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Insertion Sequence-Dependent *OmpK*36 Mutation Associated Ertapenem Resistance in Clinical *Klebsiella pneumoniae*

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Abstract

Extended-spectrum β-lactamases (ESBLs) and/or AmpC enzymes combined with deficiency of porins OmpK35 and OmpK36 are important for the development of carbapenem-resistant *Klebsiella pneumoniae*. We characterized the clinical *K. pneumoniae* human isolates and investigated the effect of meropenem induction on the *ompK*35 and *ompK*36 mutation to develop carbapenem resistance from six carbapenem-susceptible ESBL-producing *K. pneumoniae* strains. 163 clinical *K. pneumoniae* isolates were grouped mostly into the ESBL + AmpC (44.2%) and ESBL (42.9%) phenotypes. The resistance rate differed between cephalosporins (52.1% for cefepime - 97.5% for cefotaxime) and carbapenems (16% for meropenem - 28.2% for imipenem) ($P < 0.001$). The ESBL group showed the lowest resistance to cefoxitin and cefepime and all carbapenems, whereas the AmpC group exhibited the lowest resistance to cefepime and the highest resistance to all carbapenems. PCR amplification identified *bla*$_{\text{TEM}}$, *bla*$_{\text{SHV}}$, *bla*$_{\text{CTX-M-3-like}}$, and *bla*$_{\text{CTX-M-14-like}}$ of AmpA β-lactamase genes and *bla*$_{\text{DHA}}$ and *bla*$_{\text{CMY}}$ of AmpC β-lactamase genes. Compared to all 163 clinical isolates, the 56 carbapenem-resistant isolates carried less frequently of *bla*$_{\text{TEM}}$, *bla*$_{\text{CTXM-14-like}}$, and *bla*$_{\text{CTXM-3-like}}$ and more frequently of *bla*$_{\text{DHA-1}}$ and *bla*$_{\text{CMY-2}}$. The carbapenem-resistant isolates differed in prevalence against imipenem, ertapenem, and meropenem and lacked OmpK35 more frequently than OmpK36, but abnormal PCR amplicons were detected fewer in the Omp K35-deficient group than in the OmpK36-deficient group (32.5% *vs.* 68.4%,

*Equal contribution.

respectively). The carbapenem-resistant isolate mostly carried bla_{DHA} (91.1%) and three isolates carried bla_{KPC-2}. Following induction with meropenem insertion sequences in *ompK*36, not *ompK*36, were identified as IS5 for KP08, IS1 for KP15, and IS903 for KP16 isolates. OmpK36 deficiency increased resistance to ertapenem, but not imipenem and meropenem. Clinical isolates belonged mainly to ESBL + AmpC group and ESBL group with difference in resistance to cephalosporins and carbapenems, the bla genes. Carbapenem resistant isolates lacked OmpK35 expression, than the OmpK36 expression, Meropenem induction developed the carbapenem resistant isolates with insertion of different insertion sequences in *ompK*36, not *ompK*35.

Keywords

Klebsiella Pneumoniae, Carbapenem, ESBL, AmpC, Outer-Membrane Protein, Insertion Sequences

1. Introduction

The community-associated *Klebsiella pneumoniae* has gradually increased [1] and caused liver abscesses in diabetic patients [2]. The use of cephalosporin to treat *K. pneumoniae* infections stimulates mutations in cephalosprinase genes to become extended spectrum-lactamase (ESBL)-producing bacteria [3]. Therefore, carbapenems: Imipenem, meropenem, ertapenem, and doripenem are alternative drugs to treat ESBL-producing bacterial infections [4]. Emerging cabapenem resistant *K. pneumoniae* (KPC) increases mortality and is difficult to treat [5] [6] [7].

The mechanism of carbapenem resistance involves mutations in penicillin-binding proteins (PBPs) to prevent carbapenem binding [8]. The incorporation of carbapenases as NDM-1 carbapenemase can be through plasmid transfer. [9] [10] [11] [12]. The insertion or point mutations of outer membrane genes in ESBL- and/or AmpC-producing isolates to halt the entrance of carbapenem. The insertion sequences IS1 or IS10 in *ompK*35 [13] [14] and IS1, IS4, IS5, IS10, and IS903 in *ompK*36 are often acquired to develop carbapenem resistance [10] [15] [13] [14] [16] [17]. We previously reported that carbapenem usage is strongly associated with the development of carbapenem resistance in *K. pneumoniae* *[18]*.

The aim of this study was to investigate the effect of low-dosage meropenem treatment on the induction of carbapenem resistance in five carbapenem-susceptible *K. pneumoniae* strains with ESBL production with expression of OmpK35 and OmpK36 and one isolate without Ompk35 expression.

2. Materials and Methods

2.1. Bacterial Collection and Identification

163 *K. pneumoniae* isolates were collected from the Chiayi Branch of Taichung

Veterinary Hospital and identified by biochemical methods using the GDN ID-8 Kit (Bio Star, Taiwan) and PCR identification of 16S rDNA sequences [19]. This research was approved by the IRB of Taichung Veterans General Hospital (CE12034 and SE13132).

2.2. Antimicrobial Susceptibility Testing

Resistance to β-lactam antimicrobials, including ampicillin, cefoxitin (FOX), ceftriaxone (CRO), ceftazidime (CAZ), cefotaxime (CTX), and cefepime (FEP) of cephalosporins, as well as ertapenem (ETP), meropenem (MEM), and imipenem (IMP) of carbapenems, were tested using the disc diffusion method (Becton, Dickinson and Company, USA) and the guidelines of the Clinical and Laboratory Standard Institute [20]. *Escherichia coli* ATCC 25922 was used as a control. The minimal inhibitory concentration (MIC) was determined by the Etest and the broth microdilution method.

2.3. Determination of ESBL, AmpC Enzyme and Carbapenase

ESBL isolates were identified by the differences in diameter of the inhibition zones between cefotaxime and cefotaxime/clavulanic acid or between ceftazidime and ceftazidime/clavulanic acid. Active AmpC enzyme was identified using disks with 300 μg of 3-aminophenylboronic acid and carbapenemase production was detected by the Modified Hodge Test (MHT) based on the Centers for Disease Control and Prevention guidelines [21].

2.4. PCR Detection of *bla* Genes, *omp*35 and *omp*36, and Insertion Sequences

PCR amplification of AmpA bla_{TEM}, bla_{SHV}, $bla_{CTX\text{-}M\text{-}3}$, $bla_{CTX\text{-}M\text{-}14}$ and $bla_{KPC\text{-}1}$ [22] [23] [24], AmpC $bla_{CMY\text{-}2}$ and bla_{DHA} [25], carbapenem resistance genes, MBLs bla_{VIM}, bla_{IMP}, bla_{SPM}, bla_{SIM}, bla_{GIM}, bla_{KHM}, and bla_{NDM}, as well as outer membrane genes *omp*35 and *omp*36, and insertion sequences IS1, IS5, and IS903 were performed using the primers listed in **Table 1**.

2.5. Sequence Analysis

PCR products were purified using a DNA purification kit (ProTECH, Taiwan) and sequenced. DNA sequences were aligned and compared using Lasergene v7.1 software (DNASTAR Inc, USA) and the BLAST program of the National Center for Biotechnology Information.

2.6. Outer Membrane Protein Analysis of Carbapenem-Resistant Isolates Induced by Meropenem

ESBL-producing isolates KP07, KP08, KP10, KP13, KP15 and KP16 were used. Except that isolate KP15 lacked OMPK15 due to a deletion in the promoter region of *ompK*35, all isolates carried normal PCR size and protein expression of *ompK*35 and *ompK*36. These bacteria were cultured firstly in Muller-Hinton

Table 1. Primer sequences and PCR conditions.

Target		Primer sequence (5' to 3')	TA[a] (˚C)	Size (bp)
		Multiplex PCR		
bla$_{TEM}$	F	GAAGATCAGTTGGGTGCACGAGT	59	520
	R	CAACTTTATCCGCCTCCATCCAGT		
bla$_{SHV}$	F	AACGGAACTGAATGAGGCGCT	59	141
	R	TCCACCATCCACTGCAGCAGCT		
bla$_{CTX\text{-}M\text{-}3}$	F	AATCACTGCGCCAGTTCACGCT	59	479
	R	GAACGTTTCGTCTCCCAGCTGT		
bla$_{CTX\text{-}M\text{-}14}$	F	TACCGCAGATAATACGCAGGTG	59	355
	R	CAGCGTAGGTTCAGTGCGATCC		
bla$_{DHA}$	F	AACTTTCACAGGTGTGCTGGGT	59	405
	R	CCGTACGCATACTGGCTTTGC		
bla$_{VIM}$	F	GTTTGGTCGCATATCGCAAC	57	147
	R	CTTYTCAATCTCCGCGAGAAG		
bla$_{IPM\text{-}1}$	F	GGAATAGAGTGGCTTAATTCTCAATC	57	272
	R	GCTTCTAAATTTGCGTCACCC		
bla$_{IPM\text{-}2}$	R	GYAACCAAACCACTACGTTATCT	57	193
bla$_{SPM}$	F	ATGAACTCACCTAAATCGAGAGC	57	340
	R	GTGCCGTCCAAATGAAAGTG		
bla$_{SIM}$	F	GGCTTAGTAGTTCTTGACAATCAC	57	516
	R	CAATAGTGATGCGTCTCCGA		
bla$_{GIM}$	F	TGTAGCGTTGCCAGCTTTAG	57	431
	R	CAGCACCTGGATAGTAGAGC		
bla$_{KHM}$	F	CGTTTGGTCTGCTGTTGTTTAC	57	322
	R	GGAATGGACTTGGAGTTGAGAA		
bla$_{NDM}$	F	ACTTATGCCAATGCGTTGTC	57	225
	R	TCTGTCCTTGATCAGGCAG		
		Single PCR		
KP16S	F	AGCACAGAGAGCTTG	50	126
	R	ACTTTGGTCTTGCGAC		
bla$_{CMY\text{-}2}$	F	CTGACAGCCTCTTTCTCCACA	56	1005
	R	CTACGTAGCTGCCAAATCCAC		
bla$_{KPC}$	F	TGTCACTGTATCGCCGTC	54	1000
	R	CTCAGTGCTCTACAGAAAACC		
OmpK35	F	TGATCCCTGCCCTGCTGGT	56	717
	R	CCGGAGTCATGTTGTAAGTCT		

Continued

*OmpK*36	F	ACAGAGGGTTAATAACATGAA	51	1112
	R	TAGAACTGGTAAACCAGGC		
*OmpK*35-FL1	F	GTTACGCACTGTTTCGG	52	1572
	R	GGTGTACTGCAGATTAGAAC		
*OmpK*36-FL	F	GCAGCACAATGAAATAGCC	53	1265
	R	GACAAGAGTATACCAGCGAG		
*OmpK*35-Pro	F	GTTACGCACTGTTTCGG	52	502
	R	CCAGAATATTGCGCTTCATC		
*OmpK*35-Ter	F	CCAGACTTACAACATGACTC	51	349
	R	GGTGTACTGCAGATTAGAAC		
*IS*1	F	TGACTCCAACTTATTGATAGTGT	46	997
	R	CATGAATGGCGTTGGATG		
*IS*5	F	TATTTCCGGTTTTTACTGAGA	51	529
	R	CATCATGAGCCATCAACTC		
*IS*903	F	CTTTTGCTGAGTTGAAGGA	51	987
	R	TGTGTTTTCAGGCAATACG		

[a]TA: annealing temperature.

Broth (MHB) containing 0.1 μg/ml meropenem for 16 hours at 100 rpm, followed by subculture in MHB supplemented with 0.5 μg/ml, 1 μg/ml, and 2 μg/ml meropenem, subsequently. The bacterial solution was plated onto Muller-Hinton Agar (MHA), and meropenem discs were plated thereafter. Colonies in the inhibition zone were selected, and outer membrane proteins were analyzed using a previously described method [26]. Proteins were separated by 12% SDS-PAGE at 100 V, 100 mA, and 400 W. Protein profiles were recorded and analyzed using ImageJ software (National Institutes of Health, USA).

2.7. Statistical Analysis

Duncan's multiple range test and SPSS software (version 18, Chicago, IL, USA) were used to analyze differences among groups.

3. Results

3.1. Antimicrobial Resistance and Prevalence of AmpA and AmpC Genes in ESBL and AmpC Phenotypes

The 163 isolates were separated into four phenotypic groups: the ESBL + AmpC group (44.2%), the ESBL group (42.9%), the AmpC group (7.4%), and neither group (5.5%). The prevalence was 87.1% (142 isolates) for ESBL-producing isolates, and 50.3% (82 isolates) for AmpC isolates, with the different resistance patterns among the ESBL and AmpC phenotypes (**Table 2**). The resistance rate differed between cephalosporins (52.1% for cefepime - 97.5% for cefotaxime)

Table 2. Resistance to cephalosporins and carbapenems and AmpA and AmpC genes in different ESBL and AmpC pnenotypes of clinical *K. pneumonia.*

Characters	ESBL and AmpC phenotypes				
	ESBLs + AmpC	ESBLs	AmpC	Neither	Total
Number	72	70	12	9	163
Resistance to cephalosporins					
cefoxitin	68 (94.4)^{a,x}	18 (25.7)^{c,z}	11 (91.7)^{a,x}	6 (66.7)^{b,x}	103 (63.2)
ceftriaxone	71 (98.6)^{a,x}	69 (98.6)^{a,x}	7 (58.3)^{b,y}	1 (11.1)^{c,y}	148 (90.8)
ceftazidime	71 (98.6)^{a,x}	63 (90.0)^{a,x}	11 (91.7)^{a,x}	5 (55.6)^{b,x}	150 (92.0)
cefotaxime	71 (98.6)^{a,x}	69 (98.6)^{a,x}	12 (100.0)^{a,x}	7 (77.8)^{b,x}	159 (97.5)
cefepime	41 (56.9)^{a,y}	41 (58.6)^{a,y}	2 (16.7)^{b,z}	1 (11.1)^{b,y}	85 (52.1)
Resistance to carbapenem					
Imipenem	33 (45.8)^{a,x}	3 (4.3)^{b}	7 (58.3)^{a}	3 (33.3)^{a}	46 (28.2)
Meropenem	16 (22.2)^{b,y}	3 (4.3)^{b}	6 (50.0)^{a}	1 (11.1)^{b}	26 (16.0)
Ertapenem	26 (36.1)^{b,xy}	4 (5.7)^{c}	8 (66.7)^{a}	2 (22.2)	40 (24.5)
AmpA genes					
bla$_{SHV}$	72 (100.0)	70 (100.0)	12 (100.0)	9 (100.0)	163 (100)
bla$_{TEM}$	60 (83.3)	53 (75.7)	9 (75.0)	1 (11.1)	123 (75.5)
bla$_{CTX\text{-}M3}$	6 (8.3)	13 (18.6)	0 (0.0)	0 (0.0)	19 (11.7)
bla$_{CTX\text{-}M14}$	31 (43.1)	25 (35.7)	0 (0.0)	0 (0.0)	56 (34.4)
AmpC genes					
bla$_{DHA}$	67 (93.1)	13 (18.6)	9 (75.0)	4 (44.4)	93 (57.1)
bla$_{CMY2}$	7 (9.7)	3 (4.3)	3 (25.0)	0 (0.0)	13 (8.0)

$^{a\text{-}d}$Different letters indicate significant difference between different ESBL and AmpC types, and $^{x\text{-}z}$Different letters indicate significant difference between antibiotics in each ESBL and AmpC phenotypes, $P < 0.05$.

and carbapenems (16% for meropenem - 28.2% for imipenem) (P < 0.001). The ESBL + AmpC group with respect to resistance revealed no differences among ceftriaxone, cefotaxime, and cefoxitin as well as lower resistance to cefepime in cephalosporins and the highest resistance for imipenem, followed by ertapenem and r meropenem in carbapenem. The ESBL group showed the lowest resistance to cefoxitin and cefepime and all carbapenems, whereas the AmpC group exhibited the lowest resistance to cefepime and the highest resistance to all carbapenems. Nine isolates of the none group differed in resistance to cephalosporin, but some were resistant to carbapenems.

PCR amplification identified *bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{CTX\text{-}M\text{-}3\text{-}like}$, and *bla*$_{CTX\text{-}M\text{-}14\text{-}like}$ of AmpA β-lactamase genes and *bla*$_{DHA}$ and *bla*$_{CMY}$ of AmpC β-lactamase genes (**Table 2**). The prevalent AmpA genes were *bla*$_{SHV}$ (100%), *bla*$_{TEM}$ (75.5%), *bla*$_{CTXM\text{-}14\text{-}like}$ (34.4%), and *bla*$_{CTXM3\text{-}like}$ (11.7%), and were mostly detected in ESBL-producing isolates. While *bla*$_{CMY\text{-}2}$ (8.0%) was lower in the AmpC gene, *bla*$_{DHA\text{-}1}$ (57.1%) was the more prevalent with the highest prevalence in the ESBLs

+ AmpC group (93.1%), followed by the AmpC group (75%), none group (44.4%) and ESBL group (18.6%).

3.2. Characterization of the 56 Carbapenem Resistant Isolates

Of the 56 carbapenem-resistant isolates, 42.9% of the isolates were resistance to all three carbapenems and one carbapenem, respectively, while 14.3% isolates were resistant to two carbapenems (Table 3). The highest carbapenem resistance was observed in the ESBL + AmpC group (71.4%), which exhibited the highest prevalence in the isolates with single resistance to imipenem (87.5%, ertapenem (75.0%), and the AmpC group (14.3%), which showed the highest rate of resistance to all three carbapenems (25%). Although no PCR products were detected

Table 3. Characterization of 56 clinical carbapenem resistant *K. pneumoniae.*

Characters	Carbapenem resistant patterns								Total [No, (%)]
Imipenem	R	R	I	R	R	R	I	I	46 (82.1)
Meropenem	R	I	R	I	I	S	I	S	26 (46.4)
Ertapenem	R	R	R	I	S	S	R	R	40 (71.4)
No.	24	6	2		16		8		56
ESBL and AmpC phenotypes [No, (%)]									
ESBL + AmpC	15 (62.5)	4 (66.7)	1 (50.0)		14 (87.5)		6 (75.0)		40 (71.4)
ESBLs	2 (8.3)	0 (0.0)	1 (50.0)		1 (6.3)		1 (12.5)		5 (8.9)
AmpC	6 (25.0)	1 (16.6)	0 (0.0)		0 (0.0)		1 (12.5)		8 (14.3)
Neither	1 (4.2)	1 (16.6)	0 (0.0)		1 (6.3)		0 (0.0)		3 (5.4)
AmpA gnes [No, (%)]									
bla$_{TEM}$	14 (58.3)	5 (83.3)	1 (50.0)	8 (72.7)	3 (100.0)	2 (100.0)	5 (71.4)	1 (100.0)	39 (69.6)
bla$_{CTX\text{-}M3}$	0 (0.0)	1 (16.7)	1 (50.0)	1 (9.1)	0 (0.0)	0 (0.0)	1 (14.3)	0 (0.0)	4 (7.1)
bla$_{CTX\text{-}M14}$	6 (25.0)	2 (33.3)	0 (0.0)	4 (36.4)	0 (0.0)	0 (0.0)	3 (42.9)	1 (100.0)	16 (28.6)
AmpC genes [No, (%)]									
bla$_{DHA}$	20 (83.3)	6 (100.0)	1 (50.0)	11 (100.0)	3 (100.0)	2 (100.0)	7 (100.0)	1 (100.0)	51 (91.1)
bla$_{CMY2}$	5 (20.8)	1 (16.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	4 (57.1)	0 (0.0)	10 (17.9)

ESBL and AmpC phenotype	Outmembrane protein deficiency				
	OmpK35	OmpK36	OmpK35 + OmpK36	Normal	Total
ESBL + AmpC	27 (67.5)	0 (0.0)	6 (15.0)	7 (17.5)	40 (55.6)
ESBLs	3 (60.0)	0 (0.0)	2 (40.0)	0 (0.0)	5 (7.1)
AmpC	4 (50.0)	0 (0.0)	4 (50.0)	0 (0.0)	8 (66.7)
No	2 (66.7)	0 (0.0)	0 (0.0)	1 (33.3)	3 (33.3)
Total	36 (64.3)	0 (0.0)	12 (21.4)	8 (14.3)	56 (34.4)

for bla_{VIM}, bla_{IPM-1}-like, bla_{IPM-2}-like, bla_{SPM}, bla_{SIM}, bla_{GIM}, and bla_{KHM}, of the AmpB β-lactamase genes bla_{KPC} were identified in three isolates (Supplementary **Figure 1**).

Compared to all clinical isolates, the carbapenem-resistant isolates carried less frequently of bla_{TEM} (69.6% *vs.* 75.5%), $bla_{CTXM-14-like}$ (28.6% *vs.* 34.4%), and $bla_{CTXM-3-like}$ (7.1% *vs.* 11.7%) of AmpA genes and more frequently of bla_{DHA-1} (91.1% *vs.* 57.1%) and bla_{CMY-2} (17.9% *vs.* 8%) of AmpC genes, Eight isolates expressed both OmpK35 and OmpK36, 36 isolates lacked OmpK35 individually and 12 isolates lacked both OmpK35 and OmpK36; however, no isolate with single deficiency in OmpK36 was observed. These results may imply that deficiency in OmpK35 and OmpK36 may partly contribute to carbapenem resistance. PCR detected the abnormal *ompK*35 and *ompK*36 that differed among ESBL and/or AmpC phenotypes. Among 56 carbapenem-resistant isolates, abnormal PCR products were found in 29.3% (14/48) and 75.0% (9/12) of isolates with no OmpK35 and OmpK36 expression, respectively (**Table 3**). Sequence analysis of 14 abnormal PCR products of *ompK*35 revealed that one isolate contained the IS903 insertion at + 4 bp, while eight isolates contained the IS10 insertion from + 744 bp to + 1093 bp. PCR products were not detected in five isolates.

3.3. Analysis of Outer Membrane Protein Expression

K. pneumoniae ATCC 13883 was used as a control in NB broth with or without 20% sorbitol to evaluate the expression of OmpA, OmpK35 and OmpK36. Deficiency in OmpK35, OmpK36 and OmpK35/OmpK36 was observed separately in 69.4%, 1.4% and 8.3% of isolates in the ESBL + AmpC group, 60.0%, 5.7% and 2.9% of isolates in the ESBL group, 50.0%, 8.3% and 33.3% of isolates in the AmpC group, and 33.3%, 0% and 11.1% of isolates in the neither group. The analysis of 15 ESBL- and non-ESBL-producing isolates revealed that ESBL-producing I solate 92 with the AmpC phenotype and bla_{DHA-1} and deficiency in OmpK35 and OmpK36 i exhibited the highest MIC against ertapenem and imipenem, whereas non-ESBL producing isolates 110 and 114 with the AmpC

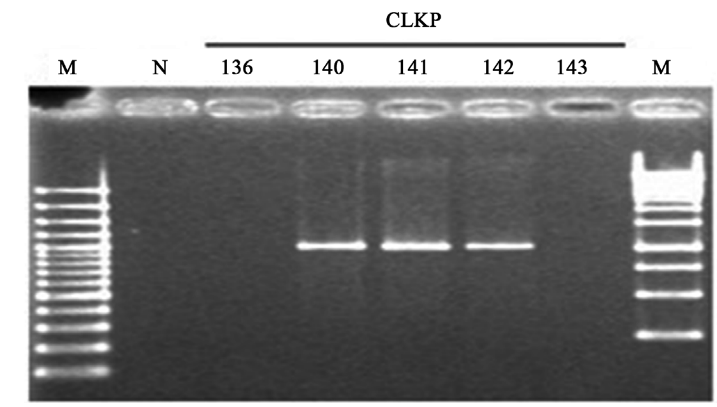

Figure 1. Electrophoresis of PCR products of KPC AmpA β-latamase gene.

phenotype and both *bla*$_{DHA-1}$ and *bla*$_{CMY-2}$ and deficiency in OmpK35 and OmpK36 exhibited the highest MIC against all three carbapenems (Table 4).

3.4. Meropenem-Induced Carbapenem Resistance Is Associated with the Insertion Sequence and Antimicrobial Variations

ESBL-producing isolates KP07, KP08, KP10, KP13, KP15 and KP16 exhibited intermediate susceptibility to carbapenem (isolates KP07, KP08, KP10 and KP16 contain *bla*$_{TEM}$, and isolates KP10 and KP16 contain *bla*$_{CTX-M-14}$ and *bla*$_{CMY}$). With the exception of isolate KP15 with a deletion in the promoter region of *ompK*35 and no OmpK35 expression normal PCR products and expression of *ompK*35 and *ompK*36 and were generated from other isolates. In total, 119 isolates were collected from KP07 (9), KP08 (17), KP10 (3), KP13 (36), KP15 (9) and KP16 (45). Only six isolates generated abnormal PCR products: KP08-1, KP08-2, KP08-12 and KP08-15 from KP08; KP15-4 from KP15; and KP16-22 from KP16. Although PCR analysis revealed three insertion sequences IS1, IS5, and IS903 present in all six isolates, only one insertion sequence was found in the *ompK*36 gene at different locations for each mutant isolate. Sequence analysis of *ompK*36 PCR products revealed an IS5 insertion at −48 bp in KP08-1 and KP08-12, at

Table 4. Characteristic of ESBL- and non-ESBL-producing clinical isolates with insertion in *ompK*35 and *ompK*36.

ESBL	Strain	□β-lactamase		Carbapenem						Outer membrane	
		AmpA	AmpC	ETP	MIC	MEM	MIC	IMP	MIC	*ompK*35	*ompK*36
	2	TEM, SHV	DHA	S	0.19	S	0.047	S	0.38	Inserted	
	13	SHV	-	S	0.094	S	0.064	S	0.19		Inserted
	55	TEM, SHV, CTXM14	-	S	0.094	S	0.016	S	0.19	Inserted	
	58	SHV	-	S	0.006	S	0.032	S	0.25		Inserted
+	67	TEM, SHV	-	S	0.064	S	0.016	S	0.094	Inserted	
	87	SHV	-	S	0.023	S	0.032	S	0.25	Inserted	
	92	SHV	DHA	R	16	I	2	R	6	Inserted	Inserted
	96	TEM, SHV, CTXM14	DHA	R	16	I	2	R	4		Inserted
	98	SHV	-	S	0.064	S	0.023	S	0.19	Inserted	
	60	SHV	DHA	S	0.25	S	0.032	S	0.75	Inserted	
	79	SHV	-	S	0.012	S	0.023	S	0.125		Inserted
	100	TEM, SHV	DHA	S	0.19	S	0.023	S	0.25	Inserted	
−	110	SHV	CMY-2, DHA	R	>32	R	12	R	>32	Inserted	Inserted
	114	TEM, SHV	CMY-2, DHA	R	>32	R	4	R	12	Inserted	Inserted
	119	TEM, SHV	CMY-2, DHA	R	4	S	0.5	I	2		Inserted

+433 bp in KP08-2, and at 78 bp in KP08-15. Additionally, an IS1 insertion at −74 bp was discovered in KP15-4, as was an IS903 insertion at +910 bp (Table 5).

Although no isolates altered antimicrobial resistance genes, the MIC value increased the highest against ertapenem, followed by meropenem and imipenem. Exception of KP08-15 with IS5 insertion in 5'-end *ompK*36, all KP08 derivatives were resistant to ertapenem and exhibited reduced susceptibility to meropenem and imipenem (Table 5). Such antimicrobial susceptibility was also confirmed by the disc diffusion method. For the *ompK*36 mutation-associated change in susceptibility to cephalosporins, all mutant isolates exhibited a reduced inhibition zone compared to the parental isolates. Importantly, isolates KP08-2 and KP15-4 developed resistance to cefepime, Instead, KP08-1, KP08-12, KP08-15 and KP16-22 developed an intermediate phenotype.

4. Discussion

The prevalence of ESBL-producing *K. pneumoniae* differs among regions and sources in Taiwan: increasing from 3.4% - 10.3% in 1995 to 11.3% in 2000 [27] [28], 20.9% in 2007 [29], 40.5% in 2010-2012 [30], 69.7% in 2006-2007 [31] and

Table 5. Insertion in OmpK36MIC and diameter of inhibition zone of carbapenem in clinical and carbapenem-induced resistance isolates.

Characters	Item	Strain								
		KP08	KP08-1	KP08-2	KP08-12	KP08-15	KP15	KP15-4	KP16	KP16-22
*ompK*36	IS family	–	IS5	IS5	IS5	IS5	–	IS1	–	IS903
	Location	–	−48 bp	433 bp	−48 bp	78 bp	–	−74 bp	–	910 bp
	Target sequence		TAAG	CTAA	AAAG	ATAA		GCCGACTG		GTAAGGATC
MIC (μg/ml)	Ertapenem	0.023	2 (R)	4 (R)	2 (R)	1.5 (R)	0.047	2 (R)	0.094	6 (R)
	ratio	1	87.0	173.9	87.0	65.2	1	42.6	1	63.8
	Meropenem	0.023	0.38 (S)	1 (S)	0.5 (S)	0.38 (S)	0.023	0.5 (S)	0.047	0.75(S)
	ratio	1	16.5	43.5	21.7	16.5	1	21.7	1	16.0
	Imipenem	0.19	0.38 (S)	0.75(S)	0.38(S)	0.38 (S)	0.125	0.25 (S)	0.38	2 or 3(I)
	ratio	1	2	3.9	2	2	1	2	1	7.9
Zome (mm)	Ertapenem	28	17	13	18	18	26	14	26	14
	Meropenem	28	22	19	22	21	25	18	25	19
	Imipenem	28	26	25	25	27	26	23	24	20
	Cefepime	25	15	13	16	17	15	9	20	15
	Ceftriazone	15	10	9	11	12	10	6	12	10
	Ceftazidime	11	8	6	9	9	6	6	8	6
	Cefotaxime	18	11	10	12	14	11	6	14	11
	Cefoxitin	23	13	12	13	12	14	6	9	6

87.1% in the current study (**Table 2**). The addition of 3-aminophenylboronic acid can enhance the accuracy to determine the ESBL-producing and the AmpC phenotypes [32] [33]. In our study, 50.7% of ESBL-producing isolates carried AmpC enzymes, which is higher than that reported previously (35.3%) (**Table 2**) [30] Furthermore, the prevalence differed between the carbapenem-resistant isolates and the total clinical isolates, with a low prevalence of AmpA genes and a high prevalence of AmpC genes (especially *bla*$_{\text{DHA-1}}$, 91.1%) in the carbapenem-resistant isolates (**Table 2** and **Table 3**). For the *bla*$_{\text{DHA}}$ and *bla*$_{\text{CMY}}$ genes of the AmpC enzyme, *bla*$_{\text{CMY}}$ can be located on a chromosome with a nonsense mutation or in a plasmid to reduce the susceptibility to third-generation cephalosporins [34], while *bla*$_{\text{DHA}}$ can increase resistance to cefoxitin (**Table 4**) [35] [36]. In our study, ESBL-producing isolates and AmpC enzyme-producing isolates exhibited different resistance patterns with higher resistance rate to ceftriaxone and cefepime *vs*. higher resistance rates to ceftazidime and cefoxitin, respectively (**Table 2**). Regardless of whether the isolate was ESBL- or non-ESBL-producing, the carbapenem-resistant isolates carried *bla*$_{\text{DHA-1}}$ and an insertion in both *ompK*35 and *ompK*36 genes, exhibiting the highest MIC to carbapenems (**Table 4**). These results indicate that porin loss and the presence of *bla*$_{\text{DHA-1}}$ are important for carbapenem resistance. However, we also observed carbapenem-resistant isolates without the ESBL and AmpC phenotypes, indicating the involvement of other mechanisms for carbapenem resistance.

Carbapenem-resistant ESBL-producing isolates are gradually increasing annually due to the use of meropenem and ertapenem for the treatment of ESBL-producing bacterial infections [29]. In recent years, carbapenem-resistant isolates have increased from 1.2% in 2003 to 11.9% in 2011[37] and have reached 13.5% against ertapenem.[15] The *bla*$_{\text{KPC}}$-carrying *K. pneumoniae* belonged to ST11 being the major clone in Taiwan [37] [38]. and worldwide [39] [40] [41]. In the present study, only three isolates carried *bla*$_{\text{KPC}}$. Furthermore, carbapenem resistance can be developed by isolates with the ESBL-type *bla*$_{\text{SHV}}$ or *bla*$_{\text{CTX-M}}$ and/or AmpC enzymes in combination with deficiency of outer membrane proteins OmpK35 and OmpK36 [42] [43]. Here, we observed one isolate with *bla*$_{\text{SHV}}$ and *bla*$_{\text{CTX-M14-like}}$ with no OmpK35 and OmpK36 expression, 13 isolates with *bla*$_{\text{SHV}}$ and *bla*$_{\text{CTX-M14-like}}$ with OmpK35 loss, and two isolates with *bla*$_{\text{SHV}}$ and *bla*$_{\text{CTX-M3-like}}$ with OmpK35 loss.

In *K. pneumoniae*, porins OmpK35 and OmpK36 play multiple roles in the development of antibiotic resistance and virulence. Isolates with deficiency in both OmpK35 and OmpK36 showed a significant decrease in virulence (*i.e.*, a slower growth rate), an increase of susceptibility to neutrophil phagocytosis [44] and a significant decrease in proinflammatory cytokine levels [45]. Further, the loss of OmpK35 is associated with resistance to lipophilic (benzylpenicillin) and large (cefepime) compounds [46]. In contrast to *ompK*35 deletion, *ompK*36 deletion caused an increased resistance to cefoxitin and cefepine associated with *bla* gene [47]. Additionally, the deletion of both ompK35 and ompK36 led to 8-

and 16-fold increases in the MICs of meropenem and cefepime, respectively. Although imipenem resistance is not directly associated with porin OmpK35/36 loss combined with ESBL production [48]. ertapenem resistance can be acquired by the loss of OmpK35/36 alone or the loss of a single porin combined with *bla*$_{\text{CTX-M-15}}$ or *bla*$_{\text{DHA-1}}$ expression [47].

Altered susceptibility to carbapenem is associated with carbapenem type, insertion sequence type, insertion location, and the MIC of the parental strain (**Table 5**). A previous study reported that the insertion sequences IS1, IS5, IS10, and IS903 insert into the promoter and coding regions of *ompK*35 and *ompK*36 (Supplementary **Table 1**). Imipenem-resistant isolates containing *bla*$_{\text{SHV-12}}$ and *bla*$_{\text{DHA-1}}$ are obtained from IS5 insertion in *ompK*36 from Korea and Taiwan [15] [49]. IS26, IS5, IS903, and IS1 insertion in *ompK*36 increases resistance to cefoxitin [50]. Following meropenem induction, all isolates with an insertion sequence in *ompK*36 exhibited reduced susceptibility to imipenem (2- to 7.9-fold increase) and meropenem (16- to 43.5-fold increase) and increased resistance to ertapenem (42.6- to 173.9-fold increase) (**Table 5**), suggesting that only OmpK36 is responsible for ertapenem resistance due to its small molecular weight. Despite the presence of IS1, IS5, and IS903 in all isolates, only KP08, KP15, and KP16 developed ertapenem resistance from a single insertion sequence in each ertapenem-resistant strain. These results imply a strain-dependent activation of the insertion sequence. Furthermore, the MIC levels of ertapenem and the inhibition zone of cephalosporins are associated with the insertion type and site.

5. Conclusion

In clinical isolates, ESBL or AmpC-producing isolates associated with carbapenem resistance were more common with deficiency in OmpK35, not OmpK36. The isolate dependent IS1, IS5, and IS903 were able to insert into *ompK*36 to cause resistance to ertapenem and reduced susceptibility to imipenem and carbapenem.

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