Characterization of ESBL-Producing Escherichia coli and Klebsiella pneumoniae Isolated from Clinical Samples in a Northern Portuguese Hospital: Predominance of CTX-M-15 and High Genetic Diversity

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Abstract: Background: Enterobacteriaceae are major players in the spread of resistance to β-lactam antibiotics through the action of CTX-M β-lactamases. We aimed to analyze the diversity and genetic characteristics of ESBL-producing Escherichia coli and Klebsiella pneumoniae isolates from patients in a Northern Portuguese hospital. Methods: A total of 62 cefotaximase/cephazidime-resistant E. coli (n = 38) and K. pneumoniae (n = 24) clinical isolates were studied. Identification was performed by MALDI-TOF MS. Antimicrobial susceptibility testing against 13 antibiotics was performed. Detection of ESBL-encoding genes and other resistance genes, phylogenetic grouping, and molecular typing (for selected isolates) was carried out by PCR/sequencing. Results: ESBL activity was detected in all 62 E. coli and K. pneumoniae isolates. Most of the ESBL-producing E. coli producing ESBL-encoding E. coli isolates carried a bla_{CTX-M} gene (37/38 isolates), being bla_{CTX-M-15} predominant (n = 32), although bla_{CTX-M-27} (n = 1) and bla_{CTX-M-1} (n = 1) were also detected. Two E. coli isolates carried the bla_{KPC2/3} gene. The lineages ST131-B2 and ST410-A were detected among the ESBL-producing blood E. coli isolates. Regarding the 24 ESBL-producing K. pneumoniae isolates, 18 carried a bla{CTX-M} gene (bla_{CTX-M-15}, 16 isolates; bla{CTX-M-55}, 2 isolates). All K. pneumoniae isolates carried blaSHV genes, including ESBL-variants (bla{SHV-12}, 14 isolates) or non-ESBL-variants (bla{SHV-11}) and bla{SHV-28}, 10 isolates; ten K. pneumoniae isolates also carried the bla{KPC2/3} gene and showed imipenem-resistance. ESBL-positive E. coli isolates were ascribable to the B2 phylogenetic group (82%), mostly associated with ST131 lineage and, at a lower rate, to ST410/A. Regarding K. pneumoniae, the three international lineages ST15, ST147, and ST280 were detected among selected isolates. Conclusions: Different ESBL variants of CTX-M (especially CTX-M-15) and SHV-type (specially SHV-12) were detected among...
CTX/CAZ$^R$ *E. coli* and *K. pneumoniae* isolates, in occasions associated with carbapenemase genes (*bla*KPC2/3 gene).

**Keywords:** antimicrobial resistance; *Klebsiella pneumoniae*; *Escherichia coli*; public health; Carbapenemases; β-lactamases; KPC2/3; CTX-M-15; human; Portugal

1. Introduction

*Escherichia coli* is a commensal microorganism of the intestinal microbiota of humans and animals. It is also involved in a great variety of intestinal and extra-intestinal infections as an opportunistic pathogen, including septicemia, as well as urinary and wound infections, among others; this microorganism presents a high facility to acquire antimicrobial-resistant genes [1,2]. In addition, *Klebsiella pneumoniae* can be found in the intestinal microbiota of healthy humans and animals although may also cause life-threatening infections; it is considered a major opportunistic pathogen implicated in nosocomial infections (as is the case of pneumonia or bloodstream infections), especially in patients of intensive-care units (UCIs) and in immunocompromised hosts with severe underlying diseases. *K. pneumoniae* strains can accumulate resistance genes, increasing their pathogenicity and causing severe infections [1,3–5]. Moreover, it is known that *K. pneumoniae* spreads easily, mainly in the hospital environment [5].

During recent years, the emergence and rapid dissemination of *Enterobacteriaceae* carrying genes encoding Extended Spectrum β-lactamases (ESBL), acquired AmpC β-lactamases (qAmpC), or carbapenemases are considered great concerns [6]. In particular, ESBLs of CTX-M-type and qAmpC enzymes (especially the CMY-2 type) were increasingly reported worldwide, specifically among clinical isolates from *E. coli* [7–9] and *K. pneumoniae* [6,10–12]. Moreover, recent studies reported ESBL-producing *K. pneumoniae* isolates among urinary tract infections and invasive infections in patients in Portugal [4,5,9], as well as among healthy Portuguese students in Lisbon [13]. Furthermore, a previous report created by our group showed the presence of ESBL-producing *E. coli* among healthy and sick cats [14] and dogs [15] in Portugal.

According to a recent review conducted by Sengodan et al. [16], more than eighty CTX-M variants have been reported worldwide; the majority of them are more active on cefotaxime than on ceftazidime. Particularly, the CTX-M-15 is frequently found among *Enterobacteriaceae* in humans in Europe [17,18], specifically in both clinical samples and healthy humans in Portugal [19–21]. Moreover, the CTX-M-15 β-lactamase is frequently associated with the uropathogenic international *E. coli* clone ST131 [22,23]. In the last decade, *E. coli* clonal group ST131 has emerged as a high-risk clone with important clinical health concerns causing multidrug-resistant (MDR) infections worldwide [11]. Particularly in Portuguese territory, ST131 clone was detected among residents of nursing homes [22], sick dogs [15], as well as in UTI clinical isolates from humans [24]. Nevertheless, other high-risk international clones have been detected among ESBL- or carbapenemase-producing *E. coli* isolates, as is the case of ST410 [25–27]. Other high-risk *E. coli* clones have been extensively reported in extraintestinal human infections [28].

According to Domokos et al. [18], high-risk *K. pneumoniae* clones, such as ST11 and ST15, have the tenacity and flexibility to accumulate resistance determinants, contributing to the increase of their pathogenicity. Regarding a recent study, the ST258, ST11, ST15, and ST147 clones spread for two decades, and recently, the CTX-M-15-producing ST307 clone in *K. pneumoniae* emerged globally [29]. Particularly, carbapenems are considered last-resort treatment but *K. pneumoniae* acquired resistance to this last resort antibiotic worldwide [30].

On the other hand, a great diversity of SHV variants have been reported, being a group of them of ESBL-type [31] being frequently detected among *K. pneumoniae* isolates, especially the SHV-5 and SHV-12 variants [1,16].
Antimicrobial resistance is commonly related to the spread of plasmids and the acquisition of resistance genes that normally occur by horizontal gene transfer (HGT). Another important aspect is the mutational events, mainly in sequences of genes encoding the target for certain antibiotics. Another mechanism of resistance (mainly in quinolones) is the alteration of the outer membrane proteins associated with active efflux pumps, which finish with the expulsion of the antibiotic out of the bacterial cell [1,16].

To our knowledge, some studies have been performed in Portugal based on the detection of ESBL-producing *Enterobacteriaceae* in hospitalized patients [3,4,12,19,32–36]. In a previous study performed by our research group, the ESBL types were determined among invasive *K. pneumoniae* isolates recovered from blood cultures in a hospital located in Northern Portugal [5]. The purpose of the present study is to expand this previous work by analyzing in the same hospital the ESBLs types and the main associated resistance mechanisms in broad-spectrum cephalosporin-resistant *K. pneumoniae* isolates obtained from different clinical origins (except blood), and also in *Escherichia coli* isolates obtained from blood and urine samples; furthermore, the genetic lineages of selected isolates were also the aim of this research.

2. Materials and Methods

2.1. Bacterial Isolates

A collection of 38 cefotaxime/ceftazidime-resistant (CTX/CAZ\textsuperscript{R}) *E. coli* isolates (18 of urine samples and 20 from blood samples) were obtained from hospitalized patients (one isolate/patient) in a Northern Portuguese hospital (Centro Hospitalar de Trás os Montes e Alto Douro, CHTMAD, Vila Real), between December 2016 and August 2018. A collection of 24 CTX/CAZ\textsuperscript{R} *K. pneumoniae* isolates (18 from the urine; three of bronchial secretion and three from pus/biopsy/catheter origins) were also collected from the same hospital between December 2016 and December 2017. The identification of the isolates was confirmed by the matrix-assisted laser desorption-ionization time-of-flight mass spectrometry method (MALDI-TOF MS) [37,38], following the instructions of the manufacturer (Bruker Daltonik, Bremen, Germany).

2.2. Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing was performed by using Kirby–Bauer disk diffusion method on Mueller-Hinton agar, according to Clinical Laboratory Standards Institute guidelines (CLSI, 2019) [39]. The susceptibility of *E. coli* and *K. pneumoniae* isolates was tested for the following antibiotics (µg/disk): amoxicillin + clavulanic acid (20 + 10), cefoxitin (30), ceftazidime (30), cefotaxime (30), imipenem (10), tetracycline (30), gentamicin (10), streptomycin (10), tobramycin (10), ciprofloxacin (5) and trimethoprim-sulfamethoxazole (1.25 + 23.75). Moreover, the susceptibility for meropenem (10) and ertapenem (10) was also tested for *K. pneumoniae* isolates. The plates were incubated for 24 h at 37 °C. *E. coli* ATCC 25922 was used as a reference strain in susceptibility testing assays.

The screening of phenotypic ESBL production was carried out by the double-disk synergy test using cefotaxime, ceftazidime, and amoxicillin/clavulanic acid discs [39]. Isolates showing resistance to three or more antibiotic classes were considered as MDR.

2.3. DNA Extraction and Quantification

Genomic DNA from CTX/CAZ\textsuperscript{R} *E. coli* isolates was extracted using the boiled method [40], selecting three to five colonies in 1mL of sterile Milli-Q water for 8 min. Moreover, genomic DNA from *K. pneumoniae* isolates was extracted using the InstaGene Matrix (Bio-Rad).

2.4. Detection of Antibiotic Resistance Genes

*E. coli* and *K. pneumoniae* ESBL-positive isolates were screened by PCR/sequencing for the presence of the genes *bla\textsubscript{CTX-M}* (different groups) [41], *bla\textsubscript{SHV}* [42], *bla\textsubscript{TEM}* [41], *bla\textsubscript{CMY-2}, bla\textsubscript{DHA-1}, bla\textsubscript{KPC-2/3}, bla\textsubscript{VIM}, bla\textsubscript{VEB}, bla\textsubscript{OXA-48}, and bla\textsubscript{NDM} [43,44]. The obtained
amplicons were sequenced and analyzed by BLAST software, available at the National Center for Biotechnology Information [45]. The isolates were also screened for the presence of the genes encoding resistance for tetracycline (tetA, tetB) [46] and colistin (mcr-1) [47]. Moreover, the presence of the intI1 gene (encoding the integrase of class 1 integrons) was analyzed on the E. coli isolates obtained from blood origin [48]. Positive controls of the University of La Rioja were used in each of the PCRs carried out in this work.

2.5. Molecular Typing of Selected E. coli and K. pneumoniae Isolates

Phylogenetic classification of all 38 E. coli isolates was performed as previously reported by Clermont et al. [49], according to the existence of arpA, chuA, yjaA, and TSPE4.C2 genes. The E. coli isolates of blood origin were further typed: (a) isolates of phylogroup B2 were screened for their affiliation to sequence type (ST) 131 by a specific PCR-region of the ST131 genome, as previously reported by Doumith et al. [50]; (b) the remaining blood isolates were typed by multilocus-sequence-typing (MLST) with seven housekeeping genes (fumC, adk, purA, icd, recA, mdh, and gyrB). The protocol described on PubMLST (Public databases for molecular typing and microbial genome diversity) was followed [51], and the allele combination was determined after sequencing of the seven genes to determine the sequence type (ST).

Moreover, the MLST was performed on selected K. pneumoniae isolates (based on the type of beta-lactamase genes they carried) by PCR/sequencing of seven housekeeping genes (gapA, phoE, infB, pgi, rpoB, tonB, and mdh) as previously indicated [52].

3. Results

3.1. Antimicrobial Resistance Phenotype in E. coli and K. pneumoniae Isolates

Regarding the 38 CTX/CAZR E. coli isolates, all of them were ESBL-producers (Table 1). High rates of antibiotic resistance were observed among these isolates for ciprofloxacin (n = 36; 94.7%), tobramycin (n = 27; 71.1%), trimethoprim/sulfamethoxazole (n = 25; 65.8%), tetracycline (n = 22; 57.9%), gentamicin (n = 22; 57.9%) and amoxicillin + clavulanic acid (n = 21; 55.3%). Importantly, two isolates showed imipenem resistance (IMP R). All E. coli isolates were categorized as MDR. The detailed antimicrobial resistance profiles of these isolates are listed in Table 1.

All of the 24 CTX/CAZR K. pneumoniae isolates were ESBL producers (100%) and they were considered for further genetic resistance analysis. Interestingly, 16 ESBL-producing K. pneumoniae isolates were also resistant to ertapenem (66.7%), 17 isolates to imipenem (70.8%), and 14 to meropenem (58.3%) (Table 2). Beside for β-lactam antimicrobials, high resistance levels were recorded towards trimethoprim/sulfamethoxazole (n = 24; 100%), ciprofloxacin (n = 22; 91.6%), tetracycline (n = 18; 75%), gentamicin (n = 18; 75%), amoxicillin + clavulanic acid (n = 17; 70.8%) and cefoxitin (n = 7; 29.2%) (Table 2). Moreover, all of the K. pneumoniae isolates showed a MDR-phenotype.
| Sample  | Origin | Date (Month Year) | Resistance Phenotype a | ESBL Production b | B-lactamases | MLST c | Resistant Genes/Integrons d | PG e |
|---------|--------|-------------------|------------------------|-------------------|--------------|--------|----------------------------|------|
| X1068   | Blood  | March 2017        | AMC, CTX, CAZ, CIP, SXT| P                 | CTX-M-15, TEM | ST131   | int1                       | B2   |
| X1080   | Blood  | July 2018         | AMC, CTX, CAZ, TET, TOB, CIP, GEN, SXT | P          | CTX-M-15, TEM | ST131   | int1, tetA                  | B2   |
| X1062   | Blood  | December 2016     | AMC, CTX, CAZ, TET, TOB, CIP, GEN, SXT | P          | CTX-M-15     | ST131   | int1, tetA                  | B2   |
| X1063   | Blood  | December 2016     | AMC, CTX, CAZ, TET, TOB, CIP, GEN, SXT | P          | CTX-M-15     | ST131   | int1, tetA                  | B2   |
| X1064   | Blood  | December 2016     | AMC, CTX, CAZ, TET, TOB, CIP, GEN, SXT | P          | CTX-M-15     | ST131   | int1                        | B2   |
| X1065   | Blood  | June 2017         | AMC, CTX, CAZ, TET, TOB, CIP, GEN, SXT | P          | CTX-M-15     | ST131   | int1, tetA                  | B2   |
| X1066   | Blood  | June 2017         | AMC, CTX, CAZ, TOB, CIP, GEN | P          | CTX-M-15     | ST131   | ND                         | B2   |
| X1067   | Blood  | February 2017     | AMC, CTX, CAZ, TET, TOB, CIP, GEN, SXT | P          | CTX-M-15     | ST131   | int1, tetA                  | B2   |
| X1069   | Blood  | March 2017        | AMC, CTX, CAZ, TET, TOB, CIP, GEN | P          | CTX-M-15     | ST131   | ND                         | B2   |
| X1070   | Blood  | March 2017        | AMC, CTX, CAZ, CIP, GEN | P          | CTX-M-15     | ST131   | ND                         | B2   |
| X1071   | Blood  | April 2017        | AMC, CTX, CAZ, TET, TOB, CIP, SXT | P          | CTX-M-15     | ST131   | int1, tetA                  | B2   |
| X1072   | Blood  | April 2017        | AMC, CTX, CAZ, TET, TOB, CIP, GEN, SXT | P          | CTX-M-15     | ST131   | int1, tetA                  | B2   |
| X1073   | Blood  | April 2017        | AMC, CTX, CAZ, TET, TOB, CIP, GEN, SXT | P          | CTX-M-15     | ST131   | int1, tetA                  | B2   |
| X1074   | Blood  | April 2017        | AMC, CTX, CAZ, TET, TOB, CIP, GEN, SXT | P          | CTX-M-15     | ST131   | int1, tetA                  | B2   |
| X1075   | Blood  | May 2017          | AMC, CTX, CAZ, CIP     | P          | CTX-M-15     | ST410   | ND                         | A    |
| X1076   | Blood  | May 2017          | AMC, CTX, CAZ, TET, TOB, CIP | P          | CTX-M-15     | ST131   | ND                         | B2   |
| X1105   | Blood  | May 2017          | AMC, CTX, CAZ, CIP     | P          | CTX-M-15     | ST410   | ND                         | A    |
| X1079   | Blood  | July 2018         | AMC, CTX, CAZ, TOB, CIP, GEN, SXT | P          | CTX-M-15     | ST131   | int1                       | B2   |
| X1081   | Blood  | August 2018       | AMC, CTX, CAZ, TET, TOB, CIP | P          | CTX-M-15     | ST131   | tetA                       | B2   |
| X1078   | Blood  | July 2018         | CTX, TET, CIP, SXT    | P          | CTX-M-27     | ST131   | ND                         | B2   |
| X3158   | Urine  | February 2017     | CTX, CAZ, TET, TOB, SXT, S | P          | CTX-M-15     | NT      | ND                         | B2   |
| X3159   | Urine  | February 2017     | CTX, CAZ, TET, TOB, CIP, GEN, S | P          | CTX-M-15     | NT      | ND                         | B2   |
| X3160   | Urine  | February 2017     | CTX, CAZ, TET, TOB, CIP, GEN, S | P          | CTX-M-15     | NT      | ND                         | B2   |
| X3161   | Urine  | March 2017        | CTX, CAZ, TET, TOB, CIP, GEN, S | P          | CTX-M-15     | NT      | ND                         | B2   |
| X3162   | Urine  | April 2017        | CTX, CAZ, TET, TOB, CIP, GEN, S | P          | CTX-M-15     | NT      | ND                         | D    |
| X3163   | Urine  | May 2017          | CTX, CAZ, TET, TOB, CIP, SXT, S | P          | CTX-M-15     | NT      | tetA, tetB                  | D    |
| X3164   | Urine  | May 2017          | CTX, CAZ, CIP, SXT, S  | P          | CTX-M-15     | NT      | ND                         | B2   |
| X3165   | Urine  | May 2017          | CTX, CAZ, CIP, S      | P          | CTX-M-15     | NT      | ND                         | B2   |
| X3167   | Urine  | June 2018         | CTX, CAZ, TET, TOB, CIP, GEN, SXT, S | P          | CTX-M-15     | NT      | tetA                       | B2   |
| X3168   | Urine  | August 2018       | CTX, CAZ, TET, TOB, CIP, GEN, SXT, S | P          | CTX-M-15     | NT      | tetA                       | B2   |
| X3169   | Urine  | August 2018       | CTX, CAZ, TET, TOB, CIP, GEN, SXT, S | P          | CTX-M-15     | NT      | tetA                       | B2   |
| X3170   | Urine  | August 2018       | CTX, CAZ, TET, CIP, S | P          | CTX-M-15     | NT      | tetB                       | B2   |
| X3173   | Urine  | June 2018         | AMC, FOX, CTX, CAZ, IMP, TET, CIP, SXT, S | P          | CTX-M-15     | NT      | tetB                       | A    |
| X3157   | Urine  | February 2017     | CTX, TET, SXT, S      | P          | CTX-M-1       | NT      | tetA                       | C    |

Table 1. Resistance phenotype and genotype in the 38 CTX/CAZ<sup>R</sup> *Escherichia coli* isolates from clinical samples in a Portuguese hospital.
Table 1. Cont.

| Sample | Origin | Date (Month Year) | Resistance Phenotype a | ESBL Production b | B-lactamases | MLST c | Resistant Genes/Integrons d | PG e |
|--------|--------|-------------------|------------------------|------------------|--------------|--------|----------------------------|------|
| X3155  | Urine  | December 2016     | CTX, TOB, CIP, SXT, S  | P                | CTX-M-variant| NT     | ND                         | B2   |
| X3166  | Urine  | May 2018          | ERT, TOB, CIP, GEN, SXT, S | P            | CTX-M-variant| NT     | ND                         | B2   |
| X3171  | Urine  | December 2016     | CTX, TOB, CIP, SXT, S  | P                | CTX-M-variant| NT     | ND                         | NC   |
| X3156  | Urine  | June 2017         | AMC, FOX, CTX, CAZ, IMP, TET, TOB, CIP, GEN, SXT, S | P            | KPC2/3       | NT     | tetA                      | B2   |

Legend: a AMC: amoxicillin + clavulanic acid; FOX: cefoxitin; CTX: cefotaxime; CAZ: ceftazidime; CHL: chloramphenicol; CIP: ciprofloxacin; TOB: tobramycin; GEN: gentamicin; SXT: trimethoprim + sulfamethoxazole; S: streptomycin; TET: tetracycline; IMP: imipenem; b P—Positive, N—Negative; c MLST—MultiLocus Sequence Typing; d NT: not tested; ND: not detected; NC: Not concluded; e Phylogroups.

Table 2. Resistance phenotype and genotype present in the 24 CTX/CAZ R Klebsiella pneumoniae isolates from clinical samples in a Portuguese hospital.

| Sample | Origin | Date (Month Year) | Antimicrobial Resistance Phenotype a | ESBL Production b | β-lactamases | MLST c | Other Genes/Int d |
|--------|--------|-------------------|------------------------|------------------|--------------|--------|------------------|
| X2175  | Urine  | June 2017         | AMC, CTX, CAZ, IMP, MRP, ERT, CIP, SXT, S | P                | CTX-M-15, KPC-2/3, SHV-12, TEM | ST15   | ND               |
| X2143  | Urine  | December 2016     | AMC, FOX, CTX, CAZ, IMP, MRP, ERT, TET, CIP, GEN, SXT, S | P                | CTX-M-15, KPC-2/3, SHV-27, TEM | ST280  | tetA             |
| X2153  | Urine  | June 2017         | AMC, FOX, CTX, CAZ, IMP, MRP, ERT, TET, CIP, GEN, SXT, S | P                | CTX-M-15, KPC-2/3, SHV-27, TEM | NT     | tetA             |
| X2155  | Urine  | February 2017     | AMC, CTX, CAZ, IMP, MRP, ERT, TET, CIP, GEN, SXT, S | P                | CTX-M-15, KPC-2/3, SHV-27, TEM | NT     | tetA             |
| X2156  | Urine  | May 2017          | AMC, CTX, CAZ, IMP, MRP, ERT, TET, CIP, GEN, SXT, S | P                | CTX-M-15, KPC-2/3, SHV-27, TEM | NT     | tetA             |
| X3098  | Urine  | December 2016     | CTX, CAZ, TET, CIP, TOB, GEN, SXT, S | P                | CTX-M-15, SHV-27 | NT     | tetA             |
| X3100  | Urine  | March 2017        | CTX, CAZ, IMP, TET, CIP, TOB, SXT, S | P                | CTX-M-15, SHV-27 | NT     | tetA             |
| X3104  | Urine  | May 2017          | AMC, CTX, CAZ, IMP, MRP, ERT, TET, CIP, TOB, SXT, S | P                | CTX-M-15, SHV-11 | NT     | ND               |
| X2157  | Urine  | April 2017        | AMC, FOX, CTX, CAZ, IMP, MRP, ERT, CHE, CIP, GEN, SXT, S | P                | CTX-M-15, KPC-2/3, SHV-28, TEM | ST15   | ND               |
| X3095  | Urine  | December 2016     | CTX, CAZ, MRP, TET, CIP, TOB, GEN, SXT, S | P                | CTX-M-15, SHV-28 | NT     | tetA             |
| X3105  | Urine  | May 2017          | AMC, CTX, CAZ, ERT, TET, CIP, TOB, GEN, SXT, S | P                | CTX-M-15, SHV-28 | NT     | tetA             |
| X3106  | Urine  | May 2017          | CTX, CAZ, ERT, TET, CIP, TOB, GEN, SXT, S | P                | CTX-M-55, SHV-11 | NT     | tetA             |
| X2142  | Urine  | December 2016     | AMC, FOX, CTX, CAZ, IMP, MRP, ERT, CHE, CIP, GEN, SXT, S | P                | SHV-12, KPC-2/3, TEM | ST147  | ND               |
| Sample | Origin          | Date (Month Year) | Antimicrobial Resistance Phenotype | ESBL Production | β-lactamases | MLST | Other Genes/Int |
|--------|-----------------|-------------------|-----------------------------------|-----------------|---------------|------|-----------------|
| X3092  | Urine           | December 2016     | AMC, CTX, CAZ, IMP, TET, TOB, CIP, GEN, SXT, S | P               | SHV-12        | NT   | ND              |
| X3096  | Urine           | December 2016     | CTX, CAZ, IMP, TET, CIP, SXT, S    | P               | SHV-12        | NT   | tetA           |
| X3097  | Urine           | December 2016     | CTX, CAZ, IMP, TET, TOB, CIP, GEN, SXT, S | P               | SHV-12        | NT   | tetA           |
| X3107  | Urine           | May 2017          | AMC, FOX, ERT, CTX, CAZ, TET, CIP, SXT, S | P               | SHV-27, KPC-2/3, TEM | ST280 | tetA |
| X2232  | Urine           | January 2017      | AMC, CTX, CAZ, IMP, MRP, ERT, TET, CIP, GEN, SXT, S | P               | CTX-M-15, KPC-2/3, SHV-11 | NT   | ND             |
| X3085  | Bronchial secretion | December 2017 | AMC, FOX, CTX, CAZ, IMP, MRP, ERT, TET, TOB, CIP, GEN, SXT, S | P               | CTX-M-15, SHV-11 | NT   | tetA           |
| X3094  | Pus             | December 2017     | CTX, CAZ, TET, TOB, GEN, SXT, S    | P               | CTX-M-15, SHV-11 | NT   | ND             |
| X3102  | Biopsy          | May 2017          | AMC, CTX, CAZ, TOB, GEN, SXT, S    | P               | CTX-M-15, SHV-11 | NT   | ND             |
| X3087  | Bronchial secretion | December 2016 | AMC, FOX, CTX, CAZ, IMP, MRP, ERT, TET, TOB, CIP, GEN, SXT, S | P               | CTX-M-15, SHV-27 | NT   | tetA           |
| X3088  | Bronchial secretion | June 2017       | AMC, CTX, CAZ, IMP, MRP, ERT, TOB, CIP, GEN, SXT, S | P               | CTX-M-15, KPC-2/3, SHV-28 | NT   | ND             |
| X3101  | Catheter        | April 2017        | AMC, CTX, CAZ, IMP, MRP, ERT, TOB, CIP, SXT, S | P               | CTX-M-55, SHV-11 | NT   | tetA           |

Legend: * AMC: amoxicillin + clavulanic acid; FOX: cefoxitin; CTX: cefotaxime; CAZ: ceftazidime; CHL: chloramphenicol; CIP: ciprofloxacin; TOB: tobramycin; GEN: gentamicin; SXT: trimethoprim + sulfamethoxazole; S: streptomycin; TET: tetracycline; IMP: imipenem; ERT: ertapenem; MRP: meropenem; b P—Positive, N—Negative; c MLST—MultiLocus Sequence Typing; d NT: not tested; ND: not detected.
3.2. Genetic Characteristics of ESBL- or Carbapenemase-Producing E. coli Isolates

Most of the 38 ESBL-producers E. coli isolates carried a bla\textsubscript{CTX-M} gene, mainly the bla\textsubscript{CTX-M-15} gene (n = 32; 84.2%) (Table 1). Furthermore, the bla\textsubscript{CTX-M-27} gene was detected in one isolate (blood origin) and the bla\textsubscript{CTX-M-1} was positive for another isolate (urine origin); the bla\textsubscript{CTX-M} variant could not be detected in three additional isolates. Interestingly, two of the ESBL-positive isolates were also IMP\textsuperscript{R} and both of them carried the bla\textsubscript{KPC-2/3} gene. Likewise, resistance to tetracycline was conferred, particularly, by the tet\textsubscript{A} (n = 16) or tet\textsubscript{B} (n = 3) genes. Moreover, the mcr-1 gene, conferring colistin resistance, was not detected among our clinical E. coli isolates. The int1 gene was found among most strains from blood origin (n = 12) (Table 1).

ESBL-positive E. coli isolates were ascribed mainly to the phylogenetic group B2 (n = 31; 75.6%), followed by A (n = 3), D (n = 2), and C (n = 1) phylogroups; according to Clermont et al. (2013) [34], the phylogenetic group associated with the remaining isolate was not conclusive. It is important to note that most of the ESBL-positive E. coli isolates from blood origin were typed as ST131/B2 (n = 18/20), although also we could identify two ST410/A isolates (Table 1).

3.3. Genetic Characteristics of ESBL-Producing K. pneumoniae Isolates

Regarding the 24 ESBL-producing K. pneumoniae isolates, the bla\textsubscript{CTX-M-15} gene was the most common among these isolates (n = 16 isolates, 66.7%), which 11 of them belong to urine origin (Table 2). Furthermore, the bla\textsubscript{CTX-M-55} gene was also found among these isolates (n = 2). A high diversity of SHV variants was detected, specifically SHV-27 (n = 8, ESBL- type), SHV-28 (n = 4), SHV-11 (n = 6, associated with CTX-M-15 or CTX-M-55), and SHV-12 (n = 6, ESBL-type and frequently associated with KPC2/3 gene) (Table 2). Regarding the K. pneumoniae isolates recovered from urine, 8 of them also carried the bla\textsubscript{TEM} gene. Moreover, the mcr-1 gene was not detected among K. pneumoniae isolates.

Three sequence types (from 5 selected isolates) belonging to major international lineages of human pathogenic β-lactamases-producing K. pneumoniae isolates were identified as follows (sequence-type/associated ESBLs): ST15/CTX-M-15 + SHV-12, ST15/CTX-M-15 + SHV-28, ST280/CTX-M-15 + SHV-27, ST280/SHV-27, and ST147/SHV-12 (Table 2).

4. Discussion

In our study, the ESBL-producing E. coli isolates were mainly associated with the carriage of the bla\textsubscript{CTX-M-15} gene (>80%) (Table 1). This finding is according to a previous result obtained in E. coli isolated from nosocomial settings in Portugal [19]. In Germany, this enzyme was also the most prevalent ESBL type (around 50% of the strains investigated) [17]. Recently, the bla\textsubscript{CTX-M-15} gene was the most frequently detected among E. coli of both sick and healthy dogs and cats in Portugal [14,15]. Nevertheless, the bla\textsubscript{CTX-M-1} was the most common ESBL gene among healthy students in Portugal (Lisbon), followed by the bla\textsubscript{CTX-M-15} gene [13].

Other Portuguese reports showed the detection of qAmpC β-lactamase-producing E. coli recovered from clinical settings (DHA and/or CMY-2) [8] and non-clinical isolates (CMY-2) [20]. Furthermore, the CMY-2 encoding gene was recently reported among healthy/sick cats in Portugal [14]; although these genes were tested, they were not found among our isolates. The ESBL-positive E. coli isolates analyzed in this study were ascribed mainly to the phylogenetic group B2 (Table 1). These findings are according to the study conducted by Zhang et al. [53], showing the high prevalence of phylogenetic group B2 among urinary E. coli isolates from human patients in the USA. Similar results were recently obtained among blood isolates in Spain [54], as well as among healthy humans [55] and nursing home residents [22], both studies performed in Portugal. Furthermore, the B2 phylogroup was the most frequent among dogs with UTI in Portugal [56]. Contrastingly, other authors reported a phylogenetic diversity among clinical isolates from humans and/or pets in Germany [17] and Switzerland [11]. The B2 group is frequently associated with virulent extra-intestinal strains of humans/animals, frequently found in human
ExPEC infections; the group D is also associated with extra-intestinal infections although at a lower rate. Contrastingly, B1 and A are ubiquitous and more associated with commensal \textit{E. coli} both in humans and vertebrate animals.

The CTX-M-15 gene is frequently associated with a specific international lineage, the epidemic clone ST131, which has spread worldwide [15,57]. According to Belas et al. (2019) [56], the \textit{E. coli} ST131/CTX-M-15/B2 is the most disseminated \textit{E. coli} clonal group worldwide associated with an extensive antimicrobial resistance profile; this lineage was found among 90\% of our \textit{E. coli} clinical isolates (Table 1), which represent a public health concern. Particularly in Portuguese territory, ST131 clone was detected among residents of nursing homes [22], sick dogs [15], as well as in healthy humans [35].

Moreover, the ST410 clone was identified among \textit{E. coli} isolates in this study (Table 1). This clone was previously reported in clinical dog samples in Liverpool [58], Switzerland [11], France [59], and Portugal [60]. According to a recent report, \textit{E. coli} ST410 should be classified as a potential new high-risk international clone [25]. Regarding clinical isolates from humans, this clone is widely distributed, mainly in Danish patients [25] and those from Southeast Asia [61].

Considering the ESBL-producing \textit{K. pneumoniae} isolates, the \textit{bla}_{\text{CTX-M-15}} gene was the most commonly detected (66.7\%) (Table 2), similarly with the results obtained in different countries in clinical samples from humans [3,5,10,62] and pets [63–65]. Moreover, this gene was the most commonly detected in \textit{K. pneumoniae} isolated from septicemias obtained from the same hospital of the present study (CHTMAD, Vila Real, Portugal) [5]. This gene was also detected among non-hospitalized patients in Portugal, according to a recent study [66]. Furthermore, the \textit{bla}_{\text{CTX-M-15}} gene was the most detected among companion animals in Italy [67] and Germany [17]. These data related with CTX-M-15 producers over-predominating is according with the majority of European countries, and this variant is widely distributed among both humans and pets.

Furthermore, a high diversity regarding SHV variants (ESBL and not ESBL ones) detected among our isolates is according to the results obtained on clinical isolates in Portugal (SHV-11/12), Spain (SHV-12), China (SHV-12/27), India (SHV-28), and Brazil (SHV-27) [4,34,68–71]. Moreover, Carvalho et al. (2021) [5] detected also different variants of the \textit{bla}_{\text{SHV}} gene (\textit{bla}_{\text{SHV-1}}, \textit{bla}_{\text{SHV-11}}, or \textit{bla}_{\text{SHV-27}}) among \textit{K. pneumoniae} isolates of blood cultures in the same hospital. It is important to note that a recent previous study showed the detection of \textit{bla}_{\text{CTX-M-15}} gene (associated in most cases with \textit{bla}_{\text{SHV-28}} gene) among \textit{K. pneumoniae} isolated from healthy and sick dogs in Portugal [65]. Curiously, the \textit{bla}_{\text{SHV-12}} (ESBL-variant) was widely reported in wildlife [31,72,73].

KPC-3-producing bacteria is endemic in many countries, but just recently appears in Portuguese hospitals. This fact is in line with our study and can be explained by the increase in carbapenems consumption. Specifically, according to the European Centre for Disease Prevention and Control (ECDC) [74], Portugal is considered one of the top carbapenem consumers in Europe (10.9\% in 2019). The \textit{bla}_{\text{KPC-2/3}} gene was also detected among Portuguese hospitalized patients [5,32,75], as well as in non-hospitalized patients [66]. According to Rodrigues et al. (2016) [66], the widespread distribution of KPC-3 among \textit{K. pneumoniae} clinical isolates in Portugal was associated with successful high-risk clones ST147 and ST15, similarly with our results.

Three sequence types belonging to major international lineages of human pathogenic \(\beta\)-lactamases-producing \textit{K. pneumoniae} were identified in this study. The ST15, associated with CTX-M-15, was detected in two isolates (Table 2). This clone was previously reported in other parts of the world among both hospital and community settings, namely in Portugal [4,5] and the Netherlands [76], indicating their global spread. Particularly, this clone was recently detected in a blood sample from the same hospital, associated with SHV-106/TEM production [5]. Furthermore, recent studies showed the presence of ST15/CTX-M-15 among healthy pets in Portugal [77], as well as sick dogs [65]. A possible explanation is the fact of HGT is caused by the proximity between humans and companion animals.
Curiously, the ST280 was found among two *K. pneumoniae* isolated from urine samples. This infrequent lineage was found among a patient in Korea [78] and in New York (in this last case, associated with the CTX-M-15 gene) [79].

On the other hand, the ST147 clone was associated with the spread of SHV-12 in a *K. pneumoniae* urine isolate in our study (Table 1); this lineage was also found among blood/urine samples in Portuguese hospitals, also associated with SHV-12 [12] or SHV-1/11+ KPC-2/3 [5].

5. Conclusions

ESBL-producing *Enterobacteriaceae* are endemic in different Portuguese clinical settings. In conclusion, the present study revealed the presence of ESBL-producing *Enterobacteriaceae* among clinical isolates from a hospital located in Northern Portugal, with the dominance of spread of CTX-M-15 co-harboring SHV-type ESBLs, and subsequently KPC-2/3 gene.

This work also showed the diversity of ESBLs associated with high-risk international clones (ST15, ST147, and ST280 clones in *K. pneumoniae*, and the ST131 and ST410 in *E. coli*), which play an important role in dissemination in hospital settings, and increased frequency in nosocomial infections with human health impact. A relentless vigilance of the evolution of the ESBL situation and the application of a One Health interdisciplinary approach is necessary to keep this problem under control.

**Author Contributions:** I.C. conceptualization, sampling, methodology, investigation, resources, data curation, writing and rewriting; J.A.C. sampling, methodology; S.M.-Á. investigation, data curation; R.C. validation, funding acquisition; C.A.-C. validation, funding acquisition; F.R. review and editing; M.S. investigation, data curation, writing—review and editing; G.I. conceptualization, methodology, validation, resources, data curation, writing—review and editing, visualization, supervision, project administration, funding acquisition; C.T. conceptualization, methodology, validation, resources, data curation, writing—review and editing, visualization, supervision, project administration, funding acquisition; P.P. conceptualization, methodology, validation, resources, data curation, writing—review and editing, visualization, supervision, project administration, funding acquisition. All authors have read and agreed to the published version of the manuscript.

**Funding:** I.C. gratefully acknowledges the financial support of “Fundaç on para a Ciência e Tecnologia” (FCT—Portugal) related to Ph.D. grant, through the reference SFRH/BD/133266/2017 (Medicina Clínica e Ciências da Saúde), as well as MCTES (Ministério da Ciência, Tecnologia e Ensino Superior) and European Union (EU), with reference to Fundo Social Europeu (FSE). The experimental work carried out in the University of La Rioja (Spain) was financed by the project SAF2016-76571-R from the Agencia Estatal de Investigación (AEI) of Spain and FEDER of EU. This work was partially supported by the *Ministerio de Ciencia, Innovación y Universidades* (Spain; grant number RTI2018-098267-R-C33), the *Junta de Castilla y León* (Consejería de Educación, Spain; grant number LE018P20) and the Associate Laboratory for Green Chemistry—LAQV which is financed by national funds from FCT/MCTES (UIDB/50006/2020 and UIDP/50006/2020).

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** The data used to support the findings of this study are available from the corresponding author upon request.

**Conflicts of Interest:** The authors declare no conflict of interest. Preliminary data was presented as a poster entitled “Genetic diversity among selected ESBL and Carbapenem-producing *Klebsiella pneumoniae* isolates from urocultures in a Portuguese hospital” in ECA 2021 Webinar congress. Part of this research was submitted to ESHG 2021 Webinar congress, with the following title “Resistance profile and genetic diversity among selected ESBL-producing *Escherichia coli* isolates from urocultures in a Portuguese hospital” and ECCMID 2021 Webinar Congress, entitled “Detection of ESBL and Carbapenem-resistant *Klebsiella pneumoniae* isolated from hospitalized patients”.


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