# Longitudinal study of ESBL/AmpC-producing Enterobacterales strains sharing between cohabiting healthy companion animals and humans in Portugal and in the United Kingdom

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## Abstract

Extended-spectrum beta-lactamase (ESBL)- and plasmid-mediated cephalosporinase (AmpC)-producing Enterobacterales (ESBL/AmpC-E) are an increasing healthcare problem in both human and veterinary medicine. The aim of this study was to investigate the possible sharing of ESBL/AmpC-E strains between healthy companion animals and humans of the same household in Portugal (PT) and the United Kingdom (UK).

In a prospective longitudinal study, between 2018-2020, faecal samples were collected from healthy dogs (*n*=90), cats (*n*=20) and their cohabiting humans (*n*=119) belonging to 41 PT and 44 UK households. Samples were screened for the presence of ESBL/AmpC-E and carbapenemase-producing bacteria. Clonal relatedness between animal and human strains was established by using REP-PCR fingerprinting method, followed by whole-genome sequencing (WGS) of selected strains.

ESBL/AmpC-E strains were detected in companion animals (PT=12.7%, *n*=8/63; UK=8.5%, *n*=4/47) and humans (PT=20.7%, *n*=12/58; UK=6.6%, *n*=4/61) in at least one timepoint. REP-PCR identified paired multidrug-resistant ESBL/AmpC-producing *Escherichia coli* strains from companion animals and owners in two Portuguese households (4.8%) and one UK household (2.3%). WGS analysis of nine *E. coli* strains from these three households confirmed that interhost sharing occurred only between the two animal-human pairs from Portugal. Three shared strains were identified: one CTX-M-15-producing *E. coli* strain in a cat-human pair (O15-H33-ST93) and two CTX-M-15- and CTX-M-55/CMY-2-producing *E. coli* strains, in a dog-human pair (O8:H9-ST410 and O11:H25-ST457, respectively) at different timepoints. These *E. coli* clonal lineages are human pandemic, highlighting the role of companion animals living in close contact with humans in the dissemination and persistence of antimicrobial resistance in the household environment.



## Keywords

One health; antimicrobial resistance transfer; CTX-M-15; CTX-M-55; CMY-2; extraintestinal pathogenic *Escherichia coli*.

## Introduction

The order Enterobacterales comprises common members of the intestinal microbiota of humans and companion animals. However, enterobacteria, most notably *Escherichia coli* and *Klebsiella pneumoniae*, can also cause both intestinal and extraintestinal diseases [1,2]. In fact, *E. coli* is the most frequent cause of urinary tract infection (UTI) and adult bacteraemia [2]. Additionally, *E. coli* is the second most common cause of neonatal meningitis [2]. Resistance to antimicrobial agents in these bacteria has become a major concern. Furthermore, the World Health Organization (WHO) priority pathogens list for research and development of new antibiotics classifies carbapenem-resistant and extended-spectrum beta-lactamase (ESBL)-producing Enterobacterales, as a Priority 1 thus belonging to the Critical group [3].

ESBL and plasmid-mediated cephalosporinase (AmpC)-producing Enterobacterales (ESBL/AmpC-E) strains are resistant to a wide range of beta-lactams, including third-generation cephalosporins (3GC), which are considered highly effective for treatment of infectious diseases [4,5]. Third- and fourth-generation cephalosporins are considered by the WHO as Highest Priority Critically Important Antimicrobials (CIAs) in human medicine [6]. Recently, this class has also been categorized by the European Medicines Agency (EMA) with the indication to 'Restrict' use (Category B) in veterinary medicine [7]. However, increasing prevalence of ESBL/AmpC-E strains has been reported during the last decades in both companion animals and humans [8–11]. The expansion of ESBL/AmpC-E strains increased hospital use of carbapenems in human medicine, which remain active against these resistant bacteria [12]. Although carbapenems are rarely used in companion animal medicine [7], the frequency of carbapenemase-producing Enterobacterales strains has been increasing worldwide in companion animals [13–15]. Due to the close companion animal-human bond, concerns have been raised that animals may act as potential reservoirs for multidrug-resistant bacteria and/or their resistance determinants [16]. Over the past years, carriage and possible transmission of ESBL/AmpC and/or carbapenemase-producing bacteria between and from companion animals to humans has been reported in several studies [15,17–19]. Yet, little is known about the dynamics of such cross-species spread when studied over time.

In this study, a prospective and longitudinal design was used to characterize the molecular epidemiology and evaluate the sharing of ESBL/AmpC and carbapenemase-producing Enterobacterales strains in healthy companion animals and humans living in the same household. This was a two-year long multicentred study conducted in Lisbon, Portugal, and South-East United Kingdom (UK).

## Materials and Methods

### *Study design*

This study was part of an international prospective longitudinal observational study conducted between 2018 and 2020 at the Small Animal Veterinary Teaching Hospital of the Faculty of Veterinary Medicine, University of Lisbon, Lisbon, Portugal and via the Royal Veterinary College, Hertfordshire, UK.

Healthy companion animals ($n$=90 dogs; $n$=20 cats) and their cohabiting humans ($n$=119) from 85 households were enrolled in this study by convenience sampling when companion animals presented for prophylaxis consultation at the veterinary hospitals. Companion animals' health status was evaluated by their assistant veterinarians. Cohabiting humans of companion animals that met the inclusion criteria were also enrolled on a voluntary basis. Inclusion criteria for enrolment of companion animals and humans were: i) no systemic antimicrobial therapy in the last three months; ii) no topical antimicrobial therapy two days before sampling; iii) have lived together for at least three months (co-habiting). To ensure that participation was anonymous, the households, humans and animals were coded.

A brief epidemiological questionnaire was filled out by the human participants containing information about all participants general health, current or previous medical treatments, travel history and exposure to healthcare facilities as well as questions to assess the closeness of the contact with their companion animals (e.g. sharing the same bed). The animals' questionnaire retrieved data on lifestyle, diet, previous antimicrobial treatments and contact with other animals. The option 'Prefer not to answer' was available for all variables on the questionnaire. Questionnaire variables and responses, by country are listed at Table S1.

Faecal samples from all participants (humans and companion animals) were collected immediately after enrolment (T0), and monthly-repeated thereafter for a period of two months (T1, T2). At each timepoint, the inclusion criteria were reviewed, and participants excluded if any change was reported. Acquisition of follow-up samples depended on the owner's willingness to continue to participate in the study. Antibiotic intake during the follow-up period resulted in exclusion from the study.

*Sample collection and processing*

Owners were given instructions and written information on how to perform their own and the animals faecal sample collection. They were asked to collect their dog's partial faecal samples (that did not touch the ground) into a sterile plastic container. Human faeces were to be collected directly into sterile plastic containers or by using 'FeCol' faeces collection papers (Alpha Laboratories Ltd, United Kingdom) and then transferred into a sterile plastic container. All materials, labels and clean laboratory gloves for sampling were provided. Samples were stored for a maximum of 48h at 4°C until processing.

One gram of homogenized faecal sample was added to 10 mL of sterile 0.85% NaCl (Merk, Germany) solution and mixed thoroughly by vortexing. Ten microliters of faecal suspension were plated onto: i) MacConkey agar plates (Biokar Diagnostics, France) with and without 1.5 µg/mL of cefotaxime (Sigma Aldrich, US) or 1 µg/mL meropenem (Sigma-Aldrich, US) supplementation; ii) MacConkey agar plates containing temocillin 30 µg disk (Mast Group, UK), used as phenotypic indicator of OXA-48-like production. To improve the detection of low number resistant bacteria, 1 g of faeces was diluted in 10 mL of sterile buffered peptone water (Biokar Diagnostics, France) and incubated at 36 ± 1 °C for 24h, followed by inoculation of 10 µL onto selective plates as described above. After overnight incubation at 36 ± 1 °C, up to five resistant suspected colonies of each different morphology were isolated and stored in 20% glycerol (Sigma-Aldrich, US) brain heart infusion broth (Biokar Diagnostics, France) at -20 °C until further processing.

*Bacterial identification and genetic profiling*

Bacterial DNA was extracted by heat lysis and centrifugation as previously described [20]. Species identification was performed by 16S rRNA gene sequencing [21]. An extended PCR scheme described by Doumith et al. (2012) was used to assign the *E. coli* isolates to one of the four major phylogroups (A, B1, B2, D) [22].

Genetic relationships between all *E. coli* isolates were initially determined by repetitive element sequence-based PCR (REP-PCR) typing [23], and persistence defined as isolation of strains with matching molecular profiles (REP-PCR and resistance determinants) from repeated samples of the same subject. Furthermore, MLST for *E. coli* [24] and *K. pneumoniae* strains [25] was performed according to the published consensus MLST scheme. The population structure of sequence types (STs) was evaluated using the goeBURST software [26].

Isolates were screened for the presence of genes encoding for ESBLs ($bla_{\text{CTX-M-type}}$, $bla_{\text{TEM}}$, $bla_{\text{SHV}}$) [27,28], AmpC variants ($bla_{\text{CIT-type}}$, $bla_{\text{FOX-type}}$, $bla_{\text{MOX-type}}$, $bla_{\text{DHA-type}}$, $bla_{\text{ACT-type}}$ and $bla_{\text{MIR-type}}$) [11] and carbapenemases ($bla_{\text{AIM}}$, $bla_{\text{DIM}}$, $bla_{\text{GIM}}$, $bla_{\text{SIM}}$, $bla_{\text{IMP}}$, $bla_{\text{VIM}}$, $bla_{\text{SPM}}$, $bla_{\text{NDM}}$, $bla_{\text{KPC}}$, $bla_{\text{BIC}}$, and $bla_{\text{OXA-48-like}}$) [29] by PCR and sequencing.

*Antimicrobial susceptibility testing*

Minimum inhibitory concentrations (MICs) were determined by broth microdilution using the MicroScan® Neg MIC Panel Type 44 (Beckman Coulter, US). Results were interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical breakpoints 2023 [30], except for amoxicillin/clavulanate and trimethoprim/sulfamethoxazole, for which criteria from the Clinical and Laboratory Standards Institute (CLSI) were used [31]. Multidrug-resistance

was defined as an isolate that was resistant to three or more antimicrobial categories [32]. For *E. coli* strains, antimicrobial susceptibility testing was only performed for one representative isolate from each REP-PCR profile detected per participant per timepoint.

*Whole genome sequencing and Bioinformatics Analysis*

Third-generation cephalosporin-resistant *E. coli* strains shared by companion animals and cohabiting humans based on REP-PCR profiling were further characterized by WGS. Genomic DNA extraction was performed with NZY Tissue gDNA Isolation kit (NZYTech, Portugal) followed by WGS library preparation using the TruSeq DNA PCR-Free preparation kit (Illumina, San Diego, California, US). These libraries were sequenced using Illumina NovaSeq 6000 platform with 2×150 bp paired-end reads at a private company (Macrogen, Seoul, Republic of Korea) making an average of $1.1x10^7$ high-quality reads per library. The raw sequence reads were assessed for quality using FastQC v0.11.9 [33] and filtered for low quality reads using PRINSEQ v0.20.4 [34] (mean base quality score of ≥20 and minimum read length of 90 nt). SPAdes v3.14.1 [35] was used to generate *de novo* genome assemblies followed by two runs of polishing with Pilon v1.24 [36] and annotation using Prokka v1.14.6 [37] a minimum of 92% of genome coverage was required. Parsnp v1.2 [38] was used to generate whole genome alignments and Gubbins [39] was used to generate phylogenies unbiased by recombination events and RaxML-NG [40] was used for bootstrap analyses (100 replicates) using *E. coli* K-12 MG1655 for comparison. The pairwise SNP distances were computed between genomes from core genome alignment using snp-dists v.0.8.2 [41]. Microreact platform [42] was used to visualize the phylogenetic tree linked to antimicrobial resistance data.

*De novo* genome assemblies presented an average L50=10 (range from 9 to 3), N50=1.7x105 (range from 1.1x105 to 2.1x105) and average depth of 317x, see Table S2 for detailed assembly statistics. These assemblies were used to confirm the presence of specific resistance genes, as well as plasmid replicon types, virulence factors, MLST and *E. coli* serotype using the Center for Genomic Epidemiology tools [43].

Based on established molecular definitions, *E. coli* strains were classified as extraintestinal pathogenic (ExPEC), if they carried ≥2 of 5 virulence makers, including *papAH* and/or *papC* (counted as one P fimbriae), *sfa/focDE* (S and F1C fimbriae), *afa/draBC* (Dr antigen-specific adhesin), *iutA* (ferric aerobactin receptor), and *kpsMII* (group 2 capsules) [44]; as uropathogenic *E. coli* (UPEC) when positive for markers, including ≥3 of 4 markers, including *chuA* (outer membrane hemin receptor), *fyuA* (Siderophore receptor), *vat* (vacuolating autotransporter toxin), and *yfcV* (adhesin) [45]; and as avian pathogenic *E. coli* (APEC) if positive for ≥4 of 5 markers, including *hlyF* (hemolysin F), *iutA*, *iroN* (enterobactin siderophore receptor), *iss* (increased serum survival), and *ompT* (outer membrane protease) [45], taking into account that these definitions did not necessarily correspond with the strain source.

*Statistical analysis*

Statistical analysis was performed using SAS statistical software package for Windows, version 9.3 (SAS Institute Inc, Cary, US). Fisher's Exact test was used for comparisons between countries and a $p$-value<0.05 was considered significant.

To identify risk factors for ESBL/AmpC-E carriage, contingency tables were generated using the collected demographic and clinical data to perform univariable logistic regression analysis.

## Results

*Study population*

Forty-one households from Portugal and 44 from the UK were enrolled (Figure 1). Participants' characteristics such as detailed demographic, social and clinical data are shown in Table S1. Household composition varied in the number of companion animals (1-5 per household) and humans (1-5 per household). Companion animals' ages ranged from 3 months to 17 years-old (median: 6.4y, $n$=110), and that of humans from 6 to 75 years-old (median: 39y, $n$=119).

Most of the companion animals lived indoors in both countries. Portuguese cats and dogs sleep more frequently in their owner's bed than dogs from the UK. Furthermore, 23.3% of dogs and 25% of cats from Portugal, and 17% from the UK took antibiotics 3-12 months prior to sampling (Table S1).

A very high number of humans reported close contact behaviours either frequently or occasionally with their companion animals, such as petting/cuddling and being kissed/licked by them. Around 37.8% of humans were healthcare professionals and 18.8% took antibiotics 3-12 months prior to sampling (Table S1).

*Prevalence of ESBL/AmpC-producing Enterobacterales carriage*

For this prospective longitudinal study, sampling was performed monthly for three months. Overall, ESBL/AmpC-E strains were isolated from 13.4% (95%CI, 7.2-19.7, $n$=16/119) of humans and 10.9% (95%CI, 4.9-16.8, $n$=12/110) companion animals from at least one timepoint sample. No carbapenemase-producing bacteria were recovered. There was no significant difference between prevalence of ESBL/AmpC-E carriage in humans and companion animals (p=0.558). At the individual level, no significant

difference was found between dogs and cats (p=0.691), dogs and humans (p=0.838) or cats and humans (p=0.466) for the isolation of ESBL/AmpC-E strains.

Comparison between countries did not reveal any difference in the prevalence of ESBL/AmpC-E strains in companion animals from Portugal (12.7%, 95%CI, 4.3-21.2, *n*=8/63) and the UK (8.5%: 95%CI, 0.2-16.8, *n*=4/47) (p=0.486). However, for humans, a significant difference was detected (PT: 20.7%, 95%CI, 9.9-31.4, *n*=12/58; UK: 6.6%, 95%CI, 0.2-12.9, *n*=4/61; p=0.031). Since results varied according to the country, the risk factor analysis for both companion animals and humans was executed separately for the two countries.

The full list of variables included in the risk factor analysis is shown in Table S3. None of the variables were significantly associated with ESBL/AmpC-E carriage either in humans or companion animals. As no variables were considered statistically significant (p-values>0.1), multivariable models could not be built.

Across all timepoints, 76 ESBL/AmpC-E strains were isolated from 28 participants (16 humans and 12 companion animals). These comprised four Enterobacterales species. Primarily *E. coli* strains, followed in frequency by *K. pneumoniae*, *Citrobacter freundii* and *Hafnia paralvei* strains (Figure 2). From a total of 71 *E. coli* strains, only 50 strains were shown to be non-duplicate by REP-PCR-profiling and were further studied. Figures S1 and S2 display strains clonality by the dendrograms generated from REP-PCR fingerprinting of PT and UK strains, respectively. Of the non-duplicate *E. coli* strains, phylogenetic group A and B2 were more common in PT, while phylogenetic group B1 and D were more frequent in the UK (Figure 2).

*Antimicrobial susceptibility*

All non-duplicate Enterobacterales strains (*n*=55) were further characterized for antimicrobial susceptibility testing, MICs for each strain are shown in Table S4. All these strains were isolated from cefotaxime supplemented MacConkey agar plates and presented MIC values for cefotaxime ranging from 4 to >32 mg/mL.

In Portugal, a high proportion of strains presented multidrug-resistance, 90.5% (*n*=19/21) from humans and 94.1% (*n*=16/17) from animals (Table 1). In the UK most strains were also multidrug-resistant (human: 75%, *n*=3/4; animals: 92.3%, *n*=12/13) (Table 1).

*Genes conferring resistance to third-generation cephalosporins*

The overall distribution of beta-lactam resistance genes differed between the companion animal and human strains (Figure 3); molecular features by strain are shown

in Table S5. Most of ESBL/AmpC-E strains harboured multiple (two or three) beta-lactamase encoding genes (Figure 3). Regarding ESBL/AmpC resistance determinants, *bla*$_{\text{CTX-M-15}}$ was the most frequent in Enterobacterales strains from Portugal, while in the UK, the *bla*$_{\text{CMY-2}}$ gene was the most common (Figure 3).

Among the 50 3GC-resistant *E. coli* strains, 20 STs were identified (Figure 4, Table S5). The dominant STs were ST131 (*n*=7/50) and ST410 (*n*=7/50), followed by ST457 (*n*=5/50). Of note, four strains belonged to *E. coli* ST10 complex (ST10, ST44 and ST617), recognized as an ExPEC lineage [46], and four to *E. coli* ST38 complex (ST38 and ST963) defined as high-risk clones owing to their multidrug-resistant profile [47] (Figure 4). Furthermore, the three *K. pneumoniae* strains from ST4476, ST348 and ST392 (Table S5), the last one belonging to the high-risk clone ST147 complex was colonising a human from Portugal [47].

*Longitudinal isolation of ESBL/AmpC-Enterobacterales strains*

Considering all included participants, at least two consecutive faecal samples were obtained from 59 households (PT=20, UK=39) (Figure 1). Among these, eight humans, two dogs and one cat from Portugal and three humans and three dogs from the UK were carriers of ESBL/AmpC-E strains in at least one timepoint. For graphical overview of each positive households over time, see Figure S3.

Repeated isolation of multidrug-resistant ESBL/AmpC-producing *E. coli* strains was observed in Portuguese humans (*n*=3) and dog (*n*=1), and in UK dogs (*n*=2) (Figure S3). Persistence of the same multidrug-resistant *E. coli* strain, harbouring the *bla*$_{\text{CTX-M-55}}$ and *bla*$_{\text{CMY-2}}$ genes, was detected by REP-PCR profiling in one Portuguese human (PT048H1) at T0 and T1 (Table 2, Figure S1). Furthermore, in the UK, two dogs from different households (UK020D1, UK030D1) had persistent isolation of a CMY-2-producing *E. coli* strain at the first (T0) and second (T1) timepoint collection, but this was no longer detectable at the third collection point (T2) (Figure S2).

The acquisition of ESBL/AmpC-E strains by participants that were negative at T0 only occurred in humans. Interestingly, none of them co-habited with companion animal carriers (Figure S3). This acquisition occurred after one month in 4.8% (*n*=1/21) of humans from Portugal and 1.9% (*n*=2/52) from the UK; and after two months in 18.2% (*n*=2/11) humans from Portugal and 2% (*n*=1/50) from the UK (Figure 1).

Overall, co-carriage of ESBL/AmpC-producing *E. coli* strains in companion animals and cohabiting humans was observed in four Portuguese households (9.7%, *n*=4/41) and one household from the UK (2.3%, *n*=1/44) (Table 2). Of these, three households (PT011, PT048, UK007) included companion animal-human *E. coli* paired strains with matching REP-PCR that were selected for WGS analysis (one representative strain from each household member per timepoint, comprising a total of nine *E. coli* strains). The REP-PCR profile analysis only revealed within-household sharing of

ESBL/AmpC-producing *E. coli* strains, indicating that *E. coli* strains were not shared by participants from different countries or households.

*Core genome relatedness between animals and humans' isolates*

Analysis of the core genomes confirmed that no distinction could be made between *E. coli* strains isolated from the two companion animal-human pairs from Portugal (PT011 and PT048) (Figure 5). Thus, within household co-carriage of the same ESBL/AmpC-producing *E. coli* strains by animals and humans was confirmed. Furthermore, the paired strains were assigned to the same ST and harboured identical resistance genes, plasmid replicons and virulence factors (Figure 5, Table S6: Full list of genetic features of sequenced strains).

At T0, the PT011 cat-human pair shared an *E. coli* O15-H33-ST93 strain, harbouring the $bla_{\text{CTX-M-15}}$ and $bla_{\text{TEM-1}}$ genes, with identical sequence displaying a single nucleotide polymorphism (SNP) difference (Figure 5).

Regarding household PT048, the dog-human pair shared two ESBL/AmpC-producing *E. coli* strains, namely, *E. coli* O8:H9-ST410 and *E. coli* O11:H25-ST457. The *E. coli* O8:H9-ST410 strains harboured $bla_{\text{CTX-M-15}}$ and $bla_{\text{OXA-1}}$ genes, displayed one SNP of difference, and were isolated from the dog at T0 and T1 and its owner at T1. These *E. coli* O8:H9-ST410 strains were classified as ExPEC due to the presence of *papC* and *iutA* genes (Figure 5) [44]. The *E. coli* O11:H25-ST457 strains harboured the $bla_{\text{CTX-M-55}}$ and $bla_{\text{CMY-2}}$ genes, had no SNP differences, and were recovered from the dog at T1 and the human at T0. These *E. coli* O11:H25-ST457 strains were also classified as ExPEC, due to the presence of *iutA* and *kpsMII* genes (Figure 5) [44]. The human from the PT048 household was a carrier at T1 of an *E. coli* strain that was not selected for WGS (PT048/1-H1F3E1, Table 2), but presented the same beta-lactam genes and REP-PCR profile as the *E. coli* O11:H25-ST457 strain isolated in T0.

Lastly, the dog-human *E. coli* pairs from UK clustered apart (>500 SNPs) and presented different resistance determinants and plasmid replicon profiles, indicating that they had not been shared within the household (Figure 5).

## Discussion

Resistance to third and fourth-generation cephalosporins is a global public health concern due to its widespread nature and the critical use of these drugs in human and veterinary medicine. In the present longitudinal study, we assessed the frequency of colonisation by ESBL/AmpC-E strains in healthy companion animals in the community and their cohabiting humans. This study was conducted in Portugal and the UK, two

countries where the prevalence of 3GC and carbapenem-resistant strains amongst clinical isolates are usually different [8,48]. WGS pairwise comparison was applied to demonstrate sharing of ESBL/AmpC-E strains between companion animals and their cohabiting humans.

The proportion of ESBL/AmpC-E strains faecal carriage in healthy companion animals in this study (PT=12.7%; UK=8.5%) agrees with previous reports from both countries [49,50] alongside data from Mexico and The Netherlands (5.7 and 10.6%, respectively) [18,51]. However, these results are lower than those obtained in healthy dogs from Chile and Germany (24 and 80%, respectively) [52,53]. Regarding Portuguese humans, the percentage of carriers (20.7%) is in line with one previous prospective study performed in healthcare students from Portugal [54] and with a meta-analysis study, in which the prevalence of carriage of ESBL strains in healthy individuals was 14% [55]. Carriage was lower in UK humans (6.6%), seemingly following the trends observed regarding the higher rate of 3GC-resistant *E. coli* reported amongst clinical isolated from Southern versus Northern European countries in 2020 [8].

ESBL/AmpC-producing *K. pneumoniae* strains were only rarely isolated from the healthy participants in this study, which is broadly in line with frequencies previously reported from healthy dogs in Northern, Portugal (2.4%) [56], and veterinary healthcare workers in The Netherlands (9.8%) [57]. Nevertheless, it should be noted that *K. pneumoniae* is a leading nosocomial agent causing a wide range of infections [1,8], that was associated with multidrug-resistant phenotypes in both this and previous studies, including resistance to highest-priority CIAs for human such as cephalosporins.

As seen in other studies, *E. coli* was the most frequently detected/isolated ESBL/AmpC-E [52,54]. Although most of the *E. coli* strains belonged to phylogroup A, the isolation of strains from group B2 and D, frequently associated with pathogenic *E. coli* strains [10], in healthy individuals from the community highlight the need for continuous monitoring. The pandemic high-risk clonal lineages *E. coli* ST38, ST69 and ST131 were detected in companion animal and humans from both countries. These clonal lineages are considered to be globally disseminated ExPEC clones, being associated with a higher number of virulence determinants [2,46,47]. Therefore, their presence in healthy companion animals and humans is of great public-health importance. Also of concern is the high proportion of ESBL/AmpC-producing *E. coli* strains isolated from healthy companion animals which were resistant to fourth-generation cephalosporins and multidrug-resistant. These results may point towards an increasing trend in multidrug-resistant bacteria carried by companion animals. Nevertheless, the absence of carbapenem-resistant isolates is reassuring.

In Portugal, 3GC-resistance was frequently associated with the $bla_{\text{CTX-M-15}}$ gene, which is the most commonly reported gene worldwide in faecal and clinical ESBL/AmpC-E strains from humans [18,57,58] and animals [18,59,60]. Yet, our study showed a decreased in the occurrence of the $bla_{\text{CTX-M-15}}$ gene in the UK in favour of the $bla_{\text{CMY-2}}$ gene. Notably, the $bla_{\text{CMY-2}}$ gene has been frequently described in 3GC-resistant Enterobacterales from dogs with urinary tract infections, including in the UK [10,60].

No specific risk factors for ESBL/AmpC-E strain carriage were identified, which may be explained by the sample size included from each country. Nevertheless, several risk factors have been associated with increased antimicrobial resistance in previous studies, such as recent hospitalization or previous antibiotic exposure in both humans and animals [18,61]. Although some enrolled participants reported taking antibiotics in the previous 3-12 months or having been recently hospitalized (≤12 months), these were not established as risk factors in this study. Considering the widespread dissemination of ESBL/AmpC-E strains [8,48,55], many routes for acquisition of these resistant Enterobacterales strains are available nowadays and so, the detection of risk-factors is increasingly more complex.

Companion animals that were not carriers of ESBL/AmpC-E strains at the start of this study remained negative throughout. However, the method used to screen for antibiotic resistant strains, bacterial culture, has a low sensitivity, and so, regardless the use of pre-enrichment technique, more frequent sampling would be necessary to reach a higher sensitivity for resistance detection [62]. Additionally, this may be related to the short duration of the present work, since in a previous six month longitudinal study, in The Netherlands, the acquisition of ESBL-producing Enterobacterales strains was detected in healthy dogs in the end of the study [18]. In the present work, acquisition of ESBL/AmpC-E strains over time by initially non-carrier humans did occur. This finding may be linked to a higher exposure of humans to different environments and foods when compared to pets. Furthermore, it may suggest that humans contribute more to the spread of different ESBL/AmpC-E strains while companion animals act as a maintenance reservoir.

Data on ESBL/AmpC-E associated to healthy animal-human co-carriage within households based on core-genome similitude by WGS are scarce. In our study, companion animal-human ESBL/AmpC-producing *E. coli* strain sharing was confirmed in 4.9% ($n$=2/41) of the Portuguese households. In a recent Dutch study, also based on the core-genome analysis, identical ESBL-producing Enterobacterales strains were detected only in 0.4% ($n$=2/550) of the households. When considering exposed households (households that had ESBL/AmpC-E positive subjects only) this sharing frequency rises to 22.2% ($n$=2/9) in our study and only to 5% in the Dutch study [18]. These differences may be related to the distinctive patterns of antimicrobial usage/resistance in the Centre versus South of Europe [8,48]. Furthermore, to best of our knowledge we found for the first-time sharing of ESBL/AmpC-E between healthy cat owners and cats in the community.

Other studies found different rates of bacterial co-carriage that might be explained by different study designs, methods and risk-factor exposure according to the country. In Brazil, 9.5% of the human-dog pairs included in the study shared multidrug-resistant *E. coli* strains [17], while in Romania co-carriage of ESBL/AmpC-E strains within human-dog pairs was found to be 6% [19]. In these studies, clonality was assessed by less discriminatory methods than WGS core-genome analysis.

The companion animal-human shared *E. coli* strains belonged to emerging clonal lineages (O15-H33-ST93, O8:H9-ST410, O11:H25-ST457), reported worldwide in association with resistance to a wide range of antibiotics. The *E. coli* ST93 lineage has been detected at the animal-human interface previously, associated with the carriage of the $bla_{\text{CTX-M}}$ and plasmid-mediated colistin-resistance (*mcr-1*) genes [63,64], and also shared between dog and owner [18]. The *E. coli* ST410 lineage is involved in the global epidemiological landscape of carbapenem-resistance [65,66], with reports of interspecies transmission of CTX-M-producing strains between humans, companion animals, wildlife and the environment [67]. The *E. coli* ST457 lineage has been shown to display a remarkable ability to capture mobile genetic elements that carry and transmit genes encoding resistance to CIAs for human medicine in a broad host range [63,68]. Notably, the *E. coli* ST410 and ST457 lineages from this study were classified as belonging to the ExPEC pathotype, highlighting their pathogenic potential. The faecal carriage and companion animal-human sharing of these successful clonal lineages harbouring ESBL/AmpC-producing *E. coli* strains is important as it broadens their dissemination and aids persistence in the household environment.

The main limitations of this study were the relatively high number of participants failing to complete the longitudinal study, despite initial good recruitment. This study might therefore underestimate the rate of persistent ESBL/AmpC-E strains carriage. Future analysis to compare the mobile genetic elements relating to antimicrobial resistance within whole faecal samples from cohabiting humans and animals would be valuable to investigate interspecies sharing of genetic material, independent of specific bacterial clones.

To our best knowledge, this is the first report of ESBL/AmpC-E strains faecal sharing in healthy dog-cat-owner pairs from Portugal. The prevalence of ESBL/AmpC-E strains carriage in healthy companion animals and humans in this study was relevant and appears to vary geographically. This study provides evidence of companion animal-human sharing of human pandemic ESBL/AmpC-producing *E. coli* clonal lineages within households. It also illustrates the importance of the healthy companion animal-human unit in the epidemiology of antibiotic-resistant high priority pathogens, and further highlights the value of a One Health approach, integrating human and animal health.

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## Conflict of interest

The authors have no relevant financial or non-financial interests to disclose.

## Availability of data and material

The sequencing data generated during the current study is available in the European Nucleotide Archive repository, under the project PRJEB51686. High resolution phylogenetic tree linked to molecular data is available at Microreact platform, https://microreact.org/project/5UAN5To1F5FudrUvKx5cGH-esbl-producing-escherichia-coli-sharing

## Code availability

Not applicable.

## Ethics approval

The study was conducted in accordance with the European Union Directive 2010/63/EU on the protection of animals used for scientific purposes and the Declaration of Helsinki. Ethical approval for collection of samples and data from humans, dogs and cats was obtained from Faculty of Veterinary Medicine, University of Lisbon Ethics Committee for Research and Education (CEBEA 027/2018) and Royal Veterinary College Ethics and Welfare Committee (URN 2017 1750-3).

## Author Contributions

Study conception and design were performed by Juliana Menezes, Anette Loeffler and Constança Pomba. All authors contributed to the material preparation and data

collection. The first draft of the manuscript was written by Juliana Menezes and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

## Consent to participate

Written informed consent was obtained prior to enrolment from each human participant included in the study for themselves and their companion animals.

## Consent for publication

Informed consent was obtained from all individual participants included in the study, for themselves and their companion animals.

**Table 1.** Antimicrobial resistance of ESBL/AmpC-producing Enterobacterales strains isolated from healthy companion animals and their cohabiting humans in Portugal and the United Kingdom.

| Antimicrobial | Portugal | | United Kingdom | |
|---|---|---|---|---|
| | Humans ESBL/AmpC-E strains (*n*= 21) | Companion animals ESBL/AmpC-E strains (*n*= 17) | Humans ESBL/AmpC-E strains (*n*= 4) | Companion animals ESBL/AmpC-E strains (*n*= 13) |
| | %R (No) | %R (No) | %R (No) | %R (No) |
| Amikacin | 0.0 (0) | 5.9 (1) | 0.0 (0) | 0.0 (0) |
| Amoxicillin/clavulanate | 52.9 (11) | 10.6 (12) | 75 (3) | 1000 (13) |
| Ampicillin | 95.2 (20) | 94.1 (16) | 100 (4) | 1000 (13) |
| Aztreonam | 85.7 (18) | 94.1 (16) | 75 (3) | 84.6 (11) |
| Ceftazidime | 66.7 (14) | 94.1 (16) | 100 (4) | 1000 (13) |
| Cefepime | 76.2 (16) | 52.9 (9) | 50 (2) | 15.4 (2) |
| Cefotaxime | 100 (21) | 100 (17) | 100 (4) | 100 (13) |
| Cefoxitin | 19.0 (4) | 52.9 (9) | 25 (1) | 84.6 (11) |
| Ciprofloxacin | 47.6 (10) | 47.1 (8) | 25 (1) | 15.4 (2) |
| Chloramphenicol | 0.0 (0) | 0.0 (0) | 25 (1) | 15.4 (2) |
| Ertapenem | 0.0 (0) | 0.0 (0) | 0.0 (0) | 0.0 (0) |
| Gentamicin | 33.3 (7) | 52.9 (9) | 25 (1) | 15.4 (2) |
| Imipenem | 0.0 (0) | 0.0 (0) | 0.0 (0) | 0.0 (0) |
| Meropenem | 0.0 (0) | 0.0 (0) | 0.0 (0) | 0.0 (0) |
| Piperacillin/tazobactam | 4.8 (1) | 17.6 (3) | 0.0 (0) | 0.0 (0) |
| Trimethoprim/sulfamethoxazole | 33.3 (7) | 17.6 (3) | 50 (2) | 53.8 ((7) |
| Multidrug-resistant | 90.5 (19) | 94.1 (16) | 75 (3) | 92.3 (12) |

Minimum inhibitory concentrations (MIC) were interpreted using the criteria of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) 2023 [30], except for amoxicillin/clavulanate and trimethoprim/sulfamethoxazole, for which criteria from the Clinical and Laboratory Standards Institute (CLSI) were used [31]. Data are reported as number (No) of resistant (R) strains; n, total number of non-duplicate strains tested.

**Table 2.** Co-carriage of ESBL/AmpC-producing Enterobacterales strains between humans and companion animals within Portuguese and United Kingdom households.

| Household number[a] | Timepoint[b] | Household member | Bacteria species | MLST | Strain code | Beta-lactam resistance genes | REP-PCR group |
|---|---|---|---|---|---|---|---|
| PT011 | T0 | Human 1 | *Escherichia coli* | ST93 | PT011/0-H1F3E1 | *bla*$_{CTX-M-15}$, *bla*$_{TEM-1}$ | A |
| | | | | ST93 | PT011/0-H1F3E4 | *bla*$_{CTX-M-15}$, *bla*$_{TEM-1}$ | B |
| | | | *Hafnia paralvei* | N/A | PT011/0-H1F3E2X | *bla*$_{CTX-M-15}$, *bla*$_{TEM-1}$, *bla*$_{ACC}$ | N/A |
| | | Cat 1 | *Escherichia coli* | ST2601 | PT011/0-C1F3E1 | *bla*$_{CTX-M-15}$ | C |
| | | | | ST93 | PT011/0-C1F3E3 | *bla*$_{CTX-M-15}$, *bla*$_{TEM-1}$ | B |
| | T1 | Human 1 | *Escherichia coli* | ST93 | PT011/1-H1F3E2 | *bla*$_{CTX-M-15}$, *bla*$_{TEM-1}$ | D |
| PT048 | T0 | Dog 1 | *Escherichia coli* | ST410 | PT048/0-D1F3E2 | *bla*$_{CTX-M-15}$, *bla*$_{SHV-12}$ | T |
| | | | | ST410 | PT048/0-D1F3E4 | *bla*$_{CTX-M-15}$ | U |
| | | | | ST457 | PT048/0-D1F3E1 | *bla*$_{CTX-M-55}$, *bla*$_{CMY-2}$ | AA |
| | | Human 1 | *Escherichia coli* | ST457 | PT048/0-H1F3E1 | *bla*$_{CTX-M-15}$, *bla*$_{CMY-2}$ | V |
| | | | | ST457 | PT048/0-H1F3E2 | *bla*$_{CTX-M-55}$, *bla*$_{CMY-2}$ | W |
| | T1 | Dog 1 | *Escherichia coli* | ST410 | PT048/1-D1F3E1 | *bla*$_{CTX-M-15}$ | Z |
| | | | | ST410 | PT048/1-D1F3E2 | *bla*$_{CTX-M-15}$ | X |
| | | | | ST457 | PT048/1-D1F3E3 | *bla*$_{CTX-M-55}$, *bla*$_{CMY-2}$ | W |
| | | | | ST410 | PT048/1-D1F3E4 | *bla*$_{CTX-M-15}$ | U |
| | | Human 1 | *Escherichia coli* | ST457 | PT048/1-H1F3E1 | *bla*$_{CTX-M-55}$, *bla*$_{CMY-2}$ | W |
| | | | | ST410 | PT048/1-H1F3E3 | *bla*$_{CTX-M-15}$ | U |
| PT056 | T0 | Dog 1 | *Escherichia coli* | ST963 | PT056/0-D1F3E1 | *bla*$_{CMY-2}$ | Q |
| | | | | ST963 | PT056/0-D1F3E2 | *bla*$_{CTX-M-32}$, *bla*$_{CMY-2}$ | AD |
| | | | | ST963 | PT056/0-D1F3E4 | *bla*$_{CMY-2}$ | AE |
| | | Human 1 | *Escherichia coli* | ST44 | PT056/0-H1F3E4 | *bla*$_{CTX-M-32}$, *bla*$_{TEM-1}$ | AB |
| | | | | ST44 | PT056/0-H1F3E6 | *bla*$_{CTX-M-32}$, *bla*$_{TEM-1}$ | AC |
| PT103 | T0 | Human 1 | *Escherichia coli* | ST5194 | PT103/0-H1F3E2 | *bla*$_{CMY-2}$ | E |
| | | Dog 2 | *Escherichia coli* | ST410 | PT103/0-D2F3E1 | *bla*$_{TEM-32}$, *bla*$_{CMY-2}$ | F |
| UK007 | T0 | Dog 1 | *Escherichia coli* | ST3902 | UK007/1-D1F3E4.1 | *bla*$_{CTX-M-1}$, *bla*$_{TEM-1}$ | AF |
| | | | | ST131 | UK007/1-D1F1(CTX)E1 | *bla*$_{CTX-M-1}$, *bla*$_{TEM-1}$, *bla*$_{CMY-2}$ | AG |
| | | Human 1 | *Escherichia coli* | ST617 | UK007/1-H1F3E4.1 | *bla*$_{CTX-M-15}$ | AF |

MLST, Multilocus sequence typing; N/A, Not applicable.

[a] First two letters in household number concerns to country of isolation: PT, Portuguese household, UK, United Kingdom household.

[b] T0 represents sampling at enrolment; T1 was performed one month after T0; T2 was performed two months after T0.

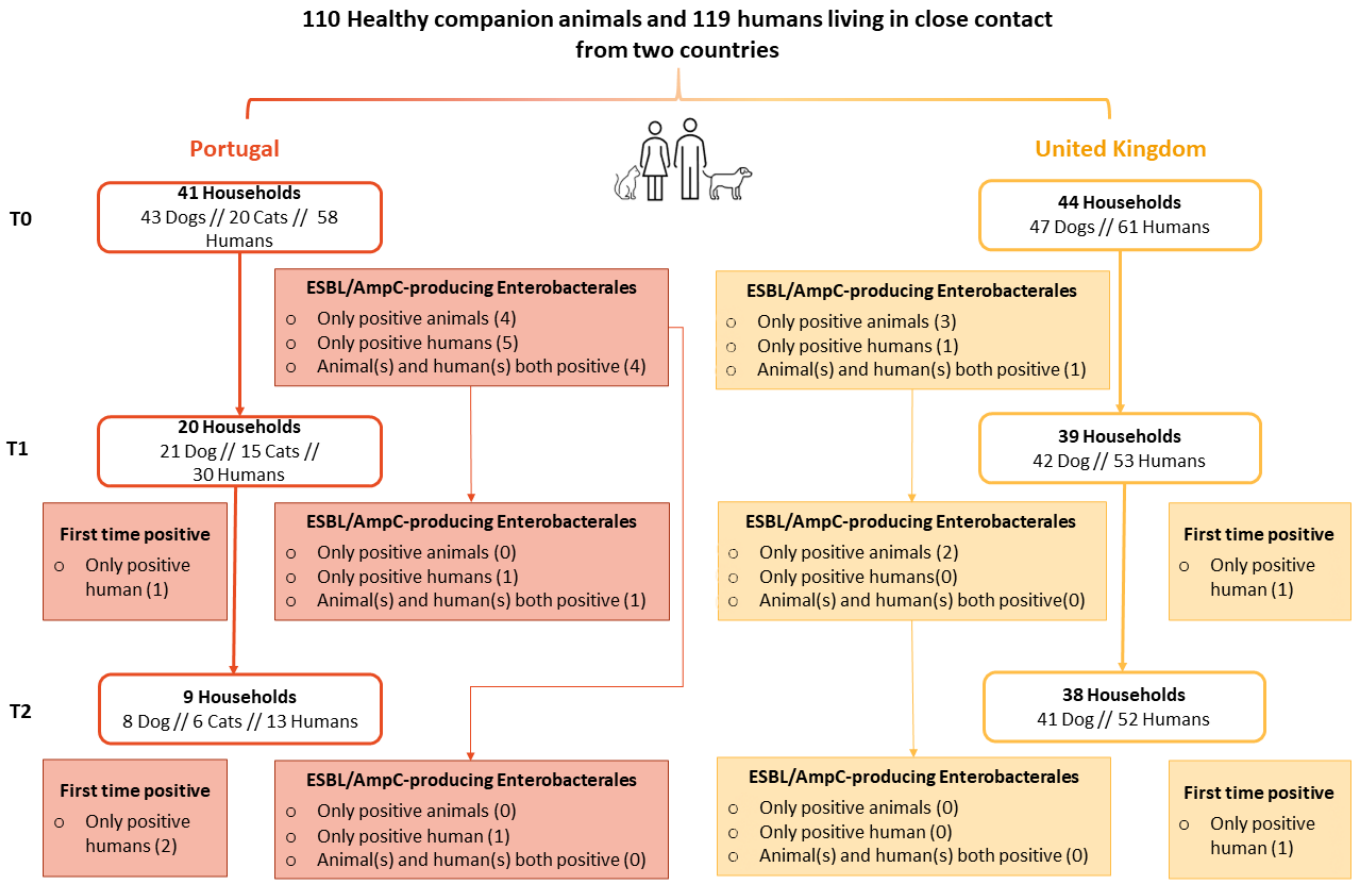


**Figure 1.** Flow chart of household participation and ESBL/AmpC-producing Enterobacterales status by country over time. T0 represents sampling at enrolment; T1 was performed one month after T0; T2 was performed two months after T0.

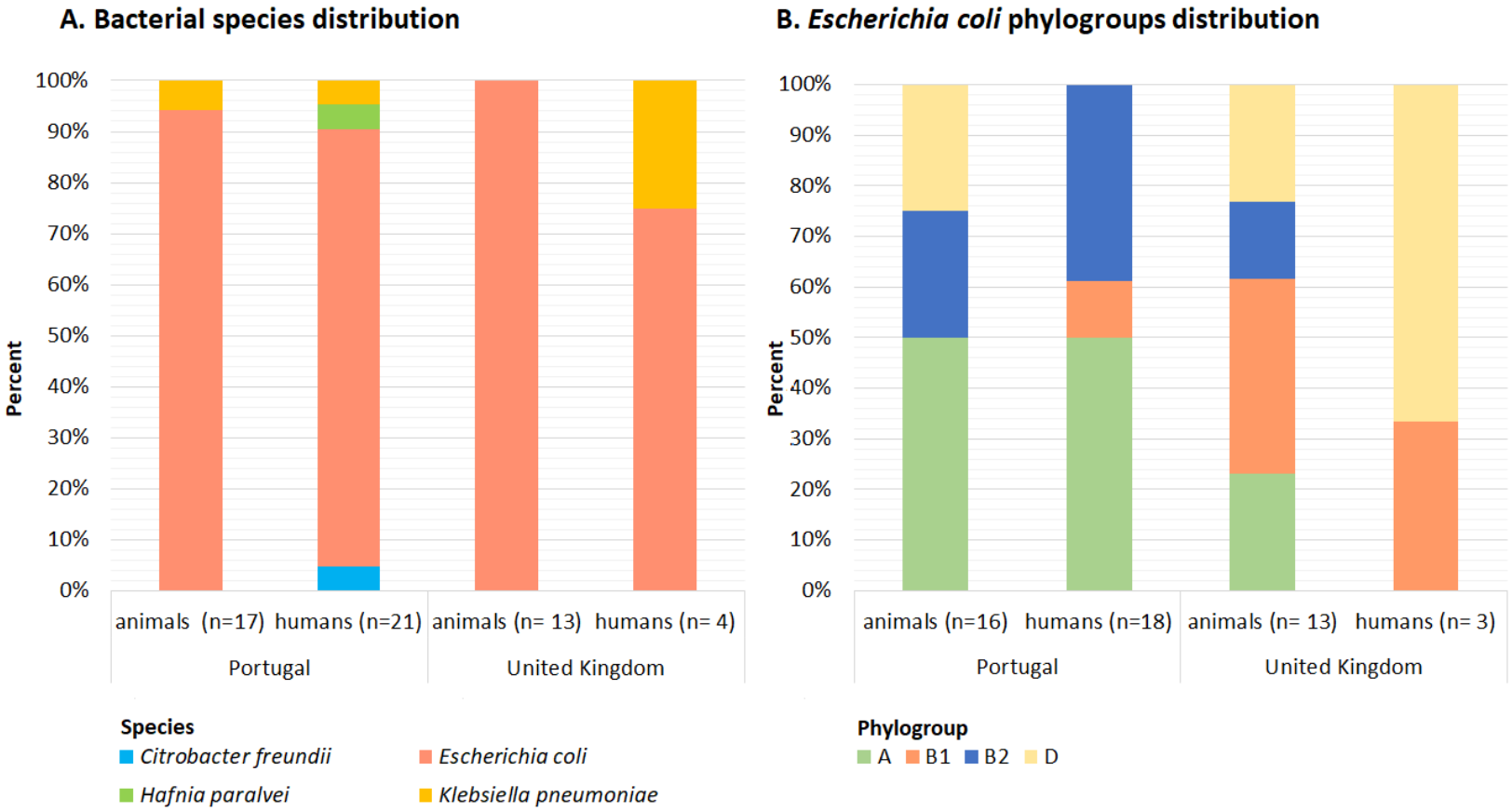


**Figure 2.** Frequency and diversity of ESBL/AmpC-producing Enterobacterales non-duplicate strains isolated from healthy companion animals and their cohabiting humans in Portugal and the United Kingdom. (A) Percentage of bacterial species by country and source. The bars are coloured by species. (B) Distribution of *Escherichia coli* phylogroups by country and source, the bars are coloured by phylogroup, as shown in the inset legend.

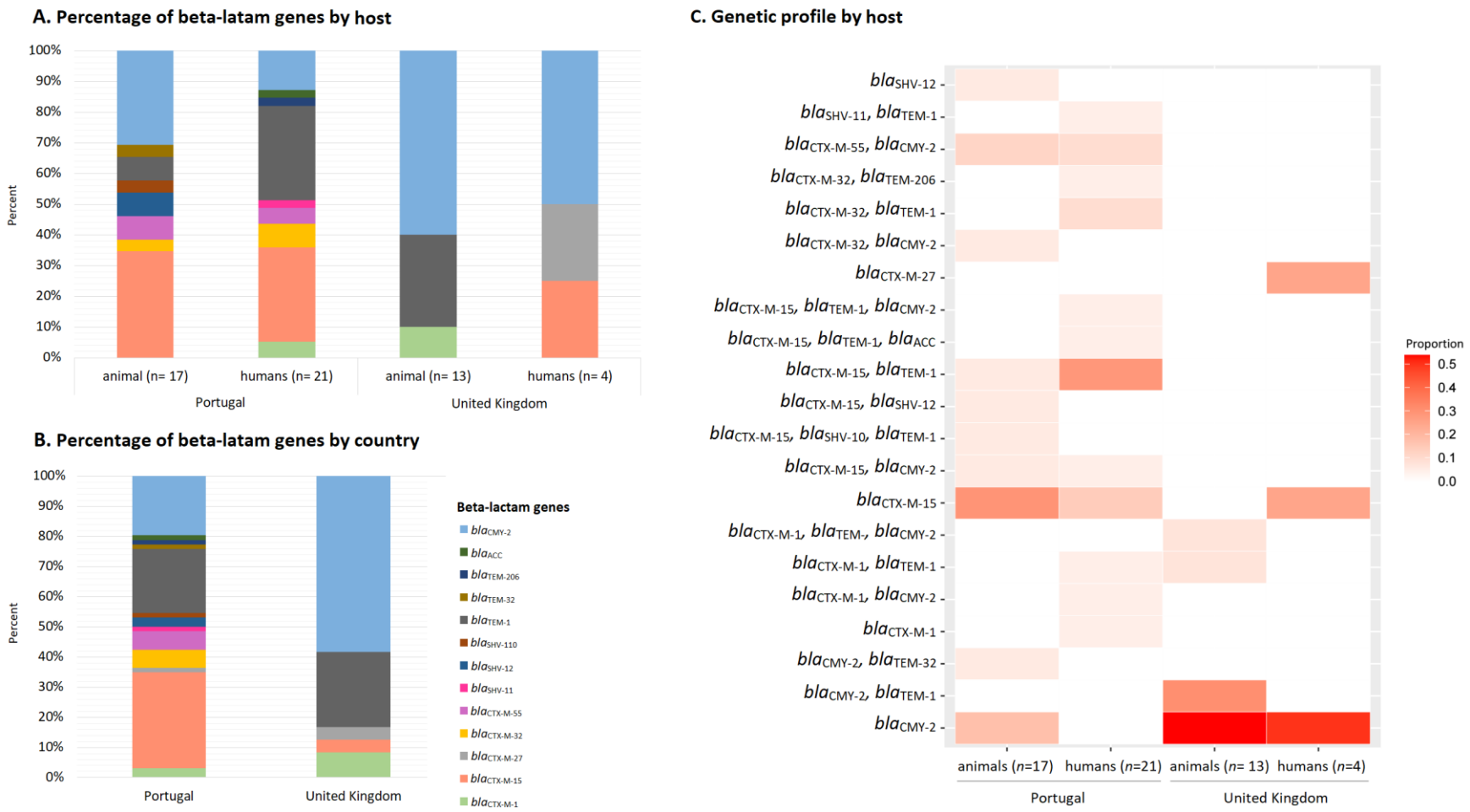


**Figure 3.** Distribution of beta-lactam genes in ESBL/AmpC-producing Enterobacterales strains isolated from healthy companion animals and their cohabiting humans in Portugal and the United Kingdom. (A) Percentage of beta-lactam genes by host and (B) country. The bars are coloured by gene as shown in the inset legend. (C) Distribution of beta-lactam gene combinations by country and host.

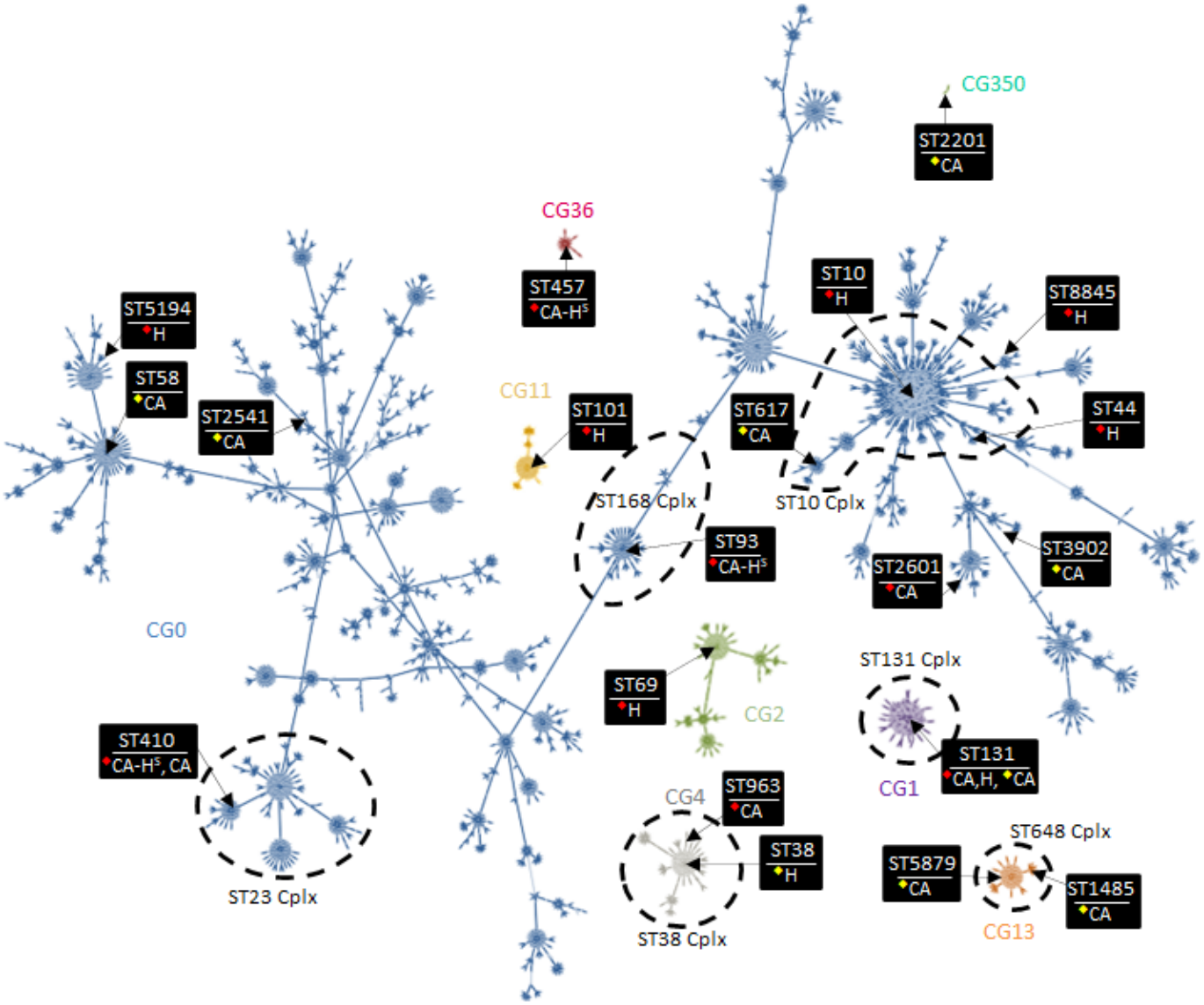
CG350
ST2201
CA
CG36
ST457
CA-H$^S$
ST5194
H
ST58
CA
ST2541
CA
CG11
ST101
H
ST168 Cplx
ST93
CA-H$^S$
ST10
H
ST617
CA
ST10 Cplx
ST8845
H
ST44
H
ST3902
CA
ST2601
CA
CG0
ST69
H
CG2
ST131 Cplx
ST131
CA,H, CA
CG1
ST410
CA-H$^S$, CA
ST23 Cplx
CG4
ST963
CA
ST38
H
ST38 Cplx
ST648 Cplx
ST5879
CA
ST1485
CA
CG13

**Figure 4.** Population snapshot of clonal groups of *Escherichia coli* strains typed in this study. The entire *E. coli* database, known as for 05 May 2023, was analysed by eBURST with the stringent group definition (single locus variants); the clonal groups (CG) that included strains typed in this study are displayed as an eBURST diagram, coloured by group (inset legend). Detected clonal complexes (Cplx) were assigned based on the predicted founder sequence type and are grouped with a dashed line. The black boxes indicate the *E. coli* sequence types found in this study. Red diamonds represent strains from Portugal and yellow diamonds, strains from the United Kingdom. CA, ESBL/AmpC-producing *E. coli* from companion animals; H, ESBL/AmpC-producing *E. coli* from humans; CA-$H^S$, ESBL/AmpC-producing *E. coli* strains shared between companion animal and owner.

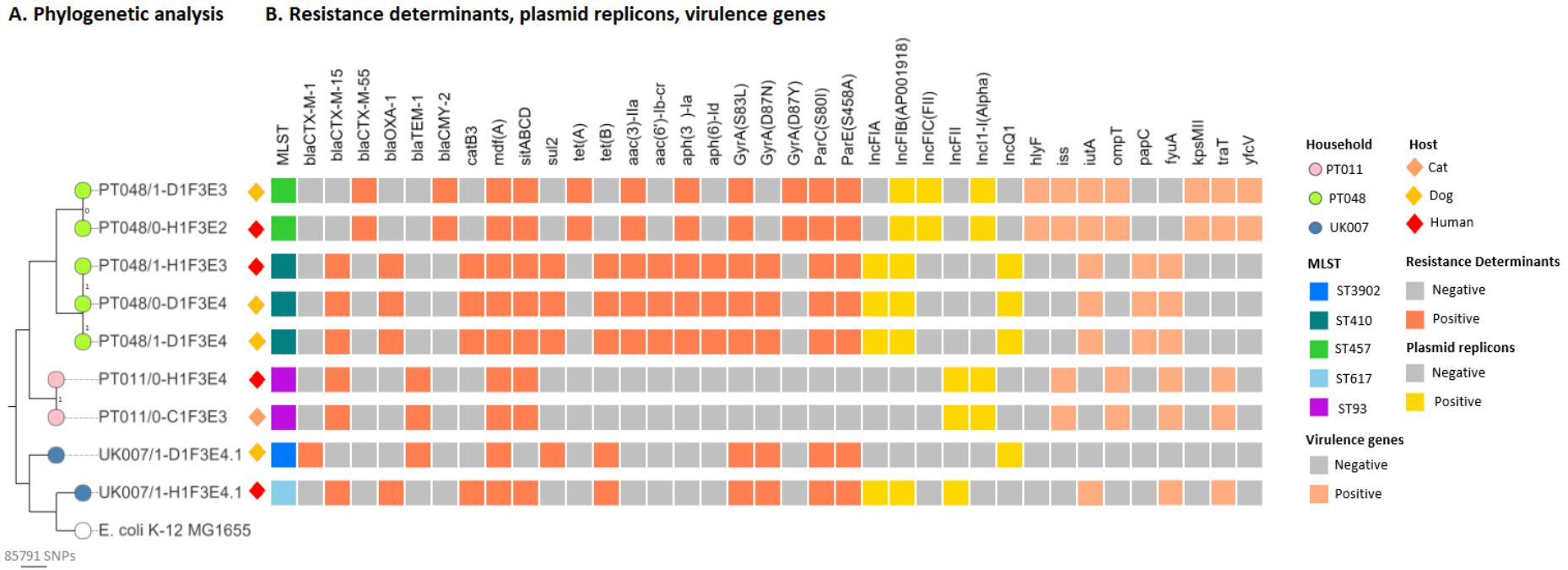


**Figure 5.** Core genome SNP analysis and genetic features of ESBL/AmpC-producing *Escherichia coli* strains from companion animals and their cohabiting humans. (A) Maximum likelihood phylogeny of the core genome of nine *E. coli* strains and the *E. coli* K-12 MG1655 strain. (B) Heatmap shows the *E. coli* sequence types, antimicrobial resistance determinants, plasmid replicons and virulence genes for each strain (see colour key on the right side of the figure).