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Prevalence of carbapenemase genes among carbapenem-nonsusceptible *Enterobacterales* collected in US hospitals in a five-year period and activity of ceftazidime/avibactam and comparator agents

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Objectives: To evaluate the prevalence of acquired β-lactamase genes and susceptibility profiles of carbapenem-nonsusceptible *Enterobacterales* (CNSE) clinical isolates collected in US hospitals during a 5-year period.

Methods: Isolates were susceptibility tested by reference broth microdilution methods. Results were interpreted using CLSI breakpoints. Isolates displaying nonsusceptible MICs for imipenem or meropenem were categorized as CNSE. CNSE isolates were screened for β-lactamase-encoding genes using whole-genome sequencing. New genes were cloned, expressed in an *Escherichia coli* background and susceptibility tested.

Results: A total of 450 (1.3%) isolates were CNSE. *Klebsiella pneumoniae* serine carbapenemase (KPC) production was the most common resistance mechanism among CNSE isolates: 281/450 (62.4%) carried bla_{KPC}, including three new variants. OXA-48-like and metallo-β-lactamase (MBL) encoding genes were detected among seven and 12 isolates, respectively. Among MBL genes, bla_{NDM-1} was the most common, but bla_{NDM-5}, bla_{VIM-1} and bla_{IMP-27} were also identified. 169 (37.6% of the CNSE) isolates did not produce carbapenemases. Ceftazidime/avibactam was the most active agent (95.0% to 100.0% susceptible) against CNSE isolates from all carbapenemase groups except MBL-producing isolates. Ceftazidime/avibactam, meropenem/vaborbactam and imipenem/relebactam inhibited 100.0%, 97.6% and 92.3% of the non-carbapenemase CNSE isolates, respectively. Among the three new bla_{KPC} variants, one conferred resistance to ceftazidime/avibactam and low meropenem MIC results while the other two had profiles similar to bla_{KPC-2} or bla_{KPC-3}.

Conclusions: A decline in carbapenemase production was noticed in US hospitals in the 5-year period analysed in this study. New β-lactam/β-lactamase inhibitor combinations tested had good activity against CNSE isolates.

Introduction

Carbapenemase production is the most common resistance mechanism against carbapenems among *Enterobacterales* species.[1,2] The most common carbapenemases detected in the USA and in most countries belong to the *Klebsiella pneumoniae* serine carbapenemase (KPC) family, mainly KPC-2 and KPC-3.[3–6] The occurrence of metallo-β-lactamases (MBL) and oxacillinases with carbapenemase activity in the US is much lower but their prevalence is not well defined.[2, 5, 7]

Carbapenemases often confer high MIC values to carbapenem agents; however, isolates with MIC values within the carbapenem intermediate or susceptible ranges have been reported.[8] Carbapenem resistance levels are multifactorial and depend on: (i) the hydrolytic profile of the enzyme; (ii) the gene copy number, which can relate to the strength of the gene promoter and/or the location of the gene; (iii) the host species; and (iv) the presence of other resistance mechanisms, among other features.[9–12] While KPC enzymes hydrolyse carbapenems well, enzymes belonging to the OXA-48 family have weaker hydrolytic activity against these agents.[13] Among MBLs, VIM enzymes have cephalosporins as their preferred substrate and their carbapenem hydrolysis is much lower when compared to the enzymes of the IMP and NDM families.[8] Last, KPC enzymes seem to have much better expression in *Klebsiella pneumoniae* when compared to other species, resulting in higher carbapenem MIC results.[14]

Carbapenem resistance can also be caused by non-enzymatic resistance mechanisms alone or in combination with

β-lactamases that have poor carbapenem hydrolysis.[15, 16] Outer membrane alterations that decrease the entry of β-lactams into the cell and increase the efflux contribute to a low number of carbapenem molecules in the periplasmic space.[17, 18] These alterations allow for the accumulation of β-lactamase molecules that could bind to the fewer carbapenem molecules available. Isolates harbouring these resistance mechanisms can have intermediate carbapenem MIC values or be resistant to some carbapenems, but not all.

Isolates displaying carbapenem-nonsusceptible MIC values include isolates categorized as resistant or intermediate to one or more carbapenems. These isolates are a challenge to conventional diagnostic methods since the presence of resistance mechanisms does not always translate into *in vitro* resistance as defined by current guidelines and breakpoints.[19] This lack of correlation between the presence of resistance mechanisms and *in vitro* resistance challenges clinical laboratories and physicians who must decide how to treat infections caused by these isolates.

In this study, we evaluated the prevalence of carbapenemase genes and other acquired β-lactamase genes among 450 carbapenem-nonsusceptible *Enterobacterales* (CNSE) isolates collected in 68 US hospitals between 2016 and 2020.

Materials and methods

Bacterial isolates and susceptibility testing

Enterobacterales isolates ($n = 34\,623$) collected in 86 US hospitals from 2016 to 2020 were submitted to a central laboratory as part of the International Network for Optimal Resistance Monitoring (INFORM) Surveillance Program.[20] Only bacterial isolates determined to be significant, i.e. the reported probable cause of an infection by local clinical and/or microbiological criteria, were included in this investigation. Participating sites were asked to submit consecutive isolates collected from patients hospitalized with pneumonia, urinary tract (UTI), bloodstream (BSI), skin and skin tissue (SSSI) or intraabdominal (IAI) infections.

Species identification was confirmed when needed by MALDI-TOF–MS using the Bruker Daltonics MALDI Biotyper (Billerica, MA, USA) following the manufacturer's instructions.

Antimicrobial susceptibility testing was performed by reference broth microdilution methods conducted according to CLSI procedures.[21] Quality control (QC) testing was performed to ensure proper test conditions. QC strains used were *Escherichia coli* ATCC 25922 and NCTC 13353, *K. pneumoniae* ATCC 700603 and ATCC BAA-1705, and *Pseudomonas aeruginosa* ATCC 27853. CLSI guidelines were used for the interpretation of susceptibility rates, with the exception of tigecycline, for which US FDA breakpoints were applied.[22, 23] Avibactam was provided by Allergan. Other agents were acquired from Sigma-Aldrich (Saint Louis, MO, USA) or U.S. Pharmacopeia (Rockville, MD, USA). Imipenem/relebactam and meropenem/vaborbactam were tested against CNSE isolates only. Relebactam and vaborbactam powders were acquired from Advanced Chemblocks (Hayward, CA, USA) or MedChemExpress (New Jersey, NY, USA).

Carbapenem-nonsusceptible enterobacterales (CNSE) definition

Enterobacterales isolates displaying nonsusceptible MIC values for imipenem and/or meropenem according to CLSI breakpoints (≥2 mg/L) were further evaluated. Imipenem MIC results were not included in the CNSE criteria for *Proteus* spp. or indole-positive Proteeae due to their intrinsically elevated MIC values for this carbapenem. Species identification for CNSE isolates was confirmed using MALDI–TOF–MS.

Characterization of β-lactam resistance mechanisms

All 450 CNSE isolates were submitted to whole-genome sequencing using the Nextera XT™ library construction protocol and index kit (Illumina, San Diego, CA, USA) following the manufacturer's instructions and then were sequenced on a MiSeq Sequencer (Illumina) with a target coverage of 30×. FASTQ format files for each sample set were assembled independently using *de novo* assembler SPAdes v.3.9.0[24] with *K* values of 21, 33, 55, 77 and 99 and careful mode on to reduce the number of mismatches. This process produced a FASTA format file of contiguous sequences with the best N50 value. In-house-designed software used the target assembled sequences[25] as queries to align against numerous resistance determinants from the NCBI Bacterial Antimicrobial Resistance Reference Gene Database (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA313047) to search for β-lactamase genes. Potential matches were generated with the criteria of >94% identity and 40% minimum coverage length.[26]

Isolates displaying ceftazidime/avibactam resistance were evaluated for transcription levels of *acrA*, *ompC*, *ompF*, *ompK35* and *ompK36*. Total mRNA was extracted and purified using the RNeasy Mini Kit (Qiagen, Germantown, MD, USA) in the QIAcube workstation according to the manufacturer's instructions. Residual DNA was eliminated by treatment with RNAse-free DNase (Promega, Madison, WI, USA) and quantification of mRNA and sample quality were assessed using the RNA 6000 Pico kit from Agilent on the Agilent 2100 Bioanalyzer, again according to the manufacturer's instructions.

The transcription levels ($\Delta\Delta C_T$) were determined in triplicate by quantitative real-time PCR reactions in the StepOne Plus instrument (Life Technologies, Foster City, CA, USA) using standard protocols.[27] Gene expression results were compared to ATCC strains (*Escherichia coli* ATCC 25922, *K. pneumoniae* ATCC 700603, *Enterobacter cloacae* ATCC 700323 and *Serratia marcescens* ATCC 8100) that displayed susceptible MIC values for cephalosporins and carbapenems.[27–29]

Transcription levels were considered significantly different if a 10-fold decrease for OMPs or a 3-fold increase for *acrA* was noted.

Characterization of β-lactamase-encoding genes

Amplicons containing the open reading frame and promoter region of the new bla_{KPC} genes were amplified using primers Forward: TTACTGCCA GTTGACGCCCAATCCC and Reverse: TTACTGCCAGTTGACGCCCAATCCC and cloned using the CloneSmart HCKan Blunt Cloning Kit (Lucigen, Middleton, WI, USA). The colonies obtained after transformation in E.cloni® *E. coli* strain (Lucigen) were selected on plates containing 30 mg/L kanamycin. The presence and orientation of inserts was confirmed by PCR and sequencing. MIC testing was performed as described previously.

Results

CNSE isolates in the US hospitals

Among 34 623 *Enterobacterales* isolates submitted to the surveillance study, 450 (1.3%) were nonsusceptible to either imipenem or meropenem. CNSE isolates were mainly collected from patients hospitalized with pneumonia (204/450 isolates; 45.3%) or UTI (122/450; 27.1%; Supplementary Figure S1, available as Supplementary data at *JAC* Online). Despite the high number of UTI isolates among CNSE, the overall prevalence of CNSE among all UTI *Enterobacterales* isolates submitted to the programme was only 0.8%. CNSE rates among IAI and SSSI isolates were 1.2% (24/450; 5.3%) and 1.1% (47/450; 10.4% of the CNSE),

respectively. Overall CNSE rates among BSI was 0.8% (53/450; 11.8%). Among patients hospitalized with pneumonia, the CNSE rate was 3.2% (data not shown). Among the CNSE isolates, 309 (68.7%) were carbapenem resistant (CRE).

The prevalence of CNSE among *Enterobacterales* isolates varied by US Census Division (Figure 1). The highest CNSE rates were observed in the Middle Atlantic (3.3%) and the lowest rates were observed in New England and the West North Central divisions (0.4% for both). Similar CNSE rates were noted in the East North Central, East South Central and South Atlantic regions (0.8 to 1.0%). CNSE numbers were slightly greater in the Pacific (1.1%) and West South Central (1.7%) divisions.

CNSE rates demonstrated a slight decline over time being 1.5% in 2016, 1.4% in 2017, 1.3% in 2018, 1.2% in 2019 and 1.1% in 2020 (chi-square *P* value = 0.014). *K. pneumoniae* (195/450; 43.3%) was the most common species among CNSE, followed by *Enterobacter cloacae* species complex (71; 15.8%), *Serratia marcescens* (54; 12.0%), *Klebsiella aerogenes* (44; 9.8%) and *E. coli* (35; 7.8%); the remaining 51 CNSE isolates were from 10 other species/species complexes (Figure 2).

Carbapenemase-encoding genes were detected in 281/450 (62.4%) CNSE isolates (Supplementary Figure S2) and 268/309 (86.7%) CRE isolates. The most common of these genes was bla_{KPC-3}, which was noted in 153 isolates from 31 hospitals. bla_{KPC-2} was the second most common gene detected among 94 CNSE isolates recovered from 23 hospitals. Most isolates carrying bla_{KPC-3} and bla_{KPC-2} were *K. pneumoniae* (153/247). Isolates harbouring these genes were noted in all US Census Divisions except New England.

Serine-carbapenemase genes bla_{KPC-4}, bla_{KPC-6} and $bla_{SME-2/-4}$ were detected in three (two *E. cloacae* and one *E. coli*), one (*E. cloacae*) and eight (*S. marcescens*) isolates, respectively. Additionally, three *K. pneumoniae* isolates carried bla_{KPC-56}, bla_{KPC-58} and bla_{KPC-59}. Since these genes were not previously described, these isolates were further characterized.

The Ambler class D carbapenemase gene bla_{OXA-48} and its variants were detected among seven isolates: $bla_{OXA-181}$, $bla_{OXA-232}$ and bla_{OXA-48} (three, three and one isolates, respectively). Additionally, one isolate harboured $bla_{OXA-232}$ and bla_{NDM-1}.

The number of MBL-producing isolates in this survey of US hospitals was relatively low. Only 12/450 (2.7%) CNSE isolates carried an MBL gene, including six bla_{NDM-1} (one of these isolates also carried $bla_{OXA-232}$), three bla_{NDM-5}, two bla_{VIM-1} and one bla_{IMP-27}. The bacterial species/species complex carrying MBL genes were four *E. cloacae* (three bla_{NDM-1} and one bla_{VIM-1}), two *E. coli* (one bla_{NDM-1} and one bla_{NDM-5}), three *K. pneumoniae* (three bla_{NDM-5} and one bla_{NDM-1} plus $bla_{OXA-232}$) and one each of *P. mirabilis* (bla_{IMP-27}) and *S. marcescens* (bla_{VIM-1}). MBL-producing isolates were detected mainly in the Middle Atlantic Division (7/12), but one MBL-producing isolate was detected in each of the following regions: New England, East South Central, West South Central, Mountain and Pacific.

A total of 169/450 (37.6%) CNSE did not carry carbapenemase genes. Most carbapenemase-negative CNSE belong to species known to hyperproduce chromosomal AmpC. These species included *K. aerogenes*, *E. cloacae*, *S. marcescens* and *Citrobacter freundii* species complex (42, 32, 31 and three isolates, respectively). Additionally, 31 *K. pneumoniae*, 13 *E. coli*, eight *P. mirabilis* and eight other species of CNSE did not harbour carbapenemase genes.

Acquired β-lactamase genes were detected among only 27.8% (47/169) of the carbapenemase-negative CNSE (Supplementary Table S1). The gene $bla_{CTX-M-15}$ was detected among 32 isolates: 22 *K. pneumoniae*, eight *E. coli* and two other species. Other bla_{CTX-M} variants were detected among five isolates alone or with other enzymes. These variants were identified in two *E. coli*, two *K. pneumoniae* and one *E. cloacae*. The ESBL genes bla_{SHV-12} and $bla_{TEM-155}$ were detected in one *K. pneumoniae* and one *P. mirabilis*, respectively. The transferable AmpCs gene bla_{CMY-2} was detected in two *E. coli*. One *Raoultella* spp. isolate harboured bla_{FOX-5} and five isolates carried bla_{TEM-1}.

All 42 *K. aerogenes*, three *C. freundii* species complex and 30 of 32 *E. cloacae* did not carry acquired β-lactamase genes.

Antimicrobial activity against CNSE isolates

Ceftazidime/avibactam was the most active agent tested against all CNSE isolates, and it inhibited 96.9% of the isolates (Table 1). Meropenem and imipenem inhibited 28.7% and 7.6% of these isolates, respectively. Other β-lactam agents alone or in combination with the β-lactamase inhibitors sulbactam and tazobactam inhibited 4.0% to 27.1% of the CNSE isolates. Among other antimicrobial classes, 95.3% and 82.4% displayed high susceptibility to tigecycline and amikacin when applying the current US FDA/CLSI breakpoints. A total of 78.2% of the CNSE isolates were categorized as intermediate to colistin.

The susceptibility of β-lactam agents was reduced among the 281 isolates producing carbapenemases, except for ceftazidime/avibactam, which inhibited 95.0% of the isolates. Cefepime and meropenem inhibited only 7.8% and 6.8% of the carbapenemase-producing CNSE isolates, respectively. Tigecycline (96.4% susceptible) was the only agent belonging to other antimicrobial classes that inhibited >75% of the carbapenemase-producing CNSE isolates (Table 1).

Ceftazidime/avibactam inhibited 98.5% of the 269 isolates producing serine carbapenemase, including isolates harbouring bla_{OXA-48}-like genes without MBLs. Furthermore, this combination agent was active against 98.9% of the 262 isolates carrying Ambler class A serine-carbapenemase genes (bla_{KPC} and bla_{SME}). All agents from non-β-lactam antimicrobial classes displayed similar activities against these groups and the overall carbapenemase-producing isolate set.

As expected, the activity of all β-lactam agents was limited against the 12 MBL producers. Aztreonam inhibited only 25.0% of these isolates. Tigecycline inhibited all MBL-producing isolates at the US FDA breakpoint. Amikacin was active against 83.3% of these isolates and 75.0% of them exhibited colistin-intermediate MIC values. One *K. pneumoniae* isolate carrying bla_{NDM-5} and one *P. mirabilis* carrying bla_{IMP-27} were susceptible to ceftazidime/avibactam.

Carbapenemase-negative CNSE isolates were tested against meropenem/vaborbactam and imipenem/relebactam in addition to the other agents tested against all CNSE. Carbapenemase-negative CNSE displayed higher susceptibility rates than carbapenemase-producing isolates to most β-lactam agents tested (Table 1). Meropenem inhibited 65.1% of these isolates while imipenem inhibited only 17.2% at 8 mg/L (CLSI susceptibility breakpoint for meropenem/vaborbactam). Ceftolozane/tazobactam had limited activity against the carbapenemase-producing isolates (0.0%

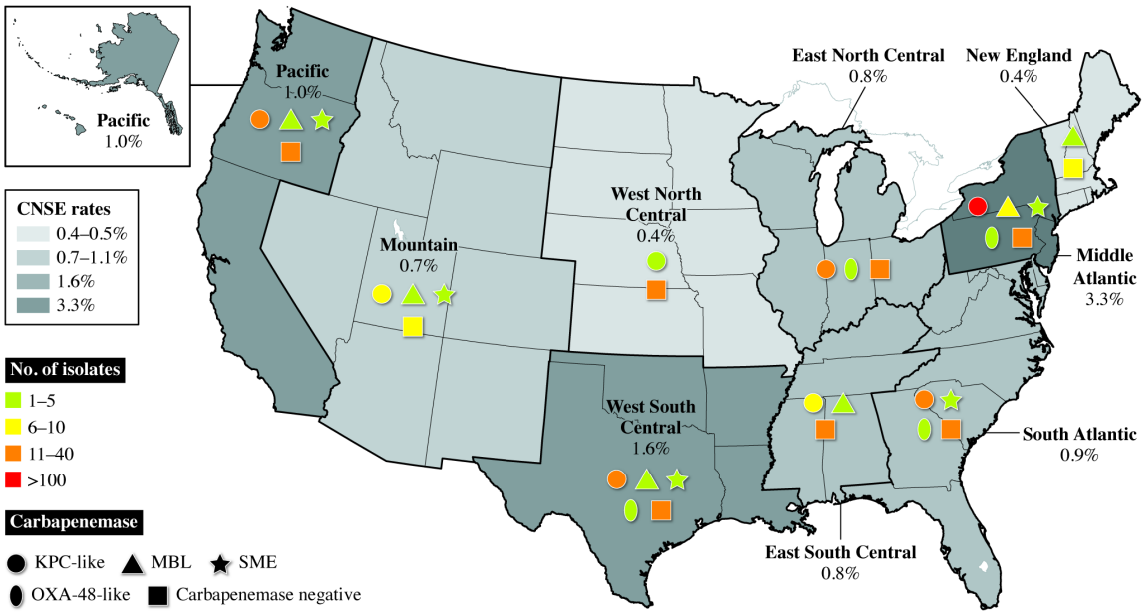

Figure 1. Distribution of carbapenemase- and non-carbapenemase-producing species across US Census Divisions.

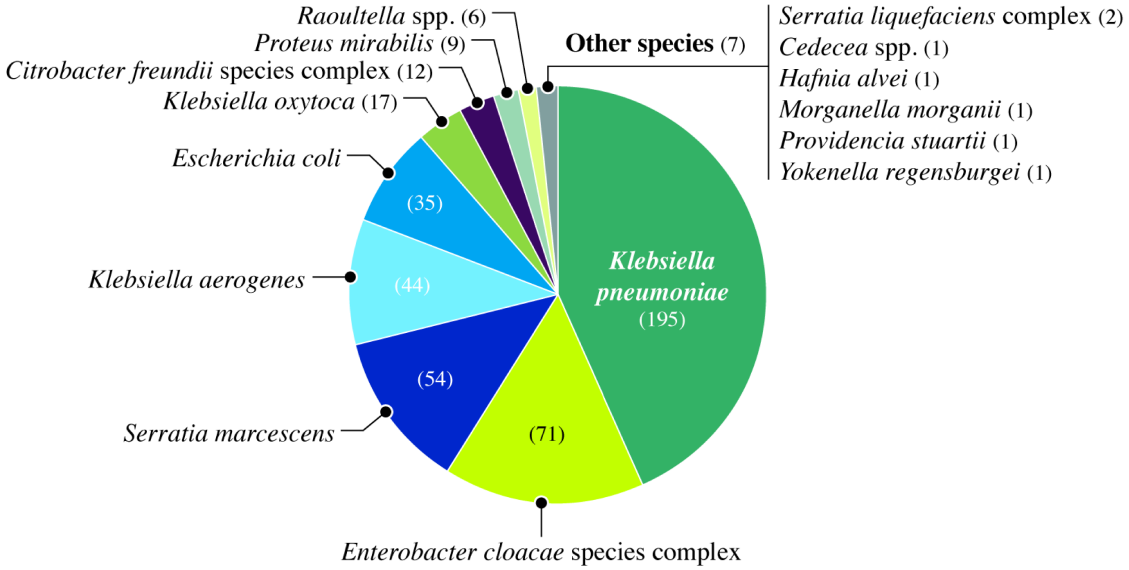

Figure 2. Bacterial species distribution of 450 CNSE isolates collected in US hospitals from 2016 to 2020.

to 5.6% inhibited at ≤2 mg/L), inhibiting 60.8% of the carbapenemase-negative CNSE. Other β-lactam agents that inhibited >50% of the carbapenemase-negative CNSE were cefepime (59.2% susceptible), aztreonam (57.4%), ceftazidime (56.8%) and piperacillin/tazobactam (53.3%). Despite the higher susceptibility rates for β-lactam agents tested against the carbapenemase-negative CNSE isolates, the newer β-lactam/β-lactamase inhibitor combinations were the most active agents against these isolates (Figure 3). Ceftazidime/avibactam inhibited all carbapenemase-negative CNSE isolates at the CLSI/EUCAST breakpoint, while meropenem/vaborbactam and imipenem/relebactam displayed activity against 85.7% and 61.9% of the carbapenemase-negative CNSE isolates, respectively. Among the non-β-lactam agents tested against the overall collection of CNSE isolates, susceptibility rates for tigecycline and amikacin were 93.5% and 97.0%, respectively. A total of 71.7% of the carbapenemase-negative CNSE isolates had intermediate MIC values for colistin.

Table 1. Activity of ceftazidime/avibactam and comparator agents tested against CNSE isolates

Antimicrobial agent	Percentage susceptible (% intermediate for colistin) according to the CLSI criteria					
	CNSE ($n=450$)	Carbapenemase producers ($n=281$)	Serine-carbapenemases (includes oxacillinases; $n=269$)	Ambler Class A serine-carbapenemases ($n=262$)	MBLs ($n=12$)	Carbapenemase-negative CNSE ($n=169$)
Ceftazidime/avibactam	96.9	95.0	98.5	98.9	16.7	100.0
Meropenem	28.7	6.8	7.1	5.7	0	65.1
Imipenem	7.6	1.8	1.9	1.1	0	17.2
Ceftazidime	24.4	5.0	4.8	5.0	8.3	56.8
Ceftriaxone	18.4	2.8	3.0	3.1	0	44.4
Cefepime	27.1	7.8	8.2	8.4	0	59.2
Aztreonam	22.2	1.1	0	0	25.0	57.4
Ampicillin/sulbactam	4.0	0.4	0	0	8.3	10.1
Piperacillin/tazobactam	21.9	3.2	3.0	3.1	8.3	53.3
Ceftolozane/ tazobactam	26.9	5.6	5.9	6.1	0	60.8
Levofloxacin	33.8	16.0	15.6	16.0	25.0	63.3
Amikacin	82.4	73.7	73.2	73.7	83.3	97.0
Gentamicin	62.4	49.8	48.7	48.9	75.0	83.4
Trimethoprim/ sulfamethoxazole	40.9	22.8	22.3	22.1	33.3	71.0
Tigecycline	95.3	96.4	96.3	96.2	100	93.5
Colistin	78.2	82.0	82.3	82.2	75.0	71.7

Lower susceptibility rates for most agents tested, including meropenem/vaborbactam and imipenem/relebactam, were noted when analysing the meropenem-nonsusceptible, carbapenemase-negative CNSE (Figure 3). These agents inhibited 93.0% and 89.5% of the meropenem-nonsusceptible, carbapenemase-negative CNSE isolates, while ceftazidime/avibactam inhibited all of these isolates at current breakpoints.

Ceftazidime/avibactam-resistant isolates and new KPC variants

Fourteen isolates displayed ceftazidime/avibactam MIC values >8 mg/L. These isolates included eight carrying bla_{NDM} and two harbouring bla_{VIM-1}. Other resistance mechanisms were also noted among the ceftazidime/avibactam-resistant, MBL-producing isolates (Table 2).

Four ceftazidime/avibactam-resistant isolates that did not harbour MBLs were one each of *E. cloacae*, *E. coli*, *K. pneumoniae* and *S. marcescens*. The *E. cloacae* isolate carried bla_{KPC-4} and exhibited a decrease in *ompC* expression. The *E. coli* harboured $bla_{OXA-181}$ among other β-lactamase genes, disruptions or indels in the two main porins and demonstrated increased AcrAB-TolC expression. The *K. pneumoniae* carried a new bla_{KPC}, named bla_{KPC-58}. The amino acid sequence of enzyme KPC-58 had an insertion of eight amino acids in position 270 (NRAPNKDD, of which RAPNKDD is a duplication) when compared to KPC-2. When expressed in an *E. coli* background, KPC-58 demonstrated an elevated ceftazidime/avibactam MIC value (2 mg/L; 16-fold higher than baseline) and low meropenem MIC values (2 mg/L; Table 3), similar to other described enzymes.[30] The susceptibility profile conferred by KPC-58 against other agents was similar to bla_{KPC} genes expressed in the same *E. coli* genetic background (Table 3). The clinical isolate carrying this enzyme was collected in a Kentucky hospital during 2019 from a 67-year-old male with a bloodstream infection (Tables 2 and 3). Nucleotide sequences for the new bla_{KPC} alleles are available in GenBank under accession numbers MT040751.1, MT463289.1 and MT463290.1.

The expression of bla_{KPC} was analysed for the KPC-4-producing *E. cloacae* and KPC-3-producing *S. marcescens* that had an expression of this gene lower than the baseline *K. pneumoniae* ATCC BAA-1705 (Table 2). A potential mechanism of ceftazidime/avibactam resistance in the *S. marcescens* isolate was not identified.

Two additional new bla_{KPC} genes were observed in this study, bla_{KPC-56} and bla_{KPC-59}. The amino acid sequence KPC-56 displayed two alterations compared to KPC-2 (H272Y and G292W). KPC-59 displayed a single amino acid change when compared to KPC-2 (G88D). These alleles expressed in an *E. coli* background displayed MIC values within ±1-fold for various β-lactam agents tested using reference broth microdilution (Table 3).

Discussion

In this 5-year survey of *Enterobacterales* isolates from US hospitals, we analysed 450 CNSE isolates, 309 of which were CRE. A decline in the prevalence of CNSE isolates was noted over the course of the study period (1.5% in 2016 to 1.1% in 2020). This decline was driven by the reduction in the prevalence of isolates carrying carbapenemases, which went from 1.0% in 2016 to 0.7% in 2020. The carbapenemase-negative CNSE rates slightly increased in 2018 (0.6%) but declined in 2020 (0.4%) compared

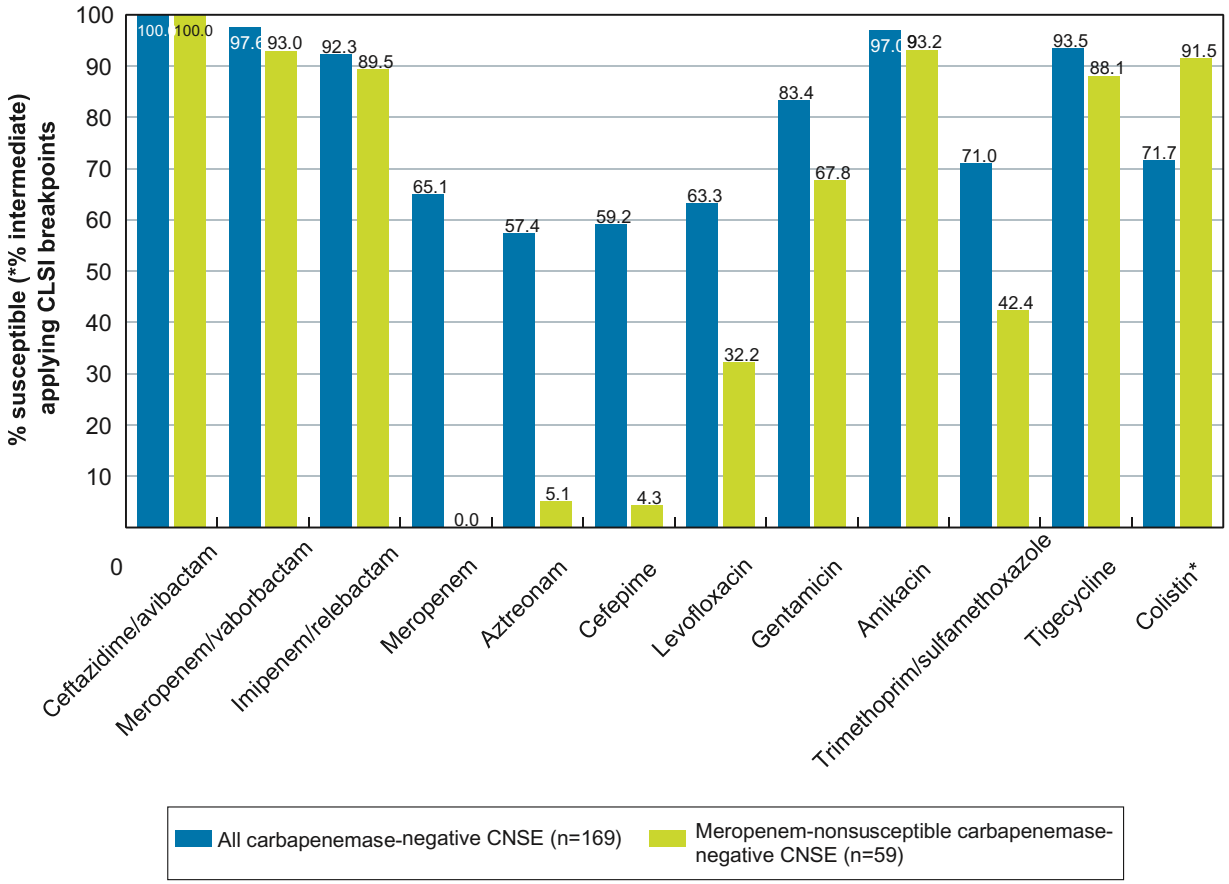

Figure 3. Susceptibility profiles of ceftazidime/avibactam, meropenem/vaborbactam, imipenem/relebactam and comparator agents against carbapenemase-negative CNSE isolates.

to 2016 (0.5%). We observed similar trends in a previous study reporting these rates.[31]

Our results demonstrated that >62% of the isolates carried carbapenemase genes and that KPC variants are still the most predominant carbapenemase in isolates from the USA. Isolates carrying genes encoding KPC-2 and KPC-3 were detected in all US Census Divisions except New England. Conversely, isolates carrying genes encoding MBLs ($n=12$) or oxacillinases with carbapenemase activity ($n=7$) were still noted in a small number of isolates, but these enzymes were detected in various locations.

Serine-carbapenemase-producing isolates were resistant to most β-lactam agents tested. Ceftazidime/avibactam displayed good activity against these isolates, inhibiting >98%. Ceftazidime/avibactam was active against six of seven isolates carrying oxacillinase enzymes with carbapenemase activity. The activity of ceftazidime/avibactam against a large collection of global *Enterobacterales* isolates producing OXA-48-like enzymes was almost 99%.[32] The ceftazidime/avibactam-resistant isolate in this study harboured $bla_{\text{OXA-181}}$ in addition to overexpression of efflux pump and alterations in two main porins, which probably contributed to its high MIC value against this combination. The activity of all β-lactams was limited against MBL-producing isolates, but 1 IMP-27-producing *P. mirabilis* and 1 NDM-5-producing *K. pneumoniae* were susceptible to ceftazidime/avibactam.

Carbapenemase-encoding genes were not detected in 37.6% of the CNSE isolates. Carbapenemase-negative *K. pneumoniae* and *E. coli* carried $bla_{\text{CTX-M}}$ genes, mainly $bla_{\text{CTX-M-15}}$, but isolates belonging to AmpC-producing species did not carry acquired β-lactamase genes. Overexpression of AmpC confers elevated imipenem MIC results,[33] consequently various carbapenemase-negative CNSE had low meropenem MIC values. Meropenem could be used for the treatment of these isolates; however, as exposure to this agent might lead to the development of resistance.[34] We noticed in a previous investigation that many carbapenemase-negative CRE isolates belonged to AmpC-producing species that accumulate resistance mechanisms such as AmpC and efflux overexpression plus porin alterations.[15] Despite recent recommendations for the treatment of AmpC-producing organisms,[35] therapies still need to be identified to prevent the development of resistance in these species.

The meropenem-nonsusceptible, carbapenemase-negative CNSE were considerably more resistant to antimicrobial agents, but ceftazidime/avibactam was active against all the carbapenemase-negative CNSE isolates regardless of their meropenem MIC values. After ceftazidime/avibactam, meropenem/vaborbactam and imipenem/relebactam were the most active agents against meropenem-nonsusceptible, carbapenemase-negative CNSE (93.0% and 89.5% susceptible, respectively).

Table 2. Characteristics and resistance mechanisms of 14 ceftazidime/avibactam-resistant CNSE

				MIC (mg/L)				Sequencing analysis[a]		Relative expression ($\Delta\Delta C_T$)			
Year	State	Organism	Infection type	Ceftazidime/ avibactam	Meropenem	Potential ceftazidime/ avibactam resistance mechanism	Other β-Lactamases	OmpC/ OmpK36	OmpF/ OmpK35	*acrA*	*ompC/ ompK36*	*ompF/ ompK35*	bla_{KPC}
2016	NY	*E. cloacae*	SSSI	16	4	KPC-4, OmpC decreased	TEM-1	alterations	alterations	0.235	3.229	0.008	<0.01
2017	NY	*S. marcescens*	PHP	32	>32	Not defined	KPC-3, OXA-10, OXA-9, TEM-1	alterations	alterations	0.86	0.469	0.633	<0.01
2018	CO	*S. marcescens*	UTI	>32	4	VIM-1		alterations	alterations	<0.01	<0.01	0.062	NT[b]
2018	KY	*E. cloacae*	UTI	>32	4	VIM-1	SHV-12, TEM-1	alterations	alterations	0.081	0.596	0.018	NT[b]
2018	TX	*E. coli*	IAI	>32	32	NDM-1	CMY-6, CTX-M-15, OXA-1, TEM-1	indels	alterations	0.833	>1000	0.137	NT[b]
2018	NY	*S. marcescens*	PHP	>32	4	NDM-1	CTX-M-15, OXA-9, TEM-1	alterations	wild-type	<0.01	0.378	0.388	NT[b]
2018	NY	*K. pneumoniae*	UTI	>32	>32	NDM-1	CTX-M-15, OXA-1, OXA-232, SHV-28, TEM-1	indels	alterations	6.97	1.501	0.151	NT[b]
2018	NJ	*E. cloacae*	BSI	>32	16	NDM-1	CTX-M-15, OXA-10-like, OXA-1, TEM-1	alterations	alterations	0.583	2.125	0.112	NT[b]
2019	NY	*E. coli*	SSSI	32	0.5	Efflux, double porin alterations	PSE-1, CMY-16-like, CTX-M-15, OXA-181, TEM-1	indels	disrupted	4.032	>1000	NT[b]	NT[b]
2019	KY	*K. pneumoniae*	BSI	32	0.5	KPC-58 (KPC-2 NRAPNKDD insertion at 270 aa)	SHV-11, TEM-1	indels	disrupted	1.621	4.389	0.785	NT[b]
2019	NY	*K. pneumoniae*	BSI	>32	>32	NDM-5	CTX-M-15, SHV-28, TEM-1	alterations	indels	3.667	4.565	4.108	NT[b]
2020	NY	*E. cloacae*	UTI	>32	16	NDM-1	CTX-M-15, OXA-1, SHV-12, TEM-1	alterations	alterations	>1000	1.30	0.120	NT[b]
2020	NY	*E. cloacae*	PHP	>32	>32	NDM-1	CTX-M-15, OXA-10, OXA-1, TEM-1	alterations	alterations	>1000	3.89	0.028	NT[b]
2020	NY	*E. coli*	BSI	>32	8	NDM-5	CMY-102-like, CTX-M-15, OXA-1	indels	alterations	14.14	>1000	>1000	NT[b]

[a]Reference sequences used to compare outer membrane proteins were *K. pneumoniae* (YP002239423.1_OMPK35, YP002237369.1_OMPK36), *E. cloacae* (CP001918.1_OMPC, CP001918.1_OMPF), *E. coli* (ASD01000043_OMPC, ASHD01000106_OMPF) and *S. marcescens* (CP012685_OMPC, CP012685_OMPF).
[b]NT = not tested due to lack of amplification.

Table 3. Characteristics of isolates carrying the new KPC variants and cloning results

Isolate characteristics	KPC alterations	Year	State	Age/ Gender	Infection type	MIC in mg/L							
						Meropenem	Imipenem	Cefepime	Ceftazidime	Aztreonam	Ampicillin/ sulbactam	Piperacillin/ tazobactam	Ceftazidime/ avibactam
Klebsiella pneumoniae clinical isolates													
KPC-56, CTX-M-15, SHV-11	KPC-2 + H272Y + G292W	2016	NY	56/F	PHP	1	4	>16	>32	>16	>32	>64	0.5
KPC-58, SHV-11, TEM-1	KPC-2 + D270insNRAPNKDD	2019	KY	67/M	BSI	0.5	4	32	>32	>16	>64	>128	32
KPC-59, CMY-2, SHV-11, SHV-12	KPC-2 + G88D	2019	LA	80/M	SSI	16	>8	>32	>32	>16	>64	>128	1
Escherichia coli transformants													
EcIoni(pSMART + KPC-56)	KPC-2 + H272Y+ G292W					1	2	4	16	>16	>64	32	0.5
EcIoni(pSMART + KPC-58)	KPC-2 + D270insNRAPNKDD					2	1	4	32	8	64	8	2
EcIoni(pSMART + KPC-59)	KPC-2 + G88D					4	>8	8	16	>16	>64	>128	0.12
EcIoni(pSMART + KPC-2)						2	4	4	8	>16	>64	32	0.25
EcIoni(pSMART + KPC-3)						4	4	8	>32	>16	>64	128	0.25
EcIoni(pSMART)						≤0.06	≤0.12	0.25	0.5	0.25	2	1	0.12

The isolates collected in this study are part of a large surveillance network. Patient data, previous use of antimicrobial agents and data regarding the therapy used to treat these infections is not available for analysis. As not all isolates in this study were tested for imipenem/relebactam and meropenem/vaborbactam, this is another limitation of this study.

The Infectious Diseases Society of America (IDSA) recently published guidelines recommending the use of the β-lactam/β-lactamase inhibitor combinations ceftazidime/avibactam, meropenem/vaborbactam and imipenem/cilastatin/relebactam for the treatment of pyelonephritis, complicated UTIs and infections outside the urinary tract caused by CREs resistant to both ertapenem and meropenem.[36] Additionally, the IDSA discouraged the use of polymyxins or combination antibiotic therapy (i.e. the use of a β-lactam agent in combination with an aminoglycoside, fluoroquinolone or polymyxin) to treat these infections.[36]

The IDSA recommendations fit the carbapenemase epidemiology pattern in US hospitals where the prevalence of MBLs is low. However, in areas with a high occurrence of MBL-producing organisms, the use of these agents requires susceptibility testing, carbapenemase-detection screening either alone or in combination, plus a good understanding of the epidemiology of these genes.[37] Unfortunately, newer β-lactam/β-lactam inhibitor combinations might not be available in countries where these isolates have higher prevalence. Despite the scarcity of MBL-producing isolates in this study, these isolates were noted in several US Census Divisions, highlighting that these measures might need to be adapted by many US institutions.

Last, despite the advances for patient treatment that these new β-lactam/β-lactam inhibitor combinations represent, resistance to them will still occur. We reported here a new bla_{KPC} gene that conferred resistance to ceftazidime/avibactam and low meropenem MIC values, similar to inhibitor-resistant KPC variants previously described.[30, 38] The enzyme encoded by this new gene has a large insertion in position 270 that has not been previously reported and kept its hydrolytic profile to all agents but meropenem.

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Transparency declarations

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Supplementary material

Supplementary material is available at *JAC* online.

References

1 Nordmann P, Poirel L. Epidemiology and diagnostics of carbapenem resistance in Gram-negative bacteria. *Clin Infect Dis* 2019; **69**: S521–8. https://doi.org/10.1093/cid/ciz824

2 Logan LK, Weinstein RA. The epidemiology of carbapenem-resistant *Enterobacteriaceae*: the impact and evolution of a global menace. *J Infect Dis* 2017; **215**: S28–36. https://doi.org/10.1093/infdis/jiw282

3 Bonomo RA, Burd EM, Conly J *et al*. Carbapenemase-producing organisms: a global scourge. *Clin Infect Dis* 2018; **66**: 1290–7. https://doi.org/10.1093/cid/cix893

4 Castanheira M, Huband MD, Mendes RE *et al*. Meropenem-vaborbactam tested against contemporary gram-negative isolates collected worldwide during 2014, including carbapenem-resistant, KPC-producing, multidrug-resistant, and extensively drug-resistant *Enterobacteriaceae*. *Antimicrob Agents Chemother* 2017; **61**: e00567. https://doi.org/10.1128/AAC.00567-17

5 Castanheira M, Deshpande LM, Mendes RE *et al*. Variations in the occurrence of resistance phenotypes and carbapenemase genes among *Enterobacteriaceae* isolates in 20 years of the SENTRY antimicrobial surveillance program. *Open Forum Infect Dis* 2019; **6**: S23–33. https://doi.org/10.1093/ofid/ofy347

6 Castanheira M, Mendes RE, Sader HS. Low frequency of ceftazidime-avibactam resistance among *Enterobacteriaceae* isolates carrying bla_{KPC} collected in U.S. Hospitals from 2012 to 2015. *Antimicrob Agents Chemother* 2017; **61**: e02369. https://doi.org/10.1128/AAC.02369-16

7 Boyd SE, Livermore DM, Hooper DC *et al*. Metallo-β-lactamases: structure, function, epidemiology, treatment options, and the development pipeline. *Antimicrob Agents Chemother* 2020; **64**: e00397. https://doi.org/10.1128/AAC.00397-20

8 Queenan AM, Bush K. Carbapenemases: the versatile beta-lactamases. *Clin Microbiol Rev* 2007; **20**: 440–58. https://doi.org/10.1128/CMR.00001-07

9 Abe R, Akeda Y, Sugawara Y *et al*. Enhanced carbapenem resistance through multimerization of plasmids carrying carbapenemase genes. *mBio* 2021; **12**: e0018621. https://doi.org/10.1128/mBio.00186-21

10 Roth AL, Kurpiel PM, Lister PD *et al*. Bla_{KPC} RNA expression correlates with two transcriptional start sites but not always with gene copy number in four genera of Gram-negative pathogens. *Antimicrob Agents Chemother* 2011; **55**: 3936–8. https://doi.org/10.1128/AAC.01509-10

11 Kitchel B, Rasheed JK, Endimiani A *et al*. Genetic factors associated with elevated carbapenem resistance in KPC-producing *Klebsiella pneumoniae*. *Antimicrob Agents Chemother* 2010; **54**: 4201–7. https://doi.org/10.1128/AAC.00008-10

12 Bulman ZP, Krapp F, Pincus NB *et al*. Genomic features associated with the degree of phenotypic resistance to carbapenems in carbapenem-resistant *Klebsiella pneumoniae*. *mSystems* 2021; **6**: e0019421. https://doi.org/10.1128/mSystems.00194-21

13 Bonomo RA. β-Lactamases: a focus on current challenges. *Cold Spring Harb Perspect Med* 2017; **7**: a025239. https://doi.org/10.1101/cshperspect.a025239

14 Nordmann P, Poirel L. The difficult-to-control spread of carbapenemase producers among *Enterobacteriaceae* worldwide. *Clin Microbiol Infect* 2014; **20**: 821–30. https://doi.org/10.1111/1469-0691.12719

15 Castanheira M, Doyle TB, Deshpande LM *et al*. Activity of ceftazidime/avibactam, meropenem/vaborbactam and imipenem/relebactam against carbapenemase-negative carbapenem-resistant Enterobacterales isolates from US hospitals. *Int J Antimicrob Agents* 2021; **58**: 106439. https://doi.org/10.1016/j.ijantimicag.2021.106439

16 Fernandez L, Hancock RE. Adaptive and mutational resistance: role of porins and efflux pumps in drug resistance. *Clin Microbiol Rev* 2012; **25**: 661–81. https://doi.org/10.1128/CMR.00043-12

17 Papp-Wallace KM, Endimiani A, Taracila MA *et al*. Carbapenems: past, present, and future. *Antimicrob Agents Chemother* 2011; **55**: 4943–60. https://doi.org/10.1128/AAC.00296-11

18 Nikaido H, Pages JM. Broad-specificity efflux pumps and their role in multidrug resistance of gram-negative bacteria. *FEMS Microbiol Rev* 2012; **36**: 340–63. https://doi.org/10.1111/j.1574-6976.2011.00290.x

19 Grundmann H, Livermore DM, Giske CG *et al*. Carbapenem-non-susceptible Enterobacteriaceae in Europe: conclusions from a meeting of national experts. *Euro Surveill* 2010; **15**: 19711. https://doi.org/10.2807/ese.15.46.19711-en

20 Sader HS, Flamm RK, Carvalhaes CG *et al*. Antimicrobial susceptibility of *Pseudomonas aeruginosa* to ceftazidime-avibactam, ceftolozane-tazobactam, piperacillin-tazobactam, and meropenem stratified by U.S. Census divisions: results from the 2017 INFORM program. *Antimicrob*

Agents Chemother 2018; **62**: e01587. https://doi.org/10.1128/AAC.01587-18

21 CLSI. *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically. M07Ed11*. 2018.

22 CLSI. *Performance Standards for Antimicrobial Susceptibility Testing. M100Ed31*. 2021.

23 USFDA. Antibacterial susceptibility test interpretive criteria. 2020. Available at https://www.fda.gov/drugs/development-resources/antibacterial-susceptibility-test-interpretive-criteria..

24 Bankevich A, Nurk S, Antipov D *et al*. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *J Comput Biol* 2012; **19**: 455–77. https://doi.org/10.1089/cmb.2012.0021

25 Camacho C, Coulouris G, Avagyan V *et al.* BLAST+: architecture and applications. *BMC Bioinf* 2009; **10**: 421. https://doi.org/10.1186/1471-2105-10-421

26 Castanheira M, Doyle TB, Kantro V *et al*. Meropenem-vaborbactam activity against carbapenem-resistant *Enterobacterales* isolates collected in U.S. Hospitals during 2016 to 2018. *Antimicrob Agents Chemother* 2020; **64**: e01951. https://doi.org/10.1128/AAC.01951-19

27 Castanheira M, Costello SE, Woosley LN *et al*. Evaluation of clonality and carbapenem resistance mechanisms among *Acinetobacter baumannii-Acinetobacter calcoaceticus* complex and Enterobacteriaceae isolates collected in European and Mediterranean countries and detection of two novel β-lactamases, GES-22 and VIM-35. *Antimicrob Agents Chemother* 2014; **58**: 7358–66. https://doi.org/10.1128/AAC.03930-14

28 Louie A, Castanheira M, Liu W *et al*. Pharmacodynamics of β-lactamase inhibition by NXL104 in combination with ceftaroline: examining organisms with multiple types of β-lactamases. *Antimicrob Agents Chemother* 2012; **56**: 258–70. https://doi.org/10.1128/AAC.05005-11

29 Castanheira M, Doyle TB, Hubler C *et al*. Ceftazidime-avibactam activity against a challenge set of carbapenem-resistant *Enterobacterales*: Ompk36 L3 alterations and beta-lactamases with ceftazidime hydrolytic activity lead to elevated MIC values. *Int J Antimicrob Agents* 2020; **56**: 106011. https://doi.org/10.1016/j.ijantimicag.2020.106011

30 Venditti C, Butera O, Meledandri M *et al*. Molecular analysis of clinical isolates of ceftazidime-avibactam-resistant *Klebsiella pneumoniae*. *Clin Microbiol Infect* 2021; **27**: 1040.e1–e6. https://doi.org/10.1016/j.cmi.2021.03.001

31 Castanheira M, Mendes RE, Jones RN *et al*. Changes in the frequencies of β-lactamase genes among *Enterobacteriaceae* isolates in U.S. Hospitals, 2012 to 2014: activity of ceftazidime-avibactam tested against beta-lactamase-producing isolates. *Antimicrob Agents Chemother* 2016; **60**: 4770–7. https://doi.org/10.1128/AAC.00540-16

32 Castanheira M, Doyle TB, Collingsworth TD *et al.* Increasing frequency of OXA-48-producing Enterobacterales worldwide and activity of ceftazidime/avibactam, meropenem/vaborbactam and comparators against these isolates. *J Antimicrob Chemother* 2021; **76**: 3125–34. https://doi.org/10.1093/jac/dkab306

33 Jacoby GA. AmpC beta-lactamases. *Clin Microbiol Rev* 2009; **22**: 161–82. https://doi.org/10.1128/CMR.00036-08

34 Rodriguez-Bano J, Gutierrez-Gutierrez B, Machuca I *et al*. Treatment of infections caused by extended-spectrum-beta-lactamase-, AmpC-, and carbapenemase-producing *Enterobacteriaceae*. *Clin Microbiol Rev* 2018; **31**: e00079. https://doi.org/10.1128/CMR.00079-17

35 Tamma PD, Doi Y, Bonomo RA *et al*. A primer on AmpC β-lactamases: necessary knowledge for an increasingly multidrug-resistant world. *Clin Infect Dis* 2019; **69**: 1446–55. https://doi.org/10.1093/cid/ciz173

36 Tamma PD, Aitken SL, Bonomo RA *et al*. Infectious Diseases Society of America guidance on the treatment of AmpC beta-lactamase-producing *Enterobacterales*, carbapenem-resistant *Acinetobacter baumannii*, and *Stenotrophomonas maltophilia* infections. *Clin Infect Dis* 2022; **74**: 2089. https://doi.org/10.1093/cid/ciab1013

37 Doi Y. Treatment options for carbapenem-resistant gram-negative bacterial infections. *Clin Infect Dis* 2019; **69**: S565–S75. https://doi.org/10.1093/cid/ciz830

38 Shields RK, Nguyen MH, Press EG *et al. In vitro* selection of meropenem resistance among ceftazidime-avibactam-resistant, meropenem-susceptible *Klebsiella pneumoniae* isolates with variant KPC-3 carbapenemases. *Antimicrob Agents Chemother* 2017; **61**: e00079. https://doi.org/10.1128/AAC.00079-17