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Phenotypic and Genomic Characterization of AmpC-Producing *Klebsiella pneumoniae* From Korea

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The prevalence of multidrug-resistant gram-negative bacteria has continuously increased over the past few years; bacterial strains producing AmpC β-lactamases and/or extended-spectrum β-lactamases (ESBLs) are of particular concern. We combined high-resolution whole genome sequencing and phenotypic data to elucidate the mechanisms of resistance to cephamycin and β-lactamase in Korean *Klebsiella pneumoniae* strains, in which no AmpC-encoding genes were detected by PCR. We identified several genes that alone or in combination can potentially explain the resistance phenotype. We showed that different mechanisms could explain the resistance phenotype, emphasizing the limitations of the PCR and the importance of distinguishing closely-related gene variants.

Key Words: AmpC, Antibiotic resistance, Whole Genome Sequencing, *Klebsiella pneumoniae*, Korea

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The AmpC β-lactamases produced by Enterobacteriaceae are clinically relevant enzymes that can hydrolyze penicillins, cephamycins such as cefoxitin, as well as broad-spectrum cephalosporins such as ceftazidime or cefotaxime. Moreover, penicillinase inhibitors are ineffective against these enzymes. The cephalosporinases encoded in the chromosome of group 3 Enterobacteriaceae were originally present only in *Enterobacter* spp., *Morganella* spp., *Citrobacter* spp., and a number of other species. Following the acquisition of *ampC* genes via transmissible plasmids, cephalosporinases began to appear in *Escherichia coli* and *Klebsiella pneumoniae* as well [1]. Distinguishing between acquired *ampC* and chromosomal *ampC* is possible by gene amplification only. Plasmid-mediated AmpC enzyme production is less common than production of extended-spectrum β-lactamases, and its detection remains difficult in the presence of other enzymes, leading to an underestimation of this resistance mechanism [2]. We identified *K. pneumoniae* strains that exhibited an AmpC phenotype in the absence of PCR-detectable *ampC* genes. Furthermore, we used genome sequencing and identified other β-lactamase genes that could possibly explain the resistance phenotype of these strains.

A total of 257 *K. pneumoniae* strains were collected from 17 hospitals in Korea. No ethical clearance was required for this study since patient data were not included and the source of the strains was anonymous. The strains were collected in 2014 and were stored at –80°C until use. Before use, the strains were subcultured on MacConkey agar and were deposited in the central laboratory (Seoul St. Mary's Hospital). Of the total isolates,

39 were intermediately resistant or resistant to cefoxitin, and 31 of these expressed a plasmid-mediated DHA-type AmpC β-lactamase; this enzyme confers resistance to cephamycins and oxyimino-cephalosporins and is most likely characterized by inducible production. Phenotypic detection of AmpC was performed using a modified disk-based method, with boronic acid as an AmpC inhibitor [3]. Briefly, the isolates were tested using a standard cefoxitin disk (30 μg) and a cefoxitin disk supplemented with 400 μg of phenylboronic acid (Sigma-Aldrich, Saint-Quentin Fallavier, France). Isolates were considered AmpC-positive if the zone of inhibition surrounding the phenylboronic acid-cefoxitin disk was ≥5 mm larger than that surrounding the cefoxitin disk.

The isolates were also tested using a multiplex PCR assay that identifies plasmid-mediated *ampC* genes [4]. Five *ampC*-PCR-negative strains exhibiting the AmpC phenotype were subjected to further phenotypic characterization and whole genome sequencing (WGS) for detailed analyses of resistance gene content. The antimicrobial susceptibilities of the strains were tested using the VITEK2 instrument (bioMérieux, Marcy-l'Étoile, France). DNA was extracted using the Mo Bio kit (QIAGEN, Hilden, Germany) and sequenced using the Illumina MiSeq system with a 2×150 bp approach and a minimum coverage of 100×. The reads were assembled using A5-miseq, and the scaffolding was performed using SSPACE. The ResFinder online tool (https://cge.cbs.dtu.dk/services/ResFinder/) was used to assess the presence of (acquired) antimicrobial resistance genes, while the BioNumerics 7 software (Applied Maths, Sint-Martens-Latem, Belgium) was used to define microbial multi locus sequence types (MLST). The same tool was used to assess mutations in the *ompK35* and *ompK36* genes, which encode porins important for antibiotic uptake; mutation of these genes can lead to impermeability to several types of β-lactam drugs [5].

The five *ampC*-PCR-negative *K. pneumoniae* strains (all originating from urine samples and collected in different cities in Korea) belonged to four different sequence types (ST) (Table 1). Phenotypic characterization showed that all strains, except for 1606284, were resistant to cefoxitin; all strains were resistant to ampicillin/sulbactam and ticarcillin/clavulanic acid; and all but two strains (1606283 and 1606285) were resistant to piperacillin/tazobactam, according to the EUCAST breakpoints (Table 2). As the ResFinder results confirmed the absence of AmpC-encoding genes, we investigated which other genes could produce an AmpC-like phenotype.

The enzymatic mechanisms conferring resistance or decreased susceptibility to β-lactamase inhibitors and cephamycins in *K. pneumoniae* include plasmid-mediated AmpC-type β-lactamase (p-AmpC) [1]; hyper-production of plasmid-mediated class A β-lactamases, such as TEM-1 and SHV-1 [6, 7]; production of inhibitor-resistant TEM (IRT) β-lactamases [8, 9]; plasmid-mediated OXA-type β-lactamase, such as OXA-1 [10]; and complex mutant TEMs (CMT), which are enzymes that combine IRT- and extended-spectrum β-lactamase (ESBL)-type substitutions [11]. ResFinder analyses identified at least one gene in each strain

Table 1. Multilocus sequence type (MLST) and metadata of the five *Klebsiella pneumoniae* strains subjected to whole genome sequencing

Strain	MLST	Country	City	Specimen source	Disease
1606283	ST17	Korea	Pusan	Urine	Urinary tract infection [chronic kidney disease]
1606284	ST11	Korea	Pusan	Urine	Urinary tract infection [acute tubulo-interstitial nephritis]
1606285	ST584	Korea	Seoul	Urine	Abscess [benign neoplasm of central nervous system]
1606286	ST307	Korea	Incheon	Urine	Other [cerebral infarction due to embolism of cerebral arteries]
1606287	ST307	Korea	Seoul	Urine	Other [upper gastrointestinal bleeding]

Table 2. Minimum inhibitory concentrations in micrograms per millilitre of the five *Klebsiealla pneumoniae* strains

Strain	Ampicillin	Ampicillin/ Sulbactam	Ticarcillin	Ticarcillin/ Clavulanate	Piperacillin	Piperacillin/ Tazobactam	Cefoxitin	Cefotaxime	Ceftazidime
1606283	≥32	≥32	≥128	=64	≥128	=16	=32	=16	≤1
1606284	≥32	≥32	≥128	≥128	≥128	≥128	≥64	=8	≥64
1606285	≥32	=16	≥128	=64	≥128	=8	≤4	=2	≥64
1606286	≥32	≥32	≥128	≥128	≥128	≥128	≥64	≥64	≥64
1606287	≥32	≥32	≥128	≥128	≥128	≥128	≥64	≥64	≥64

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Table 3. The *bla* genes conferring resistance to β-lactams identified by ResFinder and BioNumerics analysis

Strain	*tem*	*Shv*	*ctx-m*	*oxa*	*len*	*ompK*
1606283		*shv-11(2b)*	*ctx-m-14*			
1606284	*tem-1b(2b)*	*shv-11(2b)*		*oxa-1(2d)*		*ompk35* truncated
1606285	*tem-1b(2b)*				*len-12*	
1606286		*shv-28*	*ctx-m-15(2be)*	*oxa-1(2d)*		
1606287	*tem-102(2be)*	*shv-28*	*ctx-m-15(2be)*	*oxa-1(2d)*		

The Bush functional classification, when available, is indicated within parenthesis.

that could fully or partially explain the presence of a resistance phenotype in an *ampC*-PCR-negative background (Table 3).

ResFinder showed the presence of the bla_{OXA-1} gene in three of five strains (1606284, 1606286, and 1606287); two of these strains also contained $bla_{CTX-M-15}$ and bla_{SHV-28}, and the third strain also possessed bla_{TEM-1b} and bla_{SHV-11}. These three strains were associated with high minimum inhibitory concentrations (MICs) for both penicillin plus β-lactamase inhibitor combinations tested and for cefoxitin. Strain 1606283 carried $bla_{CTX-M-14}$, encoding an ESBL able to confer decreased susceptibility to β-lactamase inhibitors; this strain demonstrated resistance to sulbactam and clavulanate and intermediate resistance to tazobactam. These results and the high cefoxitin MICs suggested impermeability to cephamycins. BioNumerics analysis of the *ompK* genes did not reveal any genetic mutations leading to frameshifts resulting in a truncated protein. However, we cannot exclude the presence of mutations leading to altered functionality or limited gene expression. Strain 1606285 carried bla_{TEM-1B} together with bla_{LEN-12}; overexpression of bla_{TEM-1B} can decrease the susceptibility to combined treatment with penicillins and β-lactamase inhibitors. Overexpression of bla_{TEM-1} has been associated with promoters *p3, pa/pb, p4*, and *p5* [12]; we observed that both strains carrying the *tem-1b* gene harbored the *p3* promoter upstream of the β-lactamase gene. Finally, analysis of the *ompK* genes revealed that only the *ompK35* in strain 1606284 contained a deletion that resulted in a frameshift and, consequently, a truncated protein.

In this pilot study, we show that discrepancies between resistance phenotypes and PCR results can be elucidated and possibly corrected using genomic data. Although these results are not unexpected, our analyses demonstrate that genomics data could be a valuable addition to PCR-based targeted gene testing. We have not included functional studies to prove that the new genes identified are causal of the phenotype because this was beyond the scope of the study. Furthermore, we assume that previously reported associations between phenotypes and the presence of certain genes (such as $bla_{CTX-m-14}$, bla_{TEM-1b}, and bla_{OXA-1}) are correct and reliable [13]. Although PCR analysis is preferable in many settings because of the high cost and complexity of genomic analysis, we recommend that national reference laboratories adopt the pipeline proposed in our current analysis.

Authors' Disclosures of Potential Conflicts of Interest

All the authors, except Yeon-Joon Park, are employees of bioMérieux, a company that develops and sells diagnostic tests in the field of infectious diseases.

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