons. Regarding quinolones,
185 only one strain showed resistance against nalidixic acid, which was found to be in relation with
186 mutations in the chromosomal *gyrA* gene (S83L) (Table 2).
187 The phylogenetic analysis showed an unexpected high prevalence of B2 phylogroup among
188 commensal *E. coli* from wild boars. Specifically, the distribution of the studied *E. coli* strains in
189 the different phylogenetic groups was as follows: B2 (n=38; 47.5%), E (n=16, 20%), B1 (n=15,
190 18.7%), A (n=8, 10%) and D (n=3, 3.7%). In contrast to animals, B2 phylogroup seems to be
191 more abundant in human commensal *E. coli* strains from industrialized countries (Zhang *et al.*,
192 2002; Escobar-Páramo *et al.*, 2006; Clermont *et al.*, 2013) and has been associated with
193 extraintestinal infections (Picard *et al.*, 1999). In our collection, nearly half of the studied
194 isolates were assigned to B2 phylogroup. A previous study which compared the intestinal
195 commensal *E. coli* strains from wild boars and domestic pigs, although did not show a
196 predominance of B2 phylogroup, it did report that *E. coli* from wild boars were remarkably

more often members of B2 group than isolates recovered from healthy pigs (Römer *et al.*, 2012). On the basis of their results, authors hypothesize about the possible causes for these differences. First, they mention the probable influence of antibiotic pressure. Our results support previous studies (Alonso *et al.*, 2016) which demonstrate that AMR isolates seem to be more associated to B1 and A phylogroups. Moreover, three out of 6 isolates showing antimicrobial resistance in this collection belonged to ST155 and ST10 clonal complexes, frequently associated with resistance and even with ESBL phenotype in *E. coli* from human, animal and environmental sources (Day *et al.*, 2016). Conversely, the 38 *E. coli* isolates from wild boars assigned to B2 phylogroup were susceptible to all antimicrobial tested. This fact suggests that livestock species, more exposed to antimicrobials, may indirectly select B1 or A strains leading them preferably to colonize the gut. Another proposed explanation for the higher levels of B2 phylogroup among wild boars is in relation to the essential role played by iron, as cofactor, in many basic metabolism pathways. The presence of *E. coli* harboring the iron acquisition gene *chuA*, necessary to classify an isolate as belonging to B2, D or E phylogroups, could be increased in wild boars intestine since, unlike pigs, they do not have additional iron sources such as iron-supplemented feed (Römer *et al.*, 2012). Furthermore, other well-known factors such as diet, body mass and host habitats have been found to affect the relative abundance of phylogroups among mammals (Gordon and Cowling, 2003).

Considering the elevated rate of commensal *E. coli* of B2 phylogroup detected in the studied wild boars, we analyzed the main circulating lineages and examined whether these mammals could be reservoirs of extraintestinal pathogenic *E. coli* (ExPEC). Figure 1 shows the high genetic diversity detected among phylogroup B2 *E. coli* strains (33 unrelated PFGE patterns among 38 isolates), indicating that the dominance of this bacterial population was not due to the spread of a single or a few clones. *E. coli* of B2 phylogroup may confer a biological benefit to wild boars, its colonization capability could be favored by selective pressures or a combination of both. By MLST, we found that some of the sequence types detected among commensal *E. coli* belonging to B2 phylogroup have been previously described in humans associated with different clinical conditions, especially urinary tract infections. This is the case of clones such as

ST131/B2 (Merino *et al.*, 2016), ST28/B2 (Coque *et al.*, 2008), ST1170/B2 (Pietsch *et al.*, 2015; Porse *et al.*, 2016), ST681/B2 (Banerjee *et al.*, 2013; Hertz *et al.*, 2016) and ST625/B2 (Banerjee *et al.*, 2013). In fact, in many of the isolates belonging to these lineages, except in strains assigned to ST131 and ST28, we identified one or more of the following virulence genes usually present in ExPEC: *papC*, *papGIII*, *cnf1* and/or *hlyA*. The isolate belonging to ST681 harbored all of the mentioned virulence determinants. The genes encoding cytotoxic necrotizing factor (*cnf1*) and α-hemolysin (*hlyA*) have been described in the same pathogenicity island, which is in accordance with the association of these virulence factors observed in the present study. Regarding the isolate belonging to sequence type ST131, which was further characterized as ST131-*fimH* 22 sublineage (O25 serogroup), none of the previously mentioned virulence genes (except *fimA*, which is widely distributed and not restricted to pathogenic strains) was identified. However, one of the characteristics of this successful lineage is the apparent absence of classical ExPEC virulence factors such as P fimbriae (*pap*), cytotoxic necrotizing factor (*cnf1*) and α-hemolysin (*hlyA*) (Mathers *et al.* 2015). ST131 frequently carried other genes like *usp* (uropathogenic-specific protein), *iutA* (aerobactin receptor), *ompT* (outer membrane receptor), *malX* (pathogenicity island marker) or *sat* (secreted autotransporter toxin). In agreement to these observations, the O25-ST131 *fimH* 22 isolate detected in the present study harbored 4 of these virulence determinants (*usp*, *iutA*, *ompT* and *malX*). Other remarkable fact is that, in contrast to the highly susceptible antimicrobial phenotypes shown by ST131, ST28 and ST1170 clones in wild boars, those reported in humans are frequently more resistant and they even carry ESBL determinants (Coque *et al.*, 2008; Pietsch *et al.*, 2015; Merino *et al.*, 2016; Porse *et al.*, 2016). This may suggest a potential selection of resistant variants in environments with high antibiotic pressure.

Although wild animals like wild boars or deer have been identified as potential reservoirs of Shiga toxin producing strains (Sánchez *et al.*, 2009), none of the 80 studied *E. coli* isolates carried *stx1* or *stx2* genes. However, it is important to mention that the screening was carried out among isolates recovered from Levine Agar. No enrichment and/or selective media for the

isolation of *E. coli* 0157:H7 and other non-sorbitol fermenters was employed because the main objective of this work was not to study the prevalence of STEC.

In conclusion, even though the general rate of antimicrobial resistance found among commensal *E. coli* from wild boars is low, our results show that free-living species as representatives of the environmental setting are not exempt from AMR bacteria (usually related with B1 and A phylogroups) and transferable structures carrying resistance determinants. Moreover, we report an unexpected remarkably high prevalence of genetically diverse *E. coli* of B2 phylogroup, which includes the detection of some clones (ST131, ST28, ST1170, ST681, ST625) previously described in extraintestinal infections in humans. Further research should be carried out to better understand the flow of antibiotic and virulence genes between different ecosystems.

FUNDING

This work was supported by Project SAF2012-35474 from the Ministerio de Economía y Competitividad (MINECO) of Spain and Feder. Carla Andrea Alonso has a predoctoral fellowship FPI from the MINECO (Spain). F. Ruiz-Fons is funded by the "Ramón y Cajal" programme from MINECO (Spain).

REFERENCES

1. 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.
2. Banerjee R, Johnston B, Lohse C *et al*. The clonal distribution and diversity of extraintestinal *Escherichia coli* isolates vary according to patient characteristics. *Antimicrob Agents Chemother* 2013;**57**:5912-7.
3. Bauer RJ, Zhang L, Foxman B *et al*. Molecular epidemiology of 3 putative virulence genes for *Escherichia coli* urinary tract infection – *usp*, *iha*, and *iroN* (*E. coli*). *J Infect Dis* 2002;**185**:1521-4.

4. Belaaouaj A, Lapoumeroulie C, Caniça MM et al. Nucleotide sequences of the genes coding for the TEM-like β-lactamases IRT-1 and IRT-2 (formerly called TRI-1 and TRI-2). *FEMS Microbiol Lett* 1994;**120**:75-80.
5. Ben Said L, Jouini A, Alonso CA *et al*. Characteristics of extended-spectrum β-lactamase (ESBL)- and pAmpC beta-lactamase-producing Enterobacteriaceae of water samples in Tunisia. *Sci Total Environ* 2016;**550**:1103-9.
6. Carlos C, Pires MM, Stoppe NC *et al*. *Escherichia coli* phylogenetic group determination and its application in the identification of the major animal source of fecal contamination. *BMC Microbiol* 2010;**10**:161.
7. Cattoir V, Poires L, Rotimi V *et al.* Multiplex PCR for detection of plasmid-mediated quinolone resistance *qnr* genes in ESBL producing enterobacterial isolates. *J Antimicrob Chemother* 2007;**60**;394-7.
8. Clermont O, Johnson JR, Menard M *et al*. Determination of *Escherichia coli* O types by allele-specific polymerase chain reaction: application to the O types involved in human septicemia. *Diagn Microbiol Infect Dis* 2007;**57:**129-36.
9. Clermont O, Christenson JK, Denamur DM *et al*. The Clermont *E. coli* phylo-typing method revisited: improvement of specificity and detection of new phylo-groups. *Environ Microbiol* 2013;**5**:58-65.
10. 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.
11. Coque TM, Novais A, Carattoli A *et al*. Dissemination of clonally related *Escherichia coli* strains expressing extended-spectrum β-lactamase CTX-M-15. *Emerg Infect Dis* 2008;**14**:195-200.
12. Day MJ, Rodríguez O, van Essen-Zandbergen A *et al*. Diversity of STs, plasmids and ESBL genes among *Escherichia coli* from humans, animals and food in Germany, the Netherlands and the UK. *J Antimicrob Chemother* 2016;**71**:1178-82.

13. Escobar-Páramo P, Le Menac'h A, Le Gall T *et al*. Identification of forces shaping the comensal *Escherichia coli* genetic structure by comparing animal and human isolates. *Environm Microbiol* 2006;**8**:1975-84.
14. Gautom, RK. Rapid pulsed-field gel electrophoresis protocol for typing of *Escherichia coli* O157:H7 and other gram-negative organisms in 1 day. J Clin Microbiol 1997:35:2977-80.
15. Guardabassi L, Dijkshoorn L, Collard JM *et al*. Distribution and in-vitro transfer of tetracycline resistance determinants in clinical and aquatic *Acinetobacter* strains. *J Med Microbiol* 2000;**49**:929-36.
16. 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 Chermother* 2003;**52**:489-92.
17. Gordon DM, Cowling A. The distribution and genetic structure of *Escherichia coli* in Australian vertebrates: host and geographic effects. *Microbiology* 2003;**149**:3575-86.
18. 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.
19. Hertz FB, Nielse JB, Schønning K *et al*. Population structure of drug-susceptible, -resistant and ESBL-producing *Escherichia coli* from community-acquired urinary tract. *BMC Microbiol* 2016;**16**:63.
20. Jakobsen L, Sandvang D, Hansen LH *et al*. Characterisation, dissemination and persistence of gentamicin resistant *Escherichia coli* from a Danish university hospital to the waste water environment. *Environ Int* 2008;**34**:108-15.
21. Johnson JR, Gajewski A, Lesse AJ *et al*. Extraintestinal pathogenic *Escherichia coli* as a cause of invasive nonurinary infections. *J Clin Microbiol* 2003;**41**:5798-5802.
22. 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-lactamases in wild boars. *J Appl Microbiol* 2010;**108**:1702-11.

23. 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.
24. Maynard C, Fairbrother JM, Bekal S *et al.* Anrimicrobial resistance genes in enterotoxigenic *Escherichia coli* O149:K91 isolates obtained over a 23-year period from pigs. *Antimicrob Agents Chemother* 2003;**47**:3214-21.
25. Mathers AJ, Peirano G, Pitout JD. *Escherichia coli* ST131: The quintessential example of an international multiresistant high-risk clone. *Adv Appl Microbiol* 2015;**90**:109-54.
26. Mazel D, Dychinco B, Webb V *et al*. Antibiotic resistance in the ECOR collection: integrons and identification of a novel *aad* gene. *Antimicrob Agents Chemother* 2000;**44**:1568-74.
27. 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 surroundings. *J Sci Food Agric* 2016; DOI: 10.1002/jsfa.7709.
28. Navarro-Gonzalez N, Porrero MC, Mentaberre G *et al*. Antimicrobial resistance in indicator *Escherichia coli* isolates from free-ranging livestock and sympatric wild ungulates in a natural environment (Northeastern Spain). *Appl Environ Microbiol* 2013;**79**:6184-6.
29. Oram M, Fisher LM. Quinolones resistance mutations in the DNA gyrase of *Escherichia coli* clinical isolates identified by using the polymerase chain reaction. *Antimicrob Agents Chemother* 1991;**35**:387-9.
30. Park CH, Robicsek A, Jaboby GA *et al.* Prevalence in the United States of *aac(6')-Ib-cr* encoding a ciprofloxacin-modifying enzyme. *Antimicrob Agents Chemother* 2006;**50**:3953-55.
31. Perreten V, Boerlin P. A new sulfonamide resistance gene (*sul3*) in *Escherichia coli* is widespread in the pig population of Switzerland. Antimicrob Agents Chemother 2003;**47**:1169-72.
32. Picard B, Sevali Garcia J, Gouriou S *et al*. The link between phylogeny and virulence in *Escherichia coli* extraintestinal infection. *Infect Immun* 1999;**67**:546-53.

33. Pietsch M, Eller C, Wendt C *et al*. Molecular characterization of extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* isolates from hospital and ambulatory patients in Germany. *Vet Microbiol* 2015; DOI:10.1016/j.vetmic.2015.11.028.
34. Pitout JD, Thomson KS, Hanson ND et al. β-Lactamases responsible for resistance to expanded-spectrum cephalosporins in *Klebsiella pneumoniae*, *Escherichia coli*, and *Proteus mirabilis* isolates recovered in South Africa. *Antimicrob Agents Chemother* 1998;**42**:1350-4.
35. Poeta P, Radhouani H, Pinto L *et al*. Wild boars as reservoir of extended-spectrum beta-lactamase (ESBL) producing *Escherichia coli* of different phylogenetic groups. *J Basic Microbiol* 2009;**49**:584-8.
36. Porse A, Schønning K, Munck C *et al*. Survival and evolution of a large multidrug-resistance plasmid in new clinical bacterial hosts. *Mol Biol Evol* 2016; DOI:10.1093/molbev/msw163.
37. Rodríguez-Siek KE, Giddings CW, Doetkott T *et al.* Comparison of *Escherichia coli* isolates implicated in human urinary tract infection and avian colibacillosis. Microbiology 2005;**151**:2097-2110.
38. Römer A, Wieler LH, Schierack P. Analyses of intestinal commensal *Escherichia coli* strains from wild boars suggest adaptation to conventional pig production conditions. *Vet Microbiol* 2012;**161**:122-9.
39. Ruiz J, Simon K, Horcajada JP *et al*. Differences in virulence factors among clinical isolates of *Escherichia coli* causing cistitis and pyelonephritis in women an prostatitis in men. *J Clin Microbiol* 2002;**40**:4445-9.
40. Ruiz-Fons F. A review of the current status of relevant zoonotic pathogens in wild swine (*Sus scrofa*) populations: Changes modulating the risk of transmission to humans. *Transbound Emerg Dis* 2015; DOI:10.1111/tbed.12369.
41. Sánchez S, García-Sánchez A, Martínez R *et al*. Detection and characterisation of shiga toxin-producing *Escherichia coli* other than *Escherichia coli* O157:H7 in wild ruminants. *Vet J* 2009;**180**:384-8.

42. Stewards CD, Rasheed JK, Hubert SK *et al.* Characterization of clinical isolates of *Klebsiella pneumoniae* from 19 laboratories using the National Committee for Clinical Laboratory Standards extended-spectrum β-lactamase detection methods. *J Clin Microbiol* 2001;**39**:2864-72.
43. Velasco M, Horcajada JP, Mensa J, *et al*. Decreased invasive capacity of quinolone resistant *Escherichia coli* in urinary tract infection. *Clin Infect Dis* 2001;**33**:1682-6.
44. Vidal M, Kruger E, Durán C *et al*. Single multiplex PCR assay to identify simultaneously the six categories of diarrheagenic *Escherichia coli* associated with enteric infections. *J Clin Microbiol* 2005;**43**:5362-5.
45. Vila J, Ruiz J, Goñi P *et al*. Detection of mutations in *parC* in quinolone-resistant clinical isolates of *Escherichia coli*. *Antimicrob Agents Chemother* 1996;**40**:491-3.
46. Vinué L, Sáenz Y, Martínez S *et al*. Prevalence and diversity of extended-spectrum beta-lactamases in faecal *Escherichia coli* isolates from healthy humans in Spain. *Clin Microbiol Infect* 2008;**15**:954-7.
47. Weissman SJ, Jhonson JR, Tchesnokova, V *et al*. High-resolution two-locus clonal typing of extraintestinal pathogenic *Escherichia coli*. *Appl Environ Microbiol* 2012;**78**:1353-60.
48. Yamamoto S, Terai A, Yuri K, *et al*. Detection of virulence factors in *Escherichia coli* by multiplex polymerase chain reaction. *FEMS Immunol Med Microbiol* 1995;**12**:85-90.
49. Yamane K, Wachino J, Suzuki S *et al.* New plasmid-mediated fluoroquinolone efflux pump, QepA, found in an *Escherichia coli* clinical isolate. *Antimicrob Agents Chemother* 2007;**51**:2254-60.
50. Zhang L, Foxman B, Marrs C. Both urinary and rectal *Escherichia coli* isolates are dominant by strains of phylogenetic group B2. *J Clin Microbiol* 2002;**40**:3951-5.

Figure Legends

Fig. 1. UPGMA dendogram and PFGE patterns of Xba-I digested genomic DNA from *E. coli* isolates belonging to phylogenetic group B2. The ST131/B2 isolate was additionally screened for the presence of virulence determinants marked with an asterisk.

Table 1. Primer pairs used in this study for the characterization of antimicrobial resistance and virulence.

Target	Primers	Sequence (5'-3')	Size (bp)	Reference
Antimicrobial Resistance				
bla$_{TEM}$	TEM-F	ATTCTTGAAGACGAAAGGGC	1150	Belaaouaj *et al.*, 1994
	TEM-R	ACGCTCAGTGGAACGAAAAC		
bla$_{SHV}$	SHV-F	CACTCAAGGATGTATTGTG	885	Pitout et al., 1998
	SHV-R	TTAGCGTTGCCAGTGCTCG		
bla$_{OXA}$	OXA-F	ACACAATACATATCAACTTCGC	813	Steward *et al*., 2001
	OXA-R	AGTGTGTTTAGAATGGTGATC		
qnrA	QnrAm-F	AGAGGATTTCTCACGCCAGG	580	
	QnrAm-R	TGCCAGGCACAGATCTTGAC		
qnrB	QnrB-F	GGMATHGAAATTCGCCACTG	264	Cattoir *et al.*, 2007
	QnrB-R	TTTGCYGYYCGCCAGTCGAA		
qnrS	QnrSm-F	GCAAGTTCATTGAACAGGGT	550	
	QnrSm-R	TCTAAACCGTCGAGTTCGGCG		
aac(6')-Ib-cr	aac(6')-Ib-F	TTGCGATGCTCTATGAGTGGCTA	482	Park *et al.*, 2006
	aac(6')-Ib-R	CTCGAATGCCTGGCGTGTTT		
qepA	QepA-F	GCAGGTCCAGCAGCGGGTAG	617	Yamane *et al.*, 2007
	QepA-R	CAACTGCTTGAGCCCGTAG		
tet(A)	tetA-F	GTAATTCTGAGCACTGTCGC	937	Guardabassi *et al.*, 2000
	tetA-R	CTGCCTGGACAACATTGCTT		
tet(B)	tetB-F	CTCAGTATTCCAAGCCTTTG	416	
	tetB-R	CTAAGCACTTGTCTCCTGTT		
sul1	sul1-F	TGGTGACGGTGTTCGGCATTC	789	Mazel *et al.*, 2000
	sul1-R	GCGAGGGTTTCCGAGAAGGTG		
sul2	sul2-F	CGGCATCGTCAACATAACC	722	Maynard *et al.*, 2003
	sul2-R	GTGTGCGGATGAAGTCAG		
sul3	sul3-F	TGTCATTTACGGCATACTCG	990	Perreten *et al*., 2003
	sul3-R	ATCAGGCATCCCATTCCCAT		
gyrA	gyrA-F	TACACCGGTCAACATTGAGG	648	Oram and Fisher, 1991
	gyrA-R	TTAATGATTGCCGCCGTCGG		
parC	parC-F	AAACCTGTTCAGCGCCGCATT	395	Vila *et al*, 1996
	parC-R	GTGGTGCCGTTAAGCAAA		

Target	Primers	Sequence (5'-3')	Size (bp)	Reference
Virulence Genotyping				
stx1	stx1-F	CAGTTAATGTGGTGGCGAAGG	348	Vidal M *et al.*, 2005
	stx1-R	CACCAGACAATGTAACCGCTG		
stx2	stx2-F	ATCCTATTCCCGGGAGTTTACG	584	
	stx2-R	GCGTCATCGTATACACAGGAGC		
eae	eae-F	TCAATGCAGTTCCGTTATCAGTT	482	
	eae-R	GTAAAGTCCGTTACCCCAACCTG		
fimA	fimA-F	GTTGTTCTGTCGGCTCTGTC	447	Ruiz *et al.*, 2002
	fimA-R	ATGGTGTTGGTTCCGTTATTC		
papG allele III	papG-F	CATTTATCGTCCTCCTCAACTTAG	482	
	papG-R	AAGAAGGGATTTTGTAGCGTC		
cnf1	cnf1-F	ATGGAGTTTCCTATGCAGGAA	498	
	cnf1-R	CATTCAGAGTCCTGCCCTCATTATT		
hlyA	hlyA-F	AACAAGGATAAGCACTGTTCTGGCT	1177	Yamamoto *et al.*, 1995
	hlyA-R	ACCATATAAGCGGTCATTCCCGTCA		
papC	papC-F	GACGGCTGTACTGCAGGGTGTGGCG	328	
	papC-R	ATATCCTTTCTGCAGGGATGCAATA		
aer	aer-F	TACCGGATTGTCATATGCAGACCGT	602	
	aer-R	AATATCTTCCTCCAGTCCGGAGAAG		
bfp	bfp-F	ACAAAGATACAACAAACAAAAA	260	Velasco *et al.*, 2001
	bfp-R	TTCAGCAGGAGTAAAAGCAGTC		
usp	usp-F	ACATTCACGGCAAGCCTCAG	440	Bauer *et al.*, 2002
	usp-R	AGCGAGTTCCTGGTGAAAGC		
ompT	ompT-F	ATCTAGCCGAAGAAGGAGGC	559	Rodríguez-Siek *et al.*, 2005
	ompT-R	CCCGGGTCATAGTGTTCATC		
iutA	iutA-F	ATCGGCTGGACATCATGGGAAC	314	Jakobsen *et al.*, 2008
	iutA-R	CGCATTTACCGTCGGGAACGG		
malX	malX-F	CCACGCAATACGCCAAAGCTAA	472	
	malX-R	AACGCGTGACGATTCCTTTTGG		
sat	sat-F	ACTGGCGGACTCATGCTGT	387	Johnson *et al.*, 2003
	sat-R	AACCCTGTAAGAAGACTGAGC		

442

Table 2. Resistance determinants, integrons and phylogroups of faecal AMR *E. coli* detected among the whole collection of 80 *E. coli* of wild boars.

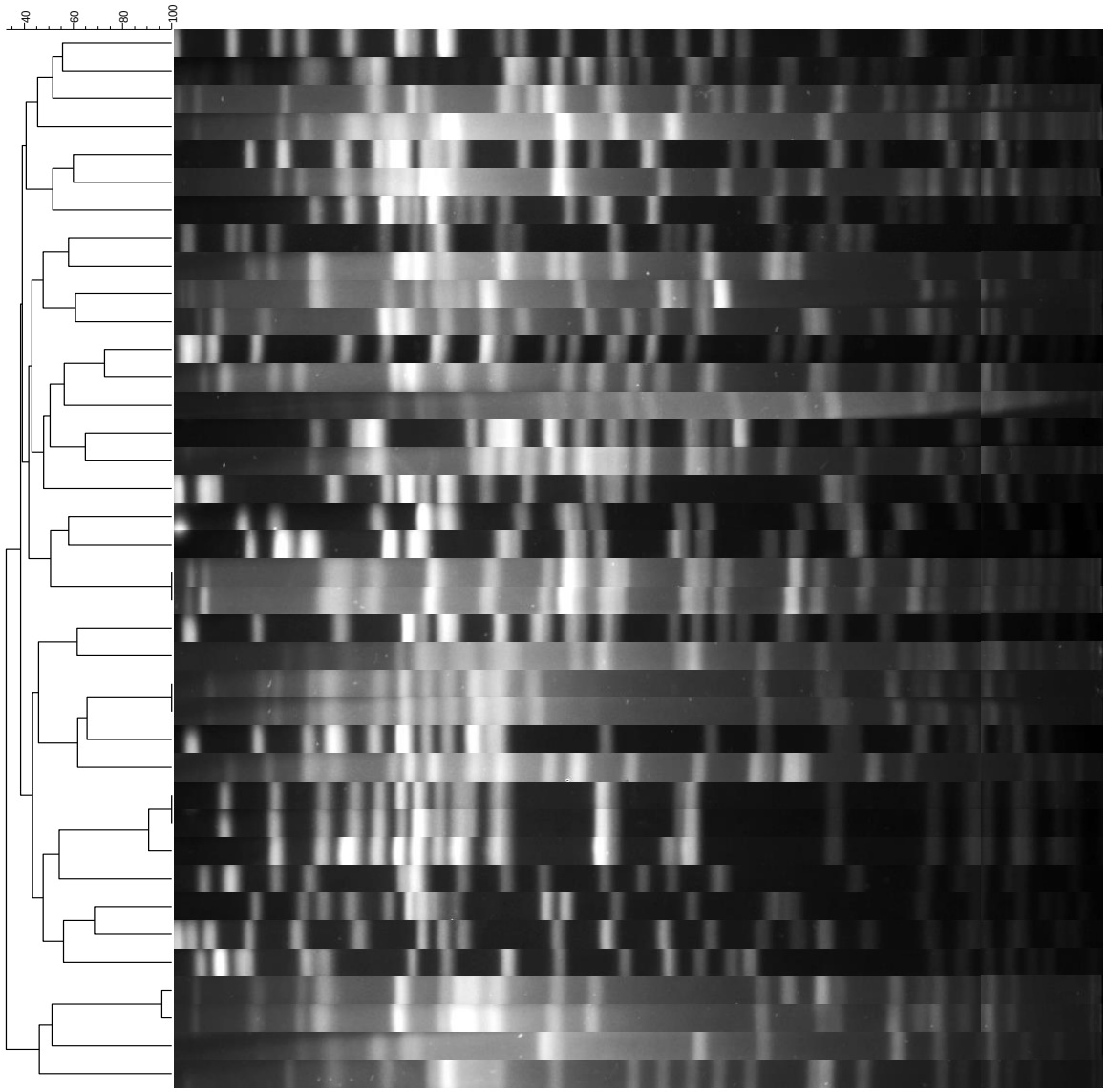
E. coli strain	Origin	Resistance phenotype[a]	Resistance genotype	Amino acid changes		Class 1 integrons (gene cassette array)	ST (ST Complex)	Phylo group
				ParC	GyrA			
C7972	*Sus scrofa*	AMP, TET, SUL	*bla*$_{TEM-1a}$, *tetA*, *sul1*	-	-	*aadA1*	ST1079	B1
C7973	*Sus scrofa*	TET	*tetB*	-	-	-	NR[b]	A
C7974	*Sus scrofa*	AMP, TET, SUL	*tetA*, *tetB*, *sul2*	-	-	-	NR[b]	A
C7989	*Sus scrofa*	TET, SUL	*tetB*, *sul1*	-	-	*aadA1*	ST10 (10)	E
C8022	*Sus scrofa*	TET, SUL, SXT	*tetA*, *sul1*, *sul2*	-	-	*dfrA1-aadA1*	ST155 (155)	B1
C8124	*Sus scrofa*	AMP, NAX, TET, SUL, SXT	*bla*$_{TEM-1b}$, *tetB*, *sul1*, *sul2*	-	S83L	-	ST58 (155)	B1

[a]AMP: ampicillin; NAX: nalidix acid; TET: tetracycline; SUL: sulphonamide; SXT: trimethoprim/sulfamethoxazole.

[a] NR: Not registered in the MLST database.

455 **Fig. 1.**

456



Strain	Virulence genes	ST (ST Complex)
C8003	*fimA*	
C8000	*fimA*	
C7992	*fimA, aer*	ST1155
C7981	*fimA*	
C8021	*fimA*	
C7991	*fimA*	
C8025	*fimA*	
C8002	*fimA*	ST83
C7979	*fimA*	ST1317 (131 Cplx)
C7993	*fimA*	
C7994	*fimA, aer*	ST567
C7997	*fimA*	ST567
C7963	*fimA, aer*	ST567
C7995	*fimA, hlyA, cnf1, papGIII, papC*	ST681
C8123	*fimA, papGIII, papC*	ST625
C7975	*fimA, papGIII, papC*	ST625
C8024	*fimA*	
C8130	*fimA*	
C8129	*eae, fimA*	ST28 (28 Cplx)
C7985	*fimA*	ST1161
C7988	*fimA*	ST1161
C8125	*fimA*	
C7983	*fimA*	
C7971	*fimA*	ST1170
C7978	*fimA*	ST1170
C8128	*fimA*	ST1170
C7970	*fimA, usp*, iutA*, ompT*, malX**	ST131 (131 Cplx)
C8121	*fimA*	ST1880
C8127	*fimA*	ST1880
C8126	*fimA, papC*	ST1880
C8015		
C8010	*fimA*	
C8017	*fimA*	
C8020	*eae, fimA*	ST2088
C7984	*fimA*	ST1170
C7986	*fimA, papC*	ST1170
C7980	*fimA, papC*	ST1385
C7977	*fimA*	