

1 **High prevalence and diversity of Extended-Spectrum β -Lactamase and emergence**
2 **of Carbapenemase producing *Enterobacteriaceae* spp in wildlife in Catalonia.**

3

4 Laila Darwich^{1,2*}, Anna Vidal¹, Chiara Seminati¹, Andreu Albamonte¹, Alba Casado¹,
5 Ferrán López¹, Rafael A. Molina-López³, and Lourdes Migura-Garcia².

6

7 ¹ Departament de Sanitat i Anatomia Animal, Universitat Autònoma de Barcelona
8 (UAB), 08193, Cerdanyola del Vallès, Spain.

9 ² IRTA, Centre de Recerca en Sanitat Animal (CReSA, IRTA-UAB), Campus de la
10 Universitat Autònoma de Barcelona, 08193, Cerdanyola del Vallès, Spain.

11 ³ Catalan Wildlife Service, Centre de Fauna Salvatge de Torreferrussa, Santa Perpètua
12 de Mogoda, Barcelona, Spain

13

14 *Corresponding author

15 E-mail: laila.darwich@uab.cat

16

17 Abstract

18

19 In wildlife, most of the studies focused on antimicrobial resistance (AMR) describe
20 *Escherichia coli* as the principal indicator of the selective pressure. In the present study,
21 new species of *Enterobacteriaceae* with a large panel of cephalosporin resistant (CR)
22 genes have been isolated from wildlife in Catalonia. A total of 307 wild animals were
23 examined to determine CR enterobacteria prevalence, AMR phenotypes and common
24 carbapenem and CR gene expression. The overall prevalence of CR-phenotype was
25 13% (40/307): 17.3% in wild mammals (18/104) and 11.5% in wild birds (22/191)
26 ($p < 0.01$). Hedgehogs presented the largest prevalence with 13.5% (14/104) of the
27 mammal specimens, followed by raptors with 7.3% (14/191) of the total bird specimens.
28 Although CR *E. coli* was obtained most frequently (45%), other CR-
29 *Enterobacteriaceae* spp like *Klebsiella pneumoniae* (20%), *Citrobacter freundii* (15%),
30 *Enterobacter cloacae* (5%), *Proteus mirabilis* (5%), *Providencia* spp (5%) and *Serratia*
31 *marcescens* (2.5%) were isolated. A high diversity of CR genes was identified among
32 the isolates, with 50% yielding *bla*CMY-2, 23% *bla*SHV-12, 20% *bla*CMY-1 and 18%
33 *bla*CTX-M-15. Additionally, new CR-gene variants and resistance to carbapenems
34 associated to OXA-48 were found. Most of the CR isolates, principally *K. pneumoniae*
35 and *C. freundii*, were multiresistant with co-resistance to fluoroquinolones, tetracycline,
36 sulphonamides and aminoglycosides. This study describes for the first time in wildlife a
37 high prevalence of *Enterobacteriaceae* spp harbouring a large variety of carbapenem
38 and CR genes frequently associated to nosocomial human infections. Implementation of
39 control measures to reduce the impact of anthropogenic pressure in the environment is
40 urgently needed.

41

42 Introduction

43 In the last decades, the prevalence of opportunistic and antimicrobial resistant (AMR)
44 bacteria associated with nosocomial infections has suffered an important increase in
45 hospital settings. The overuse of antibiotics in human and veterinary medicine have led
46 to the spread of AMR pathogens, becoming a global health problem [1].

47 Extended-spectrum β -lactamases (ESBLs) and plasmid-mediated AmpC-type β -
48 lactamases (pAmpC) are the most common enzymes that confer resistance to broad-
49 spectrum cephalosporins among members of the family *Enterobacteriaceae*. These β -
50 lactamases have extensively diversified in response to the clinical use of new generation
51 drugs: cephalosporins, carbapenems and monobactams [2]. These enzymes are mostly
52 encoded by genes located in plasmids that can be horizontally transferred to different
53 bacteria genera [1]. Carbapenems are last-line beta-lactam antibiotics with the broadest
54 spectrum of activity. Unfortunately, carbapenems nowadays are commonly used in
55 hospital settings for the treatment of life-threatening infections caused by cephalosporin
56 resistant (CR) *Enterobacteriaceae*. However, the emergence of resistance to
57 carbapenems mediated by the production of carbapenemases has led to limited
58 therapeutic options in human health [3], with the OXA-48 variant being highly
59 prevalent in human clinical infections [4].

60 The dissemination of CR has been studied widely in *Enterobacteriaceae* from humans
61 and livestock, whereas studies concerning the environment, including wildlife, are still
62 lacking [2]. In recent years, an important increase of CR *Escherichia coli* has been
63 reported in different epidemiological settings such as humans, pets, livestock, retail
64 meat and the environment [5-10]. The study of wildlife as sentinel of the AMR
65 environmental contamination has recently acquired more consideration worldwide [11].

66 However, most of the environmental-wildlife interface studies have been focused on
67 wild birds, as principal AMR disseminators by their migratory routes, with a limited
68 variety of AMR bacteria species described. Isolation of CR-carrying bacteria from wild
69 birds has been globally reported in *Escherichia coli* [12-17] and less frequently in
70 *Klebsiella pneumoniae* [18]. All these results confirm the dissemination success of
71 ESBL *bla*_{SHV-12} and *bla*_{CTX-M} variants in wild birds worldwide. More recently, presence
72 of CR *E. coli* has also been described in wild mammals, but at lower prevalence in
73 comparison with wild birds [19].

74 In the present study, we report for the first time in Spain, the presence of diverse
75 families of CR- -encoding genes in a large variety of *Enterobacteriaceae* species
76 including *E. coli*, *K. pneumoniae*, *Citrobacter freundii*, *Enterobacter cloacae*, *Serratia*
77 *marcescens* and *Proteus mirabilis*- in wild mammals and wild birds. Furthermore, we
78 describe the presence of carbapenemase resistant *E. coli* and *P. mirabilis* associated
79 with the presence of OXA-48 in isolates of wildlife origin. These bacterial species are
80 frequently associated with severe nosocomial infections in human hospitals of Catalonia
81 [20].

82 **Material and Methods**

83

84 **Study population**

85 Wild animals attended at the Wildlife Rehabilitation Centre (WRC) of Torreferrusa
86 (Catalonia, North-East Iberian Peninsula) were analysed between November 2016 and
87 May 2017. All animals were examined and tested using cloacal or rectal swabs on
88 arrival at the centre before receiving any pharmacologic or antimicrobial treatment. The
89 anthropogenic origin was confirmed as the most frequent cause of hospitalization,

90 comprising direct persecution (gunshot, poisoning, illegal captivity or traps) to
91 involuntary human induced threats (collisions with vehicles, fences or electric lines and
92 electrocution). The rehabilitation centre is under the direction of the Catalan Wildlife-
93 Service, who stipulates the management protocols and Ethical Principles according to
94 the Spanish legislation [21].

95 **Microbiological analysis**

96 Rectal and cloacal swabs were plated in MacConkey agar supplemented with
97 ceftriaxone (1mg/L). Single colonies growing on the plate were subculture and
98 identified biochemically using the API (bioMérieux, Marcy l'Etoile, France) or the
99 VITEK 2 (bioMérieux, Spain) systems. Serovar identification and phage typing of
100 *Salmonella* spp. were carried out at the Spanish National Reference Laboratory (Algete,
101 Madrid, Spain).

102 **Antimicrobial susceptibility testing**

103 Minimal inhibitory concentration (MIC) was performed using a broth microdilution
104 method (VetMIC GN-mo, SVA, Sweden) for the following antimicrobials: ampicillin
105 (Am), cefotaxime (Ctx), ceftazidime (Caz), ciprofloxacin (CIP), nalidixic acid (NAL),
106 gentamicin (GN), streptomycin (ST), kanamycin (KM), florfenicol (FF),
107 chloramphenicol (CF), tetracycline (TE), colistin (COL), sulphamethoxazole (SU) and
108 trimethoprim (TM). The *E. coli* ATCC 25922 was used as the control strain.
109 Epidemiological cut-off values were determined following the European Committee on
110 Antimicrobial Susceptibility testing (EUCAST) recommendations. For those
111 *Enterobacteriaceae* species with no cut-off values defined, cut-off values were obtained
112 from the British Society for Antimicrobial Chemotherapy (BSAC) or the Société
113 Française de Microbiologie (SFM).

114 **Characterization of antimicrobial resistance genes**

115 Molecular diagnosis of CR genes was performed for the following genes; *bla*_{SHV},
116 *bla*_{CTX-M}, *bla*_{CMY1}, *bla*_{CMY2}, and *bla*_{TEM}, [22], *bla*_{OXA} *bla*_{VIM} [23] and *mcr-1* colistin-
117 resistance genes [24].

118 PCR products were Sanger sequenced for verification at the Genomic and
119 Bioinformatics Service of the Universitat Autònoma de Barcelona (Barcelona, Spain).
120 Sequences and chromatograms were manually explored to trim bad-quality bases with
121 BioEdit 7.2. Once the assembly of the consensus sequences was done, both complete
122 and partial genomes were aligned using Clustal Omega program, and finally blasted
123 against the public database (National Center for Biotechnology Information, NCBI).

124

125 **Statistical analysis**

126 Descriptive analysis was performed under 95% confidence, using SPSS Advanced
127 Models TM 15.0 (SPSS Inc. 233 South Wacker Drive, 11th Floor Chicago, IL 60.606-
128 6412). The Chi-square test or Fisher exact test was used for comparison between
129 proportions when appropriate. Statistically significant results were considered when $P <$
130 0.05.

131 **Results**

132 The sample size comprised 307 wild animals belonging to 67 different species grouped
133 as, birds (62%), mammals (34%) and reptiles (4%) (Fig 1). Animals came from
134 different regions of Catalonia with a high density of urban areas and pig farming
135 production.

136 **Fig 1. Proportion of wildlife analysed in the study according to the zoological**
137 **category.** Animal groups: raptors (different species of birds of prey and owls), wild
138 birds (principally passerines and seagulls), hedgehogs (European and Algerian
139 hedgehogs), carnivores (mainly mustelids), and other mammals (wild boars and roe
140 deer).

141 Ceftriaxone resistant isolates were detected in 65 out of the 307 (21%) faecal samples
142 analysed. Of those, 40 harboured ESBL or pAmpC-encoding genes, representing an
143 overall prevalence of 13% (Fig 2). The prevalence of CR-carrying isolates was 17.3% in
144 wild mammals (18/104) and 11.5% in wild birds (22/191) ($p < 0.01$). Surprisingly,
145 hedgehogs presented the largest prevalence with 13.5% (14/104) of the mammal
146 specimens [67% of the Algerian (2/3) and 26% of the European (12/47) samples
147 harbouring CR-genes]. Within the bird group, raptors represented the highest prevalence
148 with 7.3% (14/191) of the total bird specimens [13% (14/108) of the raptor species
149 examined] (Fig 2).

150 **Fig 2. Prevalence of cephalosporin resistant (CR) bacteria in the different wildlife**
151 **categories.**

152 CR isolates belonged to several genres within the *Enterobacteriaceae* family, with *E.*
153 *coli* being detected most frequently (45%). Interestingly, other clinically relevant
154 enterobacteria, including *K. pneumoniae* (20%), *C. freundii* (15%), *Ent. cloacae* (5%),
155 *P. mirabilis* (5%), *Providencia* spp (5%) and *Serratia marcescens* (2.5%) were also
156 identified as carriers of CR genes. The most common ESBL or pAmpC-encoding genes
157 were *bla*_{CMY-2} (50% of the isolates), *bla*_{SHV-12} (23%), *bla*_{CMY-1} (20%), *bla*_{TEM-1b} (20%),
158 and *bla*_{CTX-M-15} (18%). However, other gene variants such as *bla*_{CTX-M-3}, *bla*_{SHV-1}, *bla*_{SHV-}
159 ₁₁, *bla*_{SHV-28} and *bla*_{SHV-167} were also detected.

160 A high genetic diversity in terms of CR encoding genes was observed in all
161 *Enterobacteriaceae* spp, with 40% (16/40) of the isolates harbouring 2 to 5 different
162 resistance genes in the same isolate (Table 1). Furthermore, carbapenemase-encoding
163 gene, OXA-48 was detected in *E. coli* and *P. mirabilis* isolated from European
164 hedgehog and Barn owl, respectively (Table 1).

165 **Table 1. Prevalence and antimicrobial resistance genotypes and phenotypes of**
166 **beta-lactamase producing *Enterobacteriaceae* spp, detected in wildlife.**

167

168 Most of the ESBL/pAmpC *Enterobacteriaceae* isolates (92%), with the exception of
169 *Ent. cloacae*, were multiresistant with a common resistance phenotype comprising β -
170 lactams-quinolones-tetracycline-sulfamethoxazole/trimethoprim (Table 1). *K.*
171 *pneumoniae* and *C. freundii* isolates both presented a multi-drug resistance profile
172 including the resistance to aminoglycosides. Moreover, 90% of the *K. pneumoniae*
173 isolates were resistant to ciprofloxacin and sulphamethoxazole, 70% to kanamycin, 55%
174 to streptomycin, and 10% to florfenicol. Additionally, all tested *C. freundii* isolates
175 exhibited resistance to trimethoprim, 90% to ciprofloxacin and 80% to nalidixic acid
176 and tetracycline (Fig 3). Although no *mcr-1* genes were detected in this study, the
177 colistin resistant phenotype was observed in *Klebsiella* spp isolated from a European
178 greenfinch and Algerian hedgehog, and in a *Providencia* spp isolated from a common
179 buzzard.

180 **Fig 3. Percentage of antimicrobial resistance in ESBL producing**
181 ***Enterobacteriaceae* isolates according to Minimal Inhibitory Concentration Test.**

182 Number of isolates tested: *E. coli* (n=16), *K. pneumoniae* (n=9), *C. freundii* (n=5).

183

184 **Discussion**

185 This study identifies for the first time a high percentage of wild mammals and wild
186 birds as carriers of different human nosocomial-like *Enterobacteriaceae* species. These
187 isolates harboured a large diversity of ESBL/pAmpC genes, presented a high prevalence
188 of resistance to fluoroquinolones (principally *K. pneumoniae* and *C. freundii* isolates)
189 and in two occasions resistance to carbapenems, all drugs of last resort for the treatment
190 of multidrug resistant infections in hospital settings [4]. Additionally, new ESBL gene
191 variants are reported in wildlife for the first time.

192 *E. coli* are the most reported ESBL/AmpC-producing enterobacteria worldwide, with
193 increasing frequency from animals, food, environmental sources and humans. In recent
194 years, CR- *E. coli* transmission has been reported in different hosts, demonstrating a
195 close human-animal ESBL/AmpC gene similarity between livestock (broilers and pigs)
196 and personnel working at the farms [10]. Additionally, similar CR genes have been
197 reported between isolates from the community and those from human clinical settings,
198 sewage water and wild birds [10]. Although ESBL transmission has been studied
199 extensively in *Enterobacteriaceae* from humans and livestock, data on antimicrobial
200 resistance in the environment is still limited [2]. Moreover, most of the studies related to
201 ESBL-carrying bacteria in wildlife are focused on the wild bird population and mainly
202 restricted to *E. coli* species [25].

203 Studies performed in the Iberian Peninsula in wildlife, have reported *bla*_{CTX-M-1} as the
204 main ESBL gene circulating [19,26]. Additionally, *bla*_{CTX-M-14a} and *bla*_{SHV-12} have also
205 been frequently detected in *E. coli* from wild animals [12,27-29] with *bla*_{CTX-M-15}
206 described in a recent study carried out in wild birds in Tunisia [17]. Interestingly, in the
207 present study, *bla*_{CTX-M-1} and *bla*_{CTX-M-14a} were never detected, whereas *bla*_{CMY-2}, *bla*_{SHV-}
208 ₁₂ and *bla*_{CTX-M-15} were frequently isolated not only in *E. coli* but also in *K. pneumoniae*
209 and *C. freundii* isolates. Furthermore, *bla*_{CTX-M-15} and *bla*_{SHV-12} are currently the most
210 predominant enzymes in human clinical specimens from community and healthcare-
211 associated infections in Spain [30,31], likely suggesting the human community as the
212 initial source of ESBL-*Enterobacteriaceae* environmental contamination.

213 In this study, 6.8% of wild mammals, principally European hedgehogs and mustelids,
214 harboured ESBL/AmpC-producing *E. coli*, the remaining 10.7% resistant isolates
215 corresponded to other *Enterobacteriaceae* spp. Our results are in agreement with
216 previous studies conducted in Spain reporting low to moderate (1.3%-10%) prevalence

217 of ESBL/AmpC-producing *E. coli* genes in wild mammals [19]. In particular, in that
218 study, hedgehogs, deer and minks were found as reservoirs of *bla*_{CMY-2} and *bla*_{SHV-12} *E.*
219 *coli* variants [19,27]. However, in the present study new gene variants *bla*_{CTX-M-3},
220 *bla*_{SHV-1}, *bla*_{SHV-11}, *bla*_{SHV-28} and *bla*_{SHV-167}, are reported in wildlife.

221 Regarding the avian species analysed, the high prevalence of *bla*_{SHV-12} detected
222 specially in raptors is in concordance with previously reported data in Spain [12]. These
223 results confirm the successful dissemination of *bla*_{SHV-12} variants among the wild bird-
224 population in Spain [12], The Netherlands [32], Poland [33] and the Czech Republic
225 [34].

226 Plasmid-mediated colistin resistance by *mcr-1* has been reported worldwide in
227 *Enterobacteriaceae* isolated from humans, livestock, companion animals, food and
228 wildlife [35]. Colistin has been used in veterinary medicine during the last decades for
229 the treatment of gastrointestinal infections in livestock, principally in pigs and poultry
230 [36]. Consequently, livestock is considered the main reservoir of *mcr-1* selection and
231 dissemination worldwide. In a recent work, whole genome sequencing based analysis
232 disclosed the relationship among *mcr-1*-harbouring *E. coli* isolates recovered from the
233 environment, pig production and human clinical isolates, demonstrating the rapidly
234 evolving epidemiology of plasmid-mediated colistin-resistant *E. coli* strains worldwide
235 and the importance of the One Health approach [37]. In our study, some *Klebsiella* and
236 *Providencia* spp isolates were phenotypically resistant to colistin, but no *mcr-1* gene
237 was detected in the isolates examined. Nevertheless, although *mcr-1* is the most
238 commonly reported gene for colistin resistance, other less frequent genes not examined
239 in the study, like *mcr-2* to -5, could not be disregarded.

240 Information about carbapenem-resistant *Enterobacteriaceae* is very scarce in wildlife.
241 There is a study conducted in Germany reporting a carbapenem-resistant *Salmonella*

242 *enterica* from a wild bird [38]. In this study, we report the presence of *bla*_{OXA-48} in *E.*
243 *coli* and *P. mirabilis* isolates from a European hedgehog and a Barn owl, respectively.
244 Since, carbapenem-resistance genes have not yet been reported in livestock in Catalonia
245 (these antibiotics are not authorized for animal production), the original source of these
246 enzymes is likely to be hospitals and healthcare settings, although transmission from
247 soil bacteria cannot be disregarded.

248 In this line, not many wildlife studies have reported the presence of other ESBL-
249 producing *Enterobacteriaceae* species rather than *E. coli* in. Within them, *K.*
250 *pneumoniae* has been described in low prevalence (1.5% on average) in wild gulls from
251 different European countries [39], Chile and Canada [40], up to 23% in gulls from
252 Alaska [41]. More recently, 8.6% wild migratory birds from Pakistan showed *bla*_{CTX-M-}
253 ₁₅ ESBL-producing *K. pneumoniae* [18]. Additionally, ESBL-producing
254 *Enterobacteriaceae* have been described in wild birds and rodents worldwide, including
255 ESBL-producing *K. pneumoniae* ST307 and *E. coli* ST38 clonal lineages recently
256 reported in an urban West African rat population [42].

257 To our knowledge, there are no reports in wildlife describing the presence of CR genes
258 in such a variety of *Enterobacteriaceae* spp, like *Citrobacter* spp, *Serratia* spp, or
259 *Enterobacter* spp. *C. freundii*, is considered an opportunistic pathogen, associated with
260 nosocomial infections, especially in patients who have been hospitalized for a
261 prolonged period of time. In the last years, this bacterium has been classified as an
262 emerging health care associated to urinary tract infections commonly diagnosed in
263 healthcare settings [43]. *Ent. cloacae* has been reported as important opportunistic and
264 multiresistant pathogen involved in outbreaks of hospital-acquired infections in Europe,
265 particularly in France [44]. ESBL- *S. marcescens* has also been classified as one of the
266 top ten priority pathogens causing infections in intensive care units [45]. The high

267 prevalence of CR *Enterobacteriaceae* encountered in this study is really concerning,
268 since wildlife is not directly exposed to any antimicrobial agents. Therefore, faecal
269 contamination of water or soil with MDR bacteria and/or antimicrobial residues can
270 lead to a selection pressure. Wastewaters from urban areas and hospitals have been
271 identified as one of the major sources of AMR environmental contamination [2]. High
272 prevalence of *bla*_{SHV-12} but also *bla*_{TEM-1} and *bla*_{CTX-M-1} alleles have been reported in
273 aquatic environments (urban waters, natural or artificial water reservoirs, seawater or
274 drinking water) in several countries worldwide, likely due to their relatively easy
275 transmission to surface water through waste water treatment plant discharges [2,46]. In
276 our study, we observed wildlife in close contact with urban and farming areas of
277 Catalonia carrying a large variety of zoonotic/nosocomial bacteria genetically resistant
278 to β -lactams-quinolones-tetracycline-sulfamethoxazole/trimethoprim-aminoglycosides
279 with similar resistant genes to those found in livestock and clinical settings. Moreover,
280 OXA-48 variants with an-extended spectrum of resistance to carbapenems were also
281 detected in our wildlife population of Catalonia. This variant is highly prevalent in
282 hospital settings in Spain [20].

283 **Conclusions**

284 This study describes for the first time a high prevalence of *Enterobacteriaceae* spp
285 harbouring a large variety of carbapenem and CR genes in the wildlife population of
286 Catalonia. Bacterial spp described in this collection are associated to nosocomial
287 infections and most of the gene-variants described here are frequently found in clinical
288 settings. Since these wild animals had not previous antimicrobial treatment, our results
289 suggest that both, antimicrobial residues and antimicrobial resistant bacteria are a spill-
290 over consequence of anthropogenic pollution. Additionally, wildlife can contribute

291 indirectly to the dissemination of resistance genes into other natural areas increasing the
292 prevalence of AMR genes in natural environments. Thus, implementation of control
293 measures to reduce the impact of anthropogenic pressure in the environment is urgently
294 needed.

295 In summary, these results support the concept that wildlife is a good sentinel of AMR
296 environmental contamination and simultaneously underline the importance of the One
297 Health approach. Further studies are needed to assess clonal relatedness among different
298 cephalosporin and carbapenem resistant enterobacteria at the human-animal-
299 environment interface.

300 **Acknowledgements**

301 Our grateful thanks to the Torreferrusa WRC staff. A. Vidal was supported by a PIF
302 grant from the Universitat Autònoma de Barcelona. Contract of LMG was supported by
303 the Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA) and
304 the European Social Fund.

305

306 **References**

307

- 308 1. Pitout JD, Laupland KB. Extended-spectrum beta-lactamase-producing
309 Enterobacteriaceae: an emerging public-health concern. *Lancet Infect Dis.*
310 2008;8(3):159-66.
- 311 2. Liakopoulos A, Mevius D, Ceccarelli D. A Review of SHV Extended-Spectrum
312 β -Lactamases: Neglected Yet Ubiquitous. *Front Microbiol.* 2016;7:1374. doi:
313 0.3389/fmicb.2016.01374.

- 314 3. Tzouvelekis LS, Markogiannakis A, Psychogiou M, Tassios PT, Daikos GL.
315 Carbapenemases in *Klebsiella pneumoniae* and other *Enterobacteriaceae*: An
316 evolving crisis of global dimensions. *Clin Microbiol Rev.* 2012;25:682–707
- 317 4. Grundmann H, Glasner C, Albiger B, Aanensen DM, Tomlinson CT,
318 Andradeviã AT, et al. Occurrence of carbapenemase-producing *Klebsiella*
319 *pneumoniae* and *Escherichia coli* in the European survey of carbapenemase-
320 producing *Enterobacteriaceae* (EuSCAPE): a prospective, multinational study.
321 *Lancet Infect Dis* 17:153–163. doi:10.1016/S1473-3099(16)30257-2.
- 322 5. Jouini A, Vinué L, Slama KB, Sáenz Y, Klibi N, Hammami S, et al.
323 Characterization of CTX-M and SHV extended-spectrum beta-lactamases and
324 associated resistance genes in *Escherichia coli* strains of food samples in
325 Tunisia. *J Antimicrob Chemother.* 2007;60:1137–1141
- 326 6. Smet A, Martel A, Persoons D, Dewulf J, Heyndrickx M, Herman L, et al.
327 Broad-spectrum β -lactamases among *Enterobacteriaceae* of animal origin:
328 molecular aspects, mobility and impact on public health. *FEMS Microbiol Rev.*
329 2010;34:295–316
- 330 7. Wieler LH, Ewers C, Guenther S, Walther B, Lübke-Becker A. Methicillin-
331 resistant staphylococci (MRS) and extended spectrum-beta-lactamases (ESBL)-
332 producing *Enterobacteriaceae* in companion animals: nosocomial infections as
333 one reason for the rising prevalence of these potential zoonotic pathogens in
334 clinical samples. *Int J Med Microbiol.* 2011;301:635–641
- 335 8. Dierikx C, van der Goot J, Fabri T, van Essen-Zandbergen A, Smith H, Mevius
336 D. Extended-spectrum- β -lactamase- and AmpC- β -lactamase-producing
337 *Escherichia coli* in Dutch broilers and broiler farmers. *J Antimicrob Chemother.*
338 2013;68:60–67

- 339 9. Sallem RB, Gharsa H, Slama KB, Rojo-Bezares B, Estepa V, Porres-Osante N,
340 et al. First detection of CTX-M-1, CMY-2, and QnrB19 resistance mechanisms
341 in fecal *Escherichia coli* isolates from healthy pets in Tunisia. *Vector Borne*
342 *Zoonotic Dis.* 2013; 13:98–102
- 343 10. Dorado-García A, Smid JH, van Pelt W, Bonten MJM, Fluit AC, van den Bunt
344 G, et al. Molecular relatedness of ESBL/AmpC-producing *Escherichia coli* from
345 humans, animals, food and the environment: a pooled analysis. *J Antimicrob*
346 *Chemother.* 2018;73(2):339-347.
- 347 11. Huijbers PM, Blaak H, de Jong MC, Graat EA, Vandenbroucke-Grauls CM, de
348 Roda Husman AM. Role of the Environment in the Transmission of
349 Antimicrobial Resistance to Humans: A Review. *Environ Sci Technol.*
350 2015;49(20):11993-2004
- 351 12. Alcalá L, Alonso CA, Simón C, González-Esteban C, Orós J, Rezusta A, et al.
352 Wild Birds, Frequent Carriers of Extended-Spectrum β -Lactamase
353 (ESBL)Producing *Escherichia coli* of CTX-M and SHV-12 Types. *Microb Ecol.*
354 2016;72(4):861-869..
- 355 13. Hasan B, Laurell K, Rakib MM, Ahlstedt E, Hernandez J, Caceres M, et al.
356 Fecal Carriage of Extended-Spectrum β -Lactamases in Healthy Humans,
357 Poultry, and Wild Birds in León, Nicaragua-A Shared Pool of bla(CTX-M)
358 Genes and Possible Interspecies Clonal Spread of Extended-Spectrum β -
359 Lactamases-Producing *Escherichia coli*. *Microb Drug Resist.* 2016;22(8):682-
360 687. Epub 2016 Mar 23. PubMed PMID: 27007258.
- 361 14. Parker D, Sniatynski MK, Mandrusiak D, Rubin JE. Extended-spectrum β -
362 lactamase producing *Escherichia coli* isolated from wild birds in Saskatoon,
363 Canada. *Lett Appl Microbiol.* 2016;63(1):11-5.

- 364 15. Atterby C, Börjesson S, Ny S, Järhult JD, Byfors S, Bonnedahl J. ESBL-
365 producing *Escherichia coli* in Swedish gulls-A case of environmental pollution
366 from humans? PLoSOne. 2017;12(12):e0190380.
367 doi:10.1371/journal.pone.0190380.
- 368 16. Mohsin M, Raza S, Schaufler K, Roschanski N, Sarwar F, Semmler T, et al.
369 High Prevalence of CTX-M-15-Type ESBL-Producing *E. coli* from Migratory
370 Avian Species in Pakistan. Front Microbiol. 2017;8:2476.
- 371 17. Ben Yahia H, Ben Sallem R, Tayh G, Klibi N, Ben Amor I, Gharsa H, et al.
372 Detection of CTX-M-15 harboring *Escherichia coli* isolated from wild birds in
373 Tunisia. BMC Microbiol. 2018;18(1):26.
- 374 18. Raza S, Mohsin M, Madni WA, Sarwar F, Saqib M, Aslam B. First Report of
375 bla(CTX-M-15)-Type ESBL-Producing *Klebsiella pneumoniae* in Wild
376 Migratory Birds in Pakistan. Ecohealth. 2017;14(1):182-186.
- 377 19. Alonso CA, Alcalá L, Simón C, Torres C. Novel sequence types of extended-
378 spectrum and acquired AmpC beta-lactamase producing *Escherichia coli* and
379 *Escherichia clade V* isolated from wild mammals. FEMS Microbiol Ecol.
380 2017;93(8). doi: 10.1093/femsec/fix097
- 381 20. Aracil-García B, Oteo-Iglesias J, Cuevas-Lobato Ó, Lara-Fuella N, Pérez-
382 Grajera I, Fernández-Romero S, et al. Spanish Group European Antimicrobial
383 Resistance Surveillance network (EARS-Net). Rapid increase in resistance to
384 third generation cephalosporins, imipenem and co-resistance in *Klebsiella*
385 *pneumoniae* from isolated from 7,140 blood-cultures (2010-2014) using EARS-
386 Net data in Spain. Enferm Infecc Microbiol Clin. 2017;35(8):480-486.
- 387 21. Spanish. R.D.1201/2005 of the Ministry of Presidency of Spain (10th October
388 2005). BOE 21st October 2005. Available:

- 389 www.boe.es/boe/dias/2005/10/21/pdfs/A34367-34391.pdf. Accessed 2018 Dec
390 27.
- 391 22. Hasman H, Mevius D, Veldman K, Olesen I, Aarestrup FM. 2005. b-Lactamases
392 among extended-spectrum b-lactamase (ESBL)-resistant Salmonella from
393 poultry, poultry products and human patients in The Netherlands. *J Antimicrob*
394 *Chemother.* 2005;56:115–121.
- 395 23. Poirel L, Walsh TR, Cuvillier V, Nordmann P. Multiplex PCR for detection of
396 acquired carbapenemase genes. *Diagn Microbiol Infect Dis.* 2011;70(1):119-23.
- 397 24. Liu YY, Wang Y, Walsh TR, Yi LX, Zhang R, Spencer J, et al. Emergence of
398 plasmid-mediated colistin resistance mechanism MCR-1 in animal and human
399 beings in China: a microbiological and molecular biological study. *Lancet*
400 *Infect Dis.* 2016;16(2):161-8.
- 401 25. Guenther S, Ewers C, Wieler LH. Extended-Spectrum Beta-Lactamases
402 Producing *E. coli* in Wildlife, yet another Form of Environmental Pollution?
403 *Front Microbiol.* 2011;2:246
- 404 26. Simões RR, Poirel L, Da Costa PM, Nordmann P. Seagulls and beaches as
405 reservoirs for multidrug-resistant *Escherichia coli*. *Emerg Infect Dis.*
406 2010;16(1):110-2.
- 407 27. Alonso CA, Michael GB, Li J, Somalo S, Simón C, Wang Y, et al. Analysis of
408 blaSHV-12-carrying *Escherichia coli* clones and plasmids from human, animal
409 and food sources. *J Antimicrob Chemother.* 2017;72(6):1589-1596.
- 410 28. Costa D, Poeta P, Sáenz Y, Vinué L, Rojo-Bezares B, Jouini A, et al. Detection
411 of *Escherichia coli* harbouring extended-spectrum beta-lactamases of the CTX-
412 M, TEM and SHV classes in faecal samples of wild animals in Portugal. *J*
413 *Antimicrob Chemother.* 2006;58(6):1311-2.

- 414 29. Cristóvão F, Alonso CA, Igrejas G, Sousa M, Silva V, Pereira JE, et al. Clonal
415 diversity of extended-spectrum beta-lactamase producing *Escherichia coli*
416 isolates in fecal samples of wild animals. *FEMS Microbiol Lett.* 2017;364(5).
- 417 30. Díaz MA, Hernández-Bello JR, Rodríguez-Baño J, Martínez-Martínez L, Calvo
418 J, Blanco J, et al. Spanish Group for Nosocomial Infections (GEIH). Diversity of
419 *Escherichia coli* strains producing extended-spectrum beta-lactamases in Spain:
420 second nationwide study. *J Clin Microbiol.* 2010;48(8):2840-5.
- 421 31. Merino I, Shaw E, Horcajada JP, Cercenado E, Mirelis B, Pallarés MA, et al.
422 ITUBRAS-GEIH-SEIMC Group. CTX-M-15-H30Rx-ST131 subclone is one of
423 the main causes of healthcare-associated ESBL-producing *Escherichia coli*
424 bacteraemia of urinary origin in Spain. *J Antimicrob Chemother.*
425 2016;71(8):2125-30
- 426 32. Veldman K, van Tulden P, Kant A, Testerink J, Mevius D. Characteristics of
427 cefotaxime-resistant *Escherichia coli* from wild birds in the Netherlands. *Appl*
428 *Environ Microbiol.* 2013;79(24):7556-61.
- 429 33. Literak I, Dolejska M, Janoszowska D, Hrusakova J, Meissner W, Rzycka H, et
430 al. Antibiotic-resistant *Escherichia coli* bacteria, including strains with genes
431 encoding the extended-spectrum beta-lactamase and QnrS, in waterbirds on the
432 Baltic Sea Coast of Poland. *Appl Environ Microbiol.* 2010;76(24):8126-34.
- 433 34. Dolejská M, Bierosová B, Kohoutová L, Literák I, Cízek A. Antibiotic-resistant
434 *Salmonella* and *Escherichia coli* isolates with integrons and extended-spectrum
435 beta-lactamases in surface water and sympatric black-headed gulls. *J Appl*
436 *Microbiol.* 2009;106(6):1941-50.
- 437 35. Skov RL, Monnet DL. Plasmid-mediated colistin resistance (*mcr-1* gene): three
438 months later, the story unfolds. *Euro Surveill.* 2016;21:30155

- 439 36. EMA. Updated Advice on the Use of Colistin Products in Animals within the
440 European Union: Development of Resistance and Possible Impact on Human
441 and Animal Health. 2016.
442 http://www.ema.europa.eu/docs/en_GB/document_library/Press_release/2016/07/WC500211081.pdf.
443
- 444 37. Guenther S, Falgenhauer L, Semmler T, Imirzalioglu C, Chakraborty T, Roesler
445 U, et al. Environmental emission of multiresistant *Escherichia coli* carrying the
446 colistin resistance gene *mcr-1* from German swine farms. *J Antimicrob*
447 *Chemother.* 2017;72(5):1289-1292.
- 448 38. Fischer J, Schmogger S, Jahn S, Helmuth R, Guerra B. NDM-1 carbapenemase-
449 producing *Salmonella enterica* subsp. *enterica* serovar *Corvallis* isolated from a
450 wild bird in Germany. *J Antimicrob Chemother.* 2013;68(12):2954-6.
- 451 39. Bonnedahl J, Stedt J, Waldenström J, Svensson L, Drobni M, Olsen B.
452 Comparison of Extended-Spectrum β -Lactamase (ESBL) CTX-M Genotypes in
453 Franklin Gulls from Canada and Chile. *PLoS One.* 2015;10(10):e0141315
- 454 40. Stedt J, Bonnedahl J, Hernandez J, Waldenström J, McMahon BJ, Tolf C, et al.
455 Carriage of CTX-M type extended spectrum β -lactamases (ESBLs) in gulls
456 across Europe. *Acta Vet Scand.* 2015;57:74.
- 457 41. Bonnedahl J, Hernandez J, Stedt J, Waldenström J, Olsen B, Drobni M.
458 Extended-spectrum β -lactamases in *Escherichia coli* and *Klebsiella pneumoniae*
459 in Gulls, Alaska, USA. *Emerg Infect Dis.* 2014;20(5):897-9.
- 460 42. Schaufler K, Nowak K, Dux A, Semmler T, Villa L, Kourouma L, et al.
461 Clinically Relevant ESBL-Producing *K. pneumoniae* ST307 and *E. coli* ST38 in
462 an Urban West African Rat Population. *Front Microbiol.* 2018;9:150.

- 463 43. Ranjan KP, Ranjan N. Citrobacter: An emerging health care associated urinary
464 pathogen. *Urology Annals*. 2013;5(4):313-314.
- 465 44. Davin-Regli A, Pagès JM. Enterobacter aerogenes and Enterobacter cloacae;
466 versatile bacterial pathogens confronting antibiotic treatment. *Frontiers*
467 *Microbiol*. 2015;6:392.
- 468 45. Rello J, Kalwaje Eshwara V, Lagunes L, Alves J, Wunderink RG, Conway-
469 Morris A, et al. A global priority list of the TOP TEn resistant Microorganisms
470 (TOTEM) study at intensive care: a prioritization exercise based on multi-
471 criteria decision analysis. *Eur J Clin Microbiol Infect Dis*. 2018. doi:
472 10.1007/s10096-018-3428-y. [Epub ahead of print]
- 473 46. Rashid M, Rakib MM, Hasan B. Antimicrobial-resistant and ESBL-producing
474 Escherichia coli in different ecological niches in Bangladesh. *Infect Ecol*
475 *Epidemiol*. 2015;5:26712.
- 476
477
478
479

Table 1. Prevalence and antimicrobial resistance genotypes and phenotypes of beta-lactamase producing *Enterobacteriaceae* spp, detected in wildlife.

<i>Scientific name</i> (common name)	Total sample	AMR genes	Bacterial spp	Drug-resistance genes	Resistance phenotype to non-B-lactams
Mammals (N=104)	N	n	Prev		
<i>Aetechinus algirus</i> (Algerian hedgehog)	3	2	67%	<i>Escherichia coli</i>	CMY-2 CIP, NAL, KM, TM
				<i>Klebsiella oxytoca</i>	CTX-M-3 GM, ST, FF, CF, TE, COL, TM
<i>Erinaceus europaeus</i> (European hedgehog)	47	12	26%	<i>Escherichia coli</i>	CMY-2 nd
				<i>Escherichia coli</i>	CMY-2 KM
				<i>Escherichia coli</i>	CMY-2 nd
				<i>Escherichia coli</i>	SHV-12 ST
				<i>Escherichia coli</i>	SHV-11,OXA-48 CIP, NAL, KM, TE, SU, TM
				<i>Klebsiella pneumoniae</i>	CMY-1,CMY-2, SHV-1,TEM-1b,CTX-M15 CIP, GM, ST, KM, TE, SU, TM
				<i>Klebsiella pneumoniae</i>	SHV-11, TEM-1b CIP, NAL, GM, ST, KM, TE, SU, TM
				<i>Klebsiella pneumoniae</i>	SHV-28 CIP, NAL, GM, ST, KM, TE, COL, SU, TM
				<i>Klebsiella pneumoniae</i>	SHV-12 CIP, NAL, KM, TE, SU, TM
				<i>Citrobacter freundii</i>	CMY-2, TEM-1b CIP, NAL, KM, TE, TM
				<i>Citrobacter freundii</i>	CMY-2, SHV-12 CIP, NAL, ST, KM, TE, COL, SU, TM
				<i>Citrobacter freundii</i>	CMY-2 CIP, NAL
<i>Capreolus capreolus</i> (European roe deer)	2	1	na	<i>Enterobacter cloacae</i>	CMY-2 SU
<i>Martes foina</i> (Beech marten)	2	1	na	<i>Citrobacter freundii</i>	CMY-2, SHV-12 CIP, NAL, GM, TE, SU, TM
<i>Meles meles</i> (European badger)	1	1	na	<i>Escherichia coli</i>	SHV-12 CIP, NAL, CF, SU, TM
<i>Mustela vison</i> (American mink)	13	1	8%	<i>Enterobacter cloacae</i>	CMY-2 SU

PREVALENCE IN MAMMALS 104 18 17.3%

CIP, Ciprofloxacin; NAL, Nalidixic acid; GN, Gentamicin; ST, Streptomycin; KM, Kanamycin; FF, Florfenicol; CF, Chloramphenicol; TE, Tetracycline; COL, Colistin; SU, Sulphametoxazole; TM, Trimethoprim. nd, not detected.

Table 1 (continuation). Prevalence and antimicrobial resistance genotypes and phenotypes of beta-lactamase producing *Enterobacteriaceae* spp, detected in wildlife

<i>Scientific name (common name)</i>	Total sample	AMR genes		Bacterial spp	Drug-resistance genes	Resistance phenotype to non-B-lactams
Raptors (n=108)	N	n	Prev			
<i>Accipiter gentilis</i> (northern goshawk)	13	3	23%	<i>Escherichia coli</i>	TEM-1b	COL
				<i>Escherichia coli</i>	CMY-2	CIP, NAL
				<i>Proteus mirabilis</i>	CMY-1, CMY-2, SHV-28, TEM-1b	CIP, NAL, GM, ST, KM, TE, SU, TM
<i>Accipiter nisus</i> (Eurasian sparrowhawk)	8	3	38%	<i>Escherichia coli</i>	CMY-1, SHV-1, TEM-1b, CTX-M15	CIP, NAL, KM, TE, SU, TM
				<i>Escherichia coli</i>	TEM-1b	CIP, TE, TM
				<i>Serratia marcescens</i>	CMY-1, CTX-M15	CIP, TE, COL, SU, TM
<i>Bubo bubo</i> (Eurasian eagle-owl)	1	1	na	<i>Escherichia coli</i>	CMY-1, SHV-167	nd
<i>Buteo buteo</i> (Common buzzard)	17	2	12%	<i>Escherichia coli</i>	SHV-12	ST, CF, TE, SU, TM
				<i>Providencia alcalifaciens</i>	SHV-12	CIP, NAL,GM,ST,KM, FF,CF, TE,SU, TM
<i>Strix aluco</i> (Tawny owl)	18	3	17%	<i>Klebsiella pneumoniae</i>	CMY-2, SHV-28	ST, SU, TM
				<i>Escherichia coli</i>	CMY-2, SHV-1	nd
				<i>Klebsiella pneumoniae</i>	SHV-12, CTX-M15	CIP
<i>Tyto alba</i> (Barn owl)	3	2	67%	<i>Escherichia coli</i>	CMY-2	CIP, NAL, ST, TE
				<i>Proteus mirabilis</i>	SHV-12,TEM-1b, OXA-48	CIP, NAL, ST, KM, CF,TE, COL, SU, TM
Other birds (n=83)	N	n	Prev			
<i>Carduelis carduelis</i> (European goldfinch)	12	1	8%	<i>Citrobacter freundii</i>	CMY-2	CIP, NAL, GM, ST, KM, CF, TE, SU, TM
<i>Carduelis choris</i> (European Greenfinch)	2	1	na	<i>Klebsiella pneumoniae</i>	CMY-1	CIP, NAL, KM, FF, CF, SU
<i>Larus michahellis</i> (Yellow-legged gull)	7	1	14%	<i>Escherichia coli</i>	CTX-M-15	CIP, NAL, GM, KM, TE, SU, TM
<i>Serinus serinus</i> (European serin)	6	1	17%	<i>Klebsiella pneumoniae</i>	CMY-1, SHV-28	CIP, NAL, ST, KM, TE, SU, TM
<i>Streptopelia decaocto</i> (Eur. collared dove)	1	1	na	<i>Citrobacter freundii</i>	CMY-2	FF, TM
<i>Sylvia melanocephala</i> (Sardinian warbler)	6	2	33%	<i>Escherichia coli</i>	CMY-2	CIP, NAL
				<i>Providencia spp</i>	CTX-M15, CMY-1	CIP, NAL, GM, ST, KM, CF, TE, SU, TM
<i>Turdus merula</i> (Common blackbird)	8	1	13%	<i>Escherichia coli</i>	CMY-2	CIP, NAL, KM, TM
PREVALENCE IN BIRDS	191	22	11.5%			

CIP, Ciprofloxacin; NAL, Nalidixic acid; GN, Gentamicin; ST, Streptomycin; KM, Kanamycin; FF, Florfenicol; CF, Chloramphenicol; TE, Tetracycline; COL, Colistin; SU, Sulphamethoxazole; TM, Trimethoprim. nd, not detected.

■ Raptors (n=108)

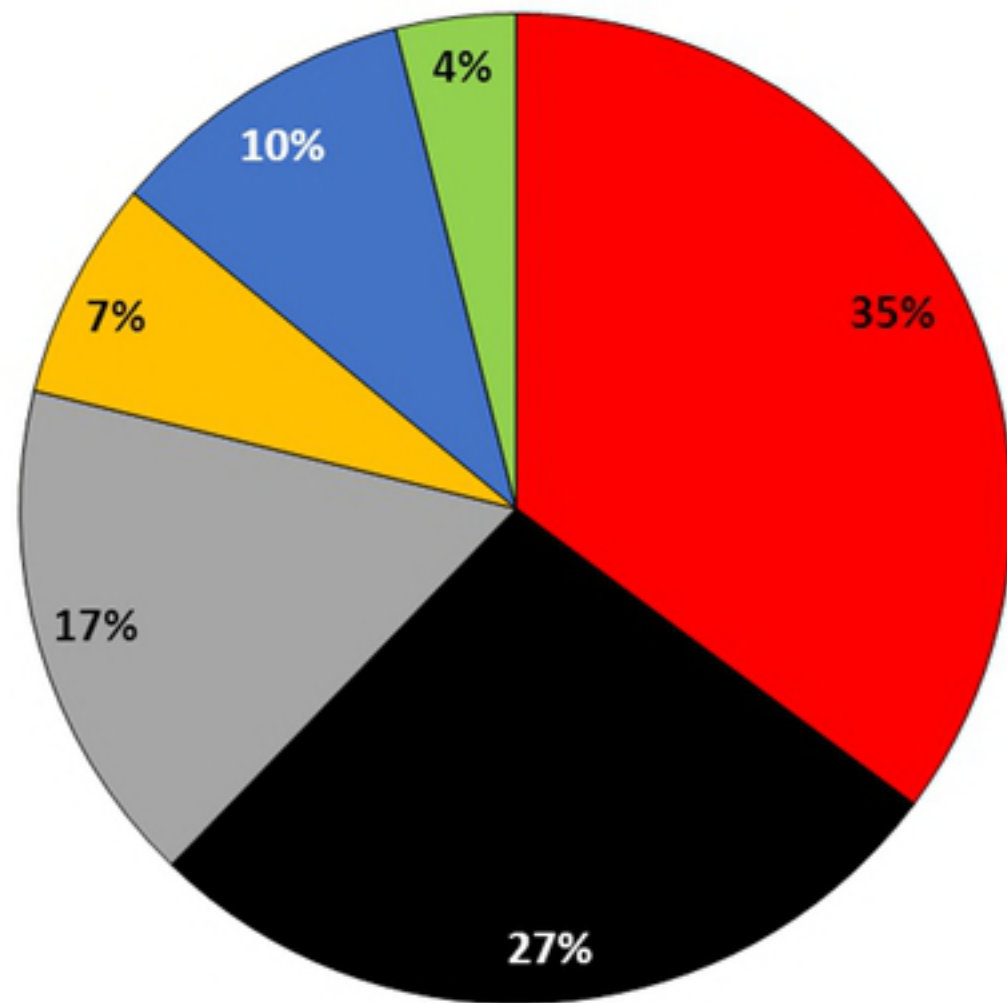
■ Wild birds (n=83)

■ Hedgehogs (n=51)

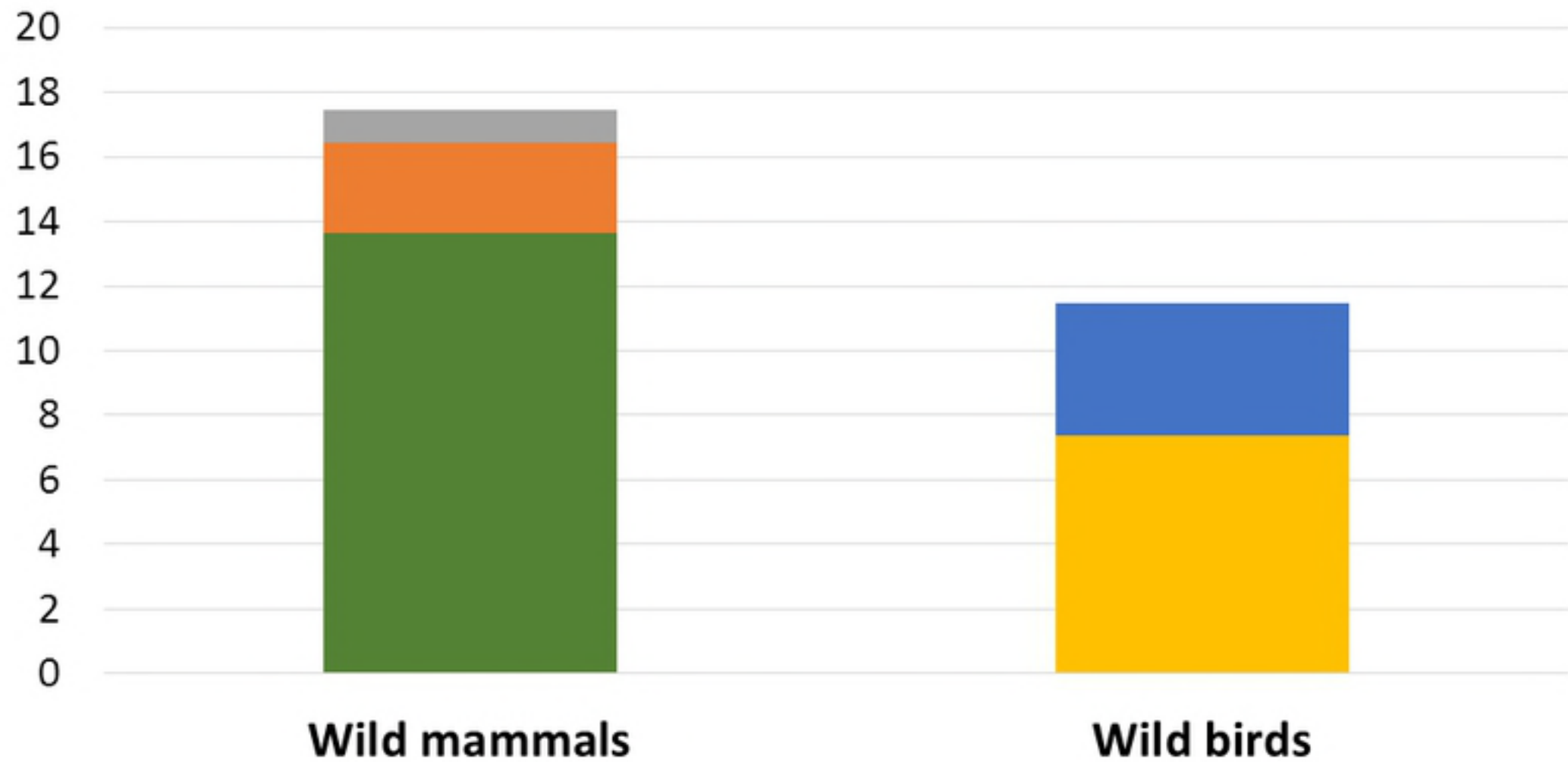
■ Carnivores (n=22)

■ Other mammals (n=31)

■ Turtles (n=12)



Figure



■ Hedgehogs

■ Mustelids

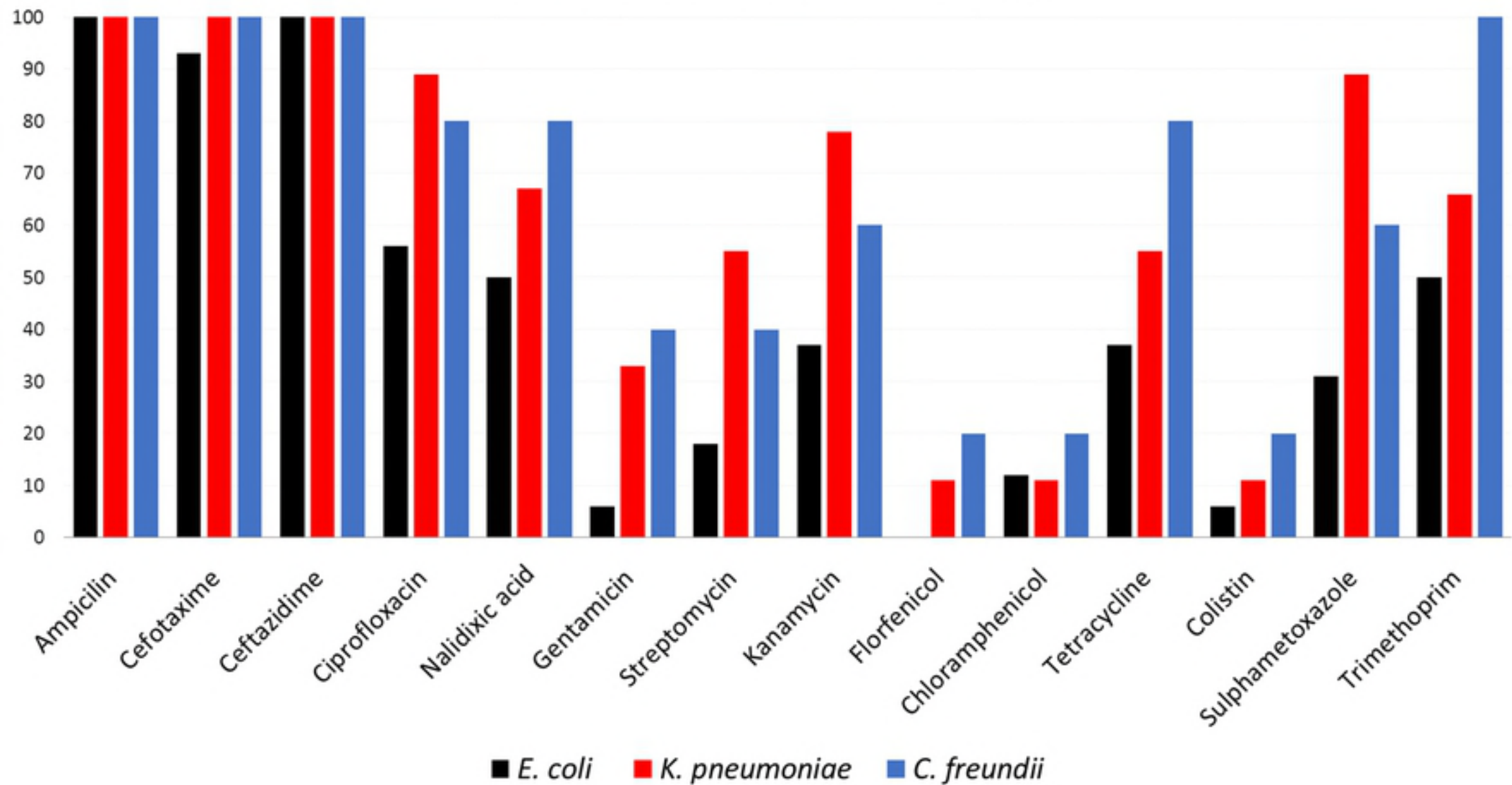
■ Deers

■ Raptors

■ Other birds

Figure

ANTIMICROBIAL RESISTANCE PROFILES (%)



Figure