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Comparative study on genotype and virulence of CTX-M-producing and non-ESBL-producing *Klebsiella pneumoniae* isolates

Short title: Virulence of CTX-M-producing *K. pneumoniae*

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Abstract

Molecular and virulence characteristics of CTX-M-producing and non-ESBL-producing *Klebsiella pneumoniae* isolates were compared. Lack of shared characteristics between the two groups suggested that most CTX-M-producing *K. pneumoniae* isolates in Korea did not occur by transfer of $bla_{\text{CTX-M}}$ into susceptible strains. Conjugation assays confirmed that the plasmid with the $bla_{\text{CTX-M-15}}$ gene confers virulence as well as antimicrobial resistance, suggesting that a CTX-M-15-producing clone such as ST11 may have a selective advantage even without antibiotic pressure.

Keywords: ST11, human serum sensitivity, virulence

In parallel with the use of antibiotic drugs, prevalence of *Escherichia coli* and *Klebsiella pneumoniae* producing CTX-M-type ESBLs has increased worldwide (1). In addition to CTX-M-15-producing *E. coli*, CTX-M-15 has increased in prevalence *K. pneumoniae* worldwide (2-4). In *K. pneumoniae*, the $bla_{\text{CTX-M-15}}$ is carried mainly by IncFII-type plasmids (5). Production of the SHV-type ESBLs in *K. pneumoniae* is associated with an increased tendency to invade epithelial cells and expression of fimbrial adhesions (10). Although highly virulent clones expressing CTX-M-type β-lactamase, such as *E. coli* ST131 have been reported (11), the association of CTX-M enzyme production with virulence and fitness in *K. pneumoniae* is not clear. In this study, we compared genotypic and phenotypic characteristics between CTX-M-producing and non-ESBL-producing *K. pneumoniae* isolates from Korea. In addition, the fitness cost of carrying the $bla_{\text{CTX-M-15}}$ gene-bearing plasmid was investigated.

In this study, 98 *K. pneumoniae* isolates, which were collected from patients with bacteremia from nine Korean hospitals as a part of multicenter surveillance study from September to December, 2008, were included (12). Thirty-three isolates were found to express $bla_{\text{CTX-M}}$ genes (18 $bla_{\text{CTX-M-14}}$ and 15 $bla_{\text{CTX-M-15}}$), while 65 isolates did not produce any ESBL. ESBL activity was confirmed using double-disc method. *In vitro* antimicrobial susceptibility testing was performed by a broth microdilution method, following the CLSI guidelines (13). MLST was performed as described previously (http://www.pasteur.fr/recherche/genopole/PF8/mlst/Kpneumoniae.html) with some modifications. PFGE was performed for all ST11 *K. pneumoniae* isolates (14). PCR assays were performed to monitor for the presence of genes previously found to be associated with virulence in *K. pneumoniae* (15,16). The string test was used to

57 determine the hypermucoviscosity phenotype (17). α-hemolysin production was
58 detected using a 5% sheep's blood agar plate (18).

59 The transfer of the plasmid carrying the $bla_{\text{CTX-M-15}}$ was accomplished using an *E. coli*
60 DH5α strain as described previously (19). The plasmid carrying the $bla_{\text{CTX-M-15}}$ gene
61 from an ST11 *K. pneumoniae* isolate, K01-12226, was used (5). The plasmid carrying
62 $bla_{\text{CTX-M-15}}$ was transferred into five non-ESBL-producing ST11 *K. pneumoniae* isolates
63 K01-1054, K01-6053, K01-7096, K01-8102, and K01-8139 from *E. coli* DH5α as a
64 donor (12). The presence of $bla_{\text{CTX-M-15}}$ in transconjugants was confirmed by PCR. A
65 serum sensitivity assay was performed on clinical isolates and transconjugants, as
66 previously described (20). Fisher's exact test was used to determine the significance of
67 differences in serum resistance between strains using SPSS version 11.5 (SPSS,
68 Chicago, IL, USA).
69

70 The CTX-M-producing *K. pneumoniae* isolates showed significantly greater
71 resistance to most antibiotics except ampicillin, amikacin, and imipenem than did non-
72 ESBL-producing isolates (p <0.05) (Table 1). While *cf29a*, a gene in *E. coli* encoding
73 adhesin CS31A, and associated with diarrhea in humans, was found in only one CTX-
74 M-14-producing, ST11 isolate, it was present in 14 non-ESBL-producing isolates (p,
75 0.018) (Table 1). Eight (72.7%) of 11 non-ESBL-producing ST11 isolates possessed
76 the *cf29a* gene, and four ST163 isolates tested positive for it. In addition to *cf29a*, *allS*,
77 which encodes an activator of the allantoin regulon, was found more frequently in non-
78 ESBL-producing isolates (p, 0.05).
79 In MLST analysis, a total of 52 different STs were identified among the 98 *K.*

pneumoniae isolates: 19 STs among the 33 CTX-M-producing isolates and 36 among the 65 non-ESBL-producing isolates (Table 2). Only three STs (ST11, ST15, and ST48) were detected in both CTX-M-producing and non-ESBL-producing isolates. This suggests that most CTX-M-producing *K. pneumoniae* isolates in Korea did not occur by transfer of the $bla_{\text{CTX-M}}$ gene into susceptible strains. In PFGE analysis, 16 ST11 isolates did not show exactly the same restriction pattern, but similarities in pattern could be identified. In particular, an approximately 388-kb fragment was found only in CTX-M-15-producing *K. pneumoniae* isolates. It was revealed to be a plasmid carrying $bla_{\text{CTX-M-15}}$, by PCR. In addition, PFGE analysis of transconjugants receiving a plasmid showed additional 388 kb band (not shown). It may be hasty to conclude that CTX-M-15-producing ST11 isolates arise by transfer of a $bla_{\text{CTX-M-15}}$-bearing plasmid into susceptible strains unique to Korea, since ST11 is distributed among CTX-M-15-producing clone worldwide (22).

Non-ESBL-producing *K. pneumoniae* isolates showed higher serum resistance than CTX-M-producing isolates (p, 0.004) (Fig. 1A). In addition, the hypermucoviscosity phenotype was more frequently identified in non-ESBL-producing isolates; only three CTX-M-producing isolates (9.1%) expressed the hypermucoviscosity phenotype as compared to 19 non-ESBL-producing isolates (29.2%) (p, 0.038). None of the *K. pneumoniae* isolates produced α-hemolysin.

To understand the effects of the plasmid on fitness measures such as growth rate and serum resistance, plasmids carrying the $bla_{\text{CTX-M-15}}$ gene of an ST11 isolate were transferred to non-ESBL-producing ST11 isolates by conjugation. All of these transconjugants showed substantial increases in MICs for cephalosporins, with one exception (cefotaxime for T-6053). The CTX-M-producing, non-ESBL-producing, and

transconjugant strains showed no significant differences in growth rates. Although serum resistance did not differ between CTX-M-producing and non-ESBL-producing ST11 *K. pneumoniae* isolates, transconjugants showed higher survival rates against serum compared with their host isolates and donors (Fig. 1B). Comparing the survival rates against serum in pairs of transconjugant and host isolates, transconjugants showed significantly higher serum resistance than did their host isolates, except for the pair that included K01-1054 and T-1054. *traT* gene in the plasmid may contribute to serum resistance, which explains the increased survival rates against serum in the transconjugants.

As a whole, non-ESBL-producing *K. pneumoniae* isolates were assumed to be more virulent than CTX-M-producing isolates. First of all, non-ESBL-producing isolates showed a higher rate of resistance against human serum than CTX-M-producing *K. pneumoniae* isolates. Second, the hypermucoviscosity phenotype was more frequently found in non-ESBL-producing isolates. In addition, while CTX-M-producing isolates contained 7.64 virulence factors on average, 8.54 virulence factors, on average, were identified in non-ESBL-producing isolates (p, 0.005).

However, serum resistance did not differ between CTX-M-15-producing and non-ESBL-producing isolates of ST11. More importantly, four out of five transconjugants showed higher serum resistance than their hosts (Fig. 1B). This suggests that plasmids with the $bla_{\text{CTX-M-15}}$ gene may confer virulence as well as antimicrobial resistance, although only one kind of plasmid (IncFII) was tested in this study. Thus, CTX-M-15-producing *K. pneumoniae* isolates may carry more than one positively selective trait, implying that antimicrobial-resistant strains could increase in prevalence even without

antimicrobial pressure. Although increased serum resistance is generally associated with decreased virulence and fitness, credible evidence to the contrary has emerged (27). Global dissemination of highly pathogenic and resistant clones would be cause for great concern (11).

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248 **Table 1.** Antimicrobial resistance and virulence factors of CTX-M-producing and non-
249 ESBL-producing *K. pneumoniae* isolates.

Antimicrobial agent	Total (n=98)	CTX-M-producing (n=33)	Non-ESBL-producing (n=65)	*P*[a]
Antimicrobial agent				
Ampicillin	97 (99.0)	33 (100)	64 (98.5)	0.474
Ceftazidime	50 (51.0)	31 (93.9)	19 (29.2)	<0.001
Cefotaxime	47 (48.0)	28 (84.8)	19 (29.2)	<0.001
Aztreonam	42 (42.9)	27 (81.8)	15 (23.1)	<0.001
Amikacin	11 (11.2)	2 (6.1)	9 (13.8)	0.327
Gentamicin	50 (51.0)	33 (100)	17 (26.2)	<0.001
Ciprofloxin	43 (43.9)	20 (60.6)	23 (35.4)	0.017
Imipenem	1 (1.0)	1 (3)	0	0.287
Trimethoprim-sulfamethoxazole	45 (45.9)	30 (90.9)	15 (23.8)	<0.001
Piperacillin-tazobactam	52 (53.1)	22 (66.7)	30 (46.2)	0.022
Virulence factor				
fimH	98 (100)	33 (100)	65 (100)	-
oxyR	98 (100)	33 (100)	65 (100)	-
ureA	98 (100)	33 (100)	65 (100)	-
kfu	97 (98.9)	32 (96.9)	65 (100)	0.330
wabG	97 (98.9)	33 (100)	64 (94.1)	0.549
uge	92 (93.8)	33 (100)	59 (90.7)	0.174
ramA	91 (92.8)	31 (93.9)	60 (92.3)	0.579
allS	31 (31.6)	4 (12.1)	27 (41.5)	0.005
iutA	29 (29.5)	8 (24.2)	21 (32.3)	0.494
rmpA	24 (24.4)	4 (12.1)	20 (30.7)	0.080
wca	22 (22.4)	5 (15.1)	17 (26.1)	0.310
cf29a	15 (15.3)	1 (3.0)	14 (21.5)	0.018
magA	13 (13.2)	2 (6.0)	11 (16.9)	0.134

ybtQ	2 (2.0)	0	2 (3.0)	0.549

[a] *p* values between CTX-M-producing and non-ESBL-producing *K. pneumoniae* isolates.

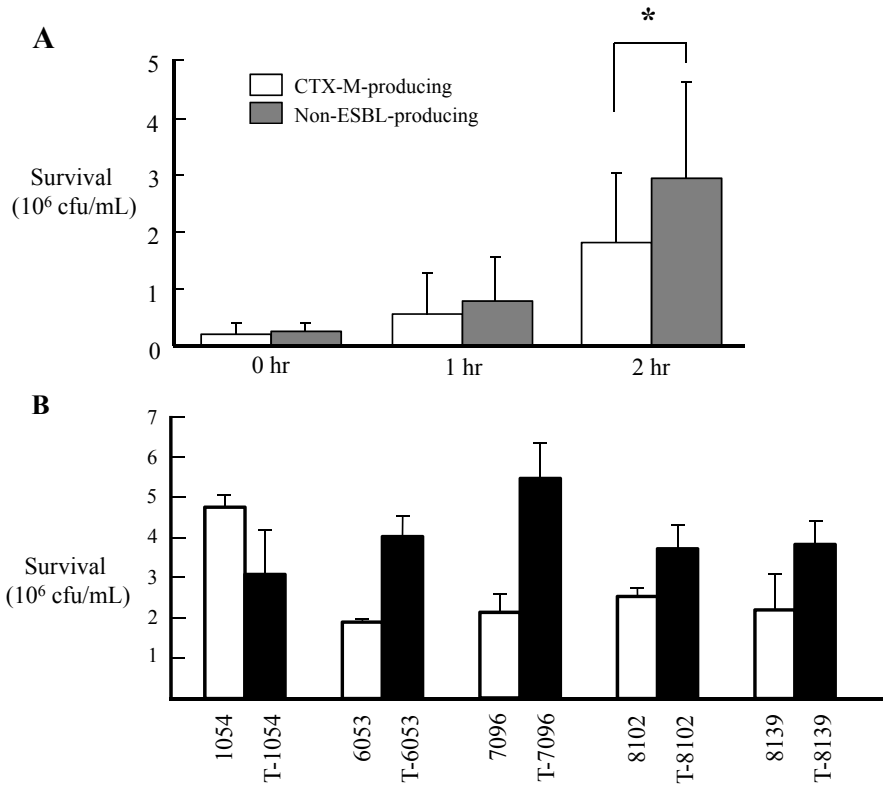
251 **Table 2.** Multi-locus sequence typing (MLST) analysis of CTX-M-producing and non-
252 ESBL-producing *K. pneumoniae* isolates.

CC	ST	Allelic profile [a]	Total (n=98)	CTX-M-producing (n=33)	Non-ESBL-producing (n=65)
CC11	11	3-3-1-1-1-1-4	16	5	11
	258	3-3-1-1-1-1-79	1	1	
	340	3-3-1-1-1-1-18	1	1	
CC631	163	2-1-1-1-9-1-12	10		10
	23	2-1-1-1-9-4-12	3	3	
	17	2-1-1-1-4-4-4	1		1
	18	2-1-1-1-4-1-4	1		1
	631	2-1-1-1-9-4-4	1		1
	1059	2-1-1-1-12-1-4	1		1
CC298	36	2-1-2-1-7-1-7	3		3
	298	2-1-2-1-1-1-7	1	1	
	966	2-1-2-1-1-1-68	1		1
CC375	375	43-1-2-1-10-4-3	2		2
	65	2-1-2-1-10-4-13	1	1	
	1053	16-1-2-1-10-4-13	1		1
CC469	469	2-1-2-1-10-1-4	2	2	
	35	2-1-2-1-10-1-19	1		1
CC1063	1063	2-3-1-1-9-4-193	1		1
	1064	2-3-1-1-1-1-193	1		1
Singleton	15	1-1-1-1-1-1-1	8	7	1
	86	9-4-2-1-1-1-27	5		5
	48	2-5-2-2-7-1-10	4	2	2
	34	2-3-6-1-9-7-4	2		2
	101	2-6-1-5-4-1-6	2		2
	12	6-3-1-1-12-1-4	1		1
	76	4-1-1-1-21-1-35	1	1	
	105	2-3-2-1-1-4-18	1	1	
	110	2-6-1-3-8-1-44	1		1
	147	2-6-1-3-8-1-44	1		1
	165	2-1-13-2-23-1-19	1	1	
	222	2-1-2-2-7-4-4	1	1	
	300	2-1-19-1-9-4-34	1	1	
	317	10-1-2-1-9-27-18	1	1	
	372	2-1-2-1-1-15-4	1	1	

380	2-1-1-1-1-4-19	1	1	
412	2-1-2-1-9-1-112	1		1
502	2-53-3-10-4-18	1		1
518	2-3-1-1-7-4-87	1		1
537	6-3-1-4-12-4-4	1	1	
538	2-1-2-20-9-1-14	1	1	
1026	2-1-2-35-10-24-19	1		1
1050	16-8-21-31-92-17-67	1		1
1051	2-1-11-1-9-10-9	1		1
1052	2-1-1-1-17-1-42	1		1
1054	2-3-1-1-12-4-12	1		1
1055	16-24-21-1-1-17-1	1		1
1056	16-62-21-40-153-40-67	1		1
1057	16-24-21-27-47-17-134	1		1
1058	43-1-2-1-10-1-12	1		1
1060	16-18-21-21-52-30-75	1		1
1061	2-3-1-20-61-4-181	1		1
1062	2-3-2-2-162-1-4	1		1

253 [a] Allelic profile, *gapA-infB-mdh-pgi-phoE-rpoB-tonB*.

Figure 1. Results of serum resistance assay are shown here as CFU viability. Error bars indicate standard deviations. (A) Serum resistance assay for all CTX-M-producing and non-ESBL-producing *K. pneumoniae* isolates. Significance is shown for the difference between CTX-M-producing and non-ESBL-producing *K. pneumoniae* isolates (*, $p < 0.05$). (B) Survival rate of each non-ESBL-producing *K. pneumoniae* isolate and their transconjugants after incubation of 2 hr.



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