Antimicrobial resistance in faecal *Escherichia coli* isolates from farmed red deer and wild small mammals. Detection of a multiresistant *E. coli* producing extended-spectrum beta-lactamase.

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26 **Abstract**

27 Eighty-nine *Escherichia coli* isolates recovered from faeces of red deer and small
28 mammals, cohabiting the same area, were analyzed to determine the prevalence and
29 mechanisms of antimicrobial resistance and molecular typing. Antimicrobial resistance
30 was detected in 6.7% of isolates, with resistances to tetracycline and quinolones being
31 the most common. An *E. coli* strain carrying $bla_{CTX-M-1}$ as well as other antibiotic
32 resistant genes included in an unusual class 1 integron (*Intl1-dfrA16–bla*$_{PSE-1}$*-aadA2-*
33 *cmlA1-aadA1-qacH*-IS*440-sul3-orf1-mef*(B)Δ-IS*26)* was isolated from a deer. The
34 $bla_{CTX-M-1}$ gene was transferred by conjugation and transconjugants also acquired an
35 IncN plasmid. This strain was typed as ST224, which seems to be well adapted to both
36 clinical and environmental settings. The phylogenetic distribution of the 89 strains
37 varied depending on the animal host. This work reveals low antimicrobial resistance
38 levels among faecal *E. coli* from wild mammals, which reflects a lower selective
39 pressure affecting these bacteria, compared to livestock. However, it is remarkable the
40 detection of a multi-resistant ESBL-*E. coli* with an integron carrying clinically relevant
41 antibiotic-resistance genes, which can contribute to the dissemination of resistance
42 determinants among different ecosystems.

43

44 *Keywords:* ESBL, antimicrobial resistance, *Escherichia coli*, deer, small mammals,
45 wildlife.

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51 **1. Introduction**

52 Antibiotic resistance is a global problem of increasing magnitude. Some
53 microorganisms, such as commensal enterococci and *Escherichia coli*, are considered
54 effective indicators for monitoring the prevalence of resistance in different populations
55 and for evaluation of the transfer of resistant bacteria between distinct ecosystems [1].
56 *E. coli* colonize the gastrointestinal tract of many animals and constitute a reservoir of
57 antimicrobial resistance genes that may play an epidemiological role in the spread of
58 resistance. In this sense, extended-spectrum beta-lactamases (ESBL) deserve special
59 attention as they confer resistance to many of the beta-lactam antibiotics commonly
60 used in human and veterinary medicine and can be easily transferred among different
61 strains due to the frequent plasmidic location of corresponding genes [2].
62 Many reports exist about antimicrobial resistant (AMR) bacteria in domestic [3-5] and
63 food-producing animals [3, 6], but fewer studies are available on wild animals. The first
64 detection of ESBL–*E. coli* in wild animals dates back to 2006 [7] and, since then,
65 increased attention has been given to identify environmental reservoirs for AMR
66 bacteria.
67 The aim of the present study was to determine the prevalence, phenotypic resistance and
68 genetic characteristics of AMR *E. coli* isolated from red deer and small mammals
69 (rodents) in Spain.
70

71 **2. Material and methods**

72 2.1. *Faecal samples and bacterial isolates*

73 A total of 217 faecal samples from red deer (*Cervus elaphus,* n=122) and small
74 mammals, including wood mice (*Apodemus sylvaticus,* n=12), black rats (*Rattus rattus,*
75 *n=40*), European rabbits (*Oryctolagus cuniculus,* n=42), and greater white-toothed

76 shrews (*Crocidura russula,* n=1), were collected from a hunting estate in Los
77 Alcornocales Natural Park (Cádiz, Southern Spain) in 2013. In the estate, wild red deer
78 and other wildlife coexist with farmed red deer. All deer samples were from farmed red
79 deer. Faeces were collected from farmed red deer with sterile disposable latex gloves
80 directly from the rectum, whereas faeces from other wildlife were collected from the
81 rectum with sterile tweezers into sterile 1.5 ml plastic tubes. Faecal samples were sent
82 to the laboratory in refrigerated conditions for processing.
83 Briefly, 0.5 g of faeces were diluted in 3 ml of sterile saline solution (0.85%). From this
84 faecal solution, 20 µl were directly seeded onto Levine agar plates. All plates were
85 examined for suspected *E. coli* colonies after overnight incubation at 37ºC. Up to two
86 colonies per plate were selected for posterior identification.
87 In addition, 100 µl of faecal solution were added to 5 ml of brain heart infusion broth
88 containing 2 µg/ml of cefotaxime (BHI-CTX). BHI-CTX broth was incubated at 37ºC
89 for 24h and 10 µl were inoculated onto MacConkey agar supplemented with cefotaxime
90 (2 µg/ml) (MacConkey-CTX). After incubation at 37ºC for 24 h, plates were examined
91 for cefotaxime-resistant (CTX^R) *E. coli* recovery. One *E. coli* isolate was selected from
92 samples in which bacterial growth was observed.
93 All the presumptive *E. coli* isolates were identified by classical biochemical methods
94 (gram–staining, triple sugar iron, indol) and by a species-specific PCR for *uidA* (beta-
95 glucuronidase enzyme) gene detection [8].
96
97 2.2. *Antimicrobial susceptibility testing*
98 Susceptibility testing was performed by the disc diffusion method, according to the
99 Clinical Laboratory Standards Institute [9]. The susceptibility of the *E. coli* isolates
100 strains was tested for 14 antibiotics: ampicillin, amoxicillin/clavulanate, ceftazidime,

101 cefotaxime, cefoxitin, imipenem, nalidixic acid, ciprofloxacin, gentamicin, amikacin,
102 tobramycin, chloramphenicol, trimethoprim/sulfamethoxazole and tetracycline. *E. coli*
103 ATCC 25922 was used as a control strain.
104 In addition, the screening of phenotypic ESBL production was carried out by the double
105 disk synergy test using cefotaxime, ceftazidime and amoxicillin/clavulanate acid discs
106 [9].
107
108 2.3. *Characterization of antimicrobial resistance genes and integrons*
109 The presence of genes encoding β-lactamase (bla_{TEM}, bla_{SHV}, bla_{CTX-M}, bla_{OXA}, bla_{PSE-1})
110 and plasmid-mediated quinolone-resistance (PMQR) (*qnrA*, *qnrB*, *qnrS*, *aac(6´)-Ib-cr*,
111 *qepA*), as well as aminoglycoside (*aac (3)-I, aac (3)-II, aac (3)-III, aac (3)-IV*) and
112 tetracycline resistance genes (*tet*(A), *tet* (B)) were tested by PCR and sequencing of
113 both strands [10, 11]. Mutations in quinolone-resistance determining region (QRDR) of
114 *gyrA* and *parC* genes were also done by PCR and DNA sequencing [11].
115 The presence of integrons was detected by PCR amplification of the integrase gene *intI1*
116 (for class 1 integrons) and *intI2* (for class 2 integrons) [12]. To characterize their genetic
117 structure, PCR "primer-walking" strategy was used in order to get the complete
118 arrangement.
119
120 2.4. *Conjugal transfer and plasmid characterization*
121 Conjugation experiments were carried out for the ESBL-*E. coli* isolate using rifampicin-
122 resistant *E. coli* strain C1520 (lactose negative) as recipient strain. MacConkey agar
123 plates containing rifampicin (100 ug/ml) and cefotaxime (2 ug/ml) were used for
124 recovery of transconjugants.

125 The plasmids of donor and transconjugant isolates were classified according to their

126 incompatibility group using the PCR-based replicon-typing method [13].

127

128 2.5. *Molecular typing of E. coli isolates*

129 All *E. coli* isolates were classified in the seven main phylogenetic groups (A, B1, B2, C,

130 D, E and F) according to Clermont *et al* [14]. *E. coli* strains belonging to B2

131 phylogroup, typically associated with higher virulence in humans, and the newly

132 described E phylogroup (which includes strains previously classified as D and B2) were

133 also studied by Pulsed-Field-Gel-Electrophoresis (PFGE) using *Xba*I enzyme. DNA

134 profiles were analyzed by the BioNumerics software 2.0 (Applied Maths, Belgium)

135 choosing the Dice coefficient.

136 ESBL-producing and other selected isolates were typed by multilocus sequence typing

137 (MLST), using seven conserved housekeeping genes (*adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA*

138 and *recA*). Amplicons were sequenced and compared with those sequences deposited in

139 MLST database (http://mlst.warwick.ac.uk/mlst/) in order to get the allele combination

140 and to determine the sequence type (ST) and clonal complex (ST Cplx).

141

142 **3. Results**

143 Faecal samples of red deer and small mammals were inoculated in Levine agar plates

144 (non-supplemented with antibiotics) and *E. coli* isolates were obtained in 72 of 122 deer

145 samples and in 12 of 95 samples of small mammals. One *E. coli* isolate per sample was

146 selected except in five deer, in which two phenotypically distinct *E. coli* strains were

147 recovered, giving in total 89 confirmed *E. coli* isolates for further study.

148 Among *E. coli* isolates from small mammals, only one strain (8.3%) showed resistance

149 to antibiotics, in particular to quinolones (nalidixic acid and ciprofloxacin). From the

150 deer isolates, 5 (6.5%) showed resistance to at least one of the antimicrobial tested: 4
151 strains to tetracycline (5.2%) and one was a multiresistant ESBL-producing *E. coli*
152 (1.3%). The ESBL-*E. coli* presented associated resistance to nalidixic acid,
153 ciprofloxacin, chloramphenicol, trimethoprim/sulfamethoxazole and tetracycline. It is
154 noteworthy that this ESBL *E. coli* grew not only on MacConkey-CTX plates but also
155 abundantly on Levine agar (>500 UFC/plate in both selective and non-selective media).
156 Overall, the prevalence of antibiotic resistance among faecal *E. coli* isolates recovered
157 from deer and small mammals was 6.7%.
158 Table 1 shows the antibiotic resistant phenotypes and genotypes for the different AMR
159 *E. coli* strains. A variety of resistance genes were detected among our strains. Three out
160 of the 4 tetracycline resistant *E. coli* harboured the *tet*(A) gene and the remaining isolate
161 contained the *tet*(B) gene. No PMQR genes were found in the two quinolone-resistant
162 strains (one isolated from a mouse and the other from a deer). Both isolates carried
163 point mutations in the QRDR of chromosomal *gyrA* and *parC* genes. Two amino acid
164 substitutions were identified in GyrA at codons 83 (serine for leucine) and 87 (aspartic
165 acid for asparagine) while one substitution (serine for isoleucine) at codon 80 was found
166 in ParC.
167 The genetic characterization of the ESBL-producer *E. coli* revealed that this strain
168 carried a non-classic class 1 integron lacking the 3'-conserved segment (*qacEΔ1* and
169 *sul1* genes). This strain presented an unusual integron structure associated with *sul3*
170 gene: *Intl1-dfrA16–bla*$_{\text{PSE-1}}$-*aadA2-cmlA1-aadA1-qacH*-IS*440-sul3-orf1-mef*(B)Δ-IS*26*
171 (Fig.1). Resistance to third generation cephalosporins was due to the presence of the
172 bla$_{\text{CTX-M-1}}$ gene, flanked downstream by an *orf477* sequence.
173 The IncN and IncK replicons were detected in C7328 ESBL-*E. coli.* In the conjugation
174 experiment, the obtained transconjugants captured the *bla*$_{\text{CTX-M-1}}$ gene in addition to

175 IncN replicon, which probably harboured this ESBL gene. These transconjugants only
176 acquired ESBL phenotype but remained susceptible to antibiotics other than β-lactams.
177 The distribution of *E. coli* strains in phylogenetic groups A, B1, B2 and E (Fig.2),
178 varied depending on the animal host source tested. No strains belonging to phylogroups
179 C, D and F were detected. All *E. coli* strains from small mammals were included into
180 the phylogenetic groups B1 and B2, but the pattern of distribution differs substantially
181 between rabbits (n=5) and the rest of small mammals (n=7). The majority of *E. coli*
182 isolates from rabbits belonged to phylogenetic group B1 (n=4, 80%), whereas isolates
183 from other small mammals were nearly equally represented in groups B1 (n=3, 42.8%)
184 and B2 (n=4, 57.2%). Most of the 77 *E. coli* isolates from deer belonged to phylogenetic
185 group B1 (n=41, 53.2%), followed by group A (n=22, 28.6%), B2 (n=5, 6.5%) and E
186 (n=9, 11.7%). Among resistant strains, 5 out of 6 were ascribed to B1 phylogroup. The
187 ESBL-producing *E. coli* strain belonged to the phylogenetic group B1 and to the
188 sequence type ST224.
189 The PFGE analysis of the *E. coli* isolates belonging to the phylogenetic groups B2 (5 of
190 deer and 5 of small mammals) and E (9 of deer) revealed 6 and 8 unrelated pulsotypes
191 among B2 and E isolates, respectively (Fig.3). Two clones were more abundant than
192 any other among the studied deer population, one of them (detected in 4 animals) was
193 ascribed to the sequence type ST812 and the other to the new ST4954. Another clone,
194 assigned to the ST104 (ST73 complex), was recovered in the faeces of two different
195 rats.
196

197 **4. Discussion**

198 From a total of 217 faecal samples collected from wild mammals (red deer and small
199 mammals) 89 *E. coli* were characterized. The relatively low *E. coli* recovery rate may

200 be due to the fact that some of the samples were conserved frozen before processing,
201 and the frozen treatment could affect the recovery of bacteria, specially of Gram-
202 negative ones [15, 16].
203 Focusing the resistance to antimicrobial agents among our *E. coli* collection, the
204 prevalence to at least one antimicrobial tested was low (6.7%). This percentage is in
205 agreement with previous studies carried out on small terrestrial mammals and wild
206 ruminants [16, 17], but contrast with the higher levels of antimicrobial resistance
207 reported in domestic [4] and food-producing animals [6, 18]. There are also several
208 studies in wild environments reporting high rates of antimicrobial resistance, especially
209 in birds [19, 20] and large mammals [17, 21, 22]. These differences in the prevalence of
210 resistance among different hosts may be explained by various factors. The exposition to
211 the selective pressure associated with the extended use of antibiotics in farming and
212 veterinary practice and the direct contact with human seems to be in relation with the
213 increasing prevalence of antimicrobial resistance bacteria among companion and food-
214 producing animals [23, 24]. Wild environments are normally not exposed to clinically
215 used antimicrobial agents but there is also number of possible exposure routes that
216 might select for resistance (environmental pollution from highly human influenced
217 settings, wastewater, manure…) [25]. There is evidence suggesting that migratory birds
218 can also play an important role in the spread of antibiotic resistance [19, 20] and the
219 studied area is in the main route of bird migration in Western Europe. In addition, some
220 host factors such as diet can affect the dynamics of the microbiota and therefore, the
221 prevalence of resistant bacteria among gut commensal bacteria [26]. The herbivore diet
222 of most of the animals involved in the present study might explain in part the low level
223 of antimicrobial resistance detected among faecal *E. coli* isolates.

224 Regarding resistant *E. coli* isolates, the highest frequency of resistance was found
225 against tetracycline. All of the tetracycline-resistant strains carried *tet*(A) and *tet(B*)
226 genes, which encode an active efflux system, suggesting that these are the most
227 common genes responsible for tetracycline resistance in wild mammals. This result is in
228 accordance with previous studies carried on in different animals [16, 27, 28, 29].
229 Among quinolone-resistant bacteria, two isolates were resistant to both nalidixic acid
230 and ciprofloxacin. For these strains, chromosomal mutations in the genes of the
231 topoisomerase type II and IV were detected. No PMQR genes were involved, limiting
232 the transference of quinolone resistance to clonal dissemination. Mutations in the
233 QRDR region are most frequently observed quinolone-resistance mechanisms, both in
234 humans and in animals [30].
235 Another important aspect to highlight is that, even though most of the *E. coli* isolated in
236 this study were susceptible to the tested antimicrobials, a multiresistant ESBL strain was
237 also obtained from a deer faecal sample. It is noteworthy that this ESBL *E. coli* grew
238 not only on MacConkey-CTX plates but also abundantly on Levine agar, suggesting a
239 high level of gut colonization. However, the study of more intestine fragments would be
240 necessary to confirm this assumption. The prevalence of ESBL-producing *E. coli* strains
241 in this study can be considered low in comparison with other wild mammals, such as
242 foxes or wild boars, where an ESBL-producing prevalence of 4% [22] and 10.4% [21]
243 have been reported, respectively. This might be explained by the fact that foxes and
244 wild boars are at the top of the food chain and can accumulate multi-resistant bacteria
245 derived from their diet [31]. ESBL-*E. coli* isolated in this work harboured the β-
246 lactamase gene $bla_{\text{CTX-M-1}}$. In the vast majority of the studies conducted in wildlife,
247 $bla_{\text{CTX-M-1}}$ was clearly predominant among ESBL isolates. In addition, CTX-M-1 is one
248 of the most widespread ESBLs in Europe and, although is more common in food-

249 producing animals, has also been frequently reported in humans [32]. This could
250 support the concept that ESBL genes may be transferred via plasmids among bacteria of
251 different origins. The transference of the $bla_{\text{CTX-M-1}}$ from the ESBL-*E. coli* isolated in
252 this study, was associated with the acquisition of the IncN replicon plasmid. Plasmids of
253 the incompatibility N and I1 groups have been reported as the major vehicles for the
254 horizontal dissemination of the $bla_{\text{CTX-M-1}}$ in *Escherichia coli* and *Salmonella* isolates
255 from human and animal sources [33, 34]. This suggests future studies to analyze in
256 more detail the genetic structure and characteristics of plasmids associated with ESBL
257 and/or multiresistance phenotypes. Furthermore, the presence of beta-lactam ($bla_{\text{PSE-1}}$),
258 streptomycin (*aadA1, aadA2*), sulfonamide (*sul3*), chloramphenicol (*cmlA1*) and
259 trimethoprim (*dfrA16*) resistance genes inside the non-classic class 1 integron of the
260 ESBL-*E. coli* strain was demonstrated. This genetic element could also contribute to the
261 selection and dissemination of multiple antibiotic resistance determinants among
262 bacteria of different ecosystems [10].
263 The phylogenetic analysis shows a great diversity of *E. coli* among wild mammals in
264 this study. Considering all the isolates, phylogroup B1 was significantly more prevalent.
265 This phylogenetic group was also found to be the predominant among most of the *E.*
266 *coli* strains that are able to persist in the environment [31]. It is also important to remark
267 the relatively high rate of strains recovered from deer and assigned to the newly
268 described phylogroup E in comparison to the percentages reported in humans [14],
269 which suggests that this phylogroup is more prevalent in animals. Even though in the
270 present study the number of *E. coli* strains obtained from deer was higher than the
271 number of isolates recovered from small mammals, relevant differences in *E. coli*
272 phylogroup distribution were observed between hosts. This result was expected based
273 on the previous studies showing that the relative abundance of phylogenetic groups

274 among mammals varies according to the host diet, body mass and climate [35]. In this
275 sense, some authors have reported a higher prevalence of B1 phylogroup among *E. coli*
276 of herbivorous mammals, which is in accordance with our result [36, 37]. It is also
277 noteworthy the detection of some indistinguishable clones ascribed to the virulence-
278 associated B2 phylogroup, which reflects the spread of clonal lineages, notably ST812
279 among deer and ST104 among small mammals.
280 The multi-resistant ESBL-producing *E. coli* isolated from a deer belonged to the
281 sequence type 224 (ST224), previously found among human clinical isolates [38, 39,
282 40], pets [41] and wild animals, such as seagulls [42] and buffalos [43]. This sequence
283 type has a worldwide distribution and, interestingly, seems to be associated with a
284 multi-resistant phenotype [38, 39], as it was observed in the present study. This fact
285 suggests the existence of a transmission pathway of phylogenetically related multi-
286 resistant strains between humans and animal populations. In addition, different strains
287 belonging to ST224 have been related to the carriage of genes encoding class A and B
288 carbapenemases [39, 40], which underlines the undesirable consequences of the
289 potential entry of these clones into wildlife.
290

291 5. Conclusions

292 In conclusion, this work shows low levels of antibiotic resistance in the commensal *E.*
293 *coli* isolates from wild mammals, which may reflect a lower selective pressure affecting
294 these commensal bacteria, compared to livestock. However, although antimicrobial
295 resistance in deer and small mammals is relatively low, it is also remarkable the
296 detection of a multi-resistant ESBL-producing *E. coli* with an unusual class 1 integron
297 carrying clinically relevant antibiotic resistance genes. The presence of these genetic
298 structures in commensal bacteria isolated from wild animals can contribute to the

299 selection and dissemination of antibiotic resistance determinants among bacteria of
300 different ecosystems. The data presented in this paper also suggests that ST224 lineage,
301 which seems to be associated with a multi-resistant phenotype, is well adapted to
302 clinical and also to environmental settings. To our knowledge, these data represent the
303 first report of AMR *E. coli* among wild mammals in Spain. Further studies should be
304 carried out in order to understand the flow of antibiotic resistance determinants between
305 different ecosystems.

306

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493 **Figure Legends**

494 Fig. 1. Gene cassette arrangement included in the non-classic class 1 integron detected
495 in the ESBL-producing *E. coli* strain. The bar line under the structure indicates the
496 length of the genes.

497 Fig. 2. Distribution of phylogenetic groups among *E. coli* isolates obtained from deer
498 and small mammals (rabbits and other wild small mammals shown in separate).

499 Fig. 3. Dendogram of pulsed-field gel electrophoresis patterns in *E. coli* isolates
500 belonging to the virulence-associated phylogenetic group B2 and E.

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515 **Fig. 1.**

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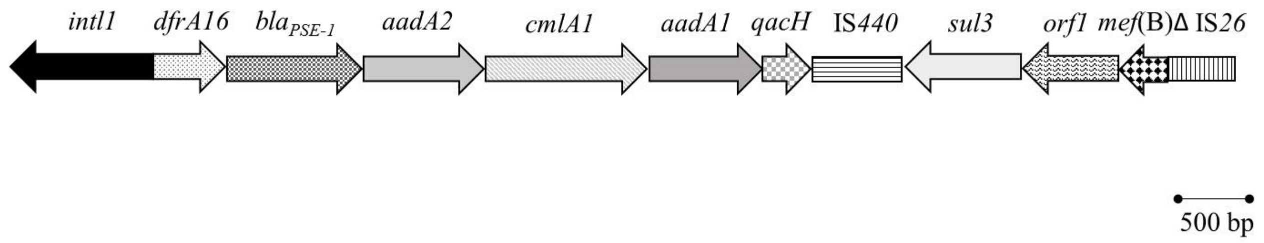

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535 **Fig. 2.**

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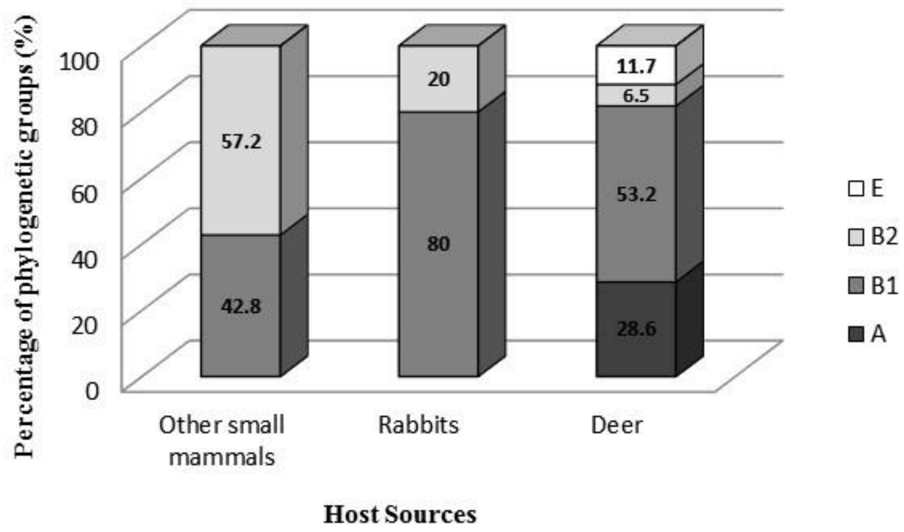

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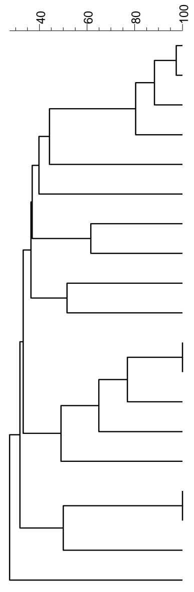
Fig. 3.



Strain	Origin	Phylogroup	ST (ST Complex)
C7341	*Cervus elaphus*	B2	ST812 (None)
C7347	*Cervus elaphus*	B2	ST812 (None)
C7334	*Cervus elaphus*	B2	ST812 (None)
C7137	*Cervus elaphus*	B2	ST812 (None)
C7358	*Cervus elaphus*	B2	
C7040	*Oryctolagus cuniculus*	B2	
C7326	*Cervus elaphus*	E	
C7145	*Cervus elaphus*	E	
C7259	*Cervus elaphus*	E	
C7343	*Cervus elaphus*	E	
C7036	*Rattus rattus*	B2	ST104 (ST73 Cplx)
C7038	*Rattus rattus*	B2	ST104 (ST73 Cplx)
C7032	*Rattus rattus*	B2	
C7035	*Rattus rattus*	B2	
C7344	*Cervus elaphus*	E	
C7261	*Cervus elaphus*	E	ST4954 (None)
C7260	*Cervus elaphus*	E	ST4954 (None)
C7327	*Cervus elaphus*	E	
C7352	*Cervus elaphus*	E	

573 **Table 1.** Determinants of resistance and molecular typing of AMR *E. coli* strains

E. coli strain	Animal host	Resistancephenotype[a]	ESBL Phenotype[b]	Resistance genes detected outside integron	Mutations in QRDR		Class 1 integron gene cassette array structure	Phylogenetic group (MLST)
					GyrA	ParC		
C7328	*Cervuselaphus*	AMP, CTX, CLO, NAL, CIP, TET, SXT	+	*bla*$_{CTX-M-1}$, *tet*(A)	S83L, D87N	S80I	*Intl1-dfrA16–bla*$_{PSE-1}$*-aadA2-cmlA1-aadA1-qacH-IS440-sul3-orf1-mef(B)Δ-IS26*	B1 (ST224)
C7136	*Cervuselaphus*	TET	-	*tet*(A)			-	B1
C7140	*Cervuselaphus*	TET	-	*tet*(A)			-	B1
C7262	*Cervuselaphus*	TET	-	*tet*(A)			-	B1
C7279	*Cervuselaphus*	TET	-	*tet*(B)			-	A
C7031	*Apodemus sylvaticus*	NAL, CIP	-		S83L, D87N	S80I	-	B1

574 [a]AMP: ampicillin; CTX: cefotaxime; CLO: chloramphenicol; NAL: nalidixacid; CIP: ciprofloxacin; TET: tetracycline; SXT:
575 trimethoprim/sulfamethoxazole

576 [b]Phenotype positive (+) or negative (-)

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