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A walk on the wild side: Wild ungulates as potential reservoirs of multi-drug resistant bacteria and genes, including *Escherichia coli* harbouring CTX-M beta-lactamases☆

Rita Tinoco Torres [a,*], Mónica V. Cunha [b,c], Débora Araujo [d], Helena Ferreira [e,f], Carlos Fonseca [a,g], Josman Dantas Palmeira [a]

[a] Departament of Biology & CESAM, University of Aveiro, Campus de Santiago, 3810-193, Aveiro, Portugal
[b] Centre for Ecology, Evolution and Environmental Changes (cE3c), Faculdade de Ciências, Universidade de Lisboa, 1749-016, Lisbon, Portugal
[c] Biosystems & Integrative Sciences Institute (BioISI), Faculdade de Ciências, Universidade de Lisboa, 1749-016, Lisbon, Portugal
[d] Faculty of Engineering of University of Porto, Porto, Portugal
[e] UCIBIO - Applied Molecular Biosciences Unit, REQUIMTE – University of Porto, Porto, Portugal
[f] Microbiology, Biological Sciences Department, Faculty of Pharmacy of University of Porto, Porto, Portugal
[g] ForestWISE - Collaborative Laboratory for Integrated Forest & Fire Management, Quinta de Prados, 5001-801, Vila Real, Portugal

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ABSTRACT

Extended-spectrum β-lactamases (ESBL)-producing *Enterobacterales* have been classified as critical priority pathogens by the World Health Organization (WHO). ESBL are universally distributed and, in 2006, were firstly reported on a wild animal. Understanding the relative contributions of wild animals to ESBL circulation in the environment is urgently needed. In this work, we have conducted a nationwide study in Portugal to investigate the occurrence of bacteria carrying clinically significant antimicrobial resistance genes (ARG), using widely distributed wild ungulates as model species. A total of 151 antimicrobial resistant-*Enterobacterales* isolates were detected from 181 wild ungulates: 50% (44/88) of isolates from wild boar (*Sus scrofa*), 40.3% (25/62) from red deer (*Cervus elaphus*), 41.4% (12/29) from fallow deer (*Dama dama*) and 100% (2/2) from mouflon (*Ovis aries* subsp. *musimon*). Selected isolates showed a diversified resistance profile, with particularly high values corresponding to ampicillin (71.5%) and tetracycline (63.6%). *Enterobacterales* strains carried bla_{TEM}, *tetA*, *tetB*, *sul2*, *sul1* or *dfrA1* ARG genes. They also carried $bla_{CTX\text{-}M}$-type genes, which are prevalent in human infections, namely CTX-M-14, CTX-M-15 and CTX-M-98. Strikingly, this is the first report of CTX-M-98 in wildlife. Almost 40% (n = 59) of *Enterobacterales* were multi-drug resistant. The diversity of plasmids carried by ESBL isolates was remarkable, including IncF, K and P. This study highlights the potential role of wild ungulates as environmental reservoirs of CTX-M ESBL-producing *E. coli* and in the spill-over of AMR bacteria and their determinants. Our findings suggest that wild ungulates are useful as strategic sentinel species of AMR in terrestrial environments, especially in response to potential sources of anthropogenic pollution, providing early warning of potential risks to human, animal and environmental health.

1. Introduction

Antibiotics are crucial for the treatment of bacterial infections in humans and have saved millions of lives (Spellberg, 2016). While antibiotics have helped to improve the quality of life and reduce overall mortality due to infectious diseases (Gould and Lawes, 2016), the intensification of antimicrobial resistance (AMR) emergence in recent decades is now jeopardizing the control of infectious diseases (Spellberg, 2016). In agreement, World Health Organization (WHO) has recognized AMR as an urgent and menacing public health problem that threatens the balance formerly achieved in the control of infectious diseases (WHO, 2014). Though the burden of AMR on human health is increasing, yet difficult to precisely quantify, recent estimates are alarming, suggesting that nearly 10 million people will die due to AMR

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* Corresponding author.
E-mail address: rita.torres@ua.pt (R.T. Torres).

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by 2050 (O'Neill, 2014). What is officially known is that AMR is responsible for 35,000 human deaths per year in the USA, 33,000 in the European Union and 58,000 in Asia (Cassini et al., 2019). Recent estimates pointed that between 2000 and 2015, in the 76 analysed countries, human antibiotic consumption increased by 65% (Klein et al., 2018). These alarming numbers suggest that global antibiotic consumption is likely to continue increasing, if no effective action is taken (Klein et al., 2018), with underlying consequences on AMR emergence.

Antibiotics are used every day in human and veterinary medicine, animal farming, aquaculture and agricultural activity, causing antibiotic residues and therefore environment pollution (Shao et al., 2021). Whilst antibiotic consumption and overuse is considered the primary driver of AMR, anthropogenic activities added an extra burden and antibiotics are now considered environmental pollutants which arrive to the environment through different sources (Singh et al., 2021), contributing to the increasing spread of AMR bacteria and antimicrobial resistance genes (ARG) (Singh et al., 2021). For example, wastewater treatment plants (WWTPs) have been considered as potential hotspots for horizontal gene transfer between bacteria from various sources (Hiller et al., 2019), as the technology and treatment stages of WWTPs have not been conceived to effectively remove antibiotics. And so, treated effluents release antibiotic residues and AMR bacteria into the environment (Danner et al., 2019). Other studies suggest that between 30 and 90% of administered antibiotics, in humans and animals, are excreted into the environment by urine and feces (Du and Liu, 2012). In fact, the chemical structure of antibiotics is hard to degrade and their residues ultimately end up in the natural environment, independently of the different input sources (Shao et al., 2021). Understanding and controlling what seems unstoppable, the current dissemination of AMR bacteria and genes, is a difficult and a challenging task for the 21st century (Larsson et al., 2018), requiring the comprehension of AMR dissemination mechanisms and routes, with emphasis on the ecosystem link through the human-animal-environment interfaces (Booton et al., 2021; Iramiot et al., 2020; Torres et al., 2019). While this triad is increasingly recognized as fundamental, often the animal compartment research is limited to food-producing animals and companion animals, overlocking a key element, the wild animals (Dolejska and Literak, 2019; Torres et al., 2021b). Fortunately, there is an increasing interest in the role of wildlife as a source and/or disperser of clinically relevant resistant bacteria to unveil the complex spread mechanisms of AMR across ecosystems (Arnold et al., 2016; Vittecoq et al., 2016).

Enterobacterales are Gram-negative bacteria naturally occurring in the intestinal tract of warm-blooded animals and comprise common human pathogens causing a wide range of diseases (*e.g.*, gastroenteritis, urinary tract infections, meningitis, pneumonia, septicaemia, among others) (Palmeira et al., 2021). *Escherichia coli*, a well-studied Enterobacterales, has been widely used as an important indicator microorganism to capture the circulation and evolution of multi-resistant bacteria in the environment and wildlife (Furness et al., 2017; Radhouani et al., 2014). β-lactams are the most common class of antibiotics prescribed to treat infections caused by these pathogens (Palmeira et al., 2021). Acquired resistance to β-lactams is chiefly mediated by extended-spectrum β-lactamases (ESBL) and its actually globally distributed. Since 2000, the spread of community-acquired ESBL-producing bacteria, mainly *Escherichia coli*, has been reported worldwide (Zhang et al., 2021) and not long after, in 2006, ESBL-producing *E. coli* in wild animals were firstly reported in Portugal (Costa et al., 2006). Since then, ESBL have been documented in wildlife and environmental ecosystems all over the world (Dolejska and Literak, 2019; Swift et al., 2019; Torres et al., 2021a). CTX-M is the most worldwide disseminated ESBL type, with high relevance to public health, since is related with outbreaks and successful spreader clones with impact to human health at hospital and community level (Bevan et al., 2017).

However, to track the spread of AMR across environments, it is vital to understand the distribution and sources of AMR in wildlife to assess their relevance as bioindicators or sentinels (Furness et al., 2017; Larsson et al., 2018; Swift et al., 2019). The lack of knowledge on the AMR life cycle contributes to the ineffective mitigation of AMR spread. In this sense, using the right wild animal species as sentinels would be extremely useful to advance knowledge and inform control options. Wild ungulates have expanded in number and distribution all over the Europe in the last decades, becoming overabundant in many scenarios (Valente et al., 2020). Due to their wide distribution, high densities, large home ranges and link between natural and anthropogenic areas, wild ungulates may become important bioindicators and sentinels to track the spread and evolution of AMR across environments (Cassini et al., 2019; Fuentes-Castillo et al., 2019; Furness et al., 2017; Swift et al., 2019; Torres et al., 2020, 2019). Additionally, wild ungulates are hunted in most countries, placing them in direct contact with humans. Following this line, it is pivotal to track and unveil the relative contribution of different sources of AMR into the environment (Larsson et al., 2018). Even though the occurrence and diversity of ESBL-producing Enterobacterales have been locally documented in some wildlife species in Portugal, data on antibiotic resistance on widely distributed wild ungulates at a national scale are limited. To fill this gap, we have conducted a national-scale study to investigate the occurrence of bacteria carrying clinically significant resistance genes in wild ungulates inhabiting Portugal. Specifically, we report the occurrence of CTX-M producing *E. coli* recovered from a diversity of wild ungulates in Portugal.

2. Materials and methods

2.1. Sampling and bacterial isolation

Faecal samples (n = 181) were collected (1–3 h after the animal death) from hunted wild boar (*Sus scrofa,* n = 88*)*, red deer (*Cervus elaphus*, n = 62), fallow deer (*Dama dama*, n = 29) and mouflon (*Ovis aries* subsp. *musimon*, n = 2) from 36 different hunting grounds during two hunting seasons (October to February 2018/2019 and 2019/2020), located in 15 of the 18 districts of continental Portugal (Fig. 1). The country's topography is characterized by pronounced differences between the north/central regions (high elevations and rough landscapes) and south/coastal regions (plains of low elevation). Mainland Portugal experiences a Mediterranean climate and the north-eastern regions are under Atlantic influences. Human population, regardless of the region, is concentrated in the west and in the centre and east is where wild ungulates densities are higher. None of the authors were involved or responsible for animals death and no animal was sacrificed for the study purposes. All institutional/national/international guidelines for the use and care of all animals have been followed.

Individual animal faecal samples were each collected directly from the rectus of the animal into a sterilized flask and were subjected to refrigeration conditions from collection until arriving at the laboratory, where they were stored at −20 °C. Microbiological analyses began with an enrichment step of 2 g of faecal samples for 40 mL of Tryptic Soy Broth (TSB) (Liofilchem – Italy) and incubation at 37 °C/overnight (without agitation). Resistant, lactose fermenting isolates of *Enterobacterales* were selected from the enrichment broth by spreading 100 μL of the enrichment *per* plate of MacConkey agar (Liofilchem – Italy) supplemented with antibiotic, including ampicillin (AMP, 100 μg/mL, Sigma-Aldrich - Germany), cefotaxime (CTX, 1 μg/mL, Labesfal - Portugal), meropenem (MRP, 0,5 μg/mL, Sigma-Aldrich - Germany), ciprofloxacin (CIP, 1 μg/mL, Acros Organics – United Kingdom) or tetracycline (TET, 100 μg/mL, Sigma-Aldrich - Germany). All plates were incubated at 37 °C/overnight. From each plate showing growth, a single lactose-fermenter colony was streaked to the same antibiotic-selection plate for phenotype confirmation, followed by incubation at 37 °C/overnight (Palmeira et al., 2020).

2.2. Antimicrobial susceptibility testing and identification

The antimicrobial resistance profile of all selected isolates was

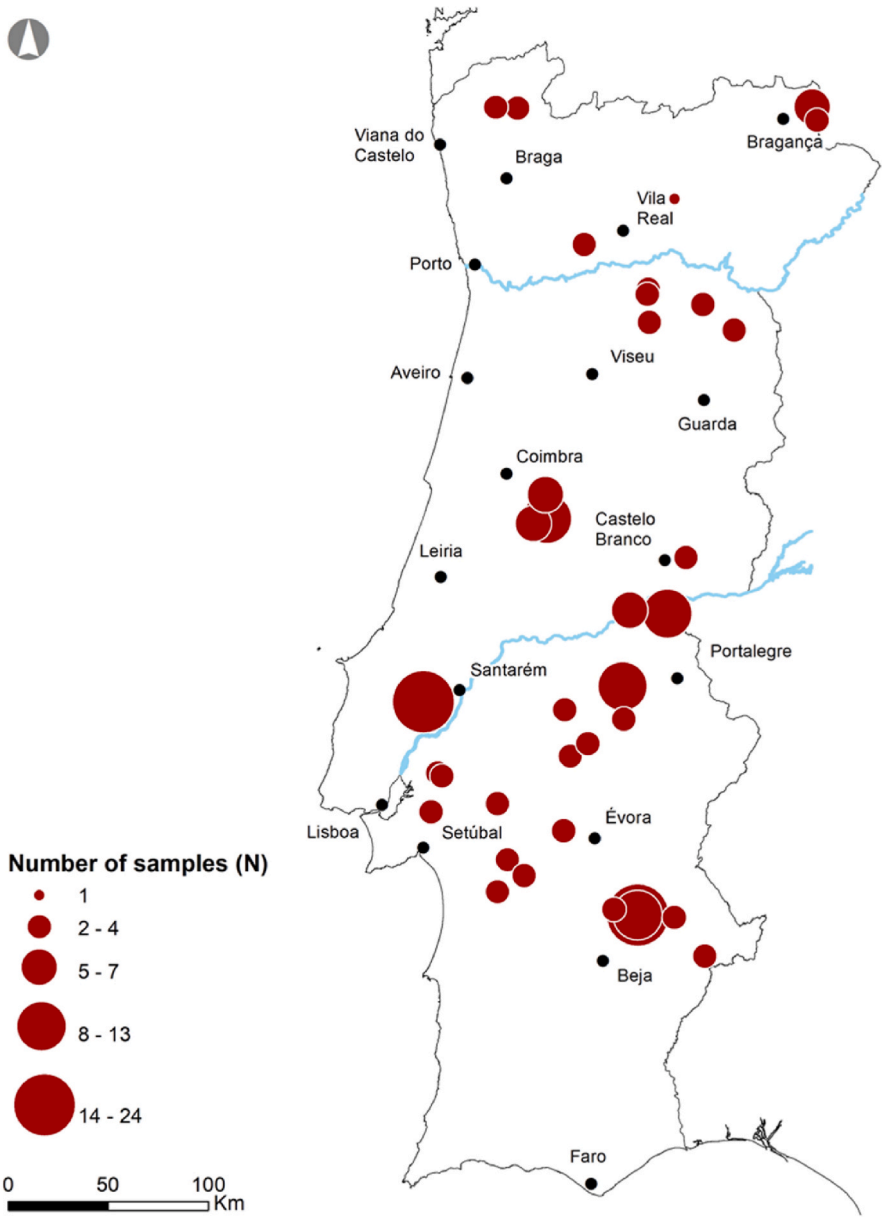

Fig. 1. Locations of sample collection (red circles correspond to the number of ungulate samples collected). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

determined by the disk-diffusion method, according to clinical breakpoints of EUCAST guidelines (2021), using antibiotic disks (Oxoid – United Kingdom) of the following antibiotics: ampicillin (AMP 10 μg), amoxicillin plus clavulanic acid (AMC, 30 μg), cefoxitin (FOX, 30 μg), cefotaxime (CTX, 5 μg), ceftazidime (CAZ, 10 μg), ceftiofur (EFT, 30 μg), cefepime (FEP, 30 μg), aztreonam (ATM, 30 μg), meropenem (MRP, 10 μg), ciprofloxacin (CIP, 5 μg), enrofloxacin (ENR, 5 μg), tetracycline (TET, 30 μg), tigecycline (TGC, 15 μg), gentamicin (GEN, 10 μg), sulfamethoxazole plus trimethoprim (SXT, 25 μg), chloramphenicol (CHL, 30 μg), fosfomycin (FOT, 200 μg) and nitrofurantoin (FUR, 100 μg). For EFT, ENR, TET and GEN the interpretation was done by CLSI standards (2020). According to the criteria of Magiorakos et al. (2012), the multidrug-resistant (MDR) status was attributed when the isolate showed resistance to one or more antimicrobials from three or more different antimicrobial categories. The bacterial identification was initially presumptive with CHROMagar Orientation (CHROMagar – France) and followed by biochemistry tests, through API20E galleries (bioMérieux, Marcy l'Etoile, France). Isolates with the same antimicrobial resistance profile and identification selected from an unique animal by different antibiotic-selective media, were considered duplicated, consequently one of them was excluded.

2.3. Antibiotic resistance genes and plasmid replicon typing

Resistant isolates were screened for ARG, selected according to their phenotype: bla_{TEM}, bla_{SHV}, bla_{OXA}, bla_{CTX-M} (resistance to β-lactams), *qnrA*, *qnrB*, *qnrS*, *aac (6′)-Ib-cr* (fluoroquinolones), *aac3′-II*, *aac3′-IV*, *ant2″*, *strA*, *strB* (aminoglycosides), *tetA*, *tetB* (tetracycline), *sul1*, *sul2*, *sul3* (sulfamethoxazole), *dfrA1*, *dfrA12*, *dfrA17* (trimethoprim), *fosA3*, *fosA5*, *fosC2* (fosfomycin), *cmlA1*, *catA* and *floR* (chloramphenicol) by PCR (Table S1). PCR amplification analysis was carried out by electrophoresis in 1,5 - 2% agarose gel (NzyTech – Portugal) under 200 A, 100 V and for 30–90 min of run conditions. PCR products purification performed through NZYGelpure kit (NzyTech – Portugal) for sequencing analysis. Amplicon sequencing of bla_{ESBL} amplicons was performed by Sanger sequencing (Eurofins Genomics – Germany). The NCBI website,

through the tool BLAST, was used for identification of the *blaESBL* gene variants (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The plasmid content of ESBL-producing isolates was determined using standard protocols based in replicon typing (Inc) (Carattoli et al., 2005). In this study, all PCR assays were performed using Super Hot Master Mix (Bioron – Germany) and Table S1 shows the details of primers and PCR conditions.

2.4. Escherichia coli typing

All ESBL-producing *E. coli* isolates were characterized in relation to phylogenetic group (A, B1, B2, C, D, E and F) by a multiplex PCR standard protocol (Clermont et al., 2013). The pathogenic potential of ESBL-producing *E. coli* isolates was evaluated by screening the six majors categories (EPEC, ATEC, STEC, ETEC, EIEC and EAEC) of intestinal pathotypes of *E. coli,* through a multiplex PCR (Müller et al., 2007) and 30 different virulence factors of *E. coli*, carried out by 5 multiplex PCR schemes (Johnson and Stell, 2000). Table S1 shows primers sequences and PCR protocols of all typing assays.

2.5. Statistical analysis

We used a 2-sample test for equality of proportions bacteria (prop. test function in R) to assess sexual (male *versus* female) and age class (adults *versus* juveniles) differences in the proportion of resistant-. To evaluate if there were any statistically differences in the detection of resistance genes between species, we used the Fisher exact test (fisher. test function in R). A p-value of <0.05 was considered statistically significant. All analysis were performed in Rstudio software version 1.4.1106.

3. Results

3.1. Resistant Enterobacterales selection

From a total of 83 out of 181 (45.8%) sampled animals, 151 resistant-*Enterobacterales* isolates were detected (Fig. 2). When analysed according to animal species, the wild boar showed 50% (44/88) of animals harbouring resistant-*Enterobacterales*, red deer 40.3% (25/62), fallow

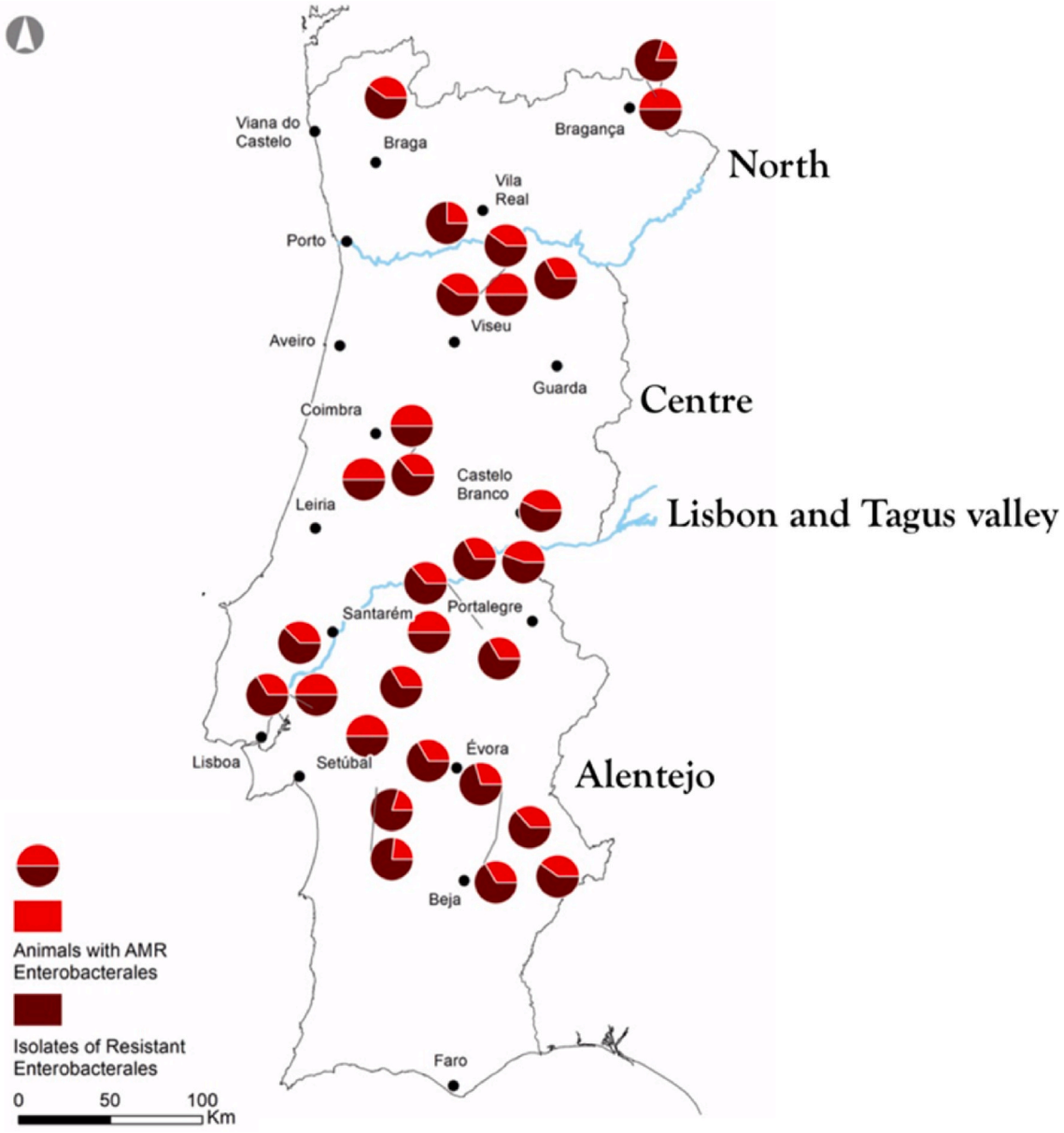

Fig. 2. Geographic distribution of resistant-*Enterobacterales* isolates among ungulates sampled in this study.

deer 41.4% (12/29) and mouflon 100% (2/2). Age analysis showed 46.7% (63/135) of adults and 43.5% (20/46) of juvenile ungulates with resistant-bacteria and males and females showed 53.6% (44/82) and 39.4% (39/99), respectively. There was no statistical significant differences between males and females (2-sample test for equality of proportions with continuity correction, $X^2 = 0.597$, df = 1, p = 0.44) and adults and juveniles (2-sample test for equality of proportions with continuity correction, $X^2 = 5.2891e\text{-}32$, df = 1, p = 1) regarding the proportion of animals with resistant-bacteria. The ratio between the number of resistant-*Enterobacterales per* colonized animal for each sampled geographical region of Portugal shows the following proportions: 1.8 resistant-isolates *per* colonized animal in the north region, 1.56 in the centre, 2.36 in Lisbon and Tagus Valley, and 1.96 in Alentejo (Fig. 2).

3.2. Antimicrobial resistance profile

The 151 resistant-isolates of *Enterobacterales* showed a diverse resistance profile to the 18 antibiotics screened in the disk diffusion susceptibility test (Table 1). Only meropenem and tigecycline were fully effective against all isolates. The higher rates of resistance were registered for ampicillin (71.5%) and tetracycline (63.6%), followed by sulfamethoxazole plus trimethoprim (23.8%), ciprofloxacin (11.3%) and chloramphenicol (10.6%). Also of note are the resistance rates to oxyimino- β-lactam antibiotics (2.6%–7.3%).

The resistance phenotype patterns were very diversified, with 41 different phenotypes (Table 2), and isolates showing resistance to one to 11 antibiotics. AMP/TET (27 isolates/151), TET (26/151), AMP (18/151) and AMP/TET/SXT (12/151) were the most prevalent resistance phenotype patterns. From 151 resistant-*Enterobacterales* isolates, a total of 59 (39.1%) were classified as MDR.

3.3. Antimicrobial resistance genes

A high diversity of ARG was detected in the genome of isolated bacteria (Table 3), showing the potential of wild ungulates as reservoir of ARG. As genes encoding resistance were screened according to the resistance phenotype, no direct prevalence should be measured. Under this observation, the most detected ARGs were bla_{TEM} (60 isolates/151), *tetA* (50/151), *tetB* (45/151), *sul2* (28/151), *sul1* (18/151) and *dfrA1* (18/151). There were no statistically differences between the ARG screened between wild boar, red deer and fallow deer (β-lactams: Fisher's exact test p-value = 0.4156; Fluoroquinolones: Fisher's exact test p-value p-value = 0.1143; Aminoglycosides: Fisher's exact test p-value p-value = 1; Tetracycline: Fisher's exact test p-value = 0.3284; Sulfamethoxazole: Fisher's exact test p-value = 0.3747; Trimethoprim: Fisher's exact test p-value = 0.5433) except for Chloramphenicol (Fisher's exact test p-value = 0.04766) where there was a significant difference only between wild boar and fallow deer (Fisher's exact test p-value = 0.02249).

Table 1
Resistance profile of 151 *Enterobacterales* isolated in wild ungulates (wild boar n = 88, red deer n = 62, fallow deer n = 29 and mouflon n = 2) from Portugal.

Antibiotic	Disk Conc. (μg)	Clinical breakpoint guideline	Resistant		
			Interpretative criteria (mm)	No. isolates	%
Ampicillin	10	EUCAST	ZD < 14	108	71.5
Amoxicillin + Clav. Ac.	30	EUCAST	ZD < 19	10	6.6
Cefoxitin	30	EUCAST	ZD < 19	14	9.3
Cefotaxime	5	EUCAST	ZD < 17	11	7.3
Ceftazidime	10	EUCAST	ZD < 19	7	4.6
Ceftiofur	30	CLSI	ZD < 18	8	5.3
Cefepime	30	EUCAST	ZD < 24	4	2.6
Aztreonam	30	EUCAST	ZD < 21	8	5.3
Meropenem	10	EUCAST	ZD < 16	0	0.0
Ciprofloxacin	5	EUCAST	ZD < 22	17	11.3
Enrofloxacin	5	CLSI	ZD < 17	9	6.0
Gentamycin	10	CLSI	ZD < 13	7	4.6
Tetracycline	30	CLSI	ZD < 12	96	63.6
Tigecycline	15	EUCAST	ZD < 18	0	0.0
Fosfomycin	200	EUCAST	ZD < 24	7	4.6
Sulfam. + Trimethoprim	25	EUCAST	ZD < 11	36	23.8
Chloramphenicol	30	EUCAST	ZD < 17	16	10.6
Nitrofurantoin	100	EUCAST	ZD < 11	1	0.7

ZD – Inhibition growth zone diameter.

Table 2
Resistance phenotype pattern of 151 resistant *Enterobacterales* isolated from wild ungulates.

Antimicrobial resistance patterns	No. Isolates	Animal host			
		Wild boar	Red deer	Fallow deer	Mouflon
AMP/TET	27	14	6	7	0
TET	26	15	9	2	0
AMP	18	11	4	2	1
AMP/TET/SXT	12	7	2	3	0
AMP/SXT	6	4	1	1	0
AMP/TET/CHL	4	3	0	1	0
TET/SXT	4	2	1	1	0
AMP/CIP/TET	3	2	1	0	0
AMP/CTX/FOX/ATM/EFT/CAZ/AMC	3	1	0	2	0
AMP/FOX	3	1	1	1	0
AMP/TET/SXT/CHL	3	3	0	0	0
TET/SXT/CHL	3	3	0	0	0
AMP/CIP	2	1	0	1	0
AMP/CIP/ENR/TET/SXT	2	0	0	2	0
AMP/FOX/FOT	2	1	1	0	0
AMP/SXT/CHL	2	1	1	0	0
AMP/TET/GEN	2	1	1	0	0
CIP/ENR	2	1	0	1	0
FOT	2	0	2	0	0
TET/FOT	2	1	0	1	0
AMP/CTX/FOX/EFT/CAZ/AMC	2	1	1	0	0
AMP/AMC/CIP/ENR/TET/SXT	1	0	0	1	0
AMP/CIP/GEN	1	0	0	0	1
AMP/CIP/ENR	1	1	0	0	0
AMP/CIP/ENR/TET	1	1	0	0	0
AMP/CIP/TET/SXT/CHL/GEN	1	0	0	1	0
AMP/GEN	1	1	0	0	0
AMP/CTX/ATM/CIP/ENR/TET/SXT	1	1	0	0	0
AMP/CTX/FEP/ATM/EFT	1	0	1	0	0
AMP/CTX/FEP/ATM/EFT/CAZ/CIP/ENR/TET/CHL/GEN	1	0	0	1	0
AMP/CTX/FEP/EFT/CAZ	1	1	0	0	0
AMP/CTX/FOX/AMC	1	1	0	0	0
AMP/CTX/FOX/ATM/AMC	1	0	1	0	0
AMP/CTX/FOX/FEP/ATM/EFT/CAZ/AMC	1	0	1	0	0
AMP/FOT	1	0	1	0	0
AMP/FOX/AMC	1	0	1	0	0
AMP/FOX/AMC/TET	1	1	0	0	0
AMP/FUR	1	1	0	0	0
AMP/TET/CHL/GEN	1	1	0	0	0
CIP/TET	1	0	1	0	0
SXT/CHL	1	1	0	0	0

AMP - ampicillin, AMC – amoxicillin plus clavulanic acid, FOX - cefoxitin, CTX – cefotaxime, CAZ - ceftazidime, EFT - ceftiofur, FEP - cefepime, ATM - aztreonam, MRP - meropenem, CIP - ciprofloxacin, ENR - enrofloxacin, TET - tetracycline, TGC - tigecycline, GEN - gentamycin, SXT - sulfamethoxazole plus trimethoprim, CHL - chloramphenicol, FOT - fosfomycin and FUR - nitrofurantoin.

Table 3
Antimicrobial resistance genes (ARG) screened according to the resistance phenotype of 151 resistant-*Enterobacterales* from wild ungulates.

Class/Antibiotic with resistant phenotype	Beta-lactams (n = 108)			
Gene	bla_{TEM}	bla_{SHV}	bla_{OXA}	bla_{CTX-M}
No. Isolates	60/108	3/108	1/108	4/108
%	55.5	2.8	0.9	3.7

Class/Antibiotic with resistant phenotype	Fluoroquinolones (n = 17)			
Gene	*qnrA*	*qnrB*	*qnrS*	*aac6′Ib-cr*
No. Isolates	0/17	1/17	4/17	3/17
%	0.0	5.9	23.5	17.6

Class/Antibiotic with resistant phenotype	Aminoglycosides (n = 7)				
Gene	*aac3′-II*	*aac3′-IV*	*ant2″*	*strA*	*strB*
No. Isolates	0/7	0/7	0/7	3/7	3/7
%	0.0	0.0	0.0	42.8	42.8

Class/Antibiotic with resistant phenotype	Tetracycline (n = 96)	
Gene	*tetA*	*tetB*
No. Isolates	50/96	45/96
%	52.1	46.9

Class/Antibiotic with resistant phenotype	Sulfamethoxazole (n = 36)		
Gene	*sul1*	*sul2*	*sul3*
No. Isolates	18/36	28/36	1/36
%	50.0	77.8	2.8

Class/Antibiotic with resistant phenotype	Trimethoprim (n = 36)		
Gene	*dfrA1*	*dfrA12*	*dfrA17*
No. Isolates	18/36	2/36	7/36
%	50.0	5.5	19.4

Class/Antibiotic with resistant phenotype	Chloramphenicol (n = 16)		
Gene	*cmlA1*	*catA*	*floR*
No. Isolates	2/16	6/16	11/16
%	12.5	37.5	68.7

Class/Antibiotic with resistant phenotype	Fosfomycin (n = 7)		
Gene	*fosA3*	*fosA5*	*fosC2*
No. Isolates	0/7	0/7	0/7
%	0.0	0.0	0.0

3.4. Extended-spectrum β-lactamases (ESBL)-producing E. coli characterization

Characteristic phenotypic synergism of ESBL producers was detected in 4 (2,65%) resistant-*Enterobacterales*, which were identified as *Escherichia coli*. Table 4 shows the features of ESBL producer isolates. These ESBL-producing *E. coli* were detected in wild boar (2/4), red deer (1/4) and fallow deer (1/4). No relation among ESBL-producing *E. coli* and animal gender or age were registered. The ESBL isolates harboured CTX-M β-lactamase, with 3 different variants: CTX-M-14 (2/4), CTX-M-15 (1/4) and CTX-M-98 (1/4). All ESBL producers were MDR, showing resistance, in addition to resistance to β-lactams, to CIP (2/4), ENR (2/4), TET (2/4), GEN (1/4), SXT (1/4) and CHL (1/4). Three isolates belonged to B1 phylogroup and one to phylogroup C. None ESBL producer isolate belonged to any intestinal pathotypes of *E. coli* evaluated. All ESBL isolates have *fimH* and *traT* as virulence factors and a high diversity of plasmids with 10 different (IncFIA, FIB, FIC, K, P, T, F, A, C and B/O) plasmids detected, according to the replicon typing characterization, but with IncF being detected in all ESBL-producing isolates.

4. Discussion

It is not new the importance of AMR to human health since its first description and significance in the 60's (Davies and Davies, 2010), but nowadays it is clear that the relevance and role of AMR spread transcend the human context, being the interface human-animal-environment strongly connected with the AMR spread and dissemination (Rousham et al., 2018). The emergence of wildlife colonized by ESBL-producing *E. coli*, with a zoonotic character, is a global public and environmental problem (Palmeira et al., 2021). Under the One Health approach, large scale surveillance is mandatory to understand the dynamics of AMR bacteria, gene transmission and the characterization of AMR through the interface human-animal-environment.

Resistant-*Enterobacterales* were present in wild ungulates from all sampled regions (North, Centre, Lisbon and Tagus Valley, and Alentejo) of mainland Portugal which emphasizes the wide AMR distribution. The proportional ratio of resistant-*Enterobacterales per* AMR harbouring animal draws attention, since Lisbon and Tagus Valley showed higher ratio proportions once this Portugal region have the higher human population density and consequently a higher anthropogenic pressure in the natural settings, suggesting that the AMR development and dispersion are closely related with human activities. Wild ungulates, due to their wide distribution, high densities and link between natural and anthropogenic areas can become important bioindicators and sentinels to track the spread and evolution of AMR across environments (Furness et al., 2017; Swift et al., 2019; Torres et al., 2019).

In our work, we found a prevalence of 45.8% of resistant-*Enterobacterales* colonizing the gastrointestinal tract of wild ungulates. No correlation between animal species and resistant-bacteria selection was established, since the ungulate species showed prevalence values (40.3–50%) without significant differences that justify any animal species-AMR correlation (the mouflon value was not considered due to the low number of samples, n = 2). In Europe, several studies have already described various resistant-bacteria in the wild ungulate species that we sampled in this study (Dias et al., 2019, 2015; Loncaric et al., 2016; Torres et al., 2020). While there was no significant difference of AMR prevalence among species, wild boar was expected to be at higher risk (Vittecoq et al., 2016). This hypothesis was based on species

Table 4
Characterization of ESBL-producing *Escherichia coli* from wild ungulates from Portugal.

Ungulate species	Sample	Sex	Age	Isolate ID	Bacterial species	ESBL gene	β-lactam resistance	Non-β-lactam resistance genes	Non-β-lactam resistance	MDR profile	Plasmid content (Inc)	PG	Virulence Factors
Wild boar	J132	F	A	U95	*E. coli*	$bla_{CTX-M-98}$	AMP, CTX, ATM	*sul1*, *sul2*, *dfrA17*	CIP, ENR, TET, SXT	MDR	FIA, FIB	C	*fimH, traT*
Wild boar	J160	M	J	U29	*E. coli*	$bla_{CTX-M-14}$	AMP, CTX, EFT, CAZ, FEP		–	MDR	F, K, P, B/O	B1	*fimH, traT*
Red deer	C57	F	A	U1	*E. coli*	$bla_{CTX-M-14}$	AMP, CTX, EFT, FEP, ATM	-	–	MDR	F, K, B/O	B1	*fimH, traT*
Fallow deer	C113	M	A	U143	*E. coli*	$bla_{CTX-M-15}$	AMP, CTX, EFT, CAZ, FEP, ATM	*strA, strB, tetA, cmlA1, floR*	CIP, ENR, GEN, TET, CHL	MDR	FIB, FIC, P, T, A, C, K	B1	*fimH, traT*

F – female; M-male; A-adult; J-juvenile; PG – Phylogenetic group.

difference feeding ecology: wild boar is an omnivorous, while the remaining species are herbivores. Omnivorous species often feed on anthropogenic garbage and wild boar represent a known epidemiological link between natural and humanized environments (Torres et al., 2020; Ramos et al., 2022).

From the total of the 151 resistant-*Enterobacterales* that we recovered, all showed resistance at least to one of the eighteen antibiotics tested. The *Enterobacterales* isolates recovered from wild ungulates showed resistance to antibiotics classified by WHO as important (nitrofurantoin), highly important (cefoxitin, tetracycline, sulfamethoxazole, trimethoprim and chloramphenicol) and critically important (ampicillin, amoxicillin plus clavulanic acid, cefotaxime, ceftazidime, cefepime, aztreonam, ciprofloxacin, gentamycin and fosfomycin) (WHO, 2019), highlighting the yet unexplored impact that these animals may exert in the dissemination of resistant-bacteria and genes that confer resistance to critically important antibiotics to human medicine, and consequently to human health. It is worth emphasizing that these free-range wild animals live in natural habitats which, according to the results presented, did not prevent that anthropogenic activities generated the pressure to create in the intestinal microbiota of these animals a real cauldron of AMR determinants with significance to human therapeutics (Dolejska and Literak, 2019; Torres et al., 2021a).

The higher resistance rates found in this study were to ampicillin (71.5%) and tetracycline (63.6%). This is alarming, as these results are similar to those obtained in Portugal in different environmental settings, such as aquacultures water and sediment, feed and trout (Novais et al., 2018) and untreated waters for human consumption (Macedo et al., 2011). Ampicillins and tetracyclines are antibiotics often used in human and veterinary settings, highlighting that resistance is already widely distributed in a wide set of environmental scenarios.

The MDR phenotype was identified in 59 (39.1%) *Enterobacterales* isolates, with a total of 26 different MDR phenotypes patterns, showing the potential of these animals to harbour bacteria doted of multiple mechanisms to promote their persistence, by promoting therapeutical failure, in an eventual infection process. MDR bacteria are one of the biggest public health problem in human healthcare settings, once they jeopardize the treatment of infections with high mortality and morbidity rates (Dadgostar, 2019). Analysing the resistance phenotype patterns per different animal host, no significant relation is present that justify the association of any specific phenotype with any animal species.

A total of 21 different types of ARG were detected and responsible for resistance to 12 antibiotic categories. Wild ungulates as reservoir of ARG have been well described in Europe, including Portugal, Spain and Germany (Darwich et al., 2021; Navarro-Gonzalez et al., 2018, 2012; Plaza-Rodríguez et al., 2021; Torres et al., 2021b). The presence of ARG itself is very important due to their capacity to confer phenotypic AMR to the carrying bacteria, but is not limited to this role, once the ARG are typically located in genetic mobile elements, such as plasmids and integrons, increasing ARG relevance due the potential of genetic horizontal transfer (Rozwandowicz et al., 2018).

In this study, we report the presence of MDR ESBL-producing *E. coli* harbouring CTX-M β-lactamases, on three different species (fallow deer, red deer and wild boar) and the presence of three different CTX-M variants: CTX-M-15, CTX-M-14 and CTX-M-98. CTX-M-15, the wider disseminated ESBL worldwide, has been linked to different *E. coli* strains, is highly related to healthcare settings infections and outbreaks (Bonnet, 2004; Cantón et al., 2012) and sporadically in pets and livestock in different European countries (Coque et al., 2008). CTX-M-15 have also been described in wildlife, namely wild birds (Guenther et al., 2010; Poeta et al., 2008; Veldman et al., 2013), highlighting this taxonomic group as potential disperser of CTX-M-15. In our study, U143 *E. coli* selected from a fallow deer can be considered a commensal according to its B1 phylogenetic profile and co-harbouring the resistance genes *strA*, *strB*, *tetA*, *cmlA1* and *floR* and the virulence factors *fimH* and *traT*. Regarding wild ungulates, CTX-M-15-producing *E. coli* has been previously described in wild boar in Algeria (Bachiri et al., 2017). Beta-lactamases have been well reported in the interface human-animal-environment, with description in wild ungulates in Austria, Algeria, Poland, Germany (Holtmann et al., 2021; Palmeira et al., 2021), and it has been previously described in wild boar in Portugal (Poeta et al., 2009).

Like CTX-M-15, CTX-M-14 is one of the most common CTX-M variants in humans (Livermore et al., 2007) and has also been reported in livestock (EFSA, 2011). At a European scale, Stedt et al. (2015) showed that the CTX-M-14 was the most common CTX-M genotype in gulls in Portugal and Spain, highlighting that this β-lactamase is also frequent in Portuguese poultry (Costa et al., 2009). Also, it has been previously found in red fox and birds of prey in Portugal (Costa et al., 2006). We report CTX-M-14-producing *E. coli* from one wild boar (U29) male and a female red deer (U1); in both cases *E. coli* showed a B1 phylogenetic profile without other ARG, but showed some virulence characteristics (e.g. *fimH* and *traT*). In Europe CTX-M-14-producing *E. coli* have just few reports in wild boar (Spain and Germany), but it is, to the best of our knowledge, the first report in red deer (Darwich et al., 2021; Holtmann et al., 2021).

CTX-M-98-producing *E. coli* was isolated from a wild boar female and this isolate did not show a B1 commensal profile, typical in animals microbiota, but showed a C phylogroup profile which is closely related to B1 (Martak et al., 2020). There are few reports of CTX-M-98 in humans and livestock (Liu et al., 2015), and to the best of our knowledge, this is the first report of CTX-M-98 in wildlife (Liu et al., 2015). The presence of virulence genes in all CTX-M-producing *E. coli* isolated in this work draws attention, since it confers the AMR reservoir status to these animals but also the reservoir status for *E. coli* with pathogenic potential (Sarowska et al., 2019).

The IncF plasmid type was found in all ESBL-producing *E. coli*, highlighting that this plasmid type is worldwide related with CTX-M genes spread (Cantón et al., 2012). CTX-M-14 is commonly related with the IncK plasmid type in southern Europe (Cantón et al., 2012), what also happened in the selected U1 and U29 CTX-M-14-producing *E. coli* coharbouring IncK plasmid, suggesting the circulation of this plasmid through different ecosystems compartment (Cantón et al., 2012). Understanding the circulation dynamics of mobile genetic elements, such as plasmids, is a key rule on AMR spread and dissemination routes with global impact, this is why it is necessary to build strategies to slowdown the AMR propagation with urgent investments in active worldwide surveillance in all compartments of interface human-animal-environment (Palmeira et al., 2020; Vrancianu et al., 2020).

5. Conclusions

We have documented that wild ungulates are environmental carriers of ESBL (CTX- M)-producing *Escherichia coli*, representing a potential risk to human, animal and environmental health. Our work contributes to the surveillance and the state of art of AMR spread in wild ungulates in Portugal. High rates of AMR were observed, showing and confirming the role of wild ungulates as reservoirs and transmitters of AMR and their determinants. Since wild ungulates have a European-wide distribution and have a direct and indirect contact with humans and livestock, the relevance and importance of surveillance of wildlife, using strategic sentinel species, cannot be overlooked, but the promotion of holistic works to reveal the real rule of wildlife in the AMR dissemination is essential. Considering the ecological impact of the transmission of resistance determinants in natural environments, the contribution of wildlife must be emphasized, needing well designed translational studies to better understand the contribution of this niche to the global spread of antibiotic resistance.

Author contributions

R.T.T: Conceptualization, Writing – original draft, Supervision,

Formal analysis, Funding acquisition, Project administration. **M.V.C.:** Writing – review & editing. **D.A.:** Methodology. **H.F.:** Resources. **C.F.:** Resources. **J.D.P.:** Methodology, Conceptualization, Formal analysis; Writing – review and editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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References

Arnold, K.E., Williams, N.J., Bennett, M., 2016. Disperse abroad in the land": the role of wildlife in the dissemination of antimicrobial resistance. Biol. Lett. 12 https://doi.org/10.1098/rsbl.2016.0137.

Bachiri, T., Bakour, S., Ladjouzi, R., Thongpan, L., Rolain, J.M., Touati, A., 2017. High rates of CTX-M-15-producing *Escherichia coli* and *Klebsiella pneumoniae* in wild boars and Barbary macaques in Algeria. J. Glob. Antimicrob. Resist. 8, 35–40. https://doi.org/10.1016/j.jgar.2016.10.005.

Bevan, E.R., Jones, A.M., Hawkey, P.M., 2017. Global epidemiology of CTX-M β-lactamases: temporal and geographical shifts in genotype. ntimicrob. J. Antimicrob. Chemother. 72 (8), 2145–2155.

Bonnet, R., 2004. Growing group of extended-spectrum β-lactamases: the CTX-M enzymes. Antimicrob. Agents Chemother. 48, 1–14. https://doi.org/10.1128/AAC.48.1.1-14.2004.

Booton, R.D., Meeyai, A., Alhusein, N., Buller, H., Feil, E., Lambert, H., Mongkolsuk, S., Pitchforth, E., Reyher, K.K., Sakcamduang, W., Satayavivad, J., Singer, A.C., Sringernyuang, L., Thamlikitkul, V., Vass, L., Avison, M.B., Chantong, B., Charoenlap, N., Couto, N., Dulyayangkul, P., Gibbon, M.J., Gould, V.C., Montrivade, V., Phoonsawad, K., Rangkadilok, N., Ratanakorn, P., Sirikanchana, K., Suriyo, T., Suwanpakdee, S., Turner, K.M.E., Wichuwaranan, K., Wiratsudakul, A., 2021. One Health drivers of antibacterial resistance: quantifying the relative impacts of human, animal and environmental use and transmission. One Heal 12. https://doi.org/10.1016/j.onehlt.2021.100220.

Cantón, R., Akóva, M., Carmeli, Y., Giske, C.G., Glupczynski, Y., Gniadkowski, M., Livermore, D.M., Miriagou, V., Naas, T., Rossolini, G.M., Samuelsen, Seifert, H., Woodford, N., Nordmann, P., Poirel, L., Bogaerts, P., Navon-Venezia, S., Cornaglia, G., 2012. Rapid evolution and spread of carbapenemases among *Enterobacteriaceae* in Europe. Clin. Microbiol. Infect. 18, 413–431. https://doi.org/10.1111/j.1469-0691.2012.03821.x.

Carattoli, A., Bertini, A., Villa, L., Falbo, V., Hopkins, K.L., Threlfall, E.J., 2005. Identification of plasmids by PCR-based replicon typing. J. Microbiol. Methods 63, 219–228. https://doi.org/10.1016/j.mimet.2005.03.018.

Cassini, A., Högberg, L.D., Plachouras, D., Quattrocchi, A., Hoxha, A., Simonsen, G.S., Colomb-Cotinat, M., Kretzschmar, M.E., Devleesschauwer, B., Cecchini, M., Ouakrim, D.A., Oliveira, T.C., Struelens, M.J., Suetens, C., Monnet, D.L., Strauss, R., Mertens, K., Struyf, T., Catry, B., Latour, K., Ivanov, I.N., Dobreva, E.G., Tambic Andrašević, A., Soprek, S., Budimir, A., Paphitou, N., Žemlicková, H., Schytte Olsen, S., Wolff Sönksen, U., Märtin, P., Ivanova, M., Lyytikäinen, O., Jalava, J., Coignard, B., Eckmanns, T., Abu Sin, M., Haller, S., Daikos, G.L., Gikas, A., Tsiodras, S., Kontopidou, F., Tóth, Á., Hajdu, Á., Guólaugsson, Ó., Kristinsson, K.G., Murchan, S., Burns, K., Pezzotti, P., Gagliotti, C., Dumpis, U., Liuimiene, A., Perrin, M., Borg, M.A., de Greeff, S.C., Monen, J.C., Koek, M.B., Elstrøm, P., Zabicka, D., Deptula, A., Hryniewicz, W., Caniça, M., Nogueira, P.J., Fernandes, P.A., Manageiro, V., Popescu, G.A., Serban, R.I., Schréterová, E., Litvová, S., Štefkovicová, M., Kolman, J., Klavs, I., Korošec, A., Aracil, B., Asensio, A., Pérez-Vázquez, M., Billström, H., Larsson, S., Reilly, J.S., Johnson, A., Hopkins, S., 2019. Attributable deaths and disability-adjusted life-years caused by infections with antibiotic-resistant bacteria in the EU and the European Economic Area in 2015: a population-level modelling analysis. Lancet Infect. Dis. 19, 56–66. https://doi.org/10.1016/S1473-3099(18)30605-4.

Clermont, O., Christenson, J.K., Denamur, E., Gordon, D.M., 2013. The Clermont *Escherichia coli* phylo-typing method revisited: improvement of specificity and detection of new phylo-groups. Environ. Microbiol. Rep. 5, 58–65. https://doi.org/10.1111/1758-2229.12019.

Coque, T.M., Novais, A., Carattoli, A., Poirel, L., Pitout, J., Peixe, L., Baquero, F., Canton, R., Nordmann, P., 2008. Dissemination of clonally related *E.coli* strains expressing ESBL CTX-M-15. Emerg. Infect. Dis. 14, 195–200.

Costa, D., Poeta, P., Sáenz, Y., Vinué, L., Rojo-Bezares, B., Jouini, A., Zarazaga, M., Rodrigues, J., Torres, C., 2006. Detection of *Escherichia coli* harbouring extended-spectrum β-lactamases of the CTX-M, TEM and SHV classes in faecal samples of wild animals in Portugal [4]. J. Antimicrob. Chemother. 58, 1311–1312. https://doi.org/10.1093/jac/dkl415.

Costa, D., Vinué, L., Poeta, P., Coelho, A.C., Matos, M., Sáenz, Y., Somalo, S., Zarazaga, M., Rodrigues, J., Torres, C., 2009. Prevalence of extended-spectrum beta-lactamase-producing *Escherichia coli* isolates in faecal samples of broilers. Vet. Microbiol. 138, 339–344. https://doi.org/10.1016/j.vetmic.2009.03.029.

Dadgostar, P., 2019. Antimicrobial resistance: implications and costs. Infect. Drug Resist. 12, 3903–3910. https://doi.org/10.2147/IDR.S234610.

Danner, M.C., Robertson, A., Behrends, V., Reiss, J., 2019. Antibiotic pollution in surface fresh waters: occurrence and effects. Sci. Total Environ. 664, 793–804. https://doi.org/10.1016/j.scitotenv.2019.01.406.

Darwich, L., Seminati, C., López-Olvera, J.R., Vidal, A., Aguirre, L., Cerdá, M., Garcias, B., Valldeperes, M., Castillo-Contreras, R., Migura-Garcia, L., Conejero, C., Mentaberre, G., 2021. Detection of beta-lactam-resistant escherichia coli and toxigenic clostridioides difficile strains in wild boars foraging in an anthropization gradient. Animals 11. https://doi.org/10.3390/ani11061585.

Davies, J., Davies, D., 2010. Origins and evolution of antibiotic resistance. Microbiol. Mol. Biol. Rev. 74, 417–433. https://doi.org/10.1128/mmbr.00016-10.

Dias, D., Caetano, T., Torres, R.T., Fonseca, C., Mendo, S., 2019. Shiga toxin-producing *Escherichia coli* in wild ungulates. Sci. Total Environ. 651, 203–209. https://doi.org/10.1016/j.scitotenv.2018.09.162.

Dias, D., Torres, R.T., Kronvall, G., Fonseca, C., Mendo, S., Caetano, T., 2015. Assessment of antibiotic resistance of *Escherichia coli* isolates and screening of Salmonella spp. in wild ungulates from Portugal. Res. Microbiol. 166, 584–593. https://doi.org/10.1016/j.resmic.2015.03.006.

Dolejska, M., Literak, I., 2019. Wildlife is overlooked in the epidemiology of medically important antibiotic-resistant bacteria. Antimicrob. Agents Chemother. 63, 1–5. https://doi.org/10.1128/AAC.01167-19.

Du, L., Liu, W., 2012. Occurrence, fate, and ecotoxicity of antibiotics in agro-ecosystems. A review. Agron. Sustain. Dev. 32, 309–327. https://doi.org/10.1007/s13593-011-0062-9.

EFSA, 2011. Scientific Opinion on the public health risks of bacterial strains producing extended-spectrum β-lactamases and/or AmpC β-lactamases in food and food-producing animals. EFSA J. 9 https://doi.org/10.2903/j.efsa.2011.2322.

Fuentes-Castillo, D., Farfán-López, M., Esposito, F., Moura, Q., Fernandes, M.R., Lopes, R., Cardoso, B., Muñoz, M.E., Cerdeira, L., Najle, I., Muñoz, P.M., Catão-Dias, J.L., González-Acuña, D., Lincopan, N., 2019. Wild owls colonized by international clones of extended-spectrum β-lactamase (CTX-M)-producing *Escherichia coli* and *Salmonella Infantis* in the Southern Cone of America. Sci. Total Environ. 674, 554–562. https://doi.org/10.1016/j.scitotenv.2019.04.149.

Furness, L.E., Campbell, A., Zhang, L., Gaze, W.H., McDonald, R.A., 2017. Wild small mammals as sentinels for the environmental transmission of antimicrobial resistance. Environ. Res. 154, 28–34. https://doi.org/10.1016/j.envres.2016.12.014.

Gould, I.M., Lawes, T., 2016. Antibiotic stewardship: prescribing social norms. Lancet 387, 1699–1701. https://doi.org/10.1016/S0140-6736(16)00007-6.

Guenther, S., Grobbel, M., Luebke-Becker, A., Goedecke, A., Friedrich, N.D., Wieler, L.H., Ewers, C., 2010. Antimicrobial resistance profiles of *Escherichia coli* from common European wild bird species. Vet. Microbiol. 144, 219–225. https://doi.org/10.1016/j.vetmic.2009.12.016.

Hiller, C.X., Hübner, U., Fajnorova, S., Schwartz, T., Drewes, J.E., 2019. Antibiotic microbial resistance (AMR) removal efficiencies by conventional and advanced wastewater treatment processes: a review. Sci. Total Environ. 685, 596–608. https://doi.org/10.1016/j.scitotenv.2019.05.315.

Holtmann, A.R., Meemken, D., Müller, A., Seinige, D., Büttner, K., Failing, K., Kehrenberg, C., 2021. Wild boars carry extended-spectrum β-lactamase-and ampc-producing escherichia coli. Microorganisms 9, 1–13. https://doi.org/10.3390/microorganisms9020367.

Iramiot, J.S., Kajumbula, H., Bazira, J., Kansiime, C., Asiimwe, B.B., 2020. Antimicrobial resistance at the human–animal interface in the pastoralist communities of Kasese district, south western Uganda. Sci. Rep. 10, 1–15. https://doi.org/10.1038/s41598-020-70517-w.

Johnson, J.R., Stell, A.L., 2000. Extended virulence genotypes of *Escherichia coli* strains from patients with urosepsis in relation to phylogeny and host compromise. J. Infect. Dis. 181, 261–272. https://doi.org/10.1086/315217.

Klein, E.Y., Van Boeckel, T.P., Martinez, E.M., Pant, S., Gandra, S., Levin, S.A., Goossens, H., Laxminarayan, R., 2018. Global increase and geographic convergence in antibiotic consumption between 2000 and 2015. Proc. Natl. Acad. Sci. U.S.A. 115, E3463–E3470. https://doi.org/10.1073/pnas.1717295115.

Larsson, D.G.J., Andremont, A., Bengtsson-Palme, J., Brandt, K.K., de Roda Husman, A. M., Fagerstedt, P., Fick, J., Flach, C.F., Gaze, W.H., Kuroda, M., Kvint, K., Laxminarayan, R., Manaia, C.M., Nielsen, K.M., Plant, L., Ploy, M.C., Segovia, C., Simonet, P., Smalla, K., Snape, J., Topp, E., van Hengel, A.J., Verner-Jeffreys, D.W., Virta, M.P.J., Wellington, E.M., Wernersson, A.S., 2018. Critical knowledge gaps and research needs related to the environmental dimensions of antibiotic resistance. Environ. Int. 117, 132–138. https://doi.org/10.1016/j.envint.2018.04.041.

Liu, H., Wang, Y., Wang, G., Xing, Q., Shao, L., Dong, X., Sai, L., Liu, Y., Ma, L., 2015. The prevalence of Escherichia coli strains with extended spectrum beta-lactamases isolated in China. Front. Microbiol. 6, 1–5. https://doi.org/10.3389/fmicb.2015.00335.

Livermore, D.M., Canton, R., Gniadkowski, M., Nordmann, P., Rossolini, G.M., Arlet, G., Ayala, J., Coque, T.M., Kern-Zdanowicz, I., Luzzaro, F., Poirel, L., Woodford, N., 2007. CTX-M: changing the face of ESBLs in Europe. J. Antimicrob. Chemother. 59, 165–174. https://doi.org/10.1093/jac/dkl483.

Loncaric, I., Beiglböck, C., Feßler, A.T., Posautz, A., Rosengarten, R., Walzer, C., Ehricht, R., Monecke, S., Schwarz, S., Spergser, J., Kübber-Heiss, A., 2016. Characterization of ESBL- and AmpC-producing and fluoroquinolone-resistant *Enterobacteriaceae* isolated from mouflons (*Ovis orientalis musimon*) in Austria and Germany. PLoS One 11, 1–9. https://doi.org/10.1371/journal.pone.0155786.

Macedo, A.S., Freitas, A.R., Abreu, C., Machado, E., Peixe, L., Sousa, J.C., Novais, C., 2011. Characterization of antibiotic resistant enterococci isolated from untreated waters for human consumption in Portugal. Int. J. Food Microbiol. 145, 315–319. https://doi.org/10.1016/j.ijfoodmicro.2010.11.024.

Magiorakos, A.P., Srinivasan, A., Carey, R.B., Carmeli, Y., Falagas, M.E., Giske, C.G., Harbarth, S., Hindler, J.F., Kahlmeter, G., Olsson-Liljequist, B., Paterson, D.L., Rice, L.B., Stelling, J., Struelens, M.J., Vatopoulos, A., Weber, J.T., Monnet, D.L., 2012. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. Clin. Microbiol. Infect. 18, 268–281. https://doi.org/10.1111/j.1469-0691.2011.03570.x.

Martak, D., Henriot, C.P., Broussier, M., Couchoud, C., Valot, B., Richard, M., Couchot, J., Bornette, G., Hocquet, D., Bertrand, X., 2020. High prevalence of human-associated *Escherichia coli* in wetlands located in eastern France. Front. Microbiol. 11, 1–7. https://doi.org/10.3389/fmicb.2020.552566.

Müller, D., Greune, L., Heusipp, G., Karch, H., Fruth, A., Tschäpe, H., Schmidt, M.A., 2007. Identification of unconventional intestinal pathogenic *Escherichia coli* isolates expressing intermediate virulence factor profiles by using a novel single-step multiplex PCR. Appl. Environ. Microbiol. 73, 3380–3390. https://doi.org/10.1128/AEM.02855-06.

Navarro-Gonzalez, N., Castillo-Contreras, R., Casas-Díaz, E., Morellet, N., Concepción Porrero, M., Molina-Vacas, G., Torres, R.T., Fonseca, C., Mentaberre, G., Domínguez, L., Lavín, S., Serrano, E., 2018. Carriage of antibiotic-resistant bacteria in urban versus rural wild boars. Eur. J. Wildl. Res. 64, 1–9. https://doi.org/10.1007/s10344-018-1221-y.

Navarro-Gonzalez, N., Mentaberre, G., Porrero, C.M., Serrano, E., Mateos, A., Lopez-Martin, J.M., 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. https://doi.org/10.1371/journal.pone.0051614.

Novais, C., Campos, J., Freitas, A.R., Barros, M., Silveira, E., Coque, T.M., Antunes, P., Peixe, L., 2018. Water supply and feed as sources of antimicrobial-resistant *Enterococcus* spp. in aquacultures of rainbow trout (*Oncorhyncus mykiss*), Portugal. Sci. Total Environ. 625, 1102–1112. https://doi.org/10.1016/j.scitotenv.2017.12.265.

O'Neill, J., 2014. Antimicrobial Resistance : Tackling a Crisis for the Health and Wealth of Nations.

Palmeira, J.D., Cunha, M.V., Carvalho, J., Ferreira, H., Fonseca, C., Torres, R.T., 2021. Emergence and spread of cephalosporinases in wildlife: a review. Animals. https://doi.org/10.3390/ani11061765, 11.

Palmeira, J.D., Haenni, M., Metayer, V., Madec, J.Y., Ferreira, H.M.N., 2020. Epidemic spread of IncI1/pST113 plasmid carrying the Extended-Spectrum Beta-Lactamase (ESBL) blaCTX-M-8 gene in *Escherichia coli* of Brazilian cattle. Vet. Microbiol. 243, 108629 https://doi.org/10.1016/j.vetmic.2020.108629.

Plaza-Rodríguez, C., Alt, K., Grobbel, M., Hammerl, J.A., Irrgang, A., Szabo, I., Stingl, K., Schuh, E., Wiehle, L., Pfefferkorn, B., Naumann, S., Kaesbohrer, A., Tenhagen, B.A., 2021. Wildlife as sentinels of antimicrobial resistance in Germany? Front. Vet. Sci. 7, 1–15. https://doi.org/10.3389/fvets.2020.627821.

Poeta, P., Radhouani, H., Igrejas, G., Gonçalves, A., Carvalho, C., Rodrigues, J., Vinué, L., Somalo, S., Torres, C., 2008. Seagulls of the Berlengas natural reserve of Portugal as carriers of fecal *Escherichia coli* harboring CTX-M and TEM extended-spectrum beta-lactamases. Appl. Environ. Microbiol. 74, 7439–7441. https://doi.org/10.1128/AEM.00949-08.

Poeta, P., Radhouani, H., Pinto, L., Martinho, A., Rego, V., Rodrigues, R., Gonçalves, 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. https://doi.org/10.1002/jobm.200900066.

Radhouani, H., Silva, N., Poeta, P., Torres, C., Correia, S., Igrejas, G., 2014. Potential impact of antimicrobial resistance in wildlife, environment, and human health. Front. Microbiol. 5, 1–12. https://doi.org/10.3389/fmicb.2014.00023.

Ramos, B., Rosalino, L.M., Palmeira, J.D., Torres, R.T., Cunha, M.V., 2022. Antimicrobial resistance in commensal Staphylococcus aureus from wild ungulates is driven by agricultural land cover and livestock farming. Environ. Pollut., 119116 https://doi.org/10.1016/j.envpol.2022.119116.

Rousham, E.K., Unicomb, L., Islam, M.A., 2018. Human, animal and environmental contributors to antibiotic resistance in low-resource settings: integrating behavioural, epidemiological and one health approaches. Proc. R. Soc. B Biol. Sci. 285 https://doi.org/10.1098/rspb.2018.0332.

Rozwandowicz, M., Brouwer, M.S.M., Fischer, J., Wagenaar, J.A., Gonzalez-Zorn, B., Guerra, B., Mevius, D.J., Hordijk, J., 2018. Plasmids carrying antimicrobial resistance genes in Enterobacteriaceae. J. Antimicrob. Chemother. 73, 1121–1137. https://doi.org/10.1093/jac/dkx488.

Sarowska, J., Futoma-Koloch, B., Jama-Kmiecik, A., Frej-Madrzak, M., Ksiazczyk, M., Bugla-Ploskonska, G., Choroszy-Krol, I., 2019. Virulence factors, prevalence and potential transmission of extraintestinal pathogenic *Escherichia coli* isolated from different sources: recent reports. Gut Pathog. 11 (1), 1–16. https://doi.org/10.1186/s13099-019-0290-0.

Shao, Y., Wang, Y., Yuan, Y., Xie, Y., 2021. A systematic review on antibiotics misuse in livestock and aquaculture and regulation implications in China. Sci. Total Environ. 798, 149205 https://doi.org/10.1016/j.scitotenv.2021.149205.

Singh, K.S., Anand, S., Dholpuria, S., Sharma, J.K., Blankenfeldt, W., Shouche, Y., 2021. Antimicrobial resistance dynamics and the one-health strategy: a review. Environ. Chem. Lett. 19, 2995–3007. https://doi.org/10.1007/s10311-021-01238-3.

Spellberg, B., 2016. The new antibiotic Mantra —" Shorter is better. JAMA Intern. Med. 176, 1254–1255. https://doi.org/10.1001/jamainternmed.2016.3633.4.

Stedt, J., Bonnedahl, J., Hernandez, J., Waldenstrom, J., McMahon, B.J., Tolf, C., Olsen, B., Drobni, M., 2015. Carriage of CTX-M type extended spectrum beta-lactamases (ESBLs) in gulls across Europe. Acta Vet. Scand. 57 https://doi.org/10.1186/s13028-015-0166-3.

Swift, B.M.C., Bennett, M., Waller, K., Dodd, C., Murray, A., Gomes, R.L., Humphreys, B., Hobman, J.L., Jones, M.A., Whitlock, S.E., Mitchell, L.J., Lennon, R.J., Arnold, K.E., 2019. Anthropogenic environmental drivers of antimicrobial resistance in wildlife. Sci. Total Environ. 649, 12–20. https://doi.org/10.1016/j.scitotenv.2018.08.180.

Torres, R.T., Carvalho, J., Cunha, M.V., Fonseca, C., 2019. Antimicrobial resistance and ecology: a dialog yet to begin. EcoHealth 16, 402–403. https://doi.org/10.1007/s10393-019-01438-y.

Torres, R.T., Carvalho, J., Cunha, M.V., Serrano, E., Palmeira, J.D., Fonseca, C., 2021a. Temporal and geographical research trends of antimicrobial resistance in wildlife – a bibliometric analysis. One Heal 11. https://doi.org/10.1016/j.onehlt.2020.100198.

Torres, R.T., Cunha, M.V., Araujo, D., Ferreira, H., Fonseca, C., Palmeira, J.D., 2021b. Emergence of colistin resistance genes (mcr-1) in *Escherichia coli* among widely distributed wild ungulates. Environ. Pollut. 291 https://doi.org/10.1016/j.envpol.2021.118136.

Torres, R.T., Fernandes, J., Carvalho, J., Cunha, M.V., Caetano, T., Mendo, S., Serrano, E., Fonseca, C., 2020. Wild boar as a reservoir of antimicrobial resistance. Sci. Total Environ. 717 https://doi.org/10.1016/j.scitotenv.2019.135001.

Valente, A.M., Acevedo, P., Figueiredo, A.M., Fonseca, C., Torres, R.T., 2020. Overabundant wild ungulate populations in Europe: management with consideration of socio-ecological consequences. Mamm Rev. 50, 353–366. https://doi.org/10.1111/mam.12202.

Veldman, K., van Tulden, P., Kant, A., Testerink, J., Mevius, D., 2013. Characteristics of cefotaxime-resistant *Escherichia coli f*rom wild birds in The Netherlands. Appl. Environ. Microbiol. 79, 7556–7561. https://doi.org/10.1128/AEM.01880-13.

Vittecoq, M., Godreuil, S., Prugnolle, F., Durand, P., Brazier, L., Renaud, N., Arnal, A., Aberkane, S., Jean-Pierre, H., Gauthier-Clerc, M., Thomas, F., Renaud, F., 2016. REVIEW: antimicrobial resistance in wildlife. J. Appl. Ecol. 53, 519–529. https://doi.org/10.1111/1365-2664.12596.

Vrancianu, C.O., Popa, L.I., Bleotu, C., Chifiriuc, M.C., 2020. Targeting plasmids to limit acquisition and transmission of antimicrobial resistance. Front. Microbiol. 11 https://doi.org/10.3389/fmicb.2020.00761.

World Health Organization, 2019. WHO List of Critically Important Antimicrobials for Human Medicine (WHO CIA List). World Health Organization, 2019.

World Health Organization, 2014. Antimicrobial Resistance: Global Report on Surveillance. World Health Organization, 2014.

Zhang, S., Abbas, M., Rehman, M.U., Wang, M., Jia, R., Chen, S., Liu, M., Zhu, D., Zhao, X., Gao, Q., Tian, B., Cheng, A., 2021. Updates on the global dissemination of colistin-resistant *Escherichia coli*: an emerging threat to public health. Sci. Total Environ. 799, 149280 https://doi.org/10.1016/j.scitotenv.2021.149280.