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Genomic Characteristics and β-Lactam/β-Lactamase Inhibitor Susceptibility Profiles of Ceftazidime–Avibactam-Resistant KPC-Producing *Klebsiella pneumoniae*

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Abstract

Background/Objectives: Ceftazidime–avibactam (CZA) resistance in KPC-producing *Klebsiella pneumoniae* is frequently associated with variant KPC enzymes, but the resulting susceptibility to other β-lactam/β-lactamase inhibitor combinations remains difficult to predict. We investigated the genomic characteristics and collateral susceptibility profiles of CZA-resistant KPC-producing *K. pneumoniae* colonizing isolates. **Methods:** This retrospective study included a selected collection of 38 isolates recovered from rectal screening samples between 2019 and 2023, with only one isolate per patient included. Isolates underwent whole-genome sequencing and susceptibility testing to aztreonam–avibactam (AZT–AVI), meropenem–vaborbactam (MEM–VAB), imipenem–relebactam (IMI–REL), and meropenem. MICs were interpreted according to EUCAST 2026 criteria. **Results:** Twelve bla_{KPC} alleles were identified. The most frequent were bla_{KPC-31} (17/38, 44.7%) and bla_{KPC-66} (7/38, 18.4%), while the remaining 14 isolates carried ten less frequent alleles. Most KPC variants involved the Ω-loop. OmpK35 and OmpK36 alterations were common and frequently occurred together. AZT–AVI was active against all isolates, whereas susceptibility to MEM–VAB, IMI–REL, and meropenem was 89.5%, 81.6%, and 44.7%, respectively. Marked MIC variability was observed among isolates carrying the same bla_{KPC} allele, including isolates with identical OmpK35 and OmpK36 profiles. Resistance to MEM–VAB and IMI–REL showed incomplete overlap across allelic backgrounds. **Conclusions:** Within this collection, isolates carrying the same bla_{KPC} allele and porin profile could exhibit different collateral susceptibility patterns, supporting direct susceptibility testing of individual β-lactam/β-lactamase inhibitor combinations.

Keywords: ceftazidime–avibactam resistance; KPC variants; *Klebsiella pneumoniae*; meropenem–vaborbactam; imipenem–relebactam; aztreonam–avibactam; intestinal colonization

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1. Introduction

Among antimicrobial-resistant Gram-negative organisms, carbapenem-resistant Enterobacterales (CREs) represent a major public health threat and are included among the

priority bacterial pathogens requiring new antimicrobial agents and improved control strategies [1,2]. In this context, *Klebsiella pneumoniae* producing *K. pneumoniae* carbapenemase (KPC), hereafter KPC-Kp, is of particular concern in countries where KPC enzymes are endemic [3,4]. Ceftazidime–avibactam (CZA) has become one of the key therapeutic options for infections caused by KPC-Kp [5], as avibactam inhibits Ambler class A β-lactamases, including KPC, as well as several class C and some class D β-lactamases, but not metallo-β-lactamases [6]. However, increasing CZA use has been accompanied by the emergence of CZA-resistant KPC-Kp isolates [4], reducing the activity of one of the most important agents currently available for these infections [7].

CZA resistance in KPC-Kp is frequently associated with diversification of ancestral $bla_{\text{KPC-2}}$ or $bla_{\text{KPC-3}}$ alleles. The latter encodes a KPC enzyme that differs from KPC-2 by the H274Y amino acid substitution. Point mutations, insertions, or deletions affecting structural regions surrounding the active site, including the Ω-loop, the 237–243 loop, and the 267–275 loop, may alter the hydrolytic spectrum of KPC enzymes and their interaction with β-lactamase inhibitors [3,8]. Depending on the specific KPC variant and the accompanying resistance determinants, isolates may retain a broad KPC-like β-lactam resistance profile, acquire a ceftazidimase-like phenotype with enhanced activity against ceftazidime, or show partial restoration of carbapenem susceptibility [9]. CZA resistance has also been reported in isolates carrying apparently unmodified ancestral bla_{KPC} alleles, including $\text{bla}_{\text{KPC-3}}$ [10,11], suggesting that accessory mechanisms, particularly alterations affecting OmpK35 and OmpK36 porins, may contribute to the resistant phenotype [12–14].

This phenotypic heterogeneity has practical implications for antimicrobial susceptibility interpretation and treatment selection, because CZA resistance does not predict a uniform susceptibility profile to other last-line agents [15–17]. Here, we report the genomic characteristics and antimicrobial susceptibility profiles of a retrospective collection of 38 nonduplicate CZA-resistant KPC-Kp isolates recovered from rectal screening samples between 2019 and 2023. We specifically examined the distribution of variant bla_{KPC} alleles and selected accessory resistance determinants in relation to susceptibility to aztreonam–avibactam (AZT–AVI), meropenem–vaborbactam (MEM–VAB), and imipenem–relebactam (IMI–REL), using meropenem as a comparator agent.

2. Results

This study included 38 CZA-resistant KPC-producing *K. pneumoniae* isolates recovered from rectal screening swabs, with only one isolate per patient included. CZA resistance was confirmed by broth microdilution in all 38 isolates: two isolates had MICs of 16 mg/L, whereas the remaining 36 had MICs > 16 mg/L. Twelve different bla_{KPC} alleles were identified by WGS (Tables 1 and S1). The most frequent were $bla_{\text{KPC-31}}$, detected in 17/38 (44.7%) isolates, and $bla_{\text{KPC-66}}$, detected in 7/38 (18.4%). The remaining 14 isolates carried $bla_{\text{KPC-3}}$ ($n = 1$), $bla_{\text{KPC-29}}$ ($n = 1$), $bla_{\text{KPC-46}}$ ($n = 1$), $bla_{\text{KPC-49}}$ ($n = 2$), $bla_{\text{KPC-61}}$ ($n = 2$), $bla_{\text{KPC-62}}$ ($n = 1$), $bla_{\text{KPC-65}}$ ($n = 2$), $bla_{\text{KPC-85}}$ ($n = 2$), $bla_{\text{KPC-98}}$ ($n = 1$), or $bla_{\text{KPC-148}}$ ($n = 1$).

Five sequence types were represented. ST512 was the most frequent (15/38, 39.5%), followed by ST307 (8/38, 21.1%), ST4615 (7/38, 18.4%), ST101 (6/38, 15.8%), and ST323 (2/38, 5.3%). The $bla_{\text{KPC-31}}$ allele was identified in four ST backgrounds, comprising ST512 ($n = 8$), ST307 ($n = 6$), ST101 ($n = 2$), and ST323 ($n = 1$). The seven $bla_{\text{KPC-66}}$ isolates belonged to ST4615 ($n = 5$) or ST101 ($n = 2$). The less frequent alleles were distributed among ST512, ST307, ST101, ST4615, or ST323, with isolate-level K- and O-locus assignments reported in Table S1.

Table 1. Genomic characteristics of CZA-resistant KPC-producing *K. pneumoniae* isolates stratified by bla_{KPC} allele.

bla_{KPC} Allele	Isolates, *n*	KPC Structural Region Affected	ST Distribution, *n*	$bla_{CTX\text{-}M\text{-}15}$-Positive Isolates, *n*/*N*	Isolates with Porin Alterations	
					OmpK35, *n*/*N*	OmpK36, *n*/*N*
$bla_{KPC\text{-}31}$	17	Ω-loop	ST512, 8; ST307, 6; ST101, 2; ST323, 1	4/17	16/17	13/17
$bla_{KPC\text{-}66}$	7	Ω-loop	ST4615, 5; ST101, 2	0/7	2/7	7/7
$bla_{KPC\text{-}3}$	1	None	ST512, 1	0/1	1/1	1/1
$bla_{KPC\text{-}29}$	1	267–275 loop	ST512, 1	0/1	1/1	1/1
$bla_{KPC\text{-}46}$	1	Ω-loop	ST323, 1	1/1	0/1	0/1
$bla_{KPC\text{-}49}$	2	Ω-loop	ST101, 1; ST512, 1	0/2	2/2	2/2
$bla_{KPC\text{-}61}$	2	Ω-loop	ST4615, 2	0/2	0/2	2/2
$bla_{KPC\text{-}62}$	1	Ω-loop	ST512, 1	0/1	1/1	1/1
$bla_{KPC\text{-}65}$	2	Ω-loop	ST307, 2	2/2	2/2	0/2
$bla_{KPC\text{-}85}$	2	Ω-loop	ST101, 1; ST512, 1	0/2	2/2	2/2
$bla_{KPC\text{-}98}$	1	Ω-loop	ST512, 1	0/1	1/1	1/1
$bla_{KPC\text{-}148}$	1	267–275 loop	ST512, 1	0/1	1/1	1/1

Abbreviations: CZA, ceftazidime–avibactam; KPC, *Klebsiella pneumoniae* carbapenemase; ST, sequence type. Notes: The two most frequent bla_{KPC} alleles are listed first; the remaining alleles are ordered numerically. "None" indicates the absence of additional amino acid alterations relative to the ancestral KPC-3 sequence. Structural-region assignments were based exclusively on the location of additional amino acid alterations relative to KPC-3. OmpK35 and OmpK36 values indicate the number of isolates carrying detected alterations. OmpK35 alterations were predicted loss-of-function frameshifts, whereas OmpK36 alterations comprised either predicted loss-of-function frameshifts or loop 3 amino acid insertions without predicted truncation; individual alterations are reported in Table S1. These classifications were based on sequence analysis and were not functionally validated.

All KPC enzymes included in the study harbored the H274Y substitution that distinguishes KPC-3 and its derivatives from KPC-2; accordingly, KPC-3 was used as the reference sequence, and H274Y was not considered an additional amino acid alteration. Additional amino acid alterations relative to KPC-3 predominantly involved the Ω-loop (35/38 isolates, 92.1%). These included D179Y in KPC-31, the L167_ΔEL_N170 deletion in KPC-66, and the L169P, R164S, S171P, L169Q, T180_ins(YT)_S181, A172V, and R164H alterations characterizing KPC-46, KPC-49, KPC-61, KPC-62, KPC-65, KPC-85, and KPC-98, respectively. KPC-29 and KPC-148 contained insertions associated with the 267–275 loop, whereas the single KPC-3 isolate had no additional amino acid alteration relative to the ancestral KPC-3 sequence (Tables 1 and S1).

The $bla_{CTX\text{-}M\text{-}15}$ gene was detected in 7/38 (18.4%) isolates, including four carrying $bla_{KPC\text{-}31}$, one carrying $bla_{KPC\text{-}46}$, and two carrying $bla_{KPC\text{-}65}$. Alterations of OmpK35 and OmpK36 were detected in 29/38 (76.3%) and 31/38 (81.6%) isolates, respectively. OmpK35 alterations consisted of predicted inactivating frameshifts, whereas OmpK36 alterations included amino acid insertions or predicted inactivating frameshifts. Both porins were altered in 24/38 (63.2%) isolates, while only two isolates retained wild-type OmpK35 and OmpK36 (one carrying $bla_{KPC\text{-}31}$ and the other $bla_{KPC\text{-}46}$) (Tables 1 and S1).

All 38 isolates were susceptible to AZT–AVI (Table 2). Overall susceptibility to MEM–VAB and IMI–REL was 89.5% (34/38) and 81.6% (31/38), respectively. According to the EUCAST 2026 criteria, 17/38 (44.7%) isolates were categorized as susceptible to meropenem, 9/38 (23.7%) as susceptible with increased exposure, and 12/38 (31.6%) as resistant. Among $bla_{KPC\text{-}31}$ isolates, susceptibility to MEM–VAB, IMI–REL, and meropenem was observed in 15/17 (88.2%), 13/17 (76.5%), and 8/17 (47.1%) isolates, respectively. All seven $bla_{KPC\text{-}66}$ isolates were susceptible to both β-lactam/β-lactamase inhibitor combinations, and six (85.7%) were susceptible to meropenem. Among isolates carrying other bla_{KPC} alleles, susceptibility to MEM–VAB, IMI–REL, and meropenem was observed in 12/14 (85.7%), 11/14 (78.6%), and 3/14 (21.4%) isolates, respectively.

Table 2. Antimicrobial susceptibility of CZA-resistant KPC-producing *K. pneumoniae* isolates stratified by analytical bla_{KPC} group.

Antimicrobial Agent	Overall, *n*/*N* (%) Susceptible	bla_{KPC-31}, *n*/*N* (%) Susceptible	bla_{KPC-66}, *n*/*N* (%) Susceptible	Other bla_{KPC} Alleles, *n*/*N* (%) Susceptible
Aztreonam–avibactam	38/38 (100)	17/17 (100)	7/7 (100)	14/14 (100)
Meropenem–vaborbactam	34/38 (89.5)	15/17 (88.2)	7/7 (100)	12/14 (85.7)
Imipenem–relebactam	31/38 (81.6)	13/17 (76.5)	7/7 (100)	11/14 (78.6)
Meropenem	17/38 (44.7)	8/17 (47.1)	6/7 (85.7)	3/14 (21.4)

Abbreviations: CZA, ceftazidime–avibactam; KPC, *Klebsiella pneumoniae* carbapenemase. Notes: Susceptibility to aztreonam–avibactam was based on reference broth microdilution results, as described in Materials and Methods. Isolate-level MICs obtained by reference broth microdilution and gradient strip diffusion are reported in Table S2. All isolates were categorized as susceptible by both methods, yielding 100% categorical agreement and 100% essential agreement.

Descriptive isolate-level analysis showed substantial MIC variability among isolates carrying the same bla_{KPC} allele (Table S2). Among bla_{KPC-31} isolates, MEM–VAB, IMI–REL, and meropenem MICs ranged from ≤0.06 to >16 mg/L, from 0.12 to 4 mg/L, and from ≤0.12 to >16 mg/L, respectively. The only bla_{KPC-31} isolate retaining wild-type OmpK35 and OmpK36 showed low MEM–VAB and meropenem MICs (≤0.06 and 0.25 mg/L, respectively). However, the eight bla_{KPC-31} isolates sharing the same OmpK35 E42fs and OmpK36 GD insertion profile exhibited MEM–VAB MICs ranging from 1 to >16 mg/L, IMI–REL MICs from 0.5 to 4 mg/L, and meropenem MICs from 2 to >16 mg/L. Similarly, meropenem MICs among bla_{KPC-66} isolates ranged from ≤0.12 to >16 mg/L. The five ST4615 isolates with wild-type OmpK35 and disrupted OmpK36 showed meropenem MICs ranging from ≤0.12 to 0.25 mg/L, whereas the two ST101 isolates sharing OmpK35 I61fs and the OmpK36 TD insertion had meropenem MICs of 2 and >16 mg/L.

Resistance to both MEM–VAB and IMI–REL was observed in four isolates: two carrying bla_{KPC-31} and one each carrying bla_{KPC-29} or bla_{KPC-49}. Three additional isolates, two carrying bla_{KPC-31} and one carrying $bla_{KPC-148}$, were resistant to IMI–REL but remained susceptible to MEM–VAB. Notably, the two bla_{KPC-49} isolates were both resistant to meropenem but showed markedly different MEM–VAB and IMI–REL MICs (>16 versus ≤0.06 mg/L and 8 versus 0.25 mg/L, respectively), despite alterations in both porins. Meropenem MICs also differed between the two bla_{KPC-65} isolates (≤0.12 and 4 mg/L), although they shared the same OmpK35 S210fs alteration and wild-type OmpK36. AZT–AVI MICs ranged from 0.03 to 2 mg/L and remained within the susceptible range irrespective of bla_{KPC} allele, CTX-M-15 status, or porin profile.

3. Discussion

Our integrated genomic and phenotypic characterization of CZA-resistant KPC-producing *K. pneumoniae* showed marked heterogeneity in both the genetic determinants of resistance and the associated antimicrobial susceptibility profiles. CZA resistance was predominantly associated with KPC-3-derived variants involving the Ω-loop, but the KPC variant alone did not account for the observed phenotype. Although MEM–VAB and IMI–REL retained activity against most isolates, resistance to one or both combinations occurred across different bla_{KPC} alleles, whereas AZT–AVI remained active against the entire collection. Moreover, substantial MIC variation was observed among isolates carrying the same allele, including isolates with apparently identical OmpK35 and OmpK36 profiles. Collectively, these findings show that, in this collection, CZA resistance mediated by KPC variants was associated with heterogeneous collateral susceptibility patterns, probably reflecting the combined effects of the variant enzyme and additional strain-dependent mechanisms.

The predominance of alterations involving the Ω-loop is consistent with the central role of this region in the emergence of CZA resistance. Amino acid substitutions, deletions, or insertions affecting residues surrounding the active site may reduce inhibition by avibactam while modifying the hydrolytic activity of KPC enzymes against carbapenems [3,4,13]. These changes can produce phenotypes ranging from preservation of the classical KPC resistance profile to increased ceftazidimase activity accompanied by partial or complete restoration of carbapenem susceptibility [8,9]. In particular, KPC-31, characterized by the D179Y substitution, has been associated with a ceftazidimase-like phenotype and reduced carbapenem MICs in standardized genetic backgrounds [9]. This effect was also evident in our clinical collection, although it was not uniform: approximately half of the KPC-31 isolates were susceptible to meropenem, whereas the remainder were categorized as susceptible with increased exposure or resistant. Similarly, six of the seven KPC-66 isolates were susceptible to meropenem, but one displayed a meropenem MIC above the resistance breakpoint. These findings illustrate the distinction between the intrinsic susceptibility profile associated with a KPC variant and the final phenotype expressed by a clinical isolate.

The high rates of susceptibility to MEM–VAB and IMI–REL observed in our collection are consistent with previous evidence that many CZA-resistant KPC variants exhibit reduced carbapenemase activity and remain susceptible to KPC-active β-lactam/β-lactamase inhibitor combinations [3,9,15]. Nevertheless, resistance to one or both combinations occurred across several allelic backgrounds, and susceptibility to MEM–VAB and IMI–REL did not completely overlap. This discordance indicates that susceptibility to one KPC-active combination should not be inferred from susceptibility to another. The different molecular structures, inhibitory properties, and dependence on entry through outer membrane porins of vaborbactam and relebactam may contribute to these discordant profiles. Experimental evolution studies have likewise shown that exposure to different β-lactam/β-lactamase inhibitor combinations may select distinct resistance trajectories: diversification of bla_{KPC} may be favored during CZA exposure, whereas resistance selected by MEM–VAB or IMI–REL may more frequently involve impaired permeability and can result in broader cross-resistance [18]. Because antimicrobial exposure data were not investigated in the present study, however, the evolutionary pathways through which the observed phenotypes emerged cannot be established.

Alterations in OmpK35 and OmpK36 were common in our collection and frequently involved both porins. Their potential contribution is biologically plausible, as reduced outer membrane permeability may increase carbapenem and β-lactam/β-lactamase inhibitor MICs by reducing the periplasmic exposure of the β-lactam and, for some combinations, its inhibitor [12,13]. Rogers et al. [19] reported that OmpK36 disruption and loop 3 GD insertions were associated with higher MEM–VAB and IMI–REL MICs, although the magnitude of these effects varied according to the KPC subtype. Functional characterization of a clinical isolate resistant to CZA, MEM–VAB, and IMI–REL similarly demonstrated that the resistant phenotype reflected the combined effects of a KPC variant and OmpK36 disruption rather than the variant enzyme alone [14]. Our isolate-level findings are compatible with such interaction: the two ST101 KPC-66 isolates carrying alterations in both porins had higher meropenem MICs than the five ST4615 isolates with wild-type OmpK35 and disrupted OmpK36. However, porin status did not provide a complete explanation. KPC-31 isolates sharing the same OmpK35 frameshift and OmpK36 GD insertion showed widely different MICs, as did the two KPC-49 isolates despite alterations of both porins. The observed associations should therefore be interpreted as evidence that porin alterations may modify the allele-associated phenotype, rather than as proof that they independently determine resistance. Clonal background may also have contributed to the observed phenotypic variability. However, because several bla_{KPC} alleles were represented by only one or two isolates or were

restricted to particular STs, the independent effects of ST, KPC allele, and porin genotype could not be separated. These observations are exploratory and require confirmation in larger collections and functional studies. Other strain-dependent mechanisms may also contribute, as increased bla_{KPC} expression and copy number have been associated with high-level CZA resistance in *K. pneumoniae* carrying variant KPC enzymes [20].

AZT–AVI was active against all isolates in our collection, despite the variable susceptibility observed with the other KPC-active β-lactam/β-lactamase inhibitor combinations. This finding is consistent with the European surveillance study by Sader et al. [16], in which all 385 KPC-producing Enterobacterales collected between 2019 and 2023 were susceptible to AZT–AVI. All CTX-M-15-positive isolates in our collection remained susceptible to AZT–AVI, consistent with the ability of avibactam to inhibit both KPC and CTX-M enzymes. Favorable AZT–AVI MIC distributions were likewise reported by Jean et al. [21] across carbapenemase-producing Enterobacterales collected from four global regions. Nevertheless, the uniform activity observed in our collection should not be interpreted as excluding the potential emergence of resistance. Satapoomin et al. [22] demonstrated that the combination of a KPC-3 V240G variant with *ramR* and *ompK36* mutations could confer resistance to AZT–AVI as well as to IMI–REL and MEM–VAB, further illustrating the cumulative contribution of enzyme modification, reduced permeability, and efflux-related mechanisms. The origin of our isolates from rectal screening samples also has important diagnostic implications. CZA-resistant KPC variants associated with restored carbapenem susceptibility may circulate in the intestinal reservoir without causing clinically recognized infection and may be missed by screening or confirmatory algorithms that depend primarily on elevated carbapenem MICs or detectable carbapenemase activity. Molecular detection of bla_{KPC}, combined with appropriate phenotypic characterization, may therefore be required to recognize isolates displaying these atypical resistance profiles [9].

This study has several limitations. The retrospectively assembled collection was selected to include CZA-resistant isolates carrying confirmed bla_{KPC} alleles and should not be considered representative of all CZA-resistant KPC-producing *K. pneumoniae* circulating at our institution. As this study was restricted to isolates collected between 2019 and 2023, the findings may reflect the local epidemiology and selective pressures of a specific period and may not capture subsequent changes in the distribution of KPC variants. The relatively small number of isolates, particularly for the less frequent alleles, precluded robust allele-specific comparisons. Accordingly, the observed genotype–phenotype relationships should be regarded as descriptive and hypothesis-generating and do not establish the independent contribution of individual KPC variants or porin alterations. In addition, genomic analysis identified plausible resistance determinants but did not assess their expression or functional contribution. Differences in bla_{KPC} copy number, promoter configuration, plasmid context, porin expression, efflux activity, or additional chromosomal mechanisms may therefore account for part of the unexplained MIC variability. Finally, because the isolates were recovered from rectal screening samples, antimicrobial exposure and clinical outcome data were not available, and the observed phenotypes cannot be linked to specific treatment-related selective trajectories. Conversely, the analysis of colonizing isolates represents a distinctive strength, as it provides insight into the intestinal reservoir of CZA-resistant KPC variants that may remain unrecognized in studies restricted to clinical infections. The isolate-level integration of KPC variants, porin alterations, and susceptibility to multiple β-lactam/β-lactamase inhibitor combinations further allowed us to examine phenotypic heterogeneity that would have been obscured by aggregate susceptibility data.

4. Materials and Methods

4.1. Study Design and Bacterial Isolates

This retrospective observational study included 38 nonduplicate CZA-resistant KPC-producing *K. pneumoniae* isolates. The isolates were recovered from rectal screening swabs collected between 2019 and 2023 through the routine surveillance program for intestinal carriage of carbapenemase-producing Enterobacterales at Fondazione Policlinico Universitario A. Gemelli IRCCS, Rome, Italy. Only one isolate per patient was included.

During routine surveillance, rectal swabs were cultured on chromID CARBA SMART agar (bioMérieux, Marcy-l'Étoile, France), a selective chromogenic medium for the detection of carbapenemase-producing Enterobacterales. Presumptive *K. pneumoniae* colonies were identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) using the MALDI Biotyper MBT Smart system (Bruker Daltonics, Bremen, Germany). Antimicrobial susceptibility testing (AST), including CZA susceptibility, was initially performed using the VITEK 2 system (bioMérieux). Carbapenemase production and type were investigated using the NG-Test CARBA 5 immunochromatographic assay (NG Biotech, Guipry-Messac, France) and/or molecular assays, according to the routine diagnostic workflow in use at the time of isolate recovery.

For the present study, stored isolates meeting these criteria were retrospectively selected from the laboratory strain collection. Because the isolates were not archived according to a prospective, consecutive protocol, the completeness of the collection could not be ensured, and the total pool of potentially eligible isolates could not be reliably reconstructed. Following revival, the available isolates underwent AST and whole-genome sequencing (WGS), as described below; isolates that could not be recovered after storage were excluded. Accordingly, the 38 isolates represented a non-exhaustive retrospective sample rather than a complete series of CZA-resistant KPC-producing *K. pneumoniae* isolates identified during the study period.

4.2. Antimicrobial Susceptibility Testing

Following revival, MICs of CZA, meropenem, MEM–VAB, and IMI–REL were determined by broth microdilution using the Thermo Scientific Sensititre Gram Negative EUMDROXF AST Plate (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's instructions. CZA testing was used to confirm the resistant phenotype initially identified by VITEK 2. Avibactam, relebactam, and vaborbactam were tested at fixed concentrations of 4, 4, and 8 mg/L, respectively. As a supplementary analysis, ertapenem MICs were examined to assess whether the carbapenem susceptibility profiles of the study isolates were compatible with their recovery on a carbapenem-selective screening medium. Ertapenem MICs were determined by broth microdilution using the Thermo Scientific Sensititre Gram Negative EUGNF Plate. AZT–AVI MICs were initially determined by gradient strip diffusion using MTS (MIC Test Strip; Liofilchem, Roseto degli Abruzzi, Teramo, Italy), an Etest-equivalent method, according to the manufacturer's instructions. At the time of testing, ready-to-use commercial broth microdilution panels containing AZT–AVI were not available, and gradient diffusion represented the only readily available commercial method for quantitative MIC determination. Previous evaluations reported acceptable agreement between AZT–AVI gradient diffusion and reference broth microdilution [23,24]. To provide reference-method confirmation, all isolates were subsequently retested by broth microdilution using in-house-prepared twofold serial dilutions of aztreonam and a fixed avibactam concentration of 4 mg/L. *Klebsiella pneumoniae* ATCC 700603 was included in each run as the quality-control strain for the commercial broth microdilution panels, the MTS method, and the in-house reference AZT–AVI broth microdilution method. All quality-control results were within the expected ranges.

MICs were interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical breakpoint tables, version 16.1 [25]. For Enterobacterales, the susceptibility and resistance MIC breakpoints were, respectively, ≤4 mg/L and >4 mg/L for AZT–AVI, ≤8 mg/L and >8 mg/L for both CZA and MEM–VAB, and ≤2 mg/L and >2 mg/L for IMI–REL. For meropenem, isolates with MICs ≤ 2 mg/L were categorized as susceptible, those with MICs of 4–8 mg/L as susceptible with increased exposure, and those with MICs > 8 mg/L as resistant. For ertapenem, isolates with MICs ≤ 0.5 mg/L and >0.5 mg/L were categorized as susceptible and resistant, respectively. Isolate-level MICs are reported in Table S2.

4.3. Whole-Genome Sequencing and Genomic Analysis

Genomic DNA was extracted from overnight bacterial cultures using the DANAGENE Microbial DNA Kit (Danagen-Bioted, Barcelona, Spain), according to the manufacturer's instructions. DNA concentration and purity were assessed using a NanoDrop One spectrophotometer (Thermo Fisher Scientific). Libraries were prepared using the Illumina DNA Prep Kit (Illumina, San Diego, CA, USA) and subjected to 250 bp paired-end sequencing on an Illumina MiSeq Dx platform. Reads were preprocessed and quality-controlled using fastp v0.24.1 and assembled de novo using Unicycler v0.5.0. Across the study collection, the median sequencing depth was 92.5-fold (range, 40–143), and the assemblies had a median N50 of 235,417 bp (range, 55,049–507,414) and a median of 184 contigs (range, 106–1047). Contigs shorter than 500 bp were excluded from downstream analyses. All raw sequence data generated in this study have been deposited in the NCBI Sequence Read Archive (SRA) under BioProject accession number PRJNA1536591.

Species identification was confirmed using KmerFinder v4.0.4. Sequence types were assigned according to the seven-locus multilocus sequence typing scheme implemented in Ridom SeqSphere+ v8.5.1. Capsular K-loci and O-antigen loci were assigned using Kleborate v3.2.4. Acquired antimicrobial resistance genes and resistance-associated mutations were identified using AMRFinderPlus v4.2.7 and independently screened using ABRicate v1.0.1 against the CARD and NCBI databases (April 2025 snapshots). Individual bla_{KPC} alleles were assigned when the corresponding reference sequence was detected with 100% sequence identity and 100% sequence coverage. bla_{KPC} alleles that could not be unambiguously assigned by AMRFinderPlus and ABRicate were further investigated by BLASTn (NCBI BLAST+ v2.16.0) alignment against the bla_{KPC-2} reference sequence (GenBank accession no. AY034847) to identify nucleotide substitutions, insertions, and deletions.

KPC amino acid alterations were defined relative to KPC-3 and described according to the standardized Ambler-based annotation proposed by Brunetti et al. [26]. Alterations were mapped to structurally relevant regions of the enzyme, including the Ω-loop (residues 164–179), the 237–243 loop, and the 267–275 loop. For the comparison of antimicrobial susceptibility profiles, isolates were stratified into three analytical bla_{KPC} groups: bla_{KPC-31}, bla_{KPC-66}, and other bla_{KPC} alleles.

OmpK35 and OmpK36 sequences were analyzed using Kleborate v3.2.4 and classified according to the predicted effect of the identified alterations. OmpK35 was classified as wild type or disrupted by a predicted loss-of-function alteration. OmpK36 was classified as wild type, altered by a loop 3 amino acid insertion, or disrupted by a predicted loss-of-function alteration. Individual porin alterations are reported in Table S1.

4.4. Data Analysis

Categorical variables were summarized as counts and percentages, and MIC distributions as ranges. Antimicrobial susceptibility was summarized overall and after stratification into the predefined analytical bla_{KPC} groups. Agreement between AZT–AVI gradient diffu-

sion and reference broth microdilution was evaluated in terms of essential and categorical agreement. Gradient diffusion MICs falling between standard twofold dilutions were rounded up to the next higher concentration before comparison. Essential agreement was defined as MIC values within ±1 twofold dilution, whereas categorical agreement was defined as assignment to the same EUCAST susceptibility category. Because of the small number of isolates carrying several individual alleles, no inferential allele-specific comparisons were performed.

5. Conclusions

CZA-resistant KPC-producing *K. pneumoniae* isolates in this collection exhibited heterogeneous collateral susceptibility profiles, including among isolates carrying the same bla_{KPC} allele and porin profile. These findings support direct susceptibility testing of each β-lactam/β-lactamase inhibitor combination, including for isolates showing restored carbapenem susceptibility. The recovery of these atypical phenotypes from rectal screening samples highlights the intestinal reservoir as a relevant target for genomic and phenotypic surveillance.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/antibiotics15111079/s1, Table S1: Isolate-level genomic characteristics of CZA-resistant KPC-producing *K. pneumoniae* isolates recovered from rectal screening swabs; Table S2: Isolate-level sequence types, porin profiles, and MICs of β-lactam/β-lactamase inhibitor combinations and carbapenems among CZA-resistant KPC-producing *K. pneumoniae* isolates.

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Informed Consent Statement: Not applicable.

Data Availability Statement: The isolate-level genomic and antimicrobial susceptibility data supporting the findings of this study are included in Tables S1 and S2. Raw sequencing reads are openly available in the NCBI Sequence Read Archive under BioProject accession number PRJNA1536591.

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Abbreviations

The following abbreviations are used in this manuscript:

AST	Antimicrobial susceptibility testing
ATCC	American Type Culture Collection
AZT–AVI	Aztreonam–avibactam
CARD	Comprehensive Antibiotic Resistance Database

CRE	Carbapenem-resistant Enterobacterales
CZA	Ceftazidime–avibactam
EUCAST	European Committee on Antimicrobial Susceptibility Testing
IMI–REL	Imipenem–relebactam
KPC	*Klebsiella pneumoniae* carbapenemase
KPC-Kp	KPC-producing *Klebsiella pneumoniae*
MALDI-TOF MS	Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
MEM-VAB	Meropenem-vaborbactam
MIC	Minimum inhibitory concentration
MTS	MIC Test Strip
NCBI	National Center for Biotechnology Information
SRA	Sequence Read Archive
ST	Sequence type
WGS	Whole-genome sequencing

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