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Occurrence of multidrug resistant Gram-negative bacteria and resistance genes in semi-aquatic wildlife - *Trachemys scripta*, *Neovison vison* and *Lutra lutra* - as sentinels of environmental health

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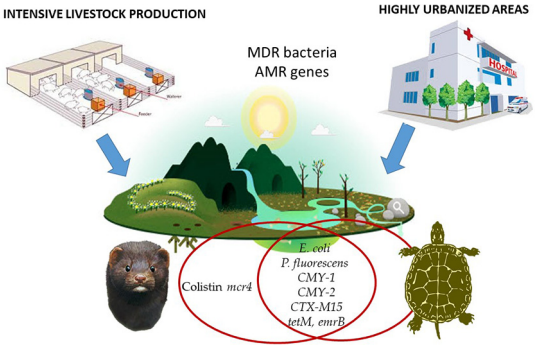
HIGHLIGHTS

- Pond sliders (25.3%), American minks (21%) and Eurasian otters (14.5%) had AMR genes.
- The highest frequency of AMR was observed against β-lactams.
- ESBL/AmpC were found in highly-populated areas and in high livestock density areas.
- Semi-aquatic wild animals are good sentinels of AMR environmental contamination
- One Health Approach is urgently needed to reduce the impact of anthropogenic actions.

GRAPHICAL ABSTRACT



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ABSTRACT

Emergence of antimicrobial resistance (AMR) in bacterial pathogens has been recognized as a major public health concern worldwide. In the present study, antimicrobial resistant Gram-negative bacteria (AMRGNB) and AMR genes were assessed in semi-aquatic wild animals from a highly populated and intensive farming region of Spain, Catalonia. Cloacal/rectal swab samples were collected from 241 animals coming from invasive species *Trachemys scripta* (n = 91) and *Neovison vison* (n = 131), and endangered-protected species *Lutra lutra* (n = 19). Accordingly, 133 (55.2%) isolates were identified as AMRGNB. *Escherichia coli* and *Pseudomonas fluorescens* were among the bacteria most frequently isolated in all animal species, but other nosocomial agents such as *Klebsiella pneumoniae*, *Salmonella* spp. or *Citrobacter freundii*, were also prevalent. The phenotypic susceptibility testing showed the highest resistance to β-lactams (91%). Molecular analysis showed 25.3% of turtles (15.4% ESBL/Ampc genes), 21% of Eurasian otters (10.5% ESBL/Ampc genes) and 14.5% of American minks (8.4% ESBL/Ampc genes) were positive to AMR genes. The genotyping frequency was *tet*M (20.6%), *bla*CMY-2 (13%), *erm*B (6.1%), *bla*CMY-1 (4.6%), *bla*CTX-M-15 (3.1%) and *mcr*-4 (0.8%). Turtles had a larger prevalence of AMRGNB and AMR genes than mustelids, but American mink carried *mcr*-4 colistin-resistance gene. Moreover, cluster analysis of AMR gene distribution revealed that an ESBL/AmpC cluster in a highly populated area comprising big metropolitan regions, and another *tet*M/*emr*B cluster in an expended area with highly intensive livestock production. Although the *mcr*-4 positive case was not included in those clusters,

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that case was found in a county with a high pig farm density. In conclusion, semi-aquatic wild animals are a good sentinel for environmental contamination with AMRGNB and AMR genes. Therefore, One Health Approach is urgently needed in highly populated regions, and with intensive livestock production like Catalonia.

1. Introduction

Antibiotics have been used to treat a wide range of bacterial infections in humans (Zinner, 2007) and animals (da Cunha et al., 2019). They have also been used as growth promoters to increase food production in animals (da Cunha et al., 2019; Van et al., 2020). β-Lactams are among the most clinically important antibiotics in both human and veterinary medicine (Li et al., 2007), being third-generation cephalosporins the most commonly used antimicrobial drugs in humans and animals (Hasman et al., 2005). Ceftriaxone is used to treat invasive Gram-negative bacterial infections in humans, and ceftiofur in food producing animals (Heider et al., 2009). However, intensive use of antibiotics in human and veterinary medicine has resulted in increased prevalence of antimicrobial resistant bacteria (AMRB), opportunistic infections in hospital settings, and global spread of resistant bacteria (Durão et al., 2018; Pitout and Laupland, 2008). Emergence of antimicrobial resistance (AMR) in most common bacterial pathogens has been recognized as a major public health concern worldwide. Resistant bacteria have emerged in hospital as well as community settings suggesting the presence of reservoirs of AMRB outside the hospital (Munita and Arias, 2016).

Emergence and spread of multidrug resistant bacteria, specifically the extended spectrum β-lactamases (ESBLs) producing strains, are often responsible for the failure of antibiotic treatment in hospital settings. ESBLs are rapidly evolving group of β-lactamases which share the ability to hydrolyze penicillins, first-, second-, and third-generation cephalosporins, and aztreonam (but not the cephamycins or carbapenems) yet are inhibited by clavulanic acid (Ghafourian et al., 2014; Paterson and Bonomo, 2005; Rawat and Nair, 2010). ESBLs are frequently plasmid encoded and can be exchanged readily among bacterial populations horizontally. Plasmids responsible for ESBL production frequently carry genes encoding resistance to other drug classes such as aminoglycosides (Paterson and Bonomo, 2005).

Studies conducted in wild birds and mammalian species showed that these animals can serve as reservoirs and potential vectors for the spread of resistant bacteria and antimicrobial resistance genes (Carroll et al., 2015; Darwich et al., 2019). Similar studies conducted in reptiles such as green sea turtles (Ahasan et al., 2017), loggerhead sea turtles (Blasi et al., 2020), Galapagos tortoises (Claudin et al., 2019), European pond turtle (Ruzauskas et al., 2016), and sea turtle (Zavala-norzagaray et al., 2015) confirmed that these animals harbored antimicrobial resistant bacteria regardless of their health status. In addition, there are few studies conducted in American minks that reported AMR mostly in pathogenic bacteria isolated from clinical cases in Denmark (Pedersen et al., 2009; Nikolaisen et al., 2017) or from a commercial mink farm in United States (Agga et al., 2021). However, to the best of our knowledge, no information has been published yet in wild mustelids.

In Catalonia (NE Spain), although there are some studies conducted on multidrug resistant bacteria (Molina-Lopez et al., 2015; Vidal et al., 2017) and antimicrobial resistance genes in wildlife (Darwich et al., 2019), there are limited data regarding the role of semi-aquatic wild animals as sentinels of environmental health. For this reason, in this study, we estimate the occurrence of AMRGNB in wild semi-aquatic animals, principally from invasive wild species such as Pond slider and American mink, and from Eurasian otters. Moreover, antimicrobial susceptibility phenotyping profile of isolates and genotyping of β-lactams, erythromycin and tetracycline-resistance genes were determined, as well as the geographical distribution of AMRGNB and genes in these wild animals in Catalonia, NE Spain.

2. Material and methods

2.1. Study area

The present study was conducted in Catalonia, an autonomous community located in the NE Spain, from January 2018 to July 2021. Catalonia is delimited in the east by Mediterranean Sea, in the north by France and in the west and south by autonomous communities of Aragon and Valencia, respectively. Catalonia has an estimated population over 7.8 million with a population density of 242.3 inhabitants per km^2 (Statistical Institute of Catalonia, 2020). Regarding livestock production, Catalonia is a region with a high density of livestock farms, mainly pig farms, leading the Spanish pig production with 21.41 million pigs slaughtered in the first 11 months of 2020 (Pig Process, 2021).

2.2. Study design

A cross-sectional study was conducted on semi-aquatic wild animals from invasive species such as *Neovison vison* (American mink) and *Trachemys scripta* (Pond slider), and protected species such as *Lutra lutra* (Eurasian otter).

The American minks and the Pond sliders are categorized as invasive species in Catalonia, and they were captured and euthanized as part of the regular population management plan for the species, according to the national legislation (Spanish RD 630/2013, August 2nd, regulation of the Spanish Catalogue of invasive exotic species). On the other hand, Eurasian otter is a protected species, thus, a rehabilitation program is always implemented. For this study, the Wildlife Rehabilitation Center (WRC) of Torreferrusa received two kind of animals: live animals (all turtles and most of the minks and otters) and dead animals (some minks and few otters). In the case of live animals, samples were always taken on arrival to the WRC before being euthanized (for invasive species) or before receiving any pharmacologic or antimicrobial treatment (for protected species). In the case of dead animals, fresh corpses (less than 24 h postmortem) found in the field (otters) or captured and euthanized animals by licensed staff in the field (minks), were submitted at the WRC and frozen at −18 °C until the necropsy and sample collection.

The most common causes of admission for Eurasian otter to the center were anthropological origins ranging from direct persecution (gunshot or poisoning) to involuntary human-induced threats (collisions with vehicles or fences).

For logistic purposes, the sampling was centralized at the WRC of Torreferrusa (Barcelona) according to the direction of the Catalan Wildlife Service - Forestal Catalana, SA (Generalitat de Catalunya Government), during a period comprised from May 2018 to May 2021. Thus, sampling methods and handling protocols used for semi-aquatic wild animals were in agreement with the management protocols, and Ethical Principles of the Spanish legislation (Spanish R.D.1201/2005 of the Ministry of Presidency of Spain, 2005).

2.3. Sample collection and processing

Cloacal or rectal swab samples were obtained using sterile swabs from all live and dead animals admitted at the WRC. Swab samples were transported on Amies transport medium (deltalab, Spain) to the Veterinary Infectious Diseases Diagnostic Laboratory located at Autonomous University of Barcelona, Spain. Upon arrival, samples were processed immediately or stored at 4 °C until being processed on the next day.

2.4. Microbiological analysis

All samples were analyzed for microbiological identification of *Enterobacteriaceae*, and some non-*Enterobacteriaceae* Gram-negative bacteria. Cloacal or rectal swabs were streaked on MacConkey agar (Oxoid, UK) and MacConkey agar supplemented with ceftriaxone (1 mg/L), and incubated at 37 °C for 24 h (Garcias et al., 2021). Bacterial cultures on MacConkey agar were used as a positive control for their corresponding culture on

ceftriaxone supplemented plates. Bacterial growth was examined for colony morphology, and ability of the bacteria to ferment lactose in the MacConkey agar. Based on their lactose fermenting ability, bacteria were classified as lactose fermenters, and non-lactose fermenters. Single colonies were subculture on MacConkey agar and incubated at 37 °C during 24 h for further biochemical identification by API® biochemical test strips (bioMérieux SA, Marcy l'Etoile, France) or by VITEK 2 systems (bioMerieux, Spain) in case of not reaching very good levels of identification with the API® (Darwich et al., 2019).

Oxidase negative bacteria were analyzed by a panel of six conventional biochemical tests including triple sugar iron, methyl red, citrate, sulfur-indole-motility, urease, and phenylalanine deaminase tests. Additionally, API® 20 E biochemical test strips were used for identification of oxidase negative isolates when the conventional biochemical tests failed to sufficiently identify the bacteria. On the other hand, oxidase positive isolates were analyzed by API® 20 NE biochemical test strips or VITEK 2 (bioMerieux, Spain) systems (Darwich et al., 2019).

After identifying the bacteria isolated from the ceftriaxone supplemented MacConkey medium, phenotypic antimicrobial susceptibility testing, and genotypic characterization of resistance genes were done. In addition, all isolates were stored in glycerol/brain heart infusion solution (30/70 v/v) at −80 °C for future use.

2.5. Phenotypic antimicrobial susceptibility testing

A Kirby-Bauer disk diffusion susceptibility test protocol (Hudzicki, 2012) was used to determine phenotypic antimicrobial susceptibility of isolates against a panel of 13 different antimicrobials. Each isolate was tested for the following antimicrobial classes using commercial disks: aminopenicillins (ampicillin and amoxicillin/clavulanic acid), cephalosporins (ceftiofur and ceftriaxone), fluoroquinolones (ciprofloxacin and enrofloxacin), aminoglycosides (gentamicin), tetracyclines (tetracycline), macrolides (erythromycin), lincosamides (lincomycin/spectinomycin), sulphonamides (sulphamethoxazole/trimethoprim), amphenicols (chloramphenicol) and, carbapenems (imipenem). The susceptibility of bacteria to each antimicrobial agent was interpreted as Susceptible (S), Intermediate (I), and Resistant (R) based on the breakpoints provided by Clinical & Laboratory Standards Institute, and the European Committee on Antimicrobial Susceptibility Testing. CLSI veterinary breakpoints were preferably used (CLSI, 2018). If not available, CLSI human (CLSI, 2020), EUCAST or CA-SFM veterinary breakpoints as previously published by Vidal et al. (2020).

2.6. Genotypic characterization of antimicrobial resistance genes

PCR was performed, as previously reported, to detect presence of most common antimicrobial resistance genes coding for ESBL/AmpC – *bla*CTX, *bla*TEM, *bla*CMY-1, *bla*CMY-2, *bla*OXA, colistin resistance – *mcr*-1 and *mcr*-4 (Darwich et al., 2019), erythromycin resistance – *erm*B, and tetracycline resistance – *tet*M (Jacob et al., 2008). DNA was extracted from bacterial culture by boiling. Briefly, bacterial growth from the MacConkey agar plates were suspended in tube containing 400 μL distilled water and boiled in water bath at 100 °C for 10 min. Then, all tubes were centrifuged at 13,000 rpm for 5 min. After centrifugation, the supernatant from each tube was recovered into a new tube and stored at −20 °C until further analysis. A PCR mix was prepared as previously described elsewhere (Vidal et al., 2020). The positive controls for all genes were provided by the Veterinary Infectious Diseases Diagnostic Laboratory of Autonomous University of Barcelona (Bellaterra, Spain). Analysis of amplified PCR products was performed in 1.5% agarose gel by electrophoresis.

Sanger DNA sequencing was conducted for verification of *bla*CTX positive PCR products at the Genomic and Bioinformatics Service of the Autonomous University of Barcelona (Spain). Sequences and chromatograms were manually explored to trim bad-quality bases with BioEdit 7.2. Once the assembly of the consensus sequences was conducted, both complete and partial sequences were aligned using the Clustal Omega program, and finally blasted against the public database (National Center for Biotechnology Information, NCBI, Bethesda, MD, USA).

2.7. Statistical analysis

All data obtained during the study were recorded on Microsoft® excel spread sheet. Recorded data included: animal's identification (including date of admission, species, location), isolated bacteria, phenotypic susceptibility profile, and antimicrobial resistance genes. Descriptive statistics was performed under 95% confidence interval and using SPSS to describe frequency of occurrence of resistant bacteria, phenotypic antimicrobial susceptibility, and genotypic antimicrobial resistance genes. Chi-square or Fisher exact test was used for comparison between proportions when appropriate. Statistical significance (unadjusted p-value) was set at 0.05.

2.8. Geographical distribution of resistant bacteria and antimicrobial resistance genes

Geographic Information System (GIS) was applied to investigate the distribution of antimicrobial resistance genes detected in bacteria isolated from semi-aquatic wildlife in Catalonia, Spain. The location of each animal was retrieved from database at the Wildlife Rehabilitation Center (WRC).

Maps were created using Quantum GIS software, version 3.18 ("QGIS Development Team. QGIS geographic information system. Open Source Geospatial Foundation Project," 2020). To evaluate area with higher probability of having animal carriers of AMR genes, cluster analysis was performed with SaTScan™ version 9.6, using a purely spatial scan analysis with a Bernoulli model. The spatial scan analysis is based in the likelihood ratio statistic based on the number of observed and expected cases in the specific zone and search for clusters using a variable circular window size to detect spatial clusters in large areas while controlling for the underlying population (Kulldorff, 2007). The number of Montecarlo simulations was set at 999 and values of p-value lower than 0.05 were consider as statistically significant.

3. Results

3.1. Study population

The study included 241 wild animals admitted at the WRC during 2018 to 2021: *Trachemys scripta* (n = 91), *Neovison vison* (n = 131) and *Lutra lutra* (n = 19).

The sampled animals were found in rivers distributed throughout the Catalan territory; turtles were taken from highly populated areas whereas mustelids came from less urbanized and rural areas (Fig. 1).

3.2. Microbiological analysis

All samples presented bacterial growth in the MacConkey control plates and 133 out of 241 (50.2%) animals tested isolates had a positive growth in the MacConkey agar supplemented with ceftriaxone. In particular, positive samples came from 80.2% (73/91) *Trachemys scripta*, 38.2% (50/131) *Neovison vison* and 52.6% (10/19) *Lutra lutra* (Table 1). For each positive sample or animal, one isolate from pure culture or majoritarian growth was further studied. Therefore, 133 bacterial isolates were subjected to antimicrobial resistance phenotyping, and 131 isolates (two samples were lost) to genotyping analysis.

The bacterial prevalence from the overall population showed that 63% and 37% of isolates were *Enterobacteriaceae* and non-*Enterobacteriaceae*, respectively. Within the *Enterobacteriaceae*, *Escherichia coli* was the most representative bacteria and *Pseudomonas fluorescens* was the most common non-enterobacteria (Table 2). Behind *E. coli* (42%), *K. pneumoniae* (17%) and *Salmonella* (8%) were highly prevalent enterobacteria in *Neovison vison*, whereas in *Lutra lutra*, *Hafnia alvei* (10%) and *Serratia marcescens* (10%) were the principal ones (Fig. 2). In turtles, *E. coli* (31%) was followed

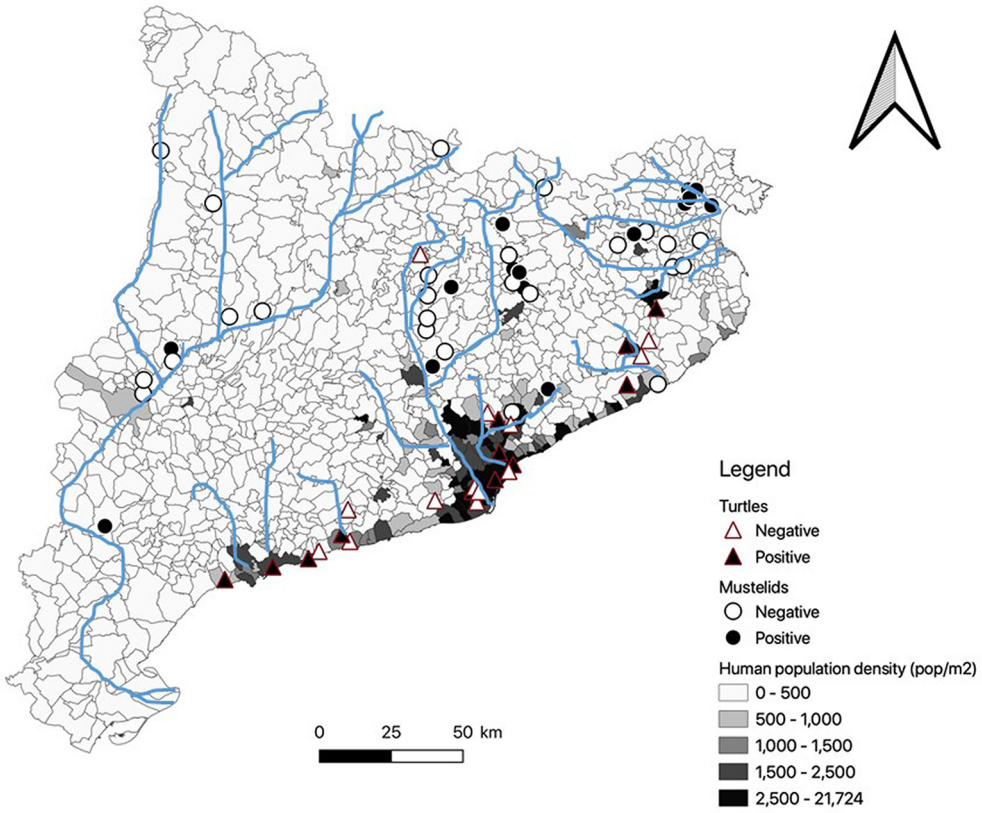

Fig. 1. The map shows the human population density, counties and principal rivers of Catalonia. The localization of the sampled animals is represented in circles (American minks and Eurasian otters) and triangles (Pond sliders). Black filled figures represent positive individuals to AMR genes whereas white figures represent negative ones.

by *Morganella morganii* (11%) and *Citrobacter freundii* (9%) among other *Enterobacteriaceae* such as *Salmonella* (7%) or *Klebsiella* spp. (Fig. 3).

3.3. Phenotypic antimicrobial susceptibility testing of isolates

Out of 133 bacterial isolates of different species that were subjected to antimicrobial susceptibility testing by disk diffusion against a panel of 13 antibiotics of different categories, highest resistance was seen against macrolides (99%), and β-lactams (91%). Varying degrees of resistance were also seen against lincosamides (39%), amphenicols (31%), tetracyclines (30%), quinolones (27%), sulphonamides (26%), and aminoglycosides (16%). None of the isolates showed resistance to imipenem (Fig. 4).

3.4. Molecular characterization of antimicrobial resistance genes of isolates

Molecular analysis of 131 bacterial isolates for the detection of AMR genes showed that 35.1% (46/131) of the bacteria isolated from Ceftriaxone-MacConkey selective medium possess at least one antimicrobial resistance gene. That represents 19.2% of the total population presented AMR genes and 11.2% of them were ESBL/AmpC genes (Table 1). Pond sliders showed higher prevalence of ESBL/AmpC genes than mustelids, 15.4% versus 9.4% respectively ($p = 0.08$).

The gene frequency results showed *tet*M (20.6%, 27/131) as the most frequent gene detected followed by *bla*CMY-2 (13.0%), *erm*B (6.1%), *bla*CMY-1 (4.6%), *bla*CTX-M-15 (3.1%) and *mcr*-4 (0.8%). None of the isolates was found positive to carbapenem resistance genes (OXA-48). One isolate of *Pseudomonas fluorescens* from an American mink was seen to harbor three different resistance genes, *bla*CMY-1, *erm*B, and *tet*M and fifteen isolates (11.5%) were found to carry two different resistance genes.

Bacterial isolates found positive for AMR genes were also analyzed for their corresponding phenotypic antimicrobial susceptibility profile against non-β-lactams (Table 3). *E. coli* was the most common enterobacteria isolated from all animal groups, showing *bla*CTX-M-15, *bla*CMY-2 or *tet*M genes. *Citrobacter* positive to *bla*CMY-2 was also found in American minks and Pond sliders. *K. pneumoniae* expressing *bla*CMY-1 and *tet*M genes was also isolated from Pond sliders. It is important to remark the presence of *Salmonella* positive to *bla*CMY-2 and *K. pneumoniae* positive to colistin *mcr*-4 in American minks (Table 3). Finally, *P. fluorescens* was the most frequent non-enterobacteria found in both mustelids and turtles presenting two or three

Table 1
Frequency of bacterial isolation on ceftriaxone supplemented MacConkey agar (CRO) and AMR genes from semi-aquatic wildlife in Catalonia, Spain.

Animal spp	Total sample	Positive animals CRO growth	Prevalence (CI95%) CRO growth	Prevalence (CI95%) AMR genes[a]	Prevalence (CI95%) ESBL/AmpC genes[a]
Trachemys scripta	91	73	80.2 (72–88.4)	25.3 (16.4–34.2)	15.4 (8.0–22.8)
Neovison vison	131	50	38.2 (29.8–46.5)	14.5 (8.5–20.5)	8.4 (3.7–13.2)
Lutra lutra	19	10	52.6 (30.2–75.1)	21.1 (2.7–39.4)	10.5 (0–24.3)
Total	241	133	55.2 (48.9–61.5)	19.2 (14.1–24.1)	11.2 (7.2–15.2)

[a] N = 131 (2 samples were not recovered for the analysis).

Table 2
Species of animals versus frequency of bacterial isolation from semi-aquatic wildlife in Catalonia, Spain (n = 133).

Species of animals	Bacteria		Number (%) of isolates
	Family	Species	
Trachemys scripta (n = 73)	*Enterobacteriaceae* (n = 45)	*Escherichia coli*	14 (19%)
		Morganella morganii	5 (6.8%)
		Citrobacter freundii	4 (5.5%)
		Citrobacter braakii	3 (4.1%)
		Hafnia alvei	3 (4.1%)
		Klebsiella oxytoca	3 (4.1%)
		Salmonella spp.	3 (4.1%)
		Klebsiella pneumoniae	2 (2.7%)
		Enterobacter cloacae	2 (2.7%)
		Enterobacter amnigenus	1 (1.4%)
		Proteus vulgaris	1 (1.4%)
		Rahnella aquatilis	1 (1.4%)
		Serratia fonticula	1 (1.4%)
		Serratia marcescens	1 (1.4%)
		Shigella spp.	1 (1.4%)
	Non-*Enterobacteriaceae* (n = 28)	*Pseudomonas fluorescens*	10 (13.7%)
		Aeromonas hydrophila	8 (11%)
		Burkholderia cepacia	4 (5.5%)
		Pseudomonas putida	3 (4.1%)
		Pseudomonas aeruginosa	2 (2.7%)
		Ralstonia pictettii	1 (1.4%)
Neovison vison (n = 50)	*Enterobacteriaceae* (n = 35)	*Escherichia coli*	15 (30%)
		Klebsiella pneumoniae	6 (12%)
		Salmonella spp.	3 (6%)
		Shigella spp.	2 (4%)
		Hafnia alvei	2 (4%)
		Morganella morganii	2 (4%)
		Proteus vulgaris	2 (4%)
		Citrobacter braakii	1 (2%)
		Citrobacter freundii	1 (2%)
		Providencia stuartii	1 (2%)
	Non-*Enterobacteriaceae* (n = 15)	*Pseudomonas fluorescens*	13 (26%)
		Ralstonia pictettii	1 (2%)
		Bordetella bronchiseptica	1 (2%)
Lutra lutra (n = 10)	*Enterobacteriaceae* (n = 4)	*Escherichia coli*	2 (20%)
		Hafnia alvei	1 (10%)
		Serratia marcescens	1 (10%)
	Non-*Enterobacteriaceae* (n = 6)	*Pseudomonas fluorescens*	5 (50%)
		Mannheimia haemolytica	1 (10%)

different AMR genes, *bla*CTX-M-15 and *tet*M among them. Turtles additionally presented other non-enterobacteria such as *Burkholderia* and *Aeromonas* with AMR genes (Table 3).

3.5. Geographic information system analysis of antimicrobial resistance genes

Geographic distribution of all AMR genes of our interest was investigated based on the location of animals from which samples were collected. For this purpose, out of 241 animals, the county (zones) of 211 animals was retrieved from database at the WRC; however, the location of 30 animals was missing. Accordingly, wild animals originated from 23 different counties in Catalonia were included in this analysis.

The geographical distribution of the AMR genes was analyzed at county level by using QGIS 3.16 (Hannover). Animals harboring bacteria with at least one AMR gene were considered positive. In some counties such as, Baix Camp, Maresme, and Ribera d'Ebre (indicated in full red circle in the map), only one animal sample was obtained, and the sample was detected positive for AMR genes (Fig. 5). There was no statistically significant association between the species of animals from which the bacteria were recovered and occurrence of AMR genes in bacteria ($\chi^2 = 4.139$; $p = 0.124$). Furthermore, the geographic distributions of ESBL/AmpC, and colistin resistance genes (*mcr*-1 and *mcr*-4), and erythromycin (*erm*B) and tetracycline (*tet*M) resistance genes were analyzed separately by using the same GIS software. Interestingly, one isolate from Osona, one of the counties with the highest density of pig farms, was found positive for *mcr*-4.

Cluster analysis using a purely spatial scan analysis to evaluate areas with higher probability of having animal carriers of AMR genes revealed that significant high-risk cluster of animals harboring a tetracycline resistance gene in the zones of Tarragona and Lleida (Relative Risk = 4.95, p-value = 0.006). Moreover, another high-risk cluster of individuals carrying ESBL/AmpC genes, which was at limit of significance, was detected in the zones of Tarragona and the metropolitan area of Barcelona (RR = 3.74, p-value = 0.07). These results are shown on Fig. 6.

4. Discussion

In the present study, different species of semi-aquatic wild animals such as Pond sliders, American minks, and Eurasian otters, were examined as

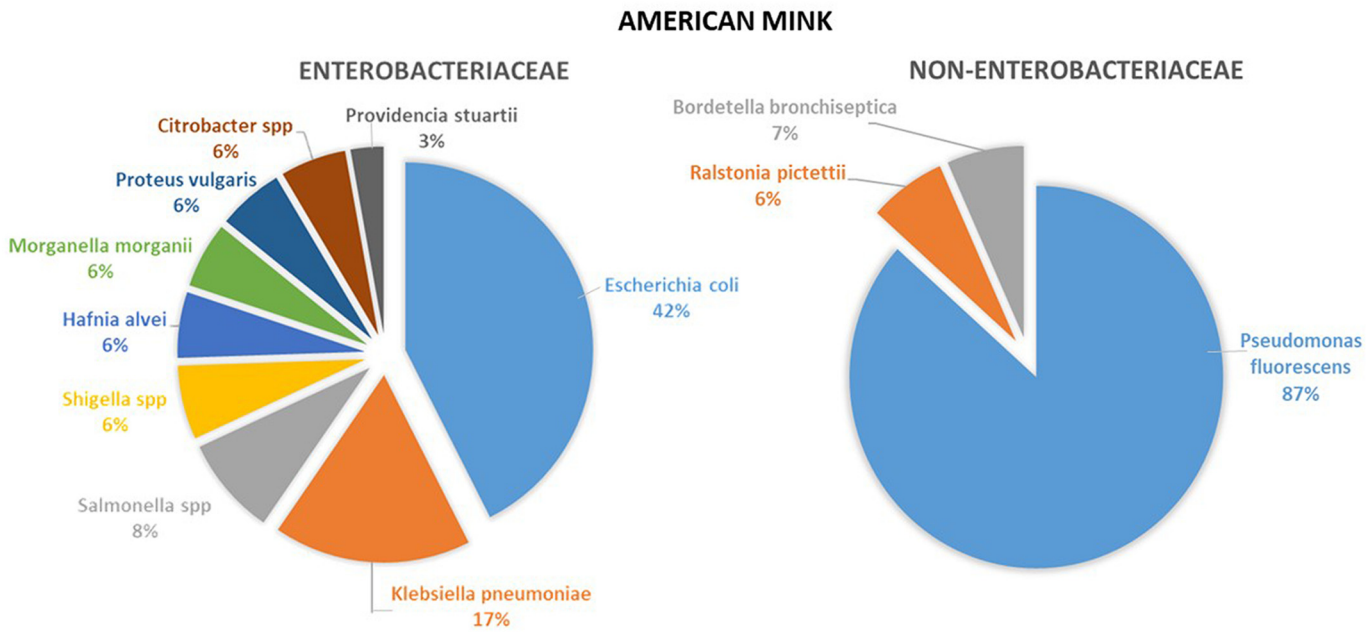

Fig. 2. Frequency of *Enterobacteriaceae* and Non-*Enterobacteriaceae* isolated from cloacal samples of *Neovison vison* in Catalonia, Spain.

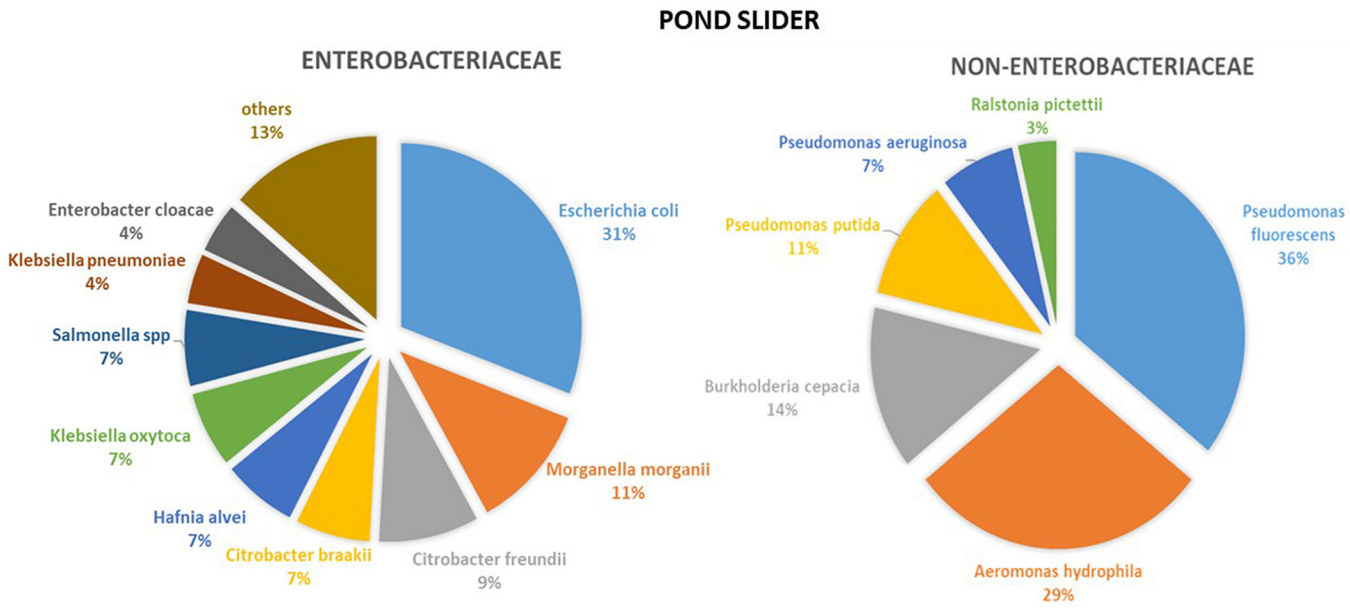

Fig. 3. Frequency of *Enterobacteriaceae* and Non-*Enterobacteriaceae* isolated from cloacal samples of *Trachemys scripta* in Catalonia, Spain.

reservoirs of AMRGNB in Catalonia, NE Spain. A wide range of antimicrobial resistant bacterial species were isolated with *E. coli* leading the *Enterobacteriaceae* group and *P. fluorescens* as the most representative non-*Enterobacteriaceae* species. Other nosocomial enterobacteria such as *Citrobacter freundii*, and *Klebsiella pneumoniae* were also frequently isolated.

E. coli was the most common AMR pathogen described in wildlife from different parts of the world. A study conducted in Poland showed that resistant *E. coli* were recovered from wild animals such as red deer, roe deer, fallow deer, European bison, and wild boar (Wasyl et al., 2018). Similarly, a study conducted in Singapore on wild birds and rodents also reported the presence of antimicrobial resistant *E. coli*, with 80.8% of these isolates were resistant to at least one antimicrobial tested (Ong et al., 2020). Tardón et al., also identified antimicrobial resistant *Enterobacteriaceae* in fractures of wild birds from wildlife rehabilitation centers in Spain (Tardón et al., 2021). Despite the fact that *E. coli* is a common normal microbiota in the large intestine (Katouli, 2010), the increase of resistant strains causing infection in animals and humans, such as urinary tract infections (Darwich et al., 2021a), may difficult empirical treatment of patients thereby posing a public health concern worldwide (Olorunmola et al., 2013).

Moreover, non-*Enterobacteriaceae* such as *Pseudomonas fluorescens* were also isolated in the present study. This result was in line with the finding of Dias et al., who reported resistant *P. fluorescens* isolated from faecal samples of different species of wild animals (Dias et al., 2018). Although *P. fluorescens* is one of the less virulent members of Pseudomonadaceae family, this agent has been implicated as a causative agent of infection in humans, such as pseudobacteraemia, especially in immunocompromised individuals (Pappas et al., 2006; Pratishtha et al., 2021).

The present study also determined AMR profiles, and types of resistance in bacterial isolates recovered from the study animals. Highest proportion of antimicrobial resistance to β-lactams (91%) was found. Varying degrees of resistance were also seen to lincosamides, amphenicols, tetracyclines, quinolones, sulphonamides, and aminoglycosides. These results were in agreement with the findings of Nikolaisen et al., who reported AMR in most pathogenic bacteria from mink in Denmark, particularly *E. coli* (Nikolaisen et al., 2017). In recent years, due to increased number of bacterial isolates carrying ESBL genes, the clinical use of carbapenems has increased. As a result, the overuse of carbapenems has led to increased number of clinical bacterial isolates producing carbapenemases (Elshamy

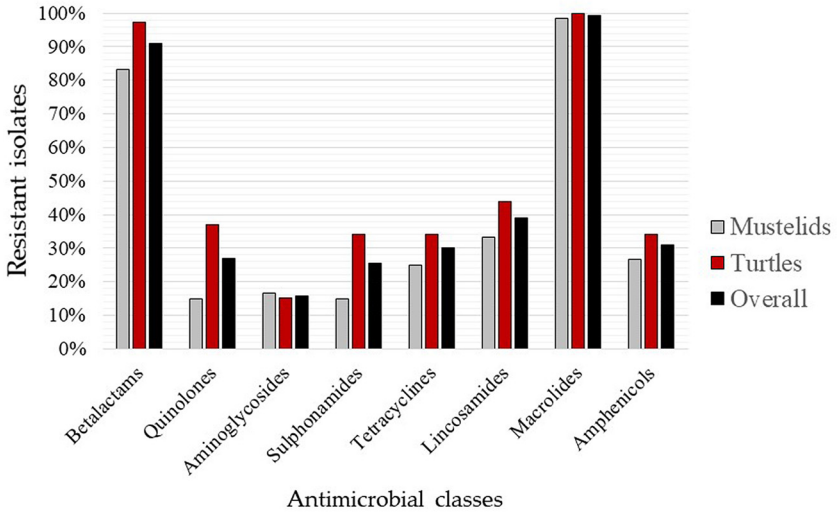

Fig. 4. Occurrence of phenotypic antimicrobial resistance profile of bacteria from semi-aquatic wildlife in Catalonia, Spain.

Table 3
Antimicrobial resistance genes and phenotypic resistance to non-β-lactams in bacterial species from semi-aquatic wildlife in Catalonia, Spain.

Animal spp.	Bacterial spp.	AMR genes	Resistance phenotype to non-β-lactams
Trachemys scripta	*Enterobacteriaceae*		
	Escherichia coli	*bla*CMY-2	CIP, ENO, GM, TE, LS
		*bla*CMY-2	XNL
		*bla*CMY-2, *tet*M	ENO, GM, TE, LS
		*bla*CMY-2, *tet*M	CIP, ENO, SXT, TE, LS, C
	Klebsiella pneumoniae	*bla*CMY-1, *tet*M	–
	Citrobacter braakii	*bla*CMY-2, *tet*M	–
		*bla*CMY-2	LS
	Hafnia alvei	*bla*CMY-2	–
	Citrobacter freundii	*bla*CMY-2	LS
		*bla*CMY-2	ENO
		*tet*M	ENO, SXT, C
	Serratia marcescens	*tet*M	GM, LS
	Serratia fonticula	*tet*M	–
	Non-*Enterobacteriaceae*		
	Pseudomonas fluorescens	*bla*CTX-M-15, *tet*M	LS
		*tet*M	CIP, ENO, GM, SXT, TE, LS
	Pseudomonas putida	*bla*CMY-1, *tet*M	ENO, SXT, C
		*bla*CMY-1	ENO, SXT, C
		*bla*CMY-1, *bla*CMY-2	ENO, SXT, LS
	Burkholderia cepacia	*erm*B	ENO, SXT, TE, C
		*tet*M	CIP, ENO, SXT, TE, LS, C
	Aeromonas hydrophila	*tet*M	GM, SXT, TE, LS
		*tet*M	TE, LS
	Ralstonia pictettii	*tet*M	CIP, SXT, TE, LS, C
Neovison vison	*Enterobacteriaceae*		
	Escherichia coli	*bla*CTX-M-15	GM, TE, LS
		*bla*CMY-2	–
		*bla*CMY-2	LS
		*bla*CMY-2, *tet*M	CIP, GM, LS
		*tet*M	–
	Citrobacter braakii	*bla*CMY-2	LS
	Citrobacter freundii	*bla*CMY-2	LS
	Salmonella spp.	*bla*CMY-2	–
	Klebsiella pneumoniae	*mcr*-4	–
		*tet*M	–
	Shigella spp.	*erm*B, *tet*M	–
		*erm*B, *tet*M	–
	Proteus vulgaris	*tet*M	–
	Non-*Enterobacteriaceae*		
	Pseudomonas fluorescens	*bla*CTX-M-15, *tet*M	ENO, GM, SXT, TE, LS
		*bla*CMY-1, *erm*B, *tet*M	–
		*bla*CMY-1, *tet*M	–
		*erm*B	C
		*erm*B, *tet*M	C
		*erm*B, *tet*M	–
Lutra lutra	*Enterobacteriaceae*		
	Escherichia coli	*erm*B	–
	Non-*Enterobacteriaceae*		
	Pseudomonas fluorescens	*bla*CTX-M-15, *tet*M	CIP, ENO, GM, SXT, TE, LS, C
		*bla*CMY-2	–
		*Tet*M	C

C, chloramphenicol; CIP, ciprofloxacin; ENO, enrofloxacin; GM, gentamicin; LS, lincomycin/spectinomycin; SXT, sulphamethoxazole/trimethoprim; TE, tetracyclin; XNL, ceftiofur.

and Aboshanab, 2020). Although carbapenem resistance has been detected in bacteria isolated from wild mammals and wild birds in Spain (Darwich et al., 2019), none of the isolates from semi-aquatic wildlife of the present study showed resistance to carbapenems.

Anthropogenic activities such as use of antimicrobials in agriculture and aquaculture, other nonhuman applications of antimicrobials, and waste disposal play an important role to create major environmental reserves of resistance, virulence genes and the organism that harbor them (Davies and Davies, 2010). Poorly treated wastes contaminated with antimicrobials from humans and livestock are often assumed to be the main sources of AMR in wildlife (Arnold et al., 2016). Waste water is a meeting place for antimicrobials, antimicrobial resistance genes, and bacteria from different sources (Karkman et al., 2018). In this study, the most frequently detected gene was *tet*M (20.6%) followed by *bla*CMY-2 (13.0%), *erm*B (6.1%), *bla*CMY-1 (4.6%), *bla*CTX-M-15 (3.1%) and *mcr*-4 (0.8%). These genes have been also reported in enterobacteria from wild birds and mammals in Spain, suggesting that are quite frequent in the environment (Darwich et al., 2019, 2021b; Garcias et al., 2021).

Other AMR genes such as *tet*M or *erm*B detected in this study, have been also reported in bacterial isolates obtained from humans, domestic and wild animals elsewhere in the world (Bryan et al., 2004). Erythromycin resistance gene – *erm*B and tetracycline resistance genes – *tet*M are among plasmid-mediated AMR genes that confer resistance to erythromycin, and tetracycline, respectively. Both antimicrobial agents are widely used in human and animal clinical practices (Bryan et al., 2004; Dönhöfer et al., 2012; Villedieu et al., 2004).The high prevalence of *tet*M and *erm*B resistance genes found in semi-aquatic wildlife could be consequence of the environmental pollution caused by faecal resistome in livestock, especially in slaughter pigs from countries with a high antimicrobial usage patterns (Munk et al., 2018).

Mobile colistin resistance (*mcr*) genes are plasmid-mediated resistance genes recently discovered in humans and food animals. The *mcr* genes are known to confer resistance to colistin sulphate – one of the last effective drugs for the treatment of multidrug resistant Gram-negative bacteria infections (Sun et al., 2018). Colistin is included within the reserve group of drugs according to the Aware list of the World Health Organization (WHO, 2021), which means that it is considered a "last-resort" drug to be used when all alternatives have failed. This antibiotic was widely used in veterinary medicine as preventive mass-medication against colibacillosis in piglets (Rhouma et al., 2016). However, nowadays there is a worldwide trend to limit colistin usage in animal production due to significant public health concern related to emergence and transmission of resistance genes (Sun et al., 2018).The *mcr*-1 and *mcr*-4 were the most frequent colistin-resistance genes reported in *Enterobacteriaceae* in pigs from Spain (Sevilla et al., 2021). The presence of *mcr*-4 in *Klebsiella pneumoniae* from *Neovison vison* in the present study could be due because Osona is one of the counties in Catalonia with the highest pig density and previous abuse of colistin sulphate in pig production as a preventive for controlling post-weaning diarrheas could happen. In fact, from 2013 to 2015, Spain was the European country with the highest sales of polymyxins for food-producing animals (EMA (European Medicine Agency), 2017), and with a significant prevalence of colistin-resistance genes, principally *mcr*-4 (Aguirre et al., 2020). The Spanish Agency for Medicines and Health Products established a drastic reduction (97%) in the use of colistin in swine from 2015 to 2018 in Spain, with a positive impact in the decrease in colistin resistance genes (Aguirre et al., 2020). In this study, we have detected the presence of *mcr*-4 in a *Neovison vison*, confirming that these genes are still present in the environment in counties with a high pig production. The presence of these colistin resistant genes in natural waters can suggest that the reduction in the use of this antimicrobial is not enough to prevent gene selection in live pigs or that river contamination come from stable *mcr* genes in old non-treated purines.

In this study, turtles had a larger prevalence of AMRGNB and AMR genes than mustelids, and colistin-resistant genes were only detected in the mustelid group. Different behaviour, feeding habits and ecological niche could explain the difference among these species. The Pond sliders, and American minks are invasive and generalist species that have spread throughout much of Europe and currently occupies the Iberian Peninsula because of their environmental release as exotic pets or their multiple escapes from mink farms, respectively (Bonesi and Palazon, 2007).

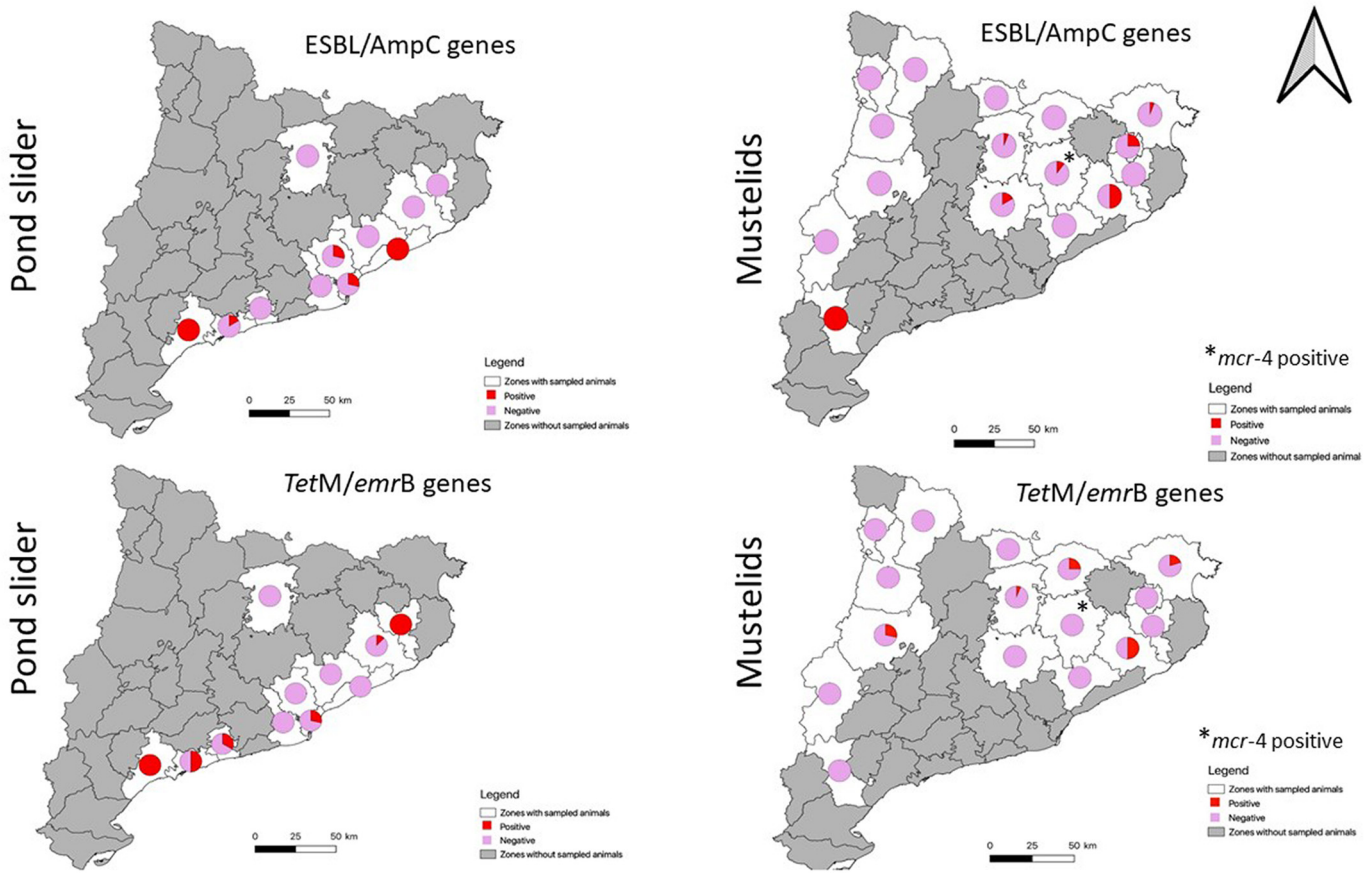

Fig. 5. Geographical distribution of antimicrobial resistance genes detected in bacteria isolated from semi-aquatic wildlife in Catalonia, Spain. Circles represent the proportion of positive animals for each county (red = positive cases; pink = negative cases).

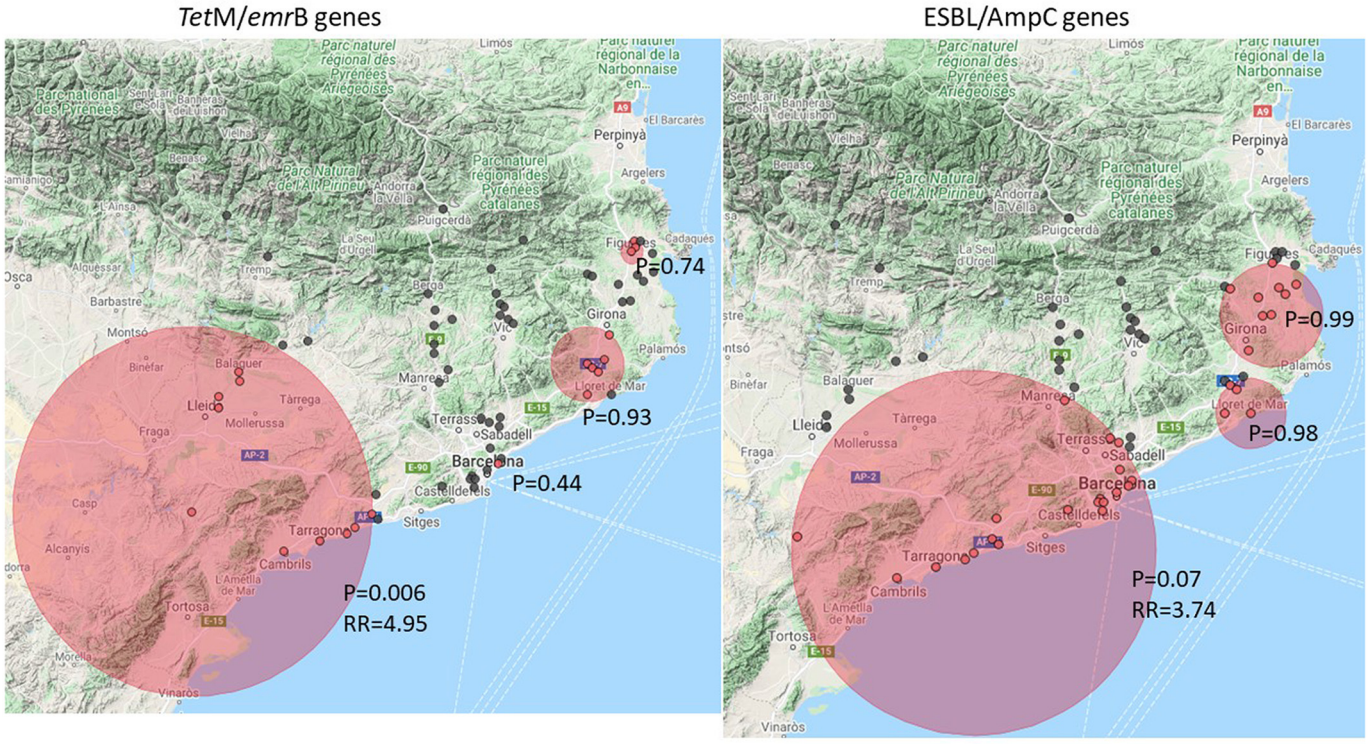

Fig. 6. Spatial cluster analysis performed with SatScan. Red circles represent high-risk clusters. Red points are locations situated into the clusters and black points are locations out of the cluster.

On the other hand, American minks and Eurasian otters are always been linked to clean and crystalline waters and their feeding habits may differ depending on the habitat. In rivers and streams, the American minks are more generalists and feeds on frogs, small mammals (rabbits, rodents and shrews), fish and waterfowl. Whereas otters are basically feeding upon fish, crustaceans and amphibians (Romero and Guitián, 2017). As regards the pond sliders, they prefer ponds, swamps, or slow-flowing portions of rivers and estuaries and they are omnivores, feeding on a wide variety of plants, insects, fish, crayfish, freshwater sponges, snails, slugs, small amphibians, and other reptiles (Bonin et al., 2006). Thus, the colonization of these invasive species could be consequence of the water pollution with AMRGNB, and AMR genes but also due to the consumption of aquatic/terrestrial contaminated preys (Fu et al., 2017; Liu et al., 2013).

The distribution of β-lactams-, erythromycin-, and tetracycline-resistance genes were homogeneous in all the regions of the study, with the exception of two clusters. The first one was found for ESBL/AmpC genes, in a highly populated area comprising big metropolitan regions of Barcelona and Tarragona with large human hospitals and also receiving the river-mouths of rivers coming from northern areas with a large pig production. Thus, Pond sliders sampled from streams, flood plains, the river-mouths of main rivers of those highly urbanized areas of Catalonia presented higher burden of AMR bacteria and AMR genes from both human and animal origin. The other cluster was observed for *tet*M/*emr*B genes covering an expended area of Tarragona and Lleida provinces with a high livestock intensive production, principally poultry and pig industries. Mustelids sampled from those rural areas with a high pig production activity, were more exposed to AMR genes related to livestock treatments. Moreover, the acquisition of colistin-resistance genes in American minks could be either by contact with water contaminated by slurry or by the consumption of small rodents that have been in contact with pig farms. The pig sector is reaching its environmental limits, principally in Catalonia. Thus, a new royal decree RD 306/2020 of the Spanish government sets the regulations for Spanish pig farming for animal health and welfare on farms and a reduction of the environmental impact, preventing the nitrate contamination from entering the groundwater and reducing emissions of ammonia and greenhouse gases. Considering the results of this study, plans for reducing the environmental impact of AMR genes should also be implemented. This signifies that the One Health Approach, which involves human-animal-environmental health to control and prevent further dissemination of resistant bacteria and resistance determinants, is urgently needed in highly populated areas with a large livestock production density like Catalonia.

5. Conclusions

This study describes AMRGNB, and resistance determinants in semi-aquatic wild animals such as Pond sliders, American minks, and Eurasian otters. In overall, 25.3% of turtles, 21% of otters and 14.5% of American minks presented AMR genes. *E. coli* and *P. fluorescens* were the most representative bacterial spp. detected in turtles and mustelids followed by other nosocomial enterobacteria such as *C. freundii* and *K. pneumoniae*. The highest frequency of AMR was observed against β-lactams, drugs frequently used in human and animal medicine. The AMR genes detected in these live semi-aquatic wild animals (*bla*CTX-M-15, *bla*CMY-1, *bla*CMY-2, *tet*M, *erm*B, and *mcr*-4 variants) have been also reported in other wild species in Catalonia. The distribution of β-lactam, erythromycin, tetracycline and colistin-resistance genes were concentrated in the metropolitan areas of big cities and in regions with a high pig farm production. Thus, semi-aquatic wild animals are good sentinel of environmental (water) pollution with AMRGNB and AMR genes. This signifies that the One Health Approach is urgently needed in highly populated areas with a large livestock production density like Catalonia.

CRediT authorship contribution statement

Conceptualization, R.A.M.-L. and L.D.; Methodology, T.S.M., B.G., G.C., C.S., R.A.M.-L., and L.D.; Software, T.S.M and B.G.; Validation, R.A.M-L., and L.D.; Formal analysis, T.S.M, B.G., G.C., and C.S.; Investigation, L.D.; Resources, L.D.; Data curation, T.S.M., B.G., R.A.M.-L., and L.D.; Writing—original draft preparation, T.S.M., B.G. and L.D.; Writing—review and editing, T.S.M., B.G., G.C., C.S., R.A.M.-L., and L.D.; Supervision, L.D.; Funding acquisition, L.D. All of the authors have read and agreed to the published version of the manuscript.

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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