Confronting Ceftolozane-Tazobactam Susceptibility in Multidrug-Resistant Enterobacterales Isolates and Whole-Genome Sequencing Results (STEP Study)

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A R T I C L E   I N F O

A B S T R A C T

Ceftolozane-tazobactam (C/T) is frequently used for infections caused by multidrug-resistant (MDR)-Enterobacterales isolates. Whole-genome sequencing (WGS, Illumina-Hiseq 4000/NovaSeq 6000, OGC, UK) was used to study the population structure, the resistome and the virulome of C/T-susceptible and -resistant MDR *Escherichia* spp. (n=30) and *Klebsiella* spp. (n=78) isolates, recovered from lower respiratory, intra-abdominal and urinary tract infections of ICU patients from 11 Portuguese Hospitals (STEP study, 2017-2018). Minimum inhibitory concentrations (MICs) were determined (ISO-broth microdilution, breakpoints EUCAST-2020). In *Escherichia* spp., a weak concordance between the phenotypic and the WGS method (*P*=0.051) was observed in the carbapenemase detection (3/30) [bla_{VIM-2} (2/3), bla_{KPC-3} (1/3)]; VIM-2-*Escherichia coli* isolates were C/T-susceptible and only the KPC-3-*Escherichia marmotae* producer showed C/T-resistance. Overall, CTX-M-15-*E. coli*-ST131-O25:H4-H30-Rx (11/30) was the most frequent subclone, followed by CTX-M-27-*E. coli*-ST131-O25:H4-H30 (4/4). Moreover, a wide resistome and virulome were detected in all *E. coli* isolates. Among *Klebsiella* spp. isolates [*K. pneumoniae* (67/78), *K. aerogenes* (7/78), *K. oxytoca* (2/78), *K. variicola* (2/78)], concordance (*P*<0.001) was observed between the phenotypic and the genomic carbapenemase detection (21/78) [bla_{KPC-3} (14/21), bla_{OXA-48} (3/21), $bla_{OXA-181}$ (3/21)]. A high correlation between C/T-resistance and carbapenemase detection was established (*P*<0.05). Overall, a high clonal diversity was observed, mainly in KPC-3-producing *K. pneumoniae* isolates. An extensive resistome was detected in *Klebsiella* spp. isolates, whereas virulence determinants were mostly identified in carbapenemase producers (*P*<0.001). WGS is a powerful tool for typing characterization and microbiological study of MDR-Enterobacterales pathogens. Furthermore, carbapenemase genes are associated with C/T-resistance in *Klebsiella* spp., but other mechanisms might also be involved.

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1. INTRODUCTION

Whole-genome sequencing (WGS) represents a promising approach for the characterization of nosocomial pathogens and its implementation in clinical laboratories for antimicrobial resistance surveillance is challenging for Public Health. The utility of genomic tools application in typing characterization, phylogenetic analysis and the determination of resistome/virulome in clinical isolates has been widely reported [1–3]. WGS has also been proposed as a powerful tool in nosocomial outbreak management, mainly in hospital departments with an increased risk of infection with multidrug-resistant (MDR) pathogens, such as the Intensive Care Units (ICU) [4,5]. Moreover, several studies have been performed to demonstrate the inference or prediction of antibiotic susceptibility profiles from the genomic data obtained using sequencing technologies and bioinformatics analysis tools [6,7].

In Portugal, as in other European countries, *Klebsiella pneumoniae* and *Escherichia coli* are the most common extended-spectrum β-lactamase (ESBL)-producing Enterobacterales species detected in both hospital and community settings [8–10]. Carbapenemase epidemiology in Portugal has been commonly related to the intestinal carriage of Gram-negative bacteria (GNB)-producing class B metallo-β-lactamases (MBLs), such as VIM, NDM and IMP enzymes [11]. Nevertheless, a recent increase of carbapenemase-producing Enterobacterales (CPE) isolates causing infections has been reported in Portuguese Hospitals due to KPC-3 (class A carbapenemase)-producing *K. pneumoniae* isolates [12,13].

Effort has recently been focused on the development of new β-lactam-β-lactamase inhibitor combinations, such as ceftolozane-tazobactam (C/T), to combat more effectively infections caused by MDR organisms. C/T shows a high antimicrobial spectrum of activity against MDR-GNB, mostly *Pseudomonas aeruginosa* and ESBL-producing Enterobacterales isolates, but has limited activity against microorganisms producing carbapenemases and a minority of AmpC β-lactamases [14]. Furthermore, resistance to C/T has already been described in ESBL-producing *K. pneumoniae* and *E. coli* isolates [15–17].

During the STEP study [18], *P. aeruginosa* and Enterobacterales clinical isolates were recovered from ICU patients admitted to 11 Portuguese hospitals in 2017 and 2018. During this study, susceptibility of C/T against Enterobacterales isolates was 84.0%. The present work comprised the study of the resistance mechanisms involved in C/T susceptibility, the population structure, the resistome and the virulome of a subset of MDR-Enterobacterales (*E. coli* and *Klebsiella* spp.) isolates from the STEP collection, with both C/T-susceptible and -resistant phenotypes, using the WGS approach.

2. MATERIAL AND METHODS

2.1. Background and selection of isolates

A total of 426 Enterobacterales (175 *E. coli*, 151 *Klebsiella* spp. and 100 other Enterobacterales) clinical isolates were collected from patients admitted to ICUs of 11 Portuguese Hospitals between June 2017 and July 2018 as part of the prospective multicenter STEP study [18]. Isolates were recovered from urinary tract (UTI), intra-abdominal (IAI) and lower respiratory tract (LRTI) infections to study the in vitro activity of C/T and comparators. The Ramón y Cajal University Hospital (Madrid, Spain) was the central laboratory for the microbiological study and subsequent genome analysis. This surveillance study was approved by the ethics committees of all participating Portuguese Hospitals. Extended-spectrum β-lactamases (ESBL) phenotype was detected in 16.6% and 28.5% of *E. coli* and *Klebsiella* spp., respectively, whereas 1.7% (*E. coli*) and 17.9% (*Klebsiella* spp.) presented a phenotype compatible with car-

bapenemase production. *E. coli* showed a high susceptibility level to C/T (98.9%), but activity was moderate in *Klebsiella* spp. (66.2%) [18].

A total of 110 Enterobacterales isolates (31 *E. coli* and 79 *Klebsiella* spp.) were selected for genome characterization using whole-genome sequencing (WGS). Selection was based on ceftolozane-tazobactam in vitro activity, ESBL and carbapenemase phenotypes, following the EUCAST-2020 criteria (http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v_10.0_Breakpoint_Tables.pdf). Phenotype distribution of selected isolates is shown in Table 1.

2.2. DNA extraction and whole-genome sequencing

Total DNA extraction was performed from 2 mL of exponential growth LB cultures using the Chemagic DNA Bacterial External Lysis Kit (PerkinElmer, Waltham, MA, USA) and following the manufacturer's instructions. Short-read sequencing was carried out using the Illumina Hiseq4000 or the Illumina NovaSeq 6000 platforms (OGC, Oxford, UK), with 2 × 150 pb paired-end reads.

2.3. Sequence processing and analysis

FastQC v.0.11.8 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and Prinseq-lite-0.20.3 (http://prinseq.sourceforge.net/) tools were used for quality control and filtering of sequences, respectively. Preprocessing short-reads *de novo* assembling and assembly evaluation were performed using SPAdes v3.11.1 and QUAST v5.0.2 (http://quast.bioinf.sbau.ru/), respectively [19]. Bacterial identification was confirmed by k-mer-based classification using Kraken v1.0 [20]. *De novo*-assembled contigs were annotated by Prokka v.1.13.3 [21].

2.4. Typing characterization

MASH (v2.1) and iTOL application (Interactive Tree Of Life, https://itol.embl.de/) were used to generate and trace a similarity tree based on neighbor-joining algorithm [22]. MLST v2.16.1 (https://github.com/tseemann/mlst) was used for in silico MLST assignment. *E. coli* phylogroups, serotypes and fimH types were determined using ClermonTyping [23], SerotypeFinder and FimTyper (https://cge.cbs.dtu.dk/services/) tools, respectively. *E. coli*-ST131-H30Rx sublineage was identified based on a specific SNV, the G723A point mutation in *ybbW*, using Blastn (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The C1-M27 clade-specific region (prophage-like region M27PP2+M27PP1) was also detected using Blastn and a reference sequence (GenBank accession number LC209430.1) [24]. In *Klebsiella* spp. isolates, *wzi* gene, capsule (K) and LPS (O) serotype prediction was performed using Kleborate software v.0.4.0-beta [25,26].

2.5. Resistome and virulome analysis

Acquired resistance mechanisms and virulence genes were identified using Abricate v0.8.11 and ResFinder, ARG-ANNOT and VFDB databases (threshold, 95% identity; 90% coverage). ARIBA tool was also used to confirm the presence/absence of carbapenem resistance genes [27]. Chromosomal point mutations related to antibiotic resistance in *E. coli* isolates were identified using PointFinder software (https://cge.cbs.dtu.dk/services/). *K. pneumoniae* integrative conjugative element (ICE*Kp*)-associated virulence loci [yersiniabactin (ybt), colibactin (clb)] and virulence plasmid-associated loci [salmochelin (iro), aerobactin (iuc), hypermucoidy (rmpA, rmpA2)] were also detected using Kleborate [28,29].

Table 1

Selection of Enterobacterales isolates for whole-genome sequencing during the STEP study [18]. Phenotypes were defined according to the minimum inhibitory concentration (MIC) values obtained by standard broth microdilution.

Bacterial spp.	C/T phenotype[1]	ESBL phenotype[2]	CP phenotype[3]	Non-ESBL-non-CP phenotype	Total
E. coli	C/T susceptible	28	2	-	**30**
	C/T resistant	-	1	-	**1**
	Total	**28**	**3**	**-**	**31**
Klebsiella spp.	C/T susceptible	23	9	7	**39**
	C/T resistant	17	22	1	**40**
	Total	**40**	**31**	**8**	**79**

C/T= ceftolozane-tazobactam; CP= carbapenemase; ESBL= extended-spectrum β-lactamase

[1] C/T resistance phenotype (MICs >2 mg/L).

[2] ESBL phenotype (MICs ≥2 mg/L for cefotaxime, ceftazidime and/or cefepime).

[3] CP phenotype (MICs >1 mg/L for imipenem and/or >0.12 mg/L for meropenem).

2.6. Statistical analysis

Concordance between in vitro antibiotic activity results and genotype data based on genome sequence analysis was evaluated using the Kappa Index (weak agreement <0.40, moderate 0.41-0.60, accurate 0.61-0.80, very accurate >0.81). Fisher's Exact test was used to estimate differences in frequencies and percentages of dichotomous variables and to calculate their odds ratio (OR) with the 95% confidence interval (95%CI). All statistical analysis was performed using R software (RStudio Team 2016 version 1.0.44, RStudio, Boston, MA, USA). A *P*-value <0.05 was considered as statistically significant.

2.7. Sequence data

All complete *Escherichia* spp. and *Klebsiella* spp. sequences were deposited at DDBJ/ENA/GenBank under the BioProject accession number PRJNA602991. Genome accession numbers are shown in Table S1.

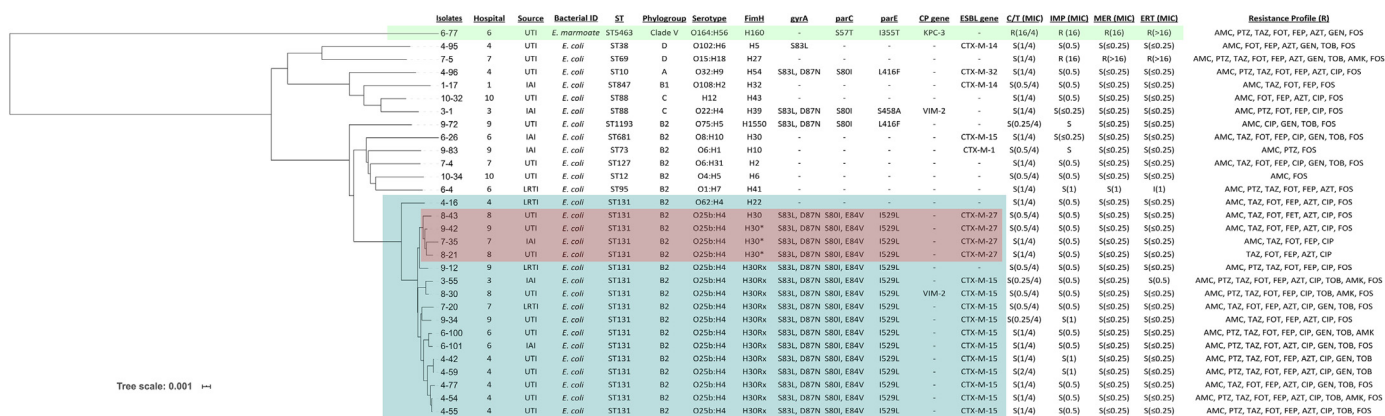## 3. RESULTS

3.1. Genome characteristics

Assembly of sequenced isolates revealed an average genome size of approximately 5.2 Mb for *Escherichia* spp. and 5.5 Mb for *Klebsiella* spp. isolates, with an average G+C content of 50.6% and 56.9%, respectively. Information about all *Escherichia* spp. and *Klebsiella* spp. genome characteristics is summarized in Table S2 and

Table S3, respectively. Two contaminated sequences were identified (1 *E. coli* and 1 *Klebsiella* spp.) and were excluded from the subsequent analysis.

3.2. Molecular epidemiology

E. coli bacterial identification was confirmed in 29 isolates (96.7%, 29/30); the remaining *Escherichia* isolate (Isolate 6-77) was identified as *Escherichia marmotae* with 99.8% identity (Figure 1). According to in silico MLST, ST131 was the most prevalent clone among *E. coli* isolates, accounting for 17 (58.6%) of the 29 studied isolates (Figure 1). A total of 94% (16/17) of the ST131-*E. coli* isolates carried the *fimH30* allele, belonged to the serotype O25:H4 and were assigned to phylogroup B2 (ST131-B2-O25:H4-H30 lineage). Among these isolates, ST131-H30Rx was the predominant subclone (12/16). Moreover, the complete C1-M27 clade–specific region (prophage-like region M27PP2+M27PP1) was identified in three ST131-H30-non-Rx isolates and a truncated M27PP1 region was detected in another (Isolate 8-43). A higher diversity of MLST-clones, serotypes, phylogroups and *fimH* alleles were identified in the remaining *E. coli* isolates of this study (Figure 1).

Among the *Klebsiella* spp. isolates, *K. pneumoniae* was the most common species (n=67) followed by *K. aerogenes* (n=7), *K. oxytoca* (n=2) and *K. variicola* (n=2). Overall, a higher clonality than in *E. coli* isolates was observed and ST307 (n=7), ST13 (n=6), ST15 (n=6), ST405 (n=5), ST17 (n=4), ST45 (n=4) and ST280 (n=4) were the predominant *K. pneumoniae* clones (Figure 2). Association was

Figure 1. Similarity tree showing the relationship among the *Escherichia* spp. isolates from the STEP study and the genomics data. Antimicrobial susceptibility results are also included [18]. Branch length is indicative of the MASH-distance. Ceftolozane-tazobactam-resistant *E. marmotae* isolate is framed in the green box. Blue box includes all ST131-*E. coli* isolates. Purple box includes the sublineage ST131-*E. coli*-H30-non-Rx. *CTX-M-27-producing ST131-*E. coli*-H30 isolates that belongs to the C1-M27 clade. ST=Sequence type; UTI= Urinary tract infection; IAI= intra-abdominal infection; LRTI= lower respiratory tract infection; CP= carbapenemase; ESBL= extended-spectrum β-lactamase; MIC= minimum inhibitory concentration; C/T= ceftolozane-tazobactam; IMP= imipenem; ERT= ertapenem; MER= meropenem; AMC= amoxicillin-clavulanic acid; PTZ= piperacillin-tazobactam; TAZ= ceftazidime; FOT= cefotaxime; FEP= cefepime; AZT= aztreonam; CIP= ciprofloxacin; GEN= gentamicin; TOB= tobramycin; AMK= amikacin; FOS= fosfomycin.
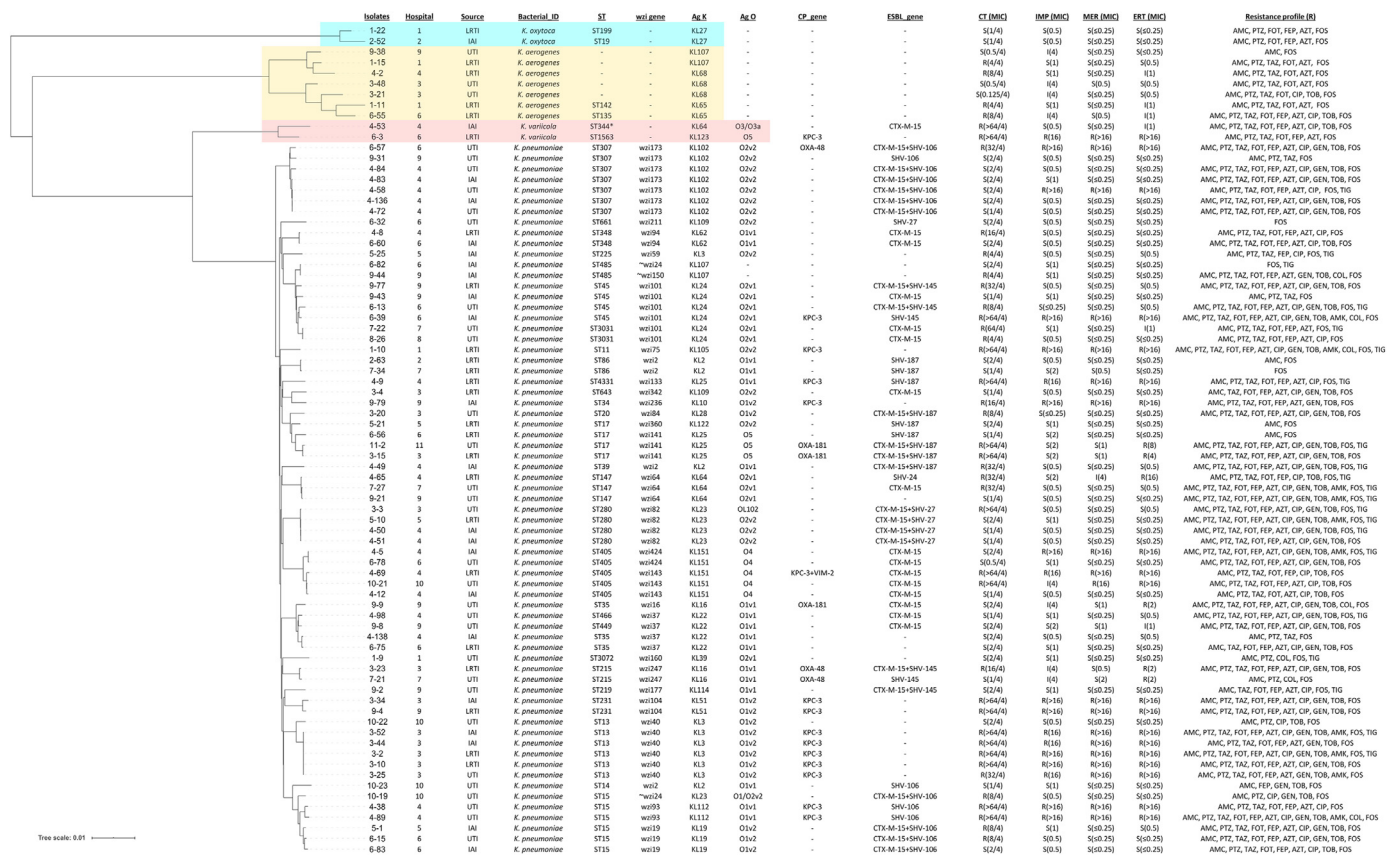
Figure 2. Similarity tree showing the relationship among the *Klebsiella* spp. isolates from the STEP study and the genomics data. Antimicrobial susceptibility results are also included [18]. Branch length is indicative of the MASH-distance. Colored box indicates the *Klebsiella* species different to *K. pneumoniae*: blue (*K. oxytoca*), orange (*K. aerogenes*) and pink (*K. variicola*). ST= Sequence type; UTI= Urinary tract infection; IAI= intra-abdominal infection; LRTI= lower respiratory tract infection; CP= carbapenemase; ESBL= extended-spectrum β-lactamase; MIC= minimum inhibitory concentration; C/T= ceftolozane-tazobactam; IMP= imipenem; ERT= ertapenem; MER= meropenem; AMC= amoxicillin-clavulanic acid; PTZ= piperacillin-tazobactam; TAZ= ceftazidime; FOT= cefotaxime; FEP= cefepime; AZT= aztreonam; CIP= ciprofloxacin; GEN= gentamicin; TOB= tobramycin; AMK= amikacin; COL= colistin; FOS= fosfomycin; TIG= tigecycline.

observed between the assigned ST and the *wzi* gene/serotype K (Antigen K)/serotype O (Antigen O) (Figure 2).

3.3. Genomic antibiotic resistance characterization

Overall, a high content of antibiotic resistance genes was found among the studied *Escherichia* spp. collection (Figure S1). Carbapenemase genes detection was demonstrated in three *Escherichia* spp. isolates (10%, 3/30) [bla_{KPC-3}-*E. marmotae* (n=1) and bla_{VIM-2}-*E. coli* (n=2)] and a weak concordance [Kappa Index (k)= 0.35; P=0.051] was observed in the carbapenemase detection between the phenotypic and WGS-based genotypic method. In this collection, the two VIM-1 *E. coli*-producers were susceptible to C/T ($MIC_{C/T}$= 0.5/4-1/4 mg/L) and only the KPC-3-producing *E. marmotae* isolate (3.3%, 1/30) showed a C/T-resistant phenotype ($MIC_{C/T}$= 16/4 mg/L). Correlation between the presence of carbapenemase genes and resistance to C/T could not be fully determined (OR= 0.0; 95%CI= 0.0-4.3; P=0.1). All ST131-*E. coli* isolates were susceptible to C/T ($MIC_{C/T}$=0.25/4-2/4 mg/L) and were mostly related to ESBL enzymes production (94.1%, 16/17). $bla_{CTX-M-15}$ and $bla_{CTX-M-27}$ genes were associated with ST131-H30Rx (11/12) (OR=116.4; 95%CI=8.2-7760.4; P<0.001) and ST131-H30-C1-M27 (4/4) (OR=0.0; 95%CI=0.0-0.9; P<0.001) sublineages, respectively. VIM-2 production was also detected in one ST131-H30-Rx-CTX-M-15 isolate. Moreover, *gyrA* (S83L, D87N), *parC* (S80I, E84V) and *parE* (I529L) fluoroquinolone resistance mutations in quinolone resistance-determining region (QRDR) were only found in ST131-

H30Rx and ST131-H30-C1-M27 isolates (OR=0.0; 95%CI= 0.0-0.06; P<0.001) (Figure 1).

An extensive resistome was also observed in the *K. pneumoniae*, *K. oxytoca* and *K. variicola* isolates (Figure S2). Carbapenemase- (26.9%, 21/78) and ESBL- (65.4%, 51/78) encoding genes were detected, mainly in the *K. pneumoniae* species. Moreover, both carbapenemase and ESBL genes were identified in 14.1% (11/78) of cases. Overall, the Kappa Index showed a very accurate agreement (k=0.88; P<0.001) between in vitro carbapenems activity and the WGS detection of carbapenemase-encoding genes. Based on sequence data, bla_{KPC-3} (n=14) was the most common carbapenemase, followed by bla_{OXA-48} (n=3) and $bla_{OXA-181}$ (n=3). Moreover, co-production of two carbapenemases (KPC-3+VIM-2) was also identified in one isolate. Most frequent associations between carbapenemase genes and *K. pneumoniae* clones were: KPC-3-ST13 (n=5), KPC-3-ST15 (n=2), KPC-3-ST231 (n=2), OXA-181-ST17 (n=2) and OXA-48-ST215 (n=2). The 90.5% of the carbapenemase-*Klebsiella* spp. isolates (19/21) showed resistance to C/T ($MIC_{C/T}$=4/4->64/4 mg/L) and correlation between C/T-resistant phenotype and carbapenemase gene detection was demonstrated (OR=15.7; 95%CI=3.3-152.5; P<0.001). Note that all KPC-3-*Klebsiella* spp. isolates were resistant to the C/T combination (OR=0.0; 95%CI=0.0-0.2; P<0.001).

Among non-carbapenemase producers (73.1%, 57/78), bla_{ESBL} genes were identified in 70.2% (40/57) of cases and correlation with resistance to ceftolozane-tazobactam (37.5%, 15/40) ($MIC_{C/T}$= 8/4->64/4 mg/L) was not established (OR=1.1; 95%CI=0.3-4.4; P>0.05). Associations between particular bla_{ESBL} genes and *K.*

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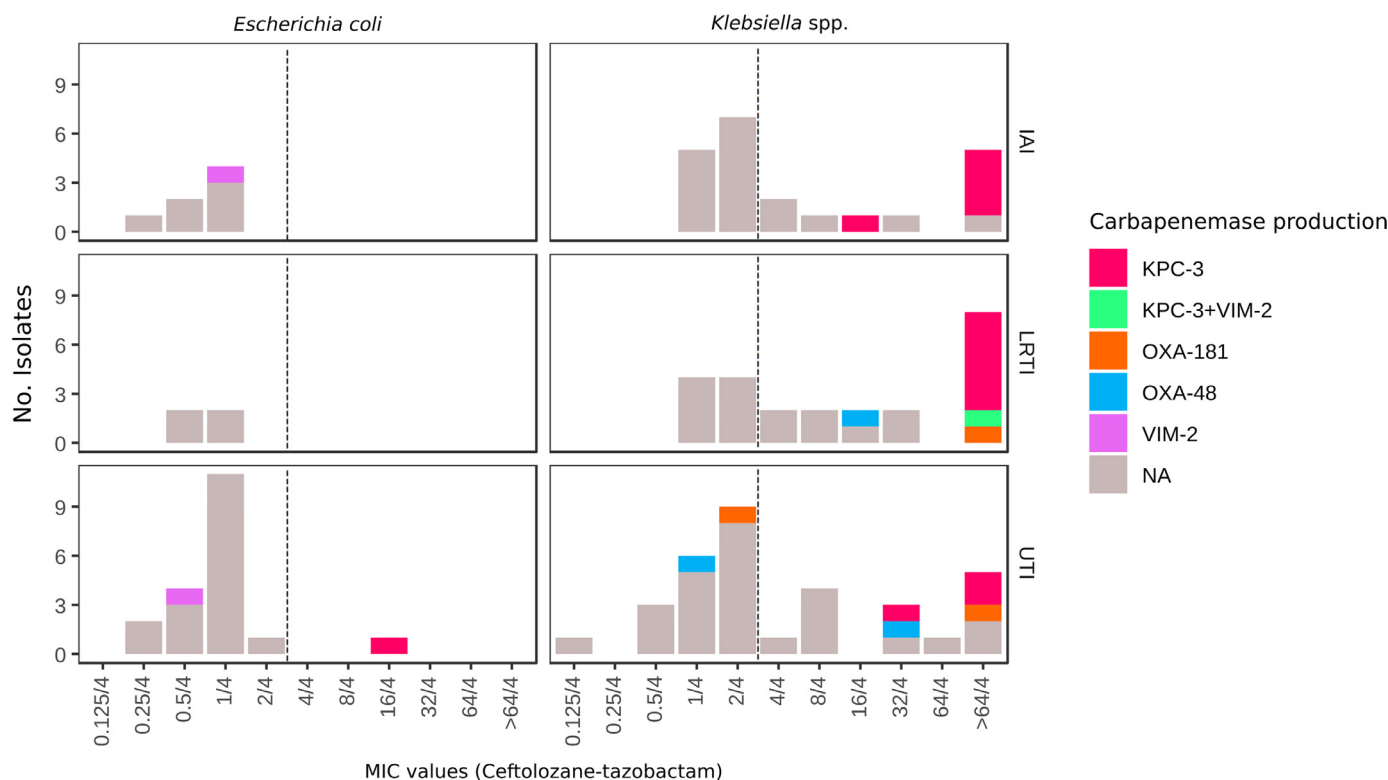
Figure 3. Distribution of both *E. coli* and *Klebsiella* spp. isolates of the STEP study according to the different minimum inhibitory concentration (MIC) values of ceftolozane-tazobactam (C/T) [18], the carbapenemase-encoding gene detected and the infection source (IAI, intra-abdominal infection; LRTI, lower respiratory tract infection; UTI, urinary tract infection). Dotted line represents the ceftolozane-tazobactam EUCAST-2020 breakpoint (S≤2 mg/L; R>2 mg/L). In *Klebsiella* spp., carbapenemase gene detection was statistically correlated to a ceftolozane-tazobactam-resistant phenotype (*P*<0.001). NA= carbapenemase-encoding gene not detected.

pneumoniae clones were also detected: CTX-M-15+SHV-106-ST307 (n=5), CTX-M-15+SHV-106-ST15 (n=4), CTX-M-15+SHV-27-ST280 (n=4), CTX-M-15-ST405 (n=4), CTX-M-15-ST45 (n=3), SHV-187-ST17 (n=2), CTX-M-15-ST3031 (n=2), CTX-M-15-ST348 (n=2) and SHV-187-ST86 (n=2). Moreover, a total of 17 *Klebsiella* spp. isolates showed a non-ESBL-non-carbapenemase genotype (21.8%, 17/78) and six of them (35.3%, 6/17) were resistant to C/T (MIC$_{C/T}$= 4/4-8/4 mg/L) (Figure 2). Distribution of both *E. coli* and *Klebsiella* spp. isolates according to the C/T minimum inhibitory concentration (MIC$_{C/T}$) values, the carbapenemase-encoding gene detected and the infection source (IAI, LRTI and UTI) is shown in Figure 3.

On the other hand, a phenotype compatible with colistin resistance (MIC=4->4 mg/L) was also found in seven *K. pneumoniae* isolates (8.9%, 7/78), five of them with a carbapenemase gene (Figure 2). However, *mcr* genes implicated in the colistin-transferable resistance mechanism were not detected (Figure S2).

3.4. Virulence determinants characterization

Overall, the *E. coli* isolates (29/30) showed a high virulence gene content (Figure S3). All C/T-susceptible *E. coli* isolates (96.7%, 29/30) presented a high number of virulence loci (n=11-26), and these were longer in the ST131-*E. coli* isolates (n=16-22). In the *E. marmotae* strain with a C/T-resistant phenotype (isolate 6-77), only the *iro* virulence locus (*iroB, iroC, iroD, iroE, iroN*) was detected (Figure S3).

Virulence determinants were identified in a lower number of *Klebsiella* spp. isolates (62.8%, 49/78) (47 *K. pneumoniae*, 1 *K. aerogenes* and 1 *K. oxytoca*) (Figure S4). Seven distinct ICE*Kp* lineages [ICEKp3 (n=7), ICEKp4 (n=15), ICEKp5 (n=1), ICEKp10 (n=5), ICEKp11 (n=2), ICEKp12 (n=7), ICEKp-unknown (n=9)] were assigned to 46 (59%, 46/78) *Klebsiella* spp. isolates (45 *K. pneumo-*

niae and 1 *K. aerogenes*) in which *ybt* (iron-scavenging siderophore yersiniabactin) (58.9%, 46/78) and *clb* (genotoxin colibactin) (7.5%, 6/78) virulence determinants were identified (Figure S4). Virulence loci encoding aerobactin (*iuc*) and salmochelin (*iro*) siderophore synthesis, in addition with hypermucoidy genes (rmpA, rmpA2), were also detected in two other *K. pneumoniae* strains (2.5%, 2/78) (Figure S4). Moreover, *ybt, clb, iuc, iro* and *rmpA* genes were more frequently detected in *K. pneumoniae* isolates that carried carbapenemase-encoding genes (OR=4.0; 95%CI=1.1-18.5; *P*=0.02). However, correlation between these virulence loci and the resistance to C/T was not established (OR=2.1; 95%CI=0.8-5.9; *P*=0.16).

4. DISCUSSION

In this work, WGS was used to study the epidemiological and microbiological characteristics of a representative collection of MDR-Enterobacterales isolates recovered from ICU patients admitted to Portuguese Hospitals as a part of the multicenter STEP study [18]. Implementation of the WGS-based approach for the prediction of antibiotic resistance phenotypes is challenging for clinical laboratories. However, although genome sequencing may be a rapid and cost-effective method to characterize MDR pathogens, the effectiveness between genotype-to-phenotype relationship is still uncertain [3]. Some studies have demonstrated a high concordance between the conventional antimicrobial susceptibility methods and the in silico antimicrobial resistance phenotype prediction using WGS [6,7]. In this work, a very accurate correlation was established between both methods in *Klebsiella* spp., but relevant discrepancies were observed in *E. coli* isolates. Moreover, in concordance with other studies [30], carbapenemase-encoding genes were not detected by the sequencing method in a low proportion of carbapenem-resistant Enterobacterales isolates and other resis-

tance mechanisms, such as mutations in resistance genes affecting permeability (i.e. *ompK35, ompK36* and *ompK37, ramR* and *acrR* genes), could also be involved (data not shown). In addition, resistance to colistin was phenotypically detected in 9% of *Klebsiella* spp. isolates, although transferable genes (i.e. *mcr-1*) related to this resistance mechanism were not identified based on the genome analysis. Resistance to colistin due to chromosomal mutations has been largely demonstrated in Enterobacterales isolates, but acquisition and dissemination of *mcr*-encoding genes plasmid-mediated colistin-resistance is also increasing [31]. However, surveillance studies performed in Denmark to detect the *mcr-1* gene in ESBL-, carbapenemase- and AmpC-producing *E. coli* isolates using WGS have demonstrated that the prevalence of this gene is very low [32].

According to our previous study, C/T exhibited a good overall spectrum activity against non-carbapenemase-producing Enterobacterales isolates [18]. In the present work, correlation between carbapenemase detection using WGS and resistance to C/T was demonstrated in *Klebsiella* spp. isolates, particularly in KPC-3-producing *K. pneumoniae* isolates. In addition, ESBL production was also identified (37.5%) among non-carbapenemase-producing *Klebsiella* spp. isolates with a C/T-resistant phenotype, although correlation was not established. In the *E. coli* collection, conclusive results were not found, probably because the STEP study did not include a high enough number of *E. coli* isolates harboring a carbapenemase gene and/or with a C/T-tazobactam-resistance phenotype.

Overall, concordant with previous data in Portugal [12,13], KPC-3-producing *K. pneumoniae* isolates were the predominant MDR-Enterobacterales pathogen in ICU patients during the STEP study. A great diversity of *K. pneumoniae* high-risk clones was observed associated with the KPC-3 carbapenemase, including some lineages first reported in Portuguese Hospitals (ST13, ST34, ST405, ST1563, ST4331) [13]. The most common carbapenemase-*K. pneumoniae* clone in this study was ST13-KPC-3, recently detected in Argentina associated also with KPC-3 production [33]. ST13-*K. pneumoniae* lineage has also been identified associated with the OXA-48 carbapenemase in clinical samples in Ireland [34] and Finland [35], and in ecological niches in Algeria [36]. The nosocomial dissemination of the ST405-*K. pneumoniae* clone has been widely detected related to different carbapenemases in other South European countries, such as Spain [37,38] and Italy [39]. According to Portuguese reports, other *K. pneumoniae* clones detected in the STEP study have been previously related not only with hospital outbreaks (ST45-KPC-3-*K. pneumoniae*), but also with non-hospitalized patients (ST231-KPC-3-*K. pneumoniae*) [12]. To the best of our knowledge this is also the first description in Portugal of the ST17 and ST215 *K. pneumoniae* clones producing OXA-181 and OXA-48 carbapenemases, respectively. Interestingly, the ST17-*K. pneumoniae* clone had been previously identified in Portugal but not in association with carbapenemase production [12]. The ST17-OXA-181-*K. pneumoniae* clone description has only been performed in hospitals from Singapore [40] and as a cause of nosocomial outbreaks in South Africa [41].

Notably in this study, carbapenemase production in *Klebsiella* spp. isolates was significantly related to the detection of the virulence genes, *ybt, clb, iuc, iro* and *rmpA* that have been previously associated with hypervirulent and invasive *K. pneumoniae* infections [28,29]. Regarding the ESBL-*K. pneumoniae* isolates, a greater diversity of clones was observed in the STEP collection than in other studies performed in Portugal [9]. These findings reveal that the global spread of both carbapenemase- and ESBL-producing *K. pneumoniae* is also happening in departments of Portuguese hospitals with a higher risk of infection, such as the ICU.

ST131 was the most common *E. coli* clone detected in ICU patients during the STEP study. In Portugal, the worldwide disseminated ST131-H30 high-risk clone was previously identified not only in fecal carriers related to CTX-M-15 and CTX-M-27 enzymes [8], but also in clinical isolates producing KPC-21 [12]. The high virulence profile of these isolates combined with the resistance to fluoroquinolones and more recently to β-lactams antibiotics have played a critical role in the dissemination of the high-risk clone ST131-H30 sublineage, and are currently the most common extra-intestinal pathogenic *E. coli* (ExPEC) clone [42]. In the present study, ST131-H30 was the most common *E. coli* sublineage, and CTX-M-15-producing ST131-H30-Rx was the predominant subclone, followed by CTX-M-27-producing ST131-H30.

In conclusion, KPC-3-producing *K. pneumoniae* and CTX-M-15-producing ST131-H30Rx-*E. coli* are the most common MDR-Enterobacterales causing infections in ICU patients from Portuguese Hospitals. Moreover, carbapenemase-encoding genes are associated with C/T resistance in *Klebsiella* spp., but other mechanisms may also be involved in other species such as *E. coli*. WGS is a powerful tool for the typing characterization and study of the resistome and virulome of these nosocomial pathogens. However, the ability to establish genotype-to-phenotype relationships in MDR-Enterobacterales isolates may still be limited for an appropriate genome sequencing clinical application.

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Competing Interests

Dr. Rafael Canton has participated in educational programmes organized by MSD and Pfizer. Dra. Margarida F. Pinto had a travel grant for ECCMID-2019 from MSD Portugal. Leonor Pássaro, Laura Paixão and João Romano are MSD Portugal employees and/or may hold stock options in Merck & Co., Inc., Kenilworth, NJ, USA. The other authors have no conflict of interests.

Ethical Approval

Not required

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2020.106259.

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