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Monitoring antimicrobial resistance trends and emerging carbapenemases in Enterobacterales causing companion animal infections: a four-year study

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ABSTRACT

This study assessed longitudinal trends in antimicrobial resistance (AMR) among Enterobacterales causing companion animal infections in Portugal between 2020 and 2023 and characterized carbapenemase-producing Enterobacterales (CPE). A collection of 4155 non-duplicate clinical Enterobacterales isolates underwent antimicrobial susceptibility testing mainly through disk diffusion, targeting β-lactams, fluoroquinolones, aminoglycosides and folate pathway inhibitors. Possible bacteria-AMR associations and temporal trends were statistically assessed. Potential CPE were selected on Brilliance™ CRE Agar, confirmed by multiplex PCR and characterized by whole-genome sequencing (Illumina NovaSeq). *In silico* MLST was performed and the clonality of the strains was evaluated through maximum likelihood phylogenetic trees and pairwise SNP matrixes. *Klebsiella* spp. and *Enterobacter* spp. exhibited a higher likelihood of AMR than *Escherichia coli* ($P < 0.001$, ORs between 2.56 and 14.32). Most AMR phenotypes remained stable or declined over time, although fluoroquinolone resistance increased toward the end of the study period. Eighteen CPE mostly associated with high-risk global STs (*E. coli* ST410 and ST457, *Klebsiella pneumoniae* ST11, ST147 and ST307, respectively) were detected, carrying bla_{KPC-3}, bla_{NDM-5}, $bla_{OXA-181}$ or $bla_{OXA-244}$. Genomic analysis showed four CPE strain pairs from epidemiologically unrelated sources had < 10 SNPs differences, indicating possible recent clonal transmission of *E. coli* ST4981 and *K. pneumoniae* ST147 and ST273 strains. Overall, this study presents longitudinal AMR surveillance data from companion animal Enterobacterales infections and characterizes the genetic diversity of CPE, having detected international high-risk clones and possible transmission events. These findings reinforce the importance of integrating companion animals into One Health surveillance systems and antimicrobial stewardship strategies.

1. Introduction

The order Enterobacterales is composed predominantly of intestinal commensal bacteria. Extraintestinal infection can be caused by endogenous bacteria acquiring virulence determinants or disruption of the host's barriers, or after introduction of exogenous strains through healthcare exposure, hospitalization, or invasive medical procedures (Geurtsen et al., 2022). Part of the challenge in the treatment of infections by Enterobacterales is the spread of antimicrobial resistance (AMR), facilitated by resistance-encoding genes in mobile genetic

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elements such as plasmids (Lepe and Martínez-Martínez, 2022). These elements can be transmitted both within the same bacterial species and across genera, often carrying genes which may encode resistance to fluoroquinolones, aminoglycosides, potentiated sulphonamides and β-lactams (Lepe and Martínez-Martínez, 2022), and creating a dynamic resistome that shifts over time and geographic regions. This is particularly relevant for the development of empirical antibiotic treatment guidelines in companion animal healthcare, where the range of authorised antimicrobials is restricted (https://eur-lex.europa.eu/eli/reg_impl/2022/1255/oj), and for the surveillance of resistance phenotypes of special interest.

The World Health Organization's Bacterial Priority Pathogens List update, issued in 2024 (World Health Organization, 2024), has 3rd generation cephalosporin (3GC) and carbapenem-resistant Enterobacterales as critical priority pathogens due to their ability to acquire and transfer resistance genes, as well as to cause severe, sometimes untreatable infections. Coupled with the significant burden caused by these infections globally, these agents have become a crucial target for surveillance. AMR is of grave concern across the One Health settings, warranting the establishment of national action plans worldwide with the aim of monitoring the evolution of AMR, antibiotic use and provide guidance towards reducing the prevalence of resistance levels across the human, animal and environmental sectors (Marco-Fuertes et al., 2022). However, most national programs thus far in Europe focus only on food-producing animals, despite evidence of transmission between companion animals and humans, causing a gap in epidemiological knowledge (Marco-Fuertes et al., 2022). This gap is of particular concern as evidence of emergence of carbapenem resistance in companion animals has been reported in several countries across different Enterobacterales species and lineages, with detected carbapenemase-encoding genes carried mostly in plasmids (Rincón-Real and Suárez-Alfonso, 2022). In a pilot study previously conducted by our research group (Moreira da Silva et al., 2024), we detected Enterobacterales carrying carbapenemase-encoding genes causing infections in companion animals, prompting this larger study to assess the scope of AMR in the companion animal setting in Portugal. As such, the main aims of this retrospective study were to monitor the frequency of AMR across 4 years in Enterobacterales isolates from infections in companion animals, as well as to characterize detected carbapenemase-producing Enterobacterales (CPE) by whole genome sequencing (WGS).

2. Materials and methods

2.1. Bacterial isolates

A total of 3959 clinical samples originating from veterinary practices across Portugal were collected from 2020 to 2023. from dogs (n = 2712/3959) and cats (n = 1256/3959), of which 4155 non-duplicate, consecutive, Enterobacterales isolates were obtained and included in this study. All samples were submitted for microbiological diagnosis of suspected infections, including bodily fluids, washes, biopsies and swabs. Isolate identification was based on phenotypical characteristics and biochemical tests, including commercial panels (API 20 E, bioMérieux, Marcy-l'Étoile, France; MicroScan Gram Negative Combo MIC Panel Type 53, Beckman Coulter, Brea, CA, USA).

At the time of collection, isolates were stored in 20% glycerol (Sigma–Aldrich, St Louis, MO, USA) brain heart infusion broth (Biokar Diagnostics) at −20ºC for up to two years, for further future analysis at the Laboratory of Antimicrobial Resistance of the Faculty of Veterinary Medicine, University of Lisbon.

2.2. Susceptibility testing

Susceptibility testing was performed during routine diagnostic analysis following the methodology of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) for disk diffusion and minimum inhibitory concentration (MIC) determination by broth microdilution on Enterobacterales (The European Committee on Antimicrobial Susceptibility Testing Breakpoint Tables for Interpretation of MICs and Zone Diameters.). *Escherichia coli* ATCC 25922 was used for quality control. Disk diffusion was the default method (n = 3495/4155). All antibiotic disks were from the same manufacturer (Oxoid Limited), except for marbofloxacin (Mast Group, Liverpool, UK). The following antibiotics were included in the susceptibility testing: amikacin, amoxicillin/clavulanate, cefotaxime, ceftazidime, ciprofloxacin, enrofloxacin, gentamicin, marbofloxacin, trimethoprim/sulfamethoxazole. MIC determination (n = 655/4155) was performed using the commercial plates MicroScan Gram Negative MIC Panel Type 44 or MicroScan Gram Negative Combo MIC Panel Type 53 (Beckman Coulter), either at the clinician's request when multidrug resistance was suspected or on the recommendation of the laboratory's microbiologists when extensive or pandrug-resistant profiles were observed on the more limited disk diffusion panel. Table S1 details the disk content for the relevant antibiotics, as well as respective breakpoint sources.

2.3. Statistical analysis

The SAS® Studio v3.81 (https://welcome.oda.sas.com/) was used for statistical analysis. In the evaluation of antimicrobial resistance, isolates classified as intermediate during susceptibility testing were considered as susceptible for statistical data analysis, following EUCAST's definition for the (I) category of "Susceptible, increased exposure" (The European Committee on Antimicrobial Susceptibility Testing Breakpoint Tables for Interpretation of MICs and Zone Diameters.). Isolates with intrinsic resistance mechanisms were excluded from analysis for the respective antimicrobial statistical analysis. 3GC were grouped together for analysis, with an isolate being considered as resistant if resistance to at least one of the compounds (cefotaxime or ceftazidime) was detected. Similarly, resistance to either ciprofloxacin, enrofloxacin or marbofloxacin was considered as representative of fluoroquinolone resistance. Finally, aminoglycoside resistance was marked by either amikacin or gentamicin.

Resistance to different antibiotic categories and multidrug resistance (resistance to at least three of the tested antibiotic categories according to Magiorakos et al (Magiorakos et al., 2012)) between bacterial genera with more than 100 isolates and *E. coli* were compared using Pearson's chi-squared test. Bacterial genera specific semestral temporal trends of antimicrobial resistance frequency were modelled using logistic regression, with an α-value of 0.05. The global temporal trends for Enterobacterales were also modelled.

2.4. Screening of carbapenemase-producing isolates

Brilliance™ CRE Agar (Oxoid Limited) was used to screen for carbapenem-resistant clinical isolates by plating the samples directly onto the agar in parallel with conventional media, and considered positive when confluent growth was observed. To detect carbapenemase gene carriage, DNA was extracted through boiling and centrifugation (Féria et al., 2002), and tested through multiplex PCR for the presence of bla_{AIM}, bla_{BIC}, bla_{DIM}, bla_{GES}, bla_{GIM}, bla_{IMP}, bla_{KPC}, bla_{NDM}, $bla_{OXA\text{-}48\text{-}like}$, bla_{SIM}, bla_{SPM}, and bla_{VIM} genes (Poirel et al., 2000, 2011). Positive samples were confirmed by Sanger sequencing at Stabvida (Caparica, Portugal), and MIC determination for meropenem and imipenem was performed using the MicroScan Gram Negative MIC Panel Type 44. Carbapenemase gene variants were identified by comparing the sequences against NCBI's Pathogen Detection Reference Gene Catalog (https://www.ncbi.nlm.nih.gov/pathogens/isolates#refgene/).

2.5. Whole genome sequencing (WGS)

All isolates identified by PCR as carrying carbapenemase-encoding

genes were subjected to WGS. DNA for genomic applications was extracted using the NZY Tissue gDNA Isolation kit (NZYTech, Lisbon, Portugal) following the standard protocol for bacteria, aside from an extension of the proteinase K lysis time to be performed overnight and applying the optional RNAse treatment. Library preparation and WGS were performed by Macrogen (Seoul, Republic of Korea), using the Illumina TruSeq DNA PCR-free preparation kit and the Novaseq platform with 2 × 150 bp paired-end reads. *De novo* assemblies were prepared using a SPAdes based pipeline as previously published in the pilot study, and previously generated assemblies for CRE agar and PCR positive strains from 2020 were included in this work, accessible at the NCBI GenBank under the BioProject Number PRJNA808048 (Moreira da Silva et al., 2024). New assemblies and raw reads were deposited in the European Nucleotide Archive, project number PRJEB95769.

Putative antimicrobial resistance genes and mutations were assessed with the online tool ResFinder v4.6.0, available at the Center for Genomic Epidemiology (https://www.genomicepidemiology.org/). Relevant virulence genes were also assessed for both *E. coli* (VirulenceFinder v2.0.5, also available at https://www.genomicepidemiology.org/), for pathotype identification, and *Klebsiella pneumoniae* strains (PathogenWatch v23.2.0, https://pathogen.watch/). *In silico* MLST was performed, using PathogenWatch for *K. pneumoniae* and PubMLST (https://pubmlst.org/) for other genera. A phylogenetic analysis was performed for each bacterial species with more than one isolate as previously published (Moreira da Silva et al., 2024), using the *K. pneumoniae* and *E. coli de novo* assemblies to generate multiple sequence alignments and construct maximum likelihood phylogenetic trees, which were visualized using the Microreact platform (https://microreact.org/). SNP distance matrixes were generated with snp-dists (https://github.com/tseemann/snp-dists).

3. Results

3.1. Description of infections caused by Enterobacterales

Between 2020 and 2023, a total of 3959 samples were obtained from companion animals presenting signs of infection. While the majority of infections were monobacterial, 26.5% (n = 1101/4155) of the samples yielded more than one bacterial agent, including multiple Enterobacterales (n = 191/1101) isolates, *Staphylococcus* spp. (n = 438/1101), *Pseudomonas* spp. (n = 311/1101), *Enterococcus* spp., (n = 197/1101), *Acinetobacter* spp. (n = 15/1101) and others (n = 47/1101). Most positive samples were obtained from dogs (68.3%, n = 2705/3959), which were also the most frequently infected with more than one agent (77.0%, n = 848/1101). Across the four years, 8.9% (n = 353/3959) of the animals were sampled at least twice on different time points for unrelated infection episodes. The most common infection site was the urinary tract (62.2%, n = 2416/3959), followed by skin and soft tissues (24.6%, n = 974/3959), and respiratory tract (5.1%, n = 203/3959) (Table S2).

Of the 4155 Enterobacterales isolates, 64.8% (n = 2693/4155) were identified as *E. coli*. Other genera with more than 100 identified isolates were *Proteus* spp. (14.4%, n = 600/4155), *Klebsiella* spp. (12.0%, n = 497/4155) and *Enterobacter* spp. (4.4%, n = 183/4155), totalling 95.6% of the isolates (n = 3973/4155). The frequency of isolation of Enterobacterales *per* host and *per* infection site is detailed in Table S3.

Table 1
Frequency of AMR per class/group and multidrug resistance phenotype.

	Susceptible (%)	Resistant (%)	*P*-value	Odds ratio (95% CI)	Effect strength
Amoxicillin/clavulanate					
Enterobacterales	2807 (67.56)	1348 (32.44)	-	-	-
E. coli	2091 (77.65)	602 (22.35)	-	-	-
Enterobacter spp.	IR	IR	IR	IR	-
Klebsiella spp.	182 (36.62)	315 (63.38)	**< 0.0001**	**6.01 (4.90–7.37)**	Positive, strong
Proteus spp.	464 (77.33)	136 (22.67)	0.8682	1.02 (0.82–1.26)	-
3rd generation cephalosporins					
Enterobacterales	3309 (79.64)	846 (20.36)	-	-	-
E. coli	2370 (88.01)	428 (15.89)	-	-	-
Enterobacter spp.	62 (33.88)	121 (66.12)	**< 0.0001**	**14.32 (10.32–19.87)**	Positive, very strong
Klebsiella spp.	227 (45.67)	270 (54.33)	**< 0.0001**	**8.73 (7.06–10.78)**	Positive, strong
Proteus spp.	519 (86.59)	81 (13.50)	0.3093	1.15 (0.88–1.49)	-
Fluoroquinolones					
Enterobacterales	3162 (76.10)	993 (23.9)	-	-	-
E. coli	2218 (82.36)	475 (17.64)	-	-	-
Enterobacter spp.	104 (56.83)	79 (43.17)	**< 0.0001**	**3.55 (2.60–4.83)**	Positive, moderate
Klebsiella spp.	260 (52.31)	237 (47.69)	**< 0.0001**	**4.26 (3.48–5.21)**	Positive, moderate
Proteus spp.	434 (72.33)	166 (27.67)	**< 0.0001**	**1.79 (1.46–2.19)**	Positive, weak
Aminoglycosides					
Enterobacterales	2952 (71.05)	1203 (28.95)	-	-	-
E. coli	2011 (74.68)	682 (25.32)	-	-	-
Enterobacter spp.	98 (53.55)	85 (46.45)	**< 0.0001**	**2.56 (1.89–3.46)**	Positive, moderate
Klebsiella spp.	264 (53.12)	233 (46.88)	**< 0.0001**	**2.60 (2.14–3.17)**	Positive, moderate
Proteus spp.	442 (73.67)	158 (26.33)	0.6083	1.05 (0.86–1.29)	-
Trimethoprim/sulfamethoxazole					
Enterobacterales	2952 (71.05)	1203 (28.95)	-	-	-
E. coli	2133 (79.21)	560 (20.79)	-	-	-
Enterobacter spp.	96 (52.46)	87 (47.54)	**< 0.0001**	**3.45 (2.55–4.68)**	Positive, moderate
Klebsiella spp.	222 (44.67)	275 (55.33)	**< 0.0001**	**4.72 (3.86–5.76)**	Positive, moderate
Proteus spp.	371 (61.83)	229 (38.17)	**< 0.0001**	**2.35 (1.95–2.84)**	Positive, moderate
Multidrug resistance					
Enterobacterales	2803 (67.46)	1352 (32.54)	-	-	-
E. coli	2044 (75.90)	649 (24.10)	-	-	-
Enterobacter spp.	97 (53.01)	86 (46.99)	**< 0.0001**	**2.79 (2.06–3.78)**	Positive, moderate
Klebsiella spp.	136 (27.36)	361 (72.64)	**< 0.0001**	**8.36 (6.74–10.38)**	Positive, strong
Proteus spp.	412 (68.67)	188 (31.33)	**0.0002**	**1.44 (1.18–1.74)**	Positive, weak

CI, confidence interval; IR, intrinsic resistance; *P*, probability. Multidrug resistance assigned if isolate is resistant to at least 3 of the tested antibiotic classes/groups, excluding intrinsic resistance. bold highlights statistically significant differences as determined by chi-squared statistical test against *E. coli*.

3.2. AMR in Enterobacterales infections and temporal regression models

Frequency of resistance in Enterobacterales to the studied antibiotic classes ranged between 32.4% (to amoxicillin/clavulanate) to 20.4% (to 3GC), with 32.5% (n = 1352/4155) of the isolates presenting multidrug resistance. A breakdown of the frequency of AMR both as a group and for each individual genus with more than 100 isolates is presented in Table 1.

Statistical analysis of the proportion of AMR to the five antibiotic categories, as well as multidrug resistance, showed significant differences for *Enterobacter* spp. and *Klebsiella* spp., with odds of resistance ranging from 2.56 to 14.32 times higher than those of *E. coli* across all examined categories ($P < 0.001$) (Table 1). *Proteus* spp. had significantly higher proportions for fluoroquinolones, trimethoprim/sulfamethoxazole and multidrug resistance compared with *E. coli*, although all odds ratios were lower than those obtained for the other two genera.

Regarding temporal trends, the Enterobacterales, *E. coli* and *Klebsiella* spp. models were mostly significant and presented slight to moderate decreases in AMR over time (Fig. 1). Across the study period, the proportion of resistance in *Klebsiella* spp. was predominantly above 40%, while *E. coli* primarily remained below 30%. The models for *Enterobacter* spp. and *Proteus* spp. were non-significant for nearly all analysed categories, suggesting a stabilization of resistance (Figure S1). The main exception was fluoroquinolone resistance as it appears to be rising in Enterobacterales, particularly in *Klebsiella* spp. Yearly counts of resistant and total isolates are provided in Table S4.

3.3. Carbapenemase-producing Enterobacterales resistance and virulence characterization

Screening using the Brilliance™ CRE agar yielded 398 isolates, of which 18 were PCR positive to either bla_{KPC}, bla_{NDM} or $bla_{OXA\text{-}48\text{-}like}$, further classified by Sanger sequencing and WGS as $bla_{KPC\text{-}3}$ (n = 5), $bla_{NDM\text{-}5}$ (n = 1), $bla_{OXA\text{-}181}$ (n = 10) and $bla_{OXA\text{-}244}$ (n = 2) (Table 2). These strains carrying carbapenemase-encoding genes were obtained from both cats and dogs (n = 7 and 11, respectively), mostly from UTI (n = 8), and were identified as *Citrobacter portucalensis* (n = 1), *E. coli* (n = 7) and *K. pneumoniae* (n = 10). Two different CPE (*E. coli* carrying $bla_{KPC\text{-}3}$ and *K. pneumoniae* carrying $bla_{OXA\text{-}181}$) were recovered from the same animal at different time points.

Phenotypical resistance profiles of the carbapenemase-positive isolates varied from resistant to all tested antibiotic categories (n = 2) to being only resistant to β-lactams (n = 1). The remaining isolates (n = 15) were all resistant to fluoroquinolones, susceptible to gentamicin/amikacin, and all but one were resistant to trimethoprim/sulfamethoxazole (Table 2). Imipenem and meropenem MICs were notably higher for the $bla_{KPC\text{-}3}$ and $bla_{NDM\text{-}5}$ carriers (mostly above 4 mg/L), while all $bla_{OXA\text{-}48\text{-}like}$ carriers were below the EUCAST susceptibility breakpoint of ≤ 2 mg/L (Table S5) (The European Committee on Antimicrobial Susceptibility Testing Breakpoint Tables for Interpretation of MICs and Zone Diameters.).

Resistance genotyping through WGS (full profiles in Table S5) revealed most isolates had acquired at least one more extended-spectrum β-lactamase (ESBL) gene besides the carbapenemase and presented a multidrug resistance profile. Among *E. coli* (Fig. 2), ST410 and ST457 harbored the largest AMR gene pools, while the ST372 strain only carried $bla_{KPC\text{-}3}$. Regarding *K. pneumoniae* (Fig. 3), the variety of acquired AMR genes within different antibiotic classes is smaller in comparison with *E. coli*, with ST273 presenting a pattern of *aadA16*, *dfrA27*, *sul2*, and *arr-3* distinct from the rest of the *K. pneumoniae* STs. A truncated *ompK35* porin gene (only 23% of the sequence remaining), involved in multidrug resistance, was detected on the ST307 strain.

The virulence profiles of the seven *E. coli* isolates were analysed and classified into pathotypes based on key virulence genes identified in the assemblies (Table S6). Both $bla_{OXA\text{-}244}$ carriers were classified as enterotoxigenic *E. coli* (ETEC). The $bla_{KPC\text{-}3}$ carriers were categorized as extraintestinal pathogenic *E. coli* (ExPEC) and further subdivided due to a divergence within the virulence profile into one avian pathogenic *E. coli* (APEC) and one uropathogenic *E. coli* (UPEC), despite both causing UTIs (in a dog and a cat, respectively). The remaining three isolates' pathotypes were non-defined. As for the *K. pneumoniae* isolates, these were screened for genes associated with hypervirulence (*rmp*, *iro*

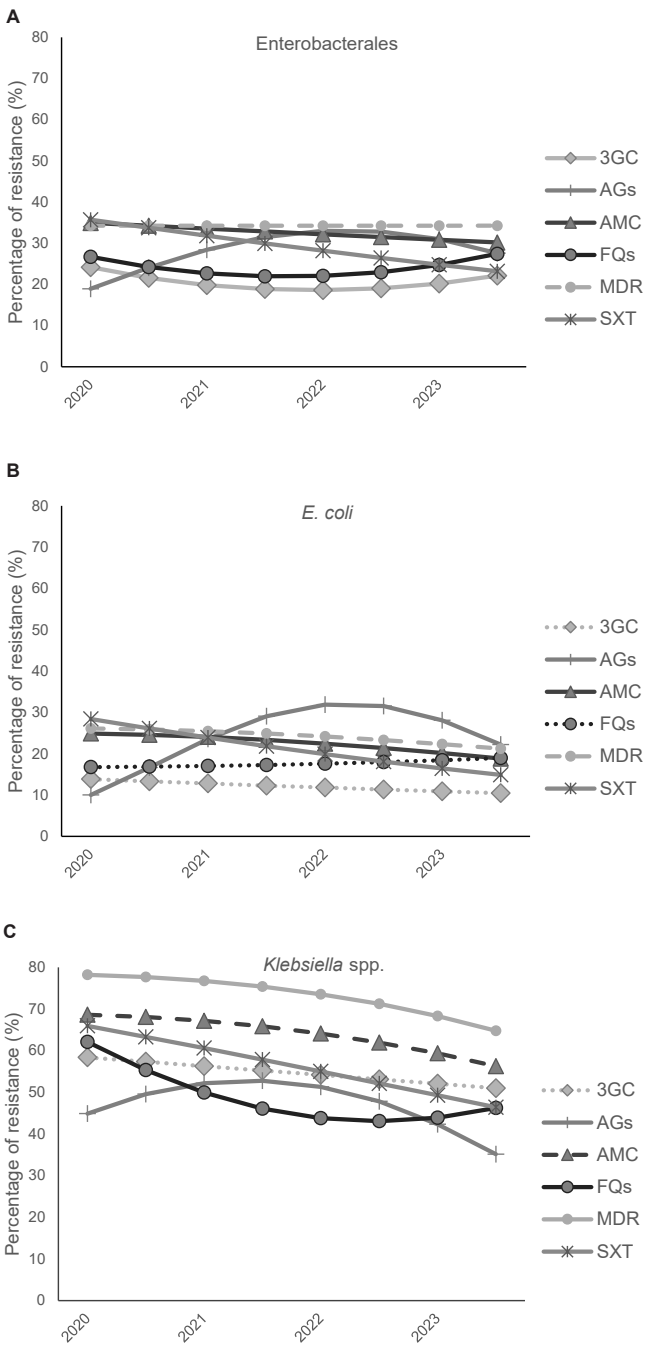

Fig. 1. Logistic regression models of antibiotic resistance of Enterobacterales as a whole (A), *E. coli* (B) and *Klebsiella* spp. (C) between 2020 and 2023. Solid lines represent statistically significant models ($P < 0.05$), dashed lines represent nearly significant models ($0.07 < P < 0.05$), and dotted lines represent non-significant models ($P > 0.07$). 3GC, 3rd generation cephalosporins; AGs, aminoglycosides; AMC, amoxicillin/clavulanate; FQs, fluoroquinolones; MDR, multidrug resistance; SXT, trimethoprim/ sulfamethoxazole. Multidrug resistance assigned if isolate is resistant to at least 3 of the tested antibiotic categories according to Magiorakos *et al* (Magiorakos et al., 2012)., excluding intrinsic resistance.

Table 2
Origin of the carbapenemase-producing isolates, antibiotic resistance profile and associated carbapenemase-encoding genes.

Species	Isolate	Year	District	Animal	Infection origin	Phenotypical resistance					Carbapenemase genes	ST	Accession number
						AMC	3GC	FQs	AGs	SXT			
Citrobacter portucalensis	5010	2021	Setúbal	Dog	UTI	R	R	R	S	R	$bla_{OXA-181}$	1333	ERS25424072
Escherichia coli	1322 A*	2021	Setúbal	Cat	UTI	R	R	S	S	S	bla_{KPC-3}	372	ERS25424073
	2969B	2021	Setúbal	Cat	Reproductive tract	R	R	R	S	R	$bla_{OXA-181}$	410	ERS25424074
	4674	2021	Oporto	Dog	UTI	R	R	R	S	R	bla_{KPC-3}	457	ERS25424075
	23867	2023	Lisbon	Cat	Cholecystitis	R	R	R	S	R	$bla_{OXA-181}$	410	ERS25424076
	30776B	2023	Setúbal	Dog	LRTI	R	R	R	R	R	$bla_{OXA-244}$	4981	ERS25424077
	31932	2023	Setúbal	Dog	UTI	R	R	R	S	R	$bla_{OXA-244}$	4981	ERS25424078
	32960	2023	Lisbon	Dog	Surgical incision	R	R	R	R	R	bla_{NDM-5}	410	ERS25424079
Klebsiella pneumoniae	482 / VG313	2020	Lisbon	Dog	UTI	R	R	R	S	R	bla_{KPC-3}	147	SAMN30806197
	487 / VG314	2020	Coimbra	Cat	Eye infection	R	R	R	S	R	bla_{KPC-3}	147	SAMN30806198
	2430 / VG117	2020	Lisbon	Cat	Oesophagostomy	R	R	R	S	R	$bla_{OXA-181}$	273	SAMN30806199
	4686 / VG380	2020	Aveiro	Dog	URTI	R	R	R	S	S	bla_{KPC-3}	392	SAMN26022687
	747*	2021	Setúbal	Cat	UTI	R	R	R	S	R	$bla_{OXA-181}$	273	ERS25424080
	2816	2021	Setúbal	Cat	UTI	R	R	R	S	R	$bla_{OXA-181}$	11	ERS25424081
	13731	2022	Setúbal	Dog	Surgical incision	R	R	R	S	R	$bla_{OXA-181}$	307	ERS25424082
	24234	2023	Évora	Dog	Otitis externa	R	R	R	S	R	$bla_{OXA-181}$	11	ERS25424083
	29065B	2023	Lisbon	Dog	UTI	R	R	R	S	R	$bla_{OXA-181}$	147	ERS25424084
	30265	2023	Lisbon	Dog	Subcutaneous abscess	R	R	R	S	R	$bla_{OXA-181}$	147	ERS25424085

3GC, 3rd generation cephalosporins; AGs, aminoglycosides; AMC, amoxicillin/clavulanate; FQs, fluoroquinolones; LRTI, lower respiratory tract infection; R, resistant; S, susceptible; SXT, trimethoprim/sulfamethoxazole; URTI, upper respiratory tract infection; UTI, urinary tract infection
* Isolates obtained from the same animal in different time points, from unrelated infection episodes.

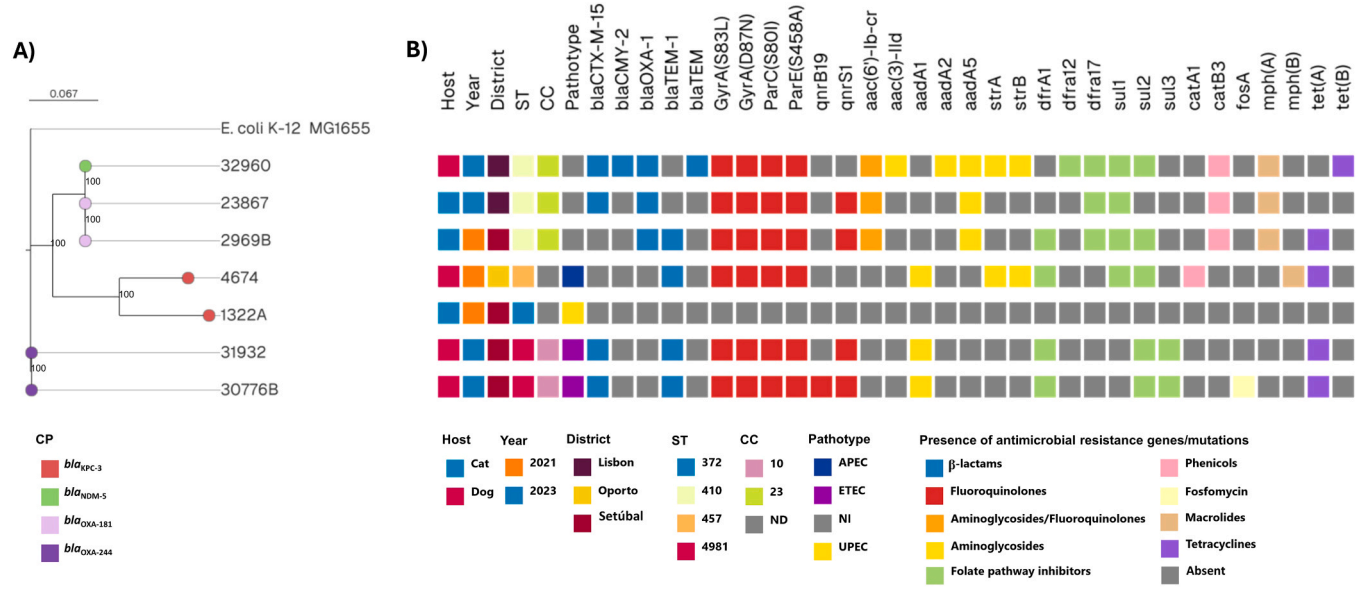

Fig. 2. A) Maximum likelihood phylogenetic tree of the core genome SNPs of carbapenemase-producing *E. coli*; **B)** Epidemiological data and antimicrobial resistance determinants heatmap. Maximum likelihood analysis used as reference the *E. coli* K-12 MG1655 genome. Bootstrap support values are shown on each node. Branch lengths represent expected substitutions per site. APEC, avian pathogenic *E. coli*; CC, clonal complex; CP, carbapenemase-encoding gene; ETEC, enterotoxigenic *E. coli*; ND, not defined; NI, non-identified; ST, sequence type; UPEC, uropathogenic *E. coli*.

and *iuc* clusters), but no hits were found by Kleborate (accessed through PathogenWatch) on the analysed assemblies (Table S7). The full virulence profiles obtained for both bacterial species can be found in Tables S6 and S7.

3.4. Genetic relatedness of carbapenemase-producing Enterobacterales

Through MLST analysis, four sequence types (STs) were identified among the *E. coli* strains. Both $bla_{OXA-244}$ *E. coli* carriers belonged to ST4981, and both the bla_{NDM-5} carrier and one of the $bla_{OXA-181}$ carriers and were assigned to ST410. The other two strains were designated as different STs (Table 2, Fig. 2). The distribution of the recombination corrected assemblies in the phylogenetic tree followed the identified STs, with more than 1500 SNPs between strains from different STs (Table S8). A close transmission event was detected among the ST4981 *E. coli* strains, differing by only 9 SNPs (a threshold of ≤17 SNPs was considered for close transmission event detection, and ≤100 SNPs for clustering more distantly linked strains) (Ludden et al., 2021; Watt et al.,

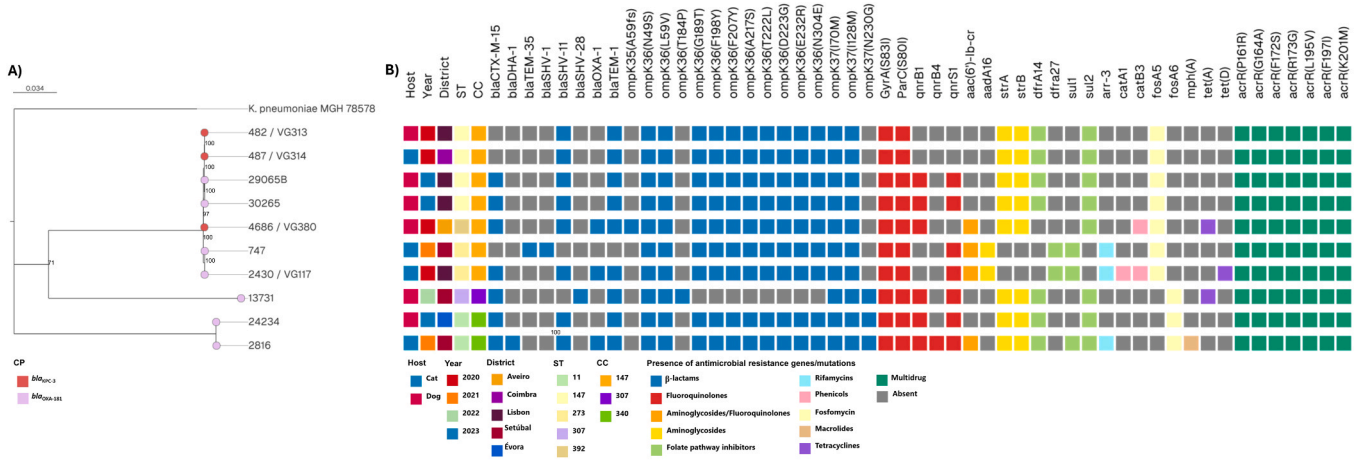

Fig. 3. A) Maximum likelihood phylogenetic tree of the core genome SNPs of carbapenemase-producing *K. pneumoniae*; **B)** Epidemiological data and antimicrobial resistance determinants heatmap. Maximum likelihood analysis used as reference the *K. pneumoniae* MGH 78578 genome. Bootstrap support values are shown on each node. Branch lengths represent expected substitutions per site. CC, clonal complex; CP, carbapenemase-encoding gene; ST, sequence type.

2025). Both strains were retrieved from samples collected within approximately one month of each other in the same veterinary hospital, from different animals. Interestingly, these ST4981 *E. coli* strains varied in two acquired resistance genes (*qnrB19* and *fosA*), pointing to possible changes in its resistome over time. A more distant relationship was also observed between two of the ST410 *E. coli* strains (2969B and 23867) which differed by 40 SNPs. These were collected from unrelated animals more than a year apart from veterinary hospitals located about 50 km from each other, presenting a weaker epidemiological connection.

The *K. pneumoniae* strains were assigned to five STs, three of which belonged to clonal complex (CC) 147 (ST147, n = 4; ST273, n = 2; and ST392, n = 1). The remainder were classified as ST11 (n = 2) and ST307 (n = 1). Three closely related isolate pairs were identified (≤10 SNP threshold for close transmission event detection) (Perdigão et al., 2020): two pairs within *K. pneumoniae* ST147 (strains 482/VG313 and 487/VG314 with 4 SNP difference; strains 29065B and 30265 with 6 SNP difference) and the *K. pneumoniae* ST273 pair (9 SNP difference) (Fig. 3, Table S9). The epidemiological linkage of these three *K. pneumoniae* pairs is unclear, as the paired strains- samples were collected in different clinics/hospitals and originated from unrelated animals.

The *C. portucalensis* strain was assigned to ST1333, with no other known strains listed under this ST in the PubMLST database at the time of accession (30th of October 2025).

4. Discussion

In this study, the current prevalence of resistance to commonly used antibiotics in the treatment of bacterial infections in companion animals was assessed over a period of 4 years. Furthermore, this study focused on the detection of carbapenemase carriers, uncovering 18 isolates, 0.4% of Enterobacterales, carrying a carbapenemase-encoding gene.

One of the major objectives of this work was to provide updated data concerning the prevalence of AMR in Enterobacterales infecting companion animals and insights on its evolution, encompassing a broader range of clinical presentations beyond urinary tract infections (Marques et al., 2018). Overall, resistance frequency in *E. coli* and Enterobacterales as a group was slightly lower than previously reported (Marques et al., 2018). This finding may reflect differences in study design and sampling scope, as the present work integrates isolates from multiple infection types and clinical settings. Conversely, clinicians are more likely to submit samples for culture in cases of recurrent infection or after failure of empirical therapy. It is therefore possible that the prevalence of AMR in our dataset is higher than in the community, as empirically-treated uncomplicated infection isolates are likely underrepresented in our research. Nonetheless, the identified AMR frequencies and trends can help inform local treatment guidelines, particularly for complicated infections, and highlight the potential role of less commonly used antibiotics, such as potentiated sulfonamides, as alternatives to classes with higher resistance rates.

Compared with the previously reported trends (Marques et al., 2018), amoxicillin/clavulanate resistance prevalence rose above that of fluoroquinolones over time. This pattern may reflect a past shift in prescribing practices away from the broader-spectrum fluoroquinolones. Based on the modelled tendency towards fluoroquinolone resistance increase in 2023, this may however have been partially reversed in recent years, although confirmation would require data on antibiotic usage.

In this study, the stabilization of multidrug resistance at approximately 35% for Enterobacterales and 25% for *E. coli* further suggests that some resistance determinants may have become partially fixed within companion animal bacterial populations. Our data also indicate that non-*E. coli* Enterobacterales may influence overall AMR prevalence. Consequently, surveillance systems that focus exclusively on *E. coli* may not adequately represent resistance patterns in Enterobacterales as a whole, particularly if other species with a high frequency of resistance account for a significant proportion of infections. The importance of the surveillance of non-*E. coli* is further highlighted by the, as Enterobacterales are categorized not only according to the resistance phenotype but also identified by particularly relevant species, with carbapenem-resistant *K. pneumoniae* at the top of the list (World Health Organization, 2024). From a total of 24 priority pathogens of the WHO's Bacterial Priority Pathogens List, nine were identified in our dataset (carbapenem-resistant *K. pneumoniae* and *E. coli*, 3GC-resistance *E. coli*, *K. pneumoniae*, *Enterobacter* spp., *Citrobacter* spp., *Proteus* spp., *Serratia* spp. and *Morganella* spp.). Overall, the presence of multiple priority pathogens in our dataset illustrates that companion animals represent an overlooked reservoir of AMR, and that surveillance systems need to include companion animals to accurately capture emerging threats within a One Health framework.

Of the 18 identified CPE, 11 were assigned to *E. coli* and *K. pneumoniae* high-risk lineages (*E. coli* ST410 and ST457; *K. pneumoniae* ST147, ST11 and ST307). These STs have been found worldwide in multidrug, carbapenem-resistant nosocomial human infections. In companion animals, *E. coli* ST410 producing NDM-5 (China, South Korea, United Kingdom), OXA-48 (Germany) and OXA-181 (Portugal, Switzerland), *K. pneumoniae* ST11 producing KPC-2 (Brazil) and OXA-48 (France, Germany, Switzerland), and ST307 producing

OXA-48 (France, Germany, Switzerland) having also been found (Rincón-Real and Suárez-Alfonso, 2022; Kocsis et al., 2022; Arcari and Carattoli, 2023; Ramírez-Castillo et al., 2023). Aside from *E. coli* ST410, in Portugal all other high-risk lineages had been previously detected colonizing or infecting companion animals as ESBL-carriers (Menezes et al., 2023; Marques et al., 2019). Yet, only the high-risk *K. pneumoniae* STs have been previously reported as carbapenemase carriers in humans in Portugal (Mendes et al., 2022) This shows an expansion of the circulating carbapenemase-carrying lineages in the country, possibly in part due to transmission of plasmids carrying carbapenemase-encoding genes across various lineages as observed in the association of bla_{KPC-3} with different plasmid incompatibility groups and the dissemination of $bla_{OXA-181}$ through IncX3 plasmids in the country (Faria et al., 2024). With EARS-NET Portuguese data reporting an increase in human invasive infections by carbapenem-resistant Enterobacterales (European Centre for Disease Prevention and Control, 2024) in a significant upwards trend, it stands to reason that the spill-over towards the environment and other One Health settings beyond human hospitals is likely intensifying. This is probably creating more interfaces within the community for colonization and, ultimately, infection by these agents, as well as horizontal spread of the mobile genetic elements such as plasmids carrying carbapenemase-encoding genes. This has been previously demonstrated across humans, livestock and companion animals in China in multiple *E. coli* STs through the dissemination of IncX3 plasmids carrying bla_{NDM} (Ramírez-Castillo et al., 2023).

The emergence of CPE in companion animals is occurring despite the prohibition of carbapenem usage under EU law in animal health (Van Duijkeren et al., 2023). A study based in a veterinary hospital in Italy reached similar conclusions (Scarpellini et al., 2025), further evidencing countries with high carbapenem-resistance prevalence, are at risk of having CPE circulation in companion animals, and thus emphasizing the need for the inclusion of the companion animal setting in AMR surveillance programs.

The use of non-carbapenem β-lactams is likely promoting the selection and maintenance of CPE in this setting as well, as illustrated by strain 1322 A, which showed resistance to amoxicillin/clavulanate and 3GC while carrying only bla_{KPC-3}. Aminopenicillins, alone or combined with clavulanic acid, fall within the hydrolysis spectrum of most carbapenemases and are widely used in companion animal medicine, so their extensive use may create selective pressure that favors the persistence and spread of CPE in the veterinary environment (Van Duijkeren et al., 2023). The same likely applies to 3GC, as *in vitro* experiments have also demonstrated that exposure to ceftiofur can foster the selection of CPE, particularly when strains co-carry genes encoding other β-lactamases, such as ESBL-encoding genes and bla_{TEM-1} (Ogunrinu et al., 2020), as is the case for the strains in our study (Table 2).

Other mechanisms are possibly influencing the emergence of CPE in the companion animal setting. The identification of *E. coli* ST372, a UPEC previously associated with canine UTI (Jousserand et al., 2025), carrying bla_{KPC-3}, together with the previous report of a dog carrying ST372 harbouring bla_{OXA-48} in France, shows the assimilation of carbapenemases into animal host-adapted lineages is possible. Such a connection could facilitate the persistence and spread of these genes in the veterinary setting, particularly if associated with chronic infections. This possibility is illustrated by the cat infected with the ST372 strain. Not only did this animal later become infected with the OXA-181-producing *K. pneumoniae* ST273 strain, but it experienced recurrent UTI's with clinical signs for 10 months, likely shedding CPE in the urine and contaminating its environment over an extended period. As observed in humans, CPE infections in companion animals may therefore enhance their role in the spread of these pathogens, both in their living environment and in veterinary healthcare facilities, especially where infection prevention and control practices are suboptimal (Rincón-Real and Suárez-Alfonso, 2022; Schmitt et al., 2021). Additional examples in this study support this concern. For instance, the two *E. coli* ST4981 OXA-244-producing isolates, with a nine SNP distance, were recovered from the same veterinary practice within one month, indicating possible recent transmission. To the best of our knowledge, this is the first report in Portugal of carbapenemase-producing *E. coli* ST372 and ST457.

While both bla_{KPC-3} and $bla_{OXA-181}$ are the most commonly detected carbapenemase-encoding genes in Portugal (Faria et al., 2024), an apparent shift from CPE carrying bla_{KPC-3} was detected within the examined timeframe, and CPE carrying $bla_{OXA-181}$ became predominant in this study. The emergence ofbla_{NDM-5} and $bla_{OXA-244}$ occurred as well. Rarer carbapenemase types such as GES, IMP and VIM have been previously described in Portuguese hospitals, aquatic environments, seagulls and bivalves, but were undetected in our study (Mó and da Silva, 2024). The introduction of both NDM-5 and OXA-244 carbapenemases in the veterinary setting is cause for special concern.

As a metallo-β-lactamase, NDM-5 has a wider range of activity against β-lactams than either KPC or OXA-48-like carbapenemases, including novel carbapenem-inhibitor combinations, making the treatment of infections by CPE producing these carbapenemases very complex (Shields and Doi, 2020). The NDM-5 *E. coli* ST410 isolate tested resistant to nearly all antibiotics in our panel but amikacin, further illustrating the difficulty in treating these infections both in human and veterinary medicine. Importantly, NDM-producers have already been reported in companion animals in multiple countries, including outbreaks or repeated detections in Europe, Asia, and North America, across different Enterobacterales species and other Gram-negative bacteria, and carried on diverse plasmid backbones (Rincón-Real and Suárez-Alfonso, 2022). This growing number of international reports highlights the ease with which NDM enzymes disseminate across species and settings, reinforcing the need for strengthened surveillance in veterinary medicine.

OXA-244 is more difficult to detect than other OXA-48-like carbapenemases because a single amino-acid substitution (R214G) markedly reduces its hydrolytic activity, often resulting in carbapenem MICs below EUCAST clinical breakpoints (Rima et al., 2021). Consequently, selective media used for carbapenemase screening may fail to detect OXA-244 producers unless additional resistance mechanisms, such as ESBL production (e.g., CTX-M) with porin loss or increased efflux, are present (Emeraud et al., 2020). With this diagnostic challenge, and facing human healthcare-associated outbreaks related to *E. coli* ST38 OXA-244-producers in Europe, the European Centre for Disease Prevention and Control has issued a rapid risk assessment, updated in July 2021, calling for improved laboratory detection and surveillance of these carbapenemases (European Centre for Disease Prevention and Control, 2021). The detection of $bla_{OXA-244}$ in *E. coli* ST4981, which has been previously isolated from humans, livestock, rainwater and dogs (European Centre for Disease Prevention and Control, 2021; Chambino et al., 2026), indicates this carbapenemase may be circulating outside the reach of human healthcare surveillance systems. Nonetheless, the recent detection of an OXA-244-producing *E. coli* ST4981 colonizing a healthy dog in another study further supports this concern (Chambino et al., 2026). Expanding OXA-244 surveillance to companion animals is therefore essential to obtain an accurate epidemiological picture of this likely under-detected carbapenemase. Finally, strain 30776B displayed an unexplained aminoglycoside resistance phenotype, possibly due to a short-read sequencing artifact or the presence of a novel unidentified resistance determinant, requiring further investigation.

C. portucalensis is a pathogen closely related to *Citrobacter freundii*, and carbapenemases have been associated to various STs worldwide, although genomic studies about this species remain scant (Nobrega et al., 2023). Concerns regarding the emergence of carbapenemase-producing *Citrobacter* spp. infections have been raised, as these organisms are less surveyed than *K. pneumoniae* or *E. coli* (Nobrega et al., 2023). The detection of an UTI caused in a dog by multidrug resistant, $bla_{OXA-181}$ carrier-*C. portucalensis* in the present study further highlights that carbapenemase production and infection by less-common Enterobacterales should not be overlooked.

Although we believe this study contributes with important data concerning the evolution of antibiotic resistance in Enterobacterales and the surveillance of carbapenemase dissemination in companion animals, there are limitations which we must address. The zone-diameter breakpoints for enrofloxacin and marbofloxacin in dogs have been recently removed from CLSI guidelines. As such, future comparisons of resistance prevalence using disk diffusion for these fluoroquinolones will no longer be possible. The corresponding MIC breakpoints have already been lowered as well, so current resistance estimates may also be under called. Furthermore, carbapenem MIC determination wasn't possible in all CRE selected isolates due to financial constraints (The European Committee on Antimicrobial Susceptibility Testing Breakpoint Tables for Interpretation of MICs and Zone Diameters.). Isolate recovery timing for the genotyping studies may have impacted the detection of carbapenemase genes as some isolates were stored for up to 2 years at −20'C. As such, some plasmids, and possibly associated carbapenemase-encoding genes, may have been lost. The use of a selective agar paired with PCR approach may miss novel variants, so a surveillance program would need a phenotypic test for agnostic carbapenemase activity detection. Agars with broad carbapenem resistance selectivity also have a low specificity (as low as 60%), not differentiating carbapenemase producers from ESBL producers or AmpC overexpression with concomitant efflux or porin alterations. Moreover, rarer carbapenemase types or weaker hydrolysers such as OXA-244 may be underrepresented since they may fail to grow on CRE agar. Finally, the history of antibiotic use in most of the sampled animals is unknown. Any possible carbapenem use before the EU regulations would have been off-label and outside the scope of the European Medicines Agency veterinary antibiotic use reports. As such, in this study, an assessment of direct selective pressure due to carbapenem use was not possible.

This study provides an updated overview of AMR in *Enterobacterales* infecting companion animals in Portugal, presenting both the current prevalence and the 4-year evolution of resistance to commonly used veterinary antibiotics. The stabilization of multidrug resistance at relatively high levels, together with divergent resistance trends between *E. coli* and other *Enterobacterales*, highlights the possible limitations of relying solely on *E. coli* as a sentinel species and underscores the need for broader, species-inclusive monitoring.

In addition, several clinical carbapenemase-carrying Enterobacterales were identified, a finding of major importance given their extensive resistance profiles and the serious therapeutic challenges they pose in animal healthcare. Furthermore, even though carbapenems are not prescribed for companion animals in Europe, the use of other broad-spectrum antimicrobials, such as aminopenicillins and 3GC, as previously discussed, can exert co-selection pressure, promoting the persistence and dissemination of carbapenemase-producing strains within the veterinary setting.

Consequently, the surveillance of resistance in companion animals is not only paramount to support informed empirical treatment decisions in the clinical context, but also to detect emerging important phenotypes. Our findings therefore support the integration of companion animals into One Health AMR surveillance programs.

Author contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by LF, JMS and JM. The first draft of the manuscript was written by LF and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

CRediT authorship contribution statement

Laura Fernandes: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Constança Pomba:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Cátia Marques:** Writing – review & editing, Validation, Methodology, Formal analysis, Conceptualization. **Cátia Caneiras:** Writing – review & editing, Supervision, Conceptualization. **Joana Moreira da Silva:** Writing – review & editing, Validation, Software, Project administration, Methodology, Investigation, Funding acquisition, Data curation. **Juliana Menezes:** Writing – review & editing, Validation, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition.

Ethics approval

This study was performed on retrospectively collected bacterial isolates in partnership with a private diagnostic laboratory with approval by the ethics commission of the Faculty of Veterinary Medicine, University of Lisbon (FMV-ULisboa), under the reference CEIE 024/2024, in accordance with the local legislation and institutional requirements.

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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. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.vetmic.2026.110987.

Data availability

The sequencing data generated by this study is available in the European Nucleotide Archive repository, under the project PRJEB95769.

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