Antimicrobial Resistance in Indicator *Escherichia coli* Isolates from Free-Ranging Livestock and Sympatric Wild Ungulates in a Natural Environment (Northeastern Spain)

N. Navarro-Gonzalez,[a] M. C. Porrero,[b] G. Mentaberre,[a] E. Serrano,[a,c] A. Mateos,[b] L. Domínguez,[b] S. Lavín[a]

Servei d' Ecopatologia de Fauna Salvatge (SEFaS), Departament de Medicina i Cirurgia Animals, Universitat Autònoma de Barcelona (UAB), Bellaterra, Barcelona, Spain[a]; Centro de Vigilancia Sanitaria Veterinaria (VISAVET), Universidad Complutense (UCM), Madrid, Spain[b]; Estadística i Investigació Operativa, Departament de Matemàtica, Universitat de Lleida (UdL), Lleida, Spain[c]

Antimicrobial resistance was assessed in indicator *Escherichia coli* isolates from free-ranging livestock and sympatric wild boar (*Sus scrofa*) and Iberian ibex (*Capra pyrenaica*) in a National Game Reserve in northeastern Spain. The frequency of antimicrobial resistance was low (0% to 7.9%). However, resistance to an extended-spectrum cephalosporin and fluoroquinolones was detected.

Antimicrobial resistance (AMR) may compromise the treatment of severe human diseases (1), and thus monitoring and reporting its occurrence is a priority for health surveillance agencies worldwide. This phenomenon has been partly associated with the use of antimicrobial agents in intensive animal food production (2); in fact, a lower occurrence of resistant bacteria has been repeatedly observed in extensive or organic farming systems than in systems employing intensive rearing (3–6).

Moreover, many studies have found similarities in the patterns of resistance in bacterial isolates from livestock and small fauna, e.g., rodents (7, 8), insects (7, 9), or birds (9), from the farm settings. Thus, we were interested in determining whether wild ungulates in close contact with free-ranging livestock carry indicator bacteria with similar resistance profiles. Indicator (commensal) *Escherichia coli* is suitable for such a study, since it is common in animal feces and provides information on resistance in a population (1).

For this purpose, we sampled both wild ungulates (wild boar [*Sus scrofa*] and Iberian ibex [*Capra pyrenaica*]) and free-ranging livestock cohabiting in a game reserve in northeastern Spain. The use of antimicrobials in this study area can be ruled out, and human activities and, thus, selective pressure are reduced. Therefore, we expect *E. coli* from these host populations to be almost free of antimicrobial resistance.

The study area is located within the National Game Reserve and Natural Park "Ports de Tortosa i Beseit" (referred to as the NGR here) in northeastern Spain. Wildlife and livestock share pastures in some canyons in the study area. See reference 10 for further information on the area and the livestock presence.

Individual fecal samples were obtained ($n = 143$) from hunter-harvested wild boars during the regular hunting season (October to January) from 2009 to 2011. Individual fecal samples ($n = 46$) were obtained from cattle (5 herds; 380 head in total), and four samples were obtained from the only horse herd in the NGR (32 head). Fecal samples were collected and stored in a sterile container and refrigerated until being sent to the laboratory within the subsequent 24 h.

Iberian ibexes ($n = 184$) were either harvested by hunters ($n = 154$) or captured ($n = 30$) from 2009 to 2011. Due to the characteristics of the hunting method, fecal samples had to be stored at −18°C until being sent to the laboratory. *E. coli* bacteria are known for their cold shock response (11); thus, we can assume that the isolation of this microorganism from feces was not highly affected by storage at this temperature.

In total, 25 g of feces was diluted in buffered peptone water (225 ml). Once diluted, one loop was cultured on MacConkey agar (direct plating) at 37°C for 18 to 20 h. One compatible colony per plate was selected and confirmed by PCR (12). This confirmed colony of indicator *E. coli* (i.e., one clone per animal) was tested for antimicrobial susceptibility (13, 14). Table 1 shows the antimicrobial agents and epidemiological cutoff values used to report microbiological resistance (1).

All isolates from livestock were tested for antimicrobial resistance ($n = 42$ [38 from cattle and 4 from horses]). A selection of *E. coli* isolates from wildlife was performed to spatially represent the whole study area; therefore, one isolate was selected per location and hunting session for wild boar and Iberian ibex (altogether, 63 and 89 isolates, respectively).

For the comparison of our data with that from intensively reared livestock, Table 2 shows the frequencies of resistant *E. coli* in cattle from Spain (26).

Frequencies of resistance in *E. coli* were compared between host species with Fisher's exact test, and the significance level was set at $\alpha = 0.05$. The P values obtained from multiple comparisons were adjusted with the strict Bonferroni correction. The statistical analyses were performed with R Software (15).

Eight wild boars (12.7%), 4 cows (10.53%), 3 Iberian ibexes (3.37%), and no horses were carriers of *E. coli* resistant to the antimicrobial agents tested (7.65% of the total tested samples). These frequencies were not statistically different (for wild boar versus cattle, adjusted P value = 1; for wild boar versus Iberian ibex, adjusted P value = 0.15; and for cattle versus Iberian ibex, adjusted P value = 0.63). No isolate resistant to colistin, amoxicillin-clavulanate, cefoxitin, amikacin, apramicin, imipenem,

TABLE 1 Antimicrobial agents and epidemiological cutoff values

Antimicrobial agent	Epidemiological cutoff value (zone diam or concn)	Source[a]
Disk diffusion		
Amoxicillin-clavulanate	17 mm	EUCAST
Cefoxitin	19 mm	EUCAST
Amikacin	18 mm	EUCAST
Apramicin	20 mm	Rosco Diagnostica
Imipenem	24 mm	EUCAST
Aztreonam	27 mm	EUCAST
Broth microdilution		
Sulfamethoxazole	64 mg/liter	EFSA
Gentamicin	2 mg/liter	EFSA
Ampicillin	8 mg/liter	EFSA
Ciprofloxacin	0.064 mg/liter	EFSA
Cefotaxime	0.25 mg/liter	EFSA
Ceftazidime	0.5 mg/liter	EFSA
Tetracycline	8 mg/liter	EFSA
Streptomycin	16 mg/liter	EFSA
Trimethoprim	2 mg/liter	EFSA
Chloramphenicol	16 mg/liter	EFSA
Florfenicol	16 mg/liter	EFSA
Kanamycin	8 mg/liter	EUCAST
Nalidixic acid	16 mg/liter	EFSA
Colistin	2 mg/liter	EFSA

[a] EFSA, EFSA Journal (14). EUCAST, www.srga.org/eucastwt/WT_EUCAST.htm.

aztreonam, gentamicin, ceftazidime, chloramphenicol, or florfenicol was found. Table 2 shows the percentage of isolates from each host group showing resistance to the rest of antimicrobial agents tested. Frequencies of resistance ranged from 0% to 7.9%. Cattle from the NGR had a significantly ($P < 0.05$) lower frequency of *E. coli* resistant to sulfamethoxazole, ampicillin, tetracycline, streptomycin, and trimethoprim than intensively reared cattle from Spain (Table 2). The same resistance profile was rarely detected more than once (Table 3).

Frequencies of AMR were less than 10%, which is defined by

TABLE 2 Frequencies of resistant *E. coli* from each host species of the National Game Reserve and from cattle from intensive rearing in Spain[a]

Antimicrobial agent	Frequency (%)			
	National Game Reserve			Intensively reared Spanish cattle[b]
	Wild boar	Iberian ibex	Cattle	
Ciprofloxacin	3.2	0	7.1	3.5
Sulfamethoxazole	6.3	1.1	**2.3**	**35.2**
Ampicillin	4.8	1.1	**2.3**	**15.6**
Cefotaxime	1.6	0	0	0
Tetracycline	7.9	3.3	**2.3**	**48.8**
Streptomycin	4.8	1.1	**2.3**	**35.5**
Trimethoprim	3.2	1.1	**0**	**17.6**
Kanamycin	6.3	0	0	2.7
Nalidixic acid	1.6	0	4.7	3.1

[a] Antimicrobial agents to which pansusceptibility in the NGR was found are referred in the text. Data in bold represent statistically significant differences between the results from free-ranging cattle from our study area and from intensively reared cattle from Spain ($P < 0.05$).
[b] Data are from reference 26.

TABLE 3 Phenotypic profile of the resistant *E. coli* strains isolated from the animal hosts of the NGR

Host species (no. of isolates)	Resistance profile[a]
Wild boar	CIPR, SMX, AMP, CEFOT, TET, NAL
Wild boar	SMX, AMP, TET, STR, KAN
Iberian ibex	SMX, AMP, TET, STR, TMP
Wild boar	SMX, TET, STR, TMP, KAN
Wild boar	CIPR, AMP, STR
Wild boar	SMX, TET, TMP
Cattle	SMX, TET, STR
Cattle	CIPR, AMP, NAL
Cattle	CIPR, NAL
Cattle	CIPR
Wild boar (2)	KAN
Iberian ibex (2), Wild boar	TET

[a] CIPR, ciprofloxacin; SMX, sulfamethoxazole; AMP, ampicillin; CEFOT, cefotaxime; TET, tetracycline; NAL, nalidixic acid; STR, streptomycin; KAN, kanamycin; TMP, trimethoprim.

the European Food Safety Authority (EFSA) (1) as a low resistance level. In the literature, great variations have been observed in AMR depending on the species, the ecosystem, and the geographic location. In some cases, this variation has been connected to the presence of farms (16) or interactions with farm waste (17), livestock rates (18), human proximity (19), or human density (20). Skurnik et al. (20) reported a resistance score in extensively reared farm animals that was higher than that seen with wildlife from the same area. However, free-ranging livestock appears not to be a main source of AMR in our study area since the resistance frequency was not higher in *E. coli* from livestock. In general, these livestock show resistance levels lower than those reported by the Spanish VAV Network for intensively reared livestock. Other potential sources of AMR may exist in the study area. Indeed, multidrug-resistant bacteria have been isolated from a range of wild animals with no known previous exposure to antimicrobial agents, a fact that suggests that resistance is not confined to the ecological niche where it emerged (21).

Resistance to an extended-spectrum cephalosporin (cefotaxime) and to fluoroquinolones was found. These agents are listed as "critically important antimicrobials for human medicine" by the WHO (22), and the carriage of resistant bacteria by wildlife is of concern for public health. In fact, wild boars have been found to be carriers of cefotaxime-resistant *E. coli* in countries as diverse as Poland (23), the Czech Republic (24), and Portugal (25). In spite of a generally low frequency of AMR, this shows that protected natural environments are not exempt from the introduction of anthropogenic AMR. Furthermore, this report shows that livestock in an extensive farming system are not important contributors to the AMR in *E. coli* in cohabiting wild ungulates.

REFERENCES

1. **European Food Safety Authority.** 2012. The European Union summary report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2010. EFSA J. **10:**2598.

2. **Allen HK, Donato J, Wang HH, Cloud-Hansen KA, Davies J, Handelsman J.** 2010. Call of the wild: antibiotic resistance genes in natural environments. Nat. Rev. Microbiol. **8:**251–259.

3. **Heuer OE, Pedersen K, Andersen JS, Madsen M.** 2002. Vancomycin-resistant enterococci (VRE) in broiler flocks 5 years after the avoparcin ban. Microb. Drug Resist. **8:**133–138.

4. **Alvarez-Fernandez E, Cancelo A, Diaz-Vega C, Capita R, Alonso-Calleja C.** 2013. Antimicrobial resistance in *E. coli* isolates from conventionally and organically reared poultry: a comparison of agar disc diffusion and Sensi Test Gram-negative methods. Food Control **30:**227–234.

5. **Blake DP, Humphry RW, Scott KP, Hillman K, Fenlon DR, Low JC.** 2003. Influence of tetracycline exposure on tetracycline resistance and the carriage of tetracycline resistance genes within commensal *Escherichia coli* populations. J. Appl. Microbiol. **94:**1087–1097.

6. **Berge AC, Hancock DD, Sischo WM, Besser TE.** 2010. Geographic, farm, and animal factors associated with multiple antimicrobial resistance in fecal *Escherichia coli* isolates from cattle in the western United States. J. Am. Vet. Med. Assoc. **236:**1338–1344.

7. **Literak I, Dolejska M, Rybarikova J, Cizek A, Strejckova P, Vyskocilova M, Friedman M, Klimes J.** 2009. Highly variable patterns of antimicrobial resistance in commensal *Escherichia coli* isolates from pigs, sympatric rodents, and flies. Microb. Drug Resist. **15:**229–237.

8. **Kozak GK, Boerlin P, Janecko N, Reid-Smith RJ, Jardine C.** 2009. Antimicrobial resistance in *Escherichia coli* isolates from swine and wild small mammals in the proximity of swine farms and in natural environments in Ontario, Canada. Appl. Environ. Microbiol. **75:**559–566.

9. **Rybaríková J, Dolejska M, Materna D, Literak I, Cizek A.** 2010. Phenotypic and genotypic characteristics of antimicrobial resistant *Escherichia coli* isolated from symbovine flies, cattle and sympatric insectivorous house martins from a farm in the Czech Republic (2006–2007). Res. Vet. Sci. **89:**179–183.

10. **Navarro-Gonzalez N, Mentaberre G, Porrero CM, Serrano E, Mateos A, Lopez-Martin JM, Lavin S, Dominguez L.** 2012. Effect of cattle on Salmonella carriage, diversity and antimicrobial resistance in free-ranging wild boar (*Sus scrofa*) in northeastern Spain. PLoS One **7:**e51614. doi:10.1371/journal.pone.0051614.

11. **Phadtare S.** 2004. Recent developments in bacterial cold-shock response. Curr. Issues Mol. Biol. **6:**125–136.

12. **Heininger A, Bider M, Schmidt S, Unertl K, Botzenhart K, Doring G.** 1999. PCR and blood culture for detection of *Escherichia coli* bacteremia in rats. J. Clin. Microbiol. **37:**2479–2482.

13. **European Food Safety Authority.** 2008. Report from the Task Force on Zoonoses Data Collection including guidance for harmonized monitoring and reporting of antimicrobial resistance in commensal *Escherichia coli* and *Enterococcus* spp. from food animals. EFSA J. **141:**1–44.

14. **European Food Safety Authority.** 2012. Technical specifications on the harmonised monitoring and reporting of antimicrobial resistance in *Salmonella*, *Campylobacter* and indicator *Escherichia coli* and *Enterococcus* spp. bacteria transmitted through food. EFSA J. **10:**2742.

15. **R Development Core Team 3.0.1.** 2013. A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org. Accessed 7 January 2013.

16. **Allen SE, Boerlin P, Janecko N, Lumsden JS, Barker IK, Pearl DL, Reid-Smith RJ, Jardine C.** 2011. Antimicrobial resistance in generic *Escherichia coli* isolates from wild small mammals living in swine farm, residential, landfill, and natural environments in southern Ontario, Canada. Appl. Environ. Microbiol. **77:**882–888.

17. **Cole D, Drum DJV, Stallknecht DE, White DG, Lee MD, Ayers S, Sobsey M, Maurer JJ.** 2005. Free-living Canada geese and antimicrobial resistance. Emerg. Infect. Dis. **11:**935–938.

18. **Guenther S, Grobbel M, Heidemanns K, Schlegel M, Ulrich RG, Ewers C, Wieler LH.** 2010. First insights into antimicrobial resistance among faecal *Escherichia coli* isolates from small wild mammals in rural areas. Sci. Total Environ. **408:**3519–3522.

19. **Wheeler E, Hong P, Bedon LC, Mackie RI.** 2012. Carriage of antibiotic-resistant enteric bacteria varies among sites in Galapagos reptiles. J. Wildl. Dis. **48:**56–67.

20. **Skurnik D, Ruimy R, Andremont A, Amorin C, Rouquet P, Picard B, Denamur E.** 2006. Effect of human vicinity on antimicrobial resistance and integrons in animal faecal *Escherichia coli*. J. Antimicrob. Chemother. **57:**1215–1219.

21. **da Costa PM, Loureiro L, Matos AJF.** 2013. Transfer of multidrug-resistant bacteria between intermingled ecological niches: the interface between humans, animals and the environment. Int. J. Environ. Res. Public Health **10:**278–294.

22. **World Health Organization.** 2007. Critically important antimicrobials for human medicine: categorization for the development of risk management strategies to contain antimicrobial resistance due to nonhuman antimicrobial use. Report of the second WHO Expert Meeting, Copenhagen, 29 to 31 May 2007. World Health Organization, Geneva, Switzerland. http://www.who.int/foodborne_disease/resistance/antimicrobials_human.pdf.

23. **Mokracka J, Koczura R, Kaznowski A.** 2012. Transferable integrons of Gram-negative bacteria isolated from the gut of a wild boar in the buffer zone of a national park. Ann. Microbiol. **62:**877–880.

24. **Literak I, Dolejska M, Radimersky T, Klimes J, Friedman M, Aarestrup FM, Hasman H, Cizek A.** 2010. 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. **108:**1702–1711.

25. **Poeta P, Radhouani H, Pinto L, Martinho A, Rego V, Rodrigues R, Goncalves A, Rodrigues J, Estepa V, Torres C, Igrejas G.** 2009. Wild boars as reservoirs of extended-spectrum beta-lactamase (ESBL) producing *Escherichia coli* of different phylogenetic groups. J. Basic Microbiol. **49:**584–588.

26. **Cabal A, Gómez-Barrero S, Porrero C, Barcena C, Lopez G, Canton R, Gortazar C, Dominguez L, Alvarez J.** 2013. Assessment of virulence factors characteristic of human *Escherichia coli* pathotypes and antimicrobial resistance in O157:H7 and non-O157:H7 isolates from livestock in Spain. Appl. Environ. Microbiol. **79:**4170–4172.