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Original Article

Comparative genetic analysis of the antimicrobial susceptibilities and virulence of hypermucoviscous and non-hypermucoviscous ESBL-producing *Klebsiella pneumoniae* in Japan

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KEYWORDS
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Abstract *Background:* Hypermucoviscous (HMV) *Klebsiella pneumoniae* produces large amounts of capsular polysaccharides, leading to high mortality. Since extended spectrum beta-lactamase (ESBL)-producing HMV *K. pneumoniae* strains have increased in Japan, we investigated and compared the antimicrobial susceptibilities and genetic characteristics of

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pneumoniae;
Hypervirulence in *K. pneumoniae* (hvKp);
Extended spectrum beta-lactamase (ESBL);
CTX-M-15;
Plasmid

HMV and non-HMV ESBL-producing *K. pneumoniae*.
Methods: We investigated 291 ESBL-producing *K. pneumoniae* collected between 2012 and 2018, and in them 54 HMV strains were identified and comparable 53 non-HMV strains were selected. Then, ESBL gene detection, plasmid replicon typing, and virulence gene detection were done by PCR amplification.
Results: Almost all of the HMV *K. pneumoniae* strains possessed *uge* (98.1%), *wabG* (96.3%), *rmpA* (94.4%), *iucA* (79.6%), *fimH* (70.4%), *iroB* (70.4%), and *peg-344* (70.4%). These genes were found less frequently in non-HMV strains (*uge* 20.8%, *wabG* 83.0%, *rmpA* 7.5%, *iucA* 3.8%, *fimH* 9.4%, *iroB* 5.7%, and *peg-344* 1.9%). K2 capsule type (40.7%) was most common in HMV strains. HMV strains showed higher resistance to cefepime ($p = 0.001$) and piperacillin/tazobactam ($p = 0.005$) than non-HMV strains. CTX-M-15 (75.9%, 60.4%) was the dominant ESBL type in both HMV and non-HMV strains, and the most common plasmid replicon type was IncFII (52.1%) in CTX-M-15-producing strains.
Conclusions: We found that HMV strains had more virulence genes and showed higher resistance to antibiotics than non-HMV strains. The most common capsule type was K2. CTX-M-15 was the most common type of ESBL gene in both HMV and non-HMV strains in Japan. The FII plasmid might be related to the spread of CTX-M-15 among *K. pneumoniae* strains.

Introduction

Klebsiella pneumoniae frequently causes hospital-acquired urinary tract infections, pneumonia, septicemia, and soft tissue infections. Hypermucoviscous (HMV) *K. pneumoniae* strains are widely known for showing hypervirulence in *K. pneumoniae* (hvKp).[1] *K. pneumoniae* has several hypervirulence factors, including capsular polysaccharides, lipopolysaccharide (LPS), adhesion, and iron uptake acquisition. HMV strains produce large amounts of capsular polysaccharides, contributing to severe infections with high mortality such as liver abscess, meningitis, and brain abscess.[1–3] While classical *K. pneumoniae* (cKp) tends to cause nosocomial outbreaks, HMV *K. pneumoniae* strains infect community-dwelling individuals who are often young and healthy.[1,2,4]

For the past decade, the prevalence of antibiotic-resistant *K. pneumoniae* strains, e.g. extended spectrum beta-lactamase (ESBL) producing bacteria, has been increasing due to the broad use of beta-lactam antibiotics.[5–7] ESBL, one of the important mechanisms in antimicrobial resistance, can hydrolyze all beta-lactams except for cephamycin and carbapenem, which limits the available choices of antibiotics for appropriate therapy. ESBL genes are classified into several types, including $bla_{CTX\text{-}M}$, bla_{SHV}, and bla_{TEM}.[8] Although the TEM and SHV types are globally widespread, the CTX-M type has emerged as the dominant ESBL type.[5,7] CTX-M-15-type ESBL-producing strains have especially increased in recent years to become the most common ESBL in many regions of the world.[3,5–9] The rate of ESBL-producing strains in *K. pneumoniae* was approximately 10% in Japan in 2017.[7] ESBL-producing antibiotic-resistant *K. pneumoniae* are difficult to treat and increase patient mortality.[5] ESBL-producing HMV *K. pneumoniae* strains have been reported recently in several countries, including Japan.[2,3,10–12] ESBL genes can spread between strains by plasmids, extrachromosomal DNA molecules, and such horizontal transfer contributes to the rapid spread of ESBL-producing *K. pneumoniae*.[5,13] The spread of ESBL genes can be determined by investigating plasmid replicon types of ESBL-producing isolates. Analyzing and tracing plasmids of ESBL-producing *K. pneumoniae* will contribute to a better understanding of the epidemiology and spread of ESBL-related antimicrobial resistance.

The epidemiology and genetic features of ESBL-producing and HMV *K. pneumoniae* have been reported globally. Liu and Guo investigated HMV strains in China, and Yamasaki et al. investigated ESBL-producing HMV strains in Indonesia.[3,10] However, despite the clinical importance of the recent surge in antibiotic resistance, few studies have been reported in Japan, resulting in an incomplete picture of antimicrobial susceptibility and the genetic characteristics of ESBL-producing *K. pneumoniae* in this region. In addition, we evaluated how HMV influences antimicrobial susceptibility and hypervirulence in *K. pneumoniae* by comparing HMV and non-HMV strains.

Methods

Bacterial isolates

In this study, a total of 291 consecutive and non-duplicate ESBL-producing *K. pneumoniae* isolates were identified from a large number of clinical specimens collected from infectious disease patients in 695 medical institutions in Hyogo Prefecture and sent to Hyogo Clinical Laboratory Corporation, Himeji, Japan between 2012 and 2018 for matrix-assisted laser desorption ionization—time of flight mass spectrometry (MALDI-TOF MS) and ESBL screening.

HMV *K. pneumoniae* strains were determined using the string test. *K. pneumoniae* strains were cultured on blood agar plates for 24 h. Strains that threaded more than 5 mm when touched with a loop were considered HMV strains, and those that threaded less than 5 mm were considered non-HMV strains.[14] Fifty-four (18.6%) HMV strains were identified.

We selected 53 strains from the 237 non-HMV *K. pneumoniae* isolates to compare their antimicrobial susceptibilities and genetic characteristics with the HMV strains and

to evaluate how HMV influences antimicrobial susceptibilities and hvKp. However, because the information on 291 ESBL-producing *K. pneumoniae* isolates was limited to source and year, these 53 non-HMV strains were selected to achieve a similar distribution of derived specimens to the HMV strains.

Antimicrobial susceptibility testing

The disc diffusion method was performed according to Clinical and Laboratory Standard Institute recommendations to determine susceptibility to ceftazidime (CAZ), cefotaxime (CTX), cefepime (CFPM), cefmetazole (CMZ), piperacillin/tazobactam (T/P), imipenem (IPM), meropenem (MEPM), sulfamethoxazole/trimethoprim (ST), minocycline (MINO), and levofloxacin (LVFX) (Becton, Dickinson and Company). *Escherichia coli* ATCC25922 was used as the precision control strain.[15] ESBL-producing strains were identified when the diameters of the inhibition zones of discs containing CAZ plus clavulanic acid (CVA) increased by 5 mm or more compared to discs containing CAZ, or when the diameters of the inhibition zones of discs containing CTX plus CVA increased by 5 mm or more compared to discs containing CTX.[15] ESBL screening was performed in the laboratory after *K. pneumoniae* isolates were collected.

Characterization of ESBL genes

After 24 h of culture on DHL agar plates, the growth colonies were added to TE buffer and heat-treated at 100 °C for 10 min, then centrifuged for DNA extraction. Polymerase chain reaction (PCR) amplification was used to detect $bla_{\text{CTX-M-1, 2, 9, 14, 15}}$, bla_{SHV}, and bla_{TEM}. The primers used are listed in Supplementary Table 1. The PCR conditions were initial denaturing at 94 °C for 3 min, followed by 25 ($bla_{\text{CTX-M}}$) and 30 (bla_{SHV} and bla_{TEM}) cycles of denaturation at 94 °C for 30 s, annealing at 60 °C ($bla_{\text{CTX-M}}$ and bla_{SHV}), 63 °C (bla_{TEM}) respectively for 30 s, extension at 72 °C for 1 min, and final extension at 72 °C for 5 min.[16] The PCR products were electrophoresized on 1% agarose gels and stained with ethidium bromide (0.5 mg/ml) in a dark room. The PCR products were purified using the QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany) and sequencing analysis was done by Eurofins genomics (Tokyo, Japan).

Plasmid replicon typing

Plasmid replicon typing was performed in all of the collected ESBL-producing *K. pneumoniae* strains to investigate the propagation styles of ESBL genes. IncF (F, FIA, FIB, FIC, FII), N, L/M, B/O, A/C, P, W, Y, H (HI1, HI2), I1, T, K, and X were determined by PCR amplification. The PCR conditions were initial denaturing at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C 30 s, extension at 72 °C for 1 min, and final extension at 72 °C for 5 min.[17]

Characterization of virulence genes

All the strains were investigated for virulence genes, including *magA*, *rmpA*, *fimH*, *wabG*, *uge*, *iucA*, *iroB*, and *peg-344* and capsular types of K1, K2, K5, K20, K54, and K57 by PCR amplification (Supplementary Tables 2 and 3). The PCR conditions are shown in Supplementary Tables 4 and 5.

Statistical analysis

Fisher's exact probability test was performed to statistically analyze correlations between ESBL genes and antimicrobial susceptibilities. In addition, antimicrobial susceptibilities, carriage of ESBL genes, virulence genes, and plasmid replicon typing were compared between HMV and non-HMV *K. pneumoniae* strains by Fisher's exact probability test. These analyses were performed by EZR, and statistical differences were considered significant when p-values were <0.05.[18]

Results

Bacterial isolates

In this study, 54 (18.6%) HMV strains were identified. These isolates were collected from various types of patient specimens, including 35 (64.8%) isolates from sputum, 16 (29.6%) from urine, and 3 (5.6%) from venous blood. In addition, 29 (54.7%) of 53 non-HMV strains were isolated from sputum, 16 (30.2%) from urine, 3 (5.6%) from venous blood, and 5 (5.7%) from other sources such as wounds, pressure ulcers, pleural effusion, ear leakage and pus. Detailed information on the 107 ESBL-producing *K. pneumoniae* isolates used in this study is shown in Supplementary Table 6.

Antimicrobial susceptibility testing

Screening confirmed that all the collected 107 *K. pneumoniae* strains produced ESBL. The results of antimicrobial susceptibility testing for ten antibiotics are shown in Fig. 1. ESBL-producing *K. pneumoniae* strains showed high susceptibility to IPM (100%), MEPM (99.1%), CMZ (97.2%), LVFX (86.9%), T/P (84.5%), and MINO (78.5%). These strains exhibited low susceptibility to CTX (0%), ST (22.4%), CFPM (29.0%), and CAZ (29.9%). HMV strains showed lower susceptibility rates to CFPM (14.8% vs. 43.4%, $p = 0.001$) and T/P (70.4% vs. 92.5%, $p = 0.005$) than non-HMV strains. Susceptibility rates to CAZ (25.9% vs. 34.0%, $p = 0.404$), ST (16.7% vs. 28.3%, $p = 0.170$) and MINO (72.2% vs. 84.9%, $p = 0.158$) were not significantly different (Fig. 2).

Characterization of ESBL genes

In the 107 ESBL-producing *K. pneumoniae* isolates, the CTX-M-15 type (68.2%) was the most commonly detected ESBL gene, followed by $bla_{\text{CTX-M-2}}$ in 6 strains (5.6%), $bla_{\text{SHV-28}}$ in 6 strains (5.6%), $bla_{\text{SHV-38}}$ in 6 strains (5.6%), bla_{TEM} in 6 strains (5.6%), $bla_{\text{CTX-M-14}}$ in 5 strains (4.7%), $bla_{\text{SHV-12}}$ in 4 strains (3.7%), $bla_{\text{SHV-187}}$ in 4 strains (3.7%), $bla_{\text{CTX-M-27}}$ in 3 strains (2.8%), and $bla_{\text{CTX-M-3}}$ in 2 strains (1.9%) (Table 1). CTX-M-15-producing strains showed higher resistance to CAZ ($p = 0.003$), ST ($p = 0.003$), and CFPM ($p = 0.002$) than non-CTX-M-15 strains (Table 2).

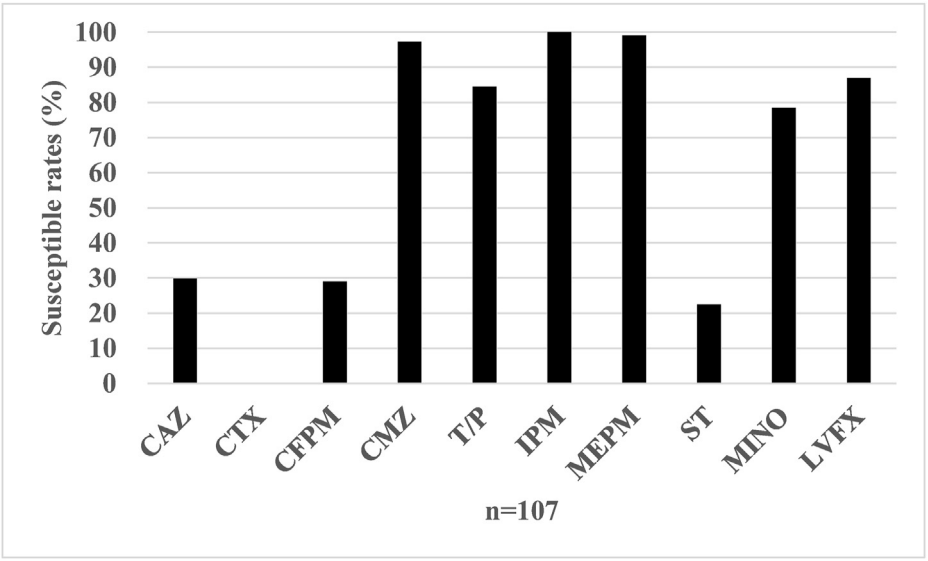

Figure 1. Antimicrobial susceptible rates of 107 ESBL-producing *K. pneumoniae* strains. The ten antibiotics were following: CAZ: ceftazidime, CTX: cefotaxime, CFPM: cefepime, CMZ: cefmetazole, T/P: piperacillin/tazobactam, IPM: imipenem, MEPM: meropenem, ST: sulfamethoxazole/trimethoprim, MINO: minocycline, and LVFX: levofloxacin.

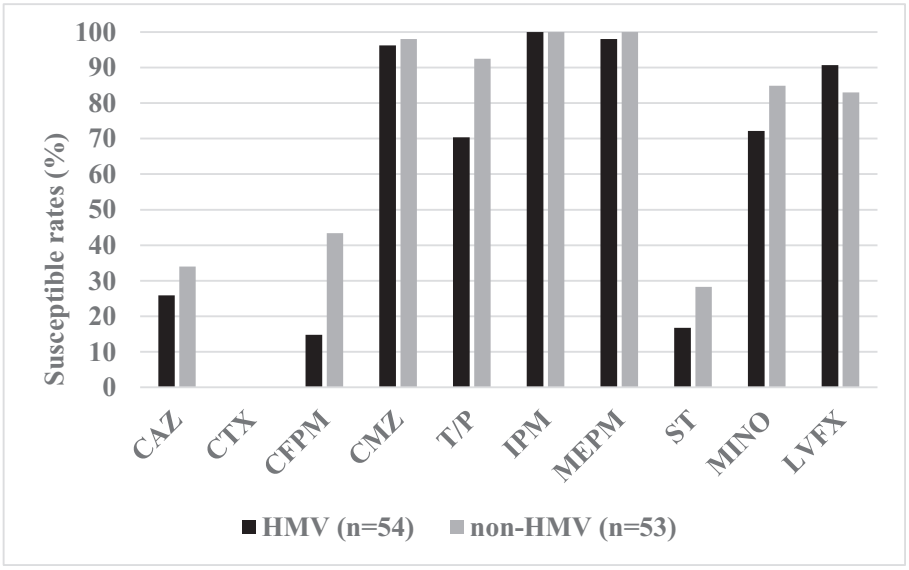

Figure 2. Antimicrobial susceptible rates of hypermucoviscous (HMV) and non-HMV *K. pneumoniae* strains in ten antibiotics. HMV strains showed lower susceptibility rates to CFPM ($p = 0.001$) and T/P ($p = 0.005$) when compared with non-HMV strains. *$p < 0.05$.

CTX-M-15 (75.9%, 60.4%) was the dominant type of ESBL gene in both HMV and non-HMV strains. In addition, bla_{SHV-38} and bla_{TEM} were significantly more frequently detected in the HMV strains than in the non-HMV strains (both 11.1% vs. 5.6%, $p = 0.027$; Table 1).

Plasmid replicon typing

Sixty-eight (63.6%) of the 107 ESBL-producing *K. pneumoniae* strains harbored IncF plasmids, of which IncFII (43.9%) was the most commonly detected (Table 3). Seven other plasmids were identified, including IncN in 30 strains (28.0%), IncY in 4 strains (3.7%), IncW in 2 strains (1.9%), IncB/O in 1 strain (0.9%), HI1 in 1 strain (0.9%), L/M in 1 strain (0.9%), and I1 in 1 strain (0.9%) (Table 3). Six known plasmids, IncA/C, IncP, IncHI2, IncT, IncK, and IncX, were not detected. Of the 54 HMV strains, 33 strains (61.1%) harbored IncF plasmids (IncF, IncFIA, IncFIB, and IncFII), followed by IncN in 30 strains (55.6%), IncY in 4 strains (7.4%), IncB/O in 1 strain (1.9%), IncHI1 in 1 strain (1.9%), IncL/M in 1 strain (1.9%), and IncW in 1 strain (1.9%) (Table 3). IncFIC, IncA/C, IncP, IncHI2, IncT, IncK, and IncX were not detected. Of the 53 non-HMV strains, IncF plasmids

Table 1 Presence of 10 extended spectrum beta-lactamase (ESBL) genes in HMV and non-HMV *K. pneumoniae* strains. *$p < 0.05$

		CTX-M-2	CTX-M-3	CTX-M-14	CTX-M-15	CTX-M-27	SHV-12	SHV-28	SHV-38	SHV-187	TEM
Number of isolates (%)	**HMV n = 54**	3 (5.6%)	0	2 (3.7%)	41 (75.9%)	1 (1.9%)	4 (7.4%)	3 (5.6%)	6 (11.1%)	0	6 (11.1%)
	non-HMV n = 53	3 (5.7%)	2 (3.8%)	3 (5.7%)	32 (60.4%)	2 (3.8%)	0	3 (5.7%)	0	4 (7.5%)	0
	Total n = 107	6 (5.6%)	2 (1.9%)	5 (4.7%)	73 (68.2%)	3 (2.8%)	4 (3.7%)	6 (5.6%)	6 (5.6%)	4 (3.7%)	6 (5.6%)
***p*-value**		1.000	0.243	1.000	0.146	0.628	0.118	1.000	**0.027**	0.0567	**0.027**

Table 2 Comparison of antimicrobial susceptibility between CTX-M-15-producing and non-producing *K. pneumoniae* strains. *$p < 0.05$

		CAZ	CTX	CPFX	CMZ	T/P	IMP	MEPM	ST	CFPM	MINO	LVFX	T/P	CMZ
Number of susceptible isolates (%)	**CTX-M-15 n = 73**	15 (20.5%)	0	14 (19.2%)	72 (98.6%)	56 (76.7%)	73 (100%)	72 (98.6%)	10 (13.7%)	14 (19.2%)	61 (83.6%)	66 (90.4%)	56 (76.7%)	72 (98.6%)
	non-CTX-M-15 n = 34	17 (50.0%)	0	17 (50.0%)	32 (94.1%)	31 (91.2%)	34 (100%)	34 (100%)	14 (41.2%)	17 (50.0%)	23 (67.6%)	27 (79.4%)	31 (91.2%)	32 (94.1%)
***p*-value**		**0.003**	-	**0.002**	0.236	0.109	-	1.000	**0.003**	**0.002**	0.070	0.132	0.109	0.236

Table 3 Plasmid replicon typing in all ESBL-producing *K. pneumoniae* isolates. $^{*}p < 0.05$

		IncN	IncF group					IncY	IncB/O	IncHI 1	IncW	IncL/M	IncI1
			Total	IncFIA	IncFIB	IncFIC	IncF	IncFII						
Number of isolates (%)	HMV n = 54	30 (55.6%)	33 (61.1%)	3 (5.6%)	15 (27.8%)	0	2 (3.7%)	27 (50.0%)	4 (7.4%)	1 (1.9%)	1 (1.9%)	1 (1.9%)	1 (1.9%)	0
	non-HMV n = 53	0	35 (66.0%)	0	27 (50.9%)	11 (20.8%)	0	20 (37.7%)	0	0	1 (1.9%)	0		1 (1.9%)
	Total n = 107	30 (28.0%)	68 (63.6%)	3 (2.8%)	42 (39.3%)	11 (10.3%)	2 (1.9%)	47 (43.9%)	4 (3.7%)	1 (0.9%)	2 (1.9%)	1 (0.9%)	1 (0.9%)	1 (0.9%)
***p*-value**		**<0.001**	0.689	0.243	**0.018**	**<0.001**	0.495	0.244	0.118	1.000	1.000	1.000	1.000	1.000

[a] Six known plasmids, IncA/C, IncP, IncHI2, IncT, IncK, and IncX, were not detected.

(IncFIB, FIC, and FII) were also the most frequently detected in 35 strains (66.0%), as well as IncW in 1 strain (1.9%) and IncI1 in 1 strain (1.9%) (Table 3). IncN, IncF, IncFIA, IncL/M, IncB/O, IncA/C, IncP, IncY, IncH, IncT, IncK, and IncX were not detected.

IncN was predominantly present in 55.6% of the HMV strains but 0% of the HMV strains ($p < 0.001$, Table 3), whereas IncFIB (27.8%, 50.9%) and IncFIC (0%, 20.8%) were significantly more frequently detected (50.9% and 20.8%) in non-HMV strains than in HMV strains (27.8% and 0%), respectively ($p = 0.018$ and $p < 0.001$, respectively, Table 3). IncFII was significantly more common in CTX-M-15-producing strains than in non-CTX-M-15 producers (52.1% vs. 26.5%, $p = 0.021$; Table 4).

Characterization of virulence genes and capsule serotypes

The string test showed that 54 (50.5%) and 53 (49.5%) of the 107 ESBL-producing *K. pneumoniae* isolates were HMV strains and non-HMV strains, respectively. The number of HMV strains increased yearly, with 7.4% of 54 HMV strains from 2012 to 2013, 37.0% from 2014 to 2015, and 55.6% from 2017 to 2018 (Supplementary Table 1). The virulence genes *uge* (98.1% vs. 20.8%), *wabG* (96.3% vs. 83.0%), *rmpA* (94.4% vs. 7.5%), *iucA* (79.6% vs. 3.8%), *fimH* (70.4% vs. 9.4%), *iroB* (70.4% vs. 5.7%), and *peg-344* (70.4% vs. 1.9%) were significantly more prevalent in the HMV strains compared with the non-HMV strains ($p < 0.001$, $p = 0.029$, $p < 0.001$, $p < 0.001$, $p < 0.001$, $p < 0.001$, and $p < 0.001$, respectively; Table 5). In the 53 non-HMV strains, *wabG* was the most frequently detected virulence gene (44 (83.0%) strains), followed by *uge* in 11 strains (20.8%), *fimH* in 5 strains (9.4%), *rmpA* in 4 strains (7.5%), *iroB* in 3 strains (5.7%), *iucA* and *magA* in 2 strains (3.8%), and *peg-344* in 1 strain (1.9%). Moreover, 41 (75.9%) of the 54 HMV strains exhibited either one of the three capsule serotypes K1, K2, K5, K20, or K54 with K2 as the most common capsule serotype (40.7%) followed by K20 (20.4%), K1 (7.4%), K5 (5.6%), and K54 (3.7%) (Table 5). Only 9 (17.0%) of the 53 non-HMV strains showed positive capsule serotypes, including K1 (3.8%), K2 (13.2%), K20 (3.8%), K54 (5.7%), and K57 (3.8%) (Table 5). All 6 strains exhibiting *magA* were K1 capsule serotype.

Discussion

A total of 291 *K. pneumoniae* isolates were collected from medical institutions throughout Hyogo Prefecture, Japan for this study. Though several studies have investigated the epidemiology of HMV *K. pneumoniae* in Japan,[12,19] no reports have investigated HMV strains to compare the antimicrobial susceptibility and genetic characteristics of ESBL-producing *K. pneumoniae* between HMV and non-HMV strains. In our study, 54 of 291 (18.6%) ESBL-producing *K. pneumoniae* isolates in Hyogo Prefecture were determined as HMV strains. Other studies in Osaka Prefecture, Japan (21.1%), Indonesia (17.0%), and Taiwan (22.3%) showed similar trends.[3,19,20] However, 31 (31.0%) of 100 ESBL-producing *K. pneumoniae* isolates throughout Japan in 2018 were identified as HMV, suggesting that HMV strains

Table 4 Difference of plasmid replicon typing in CTX-M-15-producing and non-producing strains. *$p < 0.05$

		IncN	IncF group						IncY	IncB/O	IncHI1	IncW	IncL/M	IncI1
			Total	IncFIA	IncFIB	IncFIC	IncF	IncFII						
Number of isolates (%)	**CTX-M-15 n = 73**	22 (30.1%)	50 (68.5%)	2 (2.7%)	30 (41.1%)	8 (11.0%)	0	38 (52.1%)	1 (1.4%)	1 (1.4%)	1 (1.4%)	1 (1.4%)	1 (1.4%)	1 (1.4%)
	non-CTX-M-15 n = 34	8 (23.5%)	18 (52.9%)	1 (2.9%)	12 (35.3)	3 (8.8%)	2 (5.9%)	9 (26.5%)	3 (8.8%)	0	0	1 (2.9%)	0	0
***p*-value**		0.644	0.135	1.000	0.672	1.000	0.099	**0.021**	0.094	1.000	1.000	0.537	1.000	1.000

[a] Six known plasmids, IncA/C, IncP, IncHI2, IncT, IncK, and IncX, were not detected.

Table 5 Distribution of 8 virulence genes and 6 capsule serotypes in HMV and non-HMV *K. pneumoniae* strains. *$p < 0.05$

		Virulence gene								Capsule serotype					
		magA	*rmpA*	*fimH*	*uge*	*wabG*	*iucA*	*iroB*	*peg-344*	K1	K2	K5	K20	K54	K57
Number of isolates (%)	**HMV n = 54**	4 (7.4%)	51 (94.4%)	38 (70.4%)	53 (98.1%)	52 (96.3%)	43 (79.6%)	38 (70.4%)	38 (70.4%)	4 (7.4%)	22 (40.7%)	3 (5.6%)	11 (20.4%)	2 (3.7%)	0
	non-HMV n = 53	2 (3.8%)	4 (7.5%)	5 (9.4%)	11 (20.8%)	44 (83.0%)	2 (3.8%)	3 (5.7%)	1 (1.9%)	2 (3.8%)	7 (13.2%)	0	2 (3.8%)	3 (5.7%)	2 (3.8%)
	Total n = 107	6 (5.6%)	55 (51.4%)	43 (40.2%)	64 (59.8%)	96 (89.7%)	45 (42.1%)	41 (38.3%)	39 (36.4%)	6 (5.6%)	29 (27.1%)	3 (2.8%)	13 (12.1%)	5 (4.8%)	2 (1.9%)
***p*-value**		0.678	**<0.001**	**<0.001**	**<0.001**	**0.029**	**<0.001**	**< 0.001**	**<0.001**	0.678	**0.002**	0.243	**0.015**	0.678	0.243

may be increasing year by year.[12] In this study, the number of HMV strains increased yearly, with 7.4% of 54 HMV strains from 2012 to 2013, 37.0% from 2014 to 2015, and 55.6% from 2017 to 2018 (Supplementary Table 1).

ESBL-producing *K. pneumoniae* isolates showed low susceptibility to cephalosporins CTX (0%), CFPM (29.0%) and CAZ (29.9%). These strains were mostly susceptible to carbapenems (IPM 100%, MEPM 99.1%), cephamycin (CMZ 97.2%), and quinolone (LVFX 86.9%). Although carbapenems are mostly effective on ESBL-producing bacteria, their unlimited or broad use may cause the emergence and increase of carbapenem-resistant strains in *Enterobacteriaceae*.[21] Cephamycins are stable against hydrolysis by ESBL *in vitro* and can work as efficacious alternative antibiotics.[22] ESBL-producing *K. pneumoniae* strains usually had concomitant resistance to quinolone, with low susceptibility rates to LVFX in Japan (12.5%) and Indonesia (37.2%).[3,23] However, recent reports of high LVFX susceptibility in Japan (84.0%) and China (73.3%) highlight the urgent necessity of monitoring quinolone resistance.[12,24] In our study, the HMV strains had lower susceptibilities to CFPM and T/P than non-HMV strains, suggesting an association between HMV characteristics and antimicrobial resistance in ESBL-producing *K. pneumoniae*. Although HMV *K. pneumoniae* strains were reported with high susceptibilities to antibiotics in the past, antibiotic resistance has increased recently.[1,12,24] HMV strains were reported to show low susceptibilities to CFPM (Italy: 0%, India: 0%, Egypt: 13.6%) and T/P (India: 0%, Italy: 5.6%) in previous studies.[25–27] In addition, several studies in China identified carbapenem-resistant hvKp (CR-hvKp), and the prevalence of multidrug-resistant hvKp (MDR-hvKp) and extensively drug-resistant hvKp (XDR-hvKp) isolates were reported in Iran.[28–30] HMV strains also showed higher resistance to some antimicrobials than non-HMV strains in Japan.[19] On the other hand, although ESBL-producing HMV strains in Japan showed high resistance to CFPM (80.0%), the same isolates had low resistance to T/P (20.0%).[31] Susceptibilities to CFPM and T/P were not significantly different between HMV and non-HMV ESBL-producing strains in Indonesia.[3] Therefore, the results of our study indicate that HMV *K. pneumoniae* strains have acquired antibiotic resistance more frequently than non-HMV strains in Japan.

CTX-M-15 (68.2%) was the most predominant ESBL type in our HMV (75.9%) and non-HMV (60.4%) strains, which is similar to other reports globally. High rates of CTX-M-15 producing *K. pneumoniae* were reported in Iran (96.1%), Denmark (77.0%), and Indonesia (89.4%).[3,32,33] The carriage rate of $bla_{CTX\text{-}M\text{-}15}$ has increased compared to previous studies in Japan, from 26.7% to 68.2% from 2013-2017.[34] Moreover, our study indicated that CTX-M-15-producing *K. pneumoniae* strains exhibited significantly high resistance to CAZ, ST, and CFPM, which is consistent with the other reports of CTX-M-15-producing strains with higher resistance to CAZ than other CTX-M types in Japan and China.[12,23,35] In Japan, CTX-M-type-ESBL-producing strains also showed higher resistance to CFPM than other ESBLs.[31,36] In addition, CTX-M-15-producing *K. pneumoniae* strains in Tunisia showed high resistance to ST.[37] Moreover, CTX-M-15-producing *K. pneumoniae* strains were reported to show significantly higher resistance to T/P than other ESBLs in Japan and Indonesia.[31,38] Our study suggested other antibiotic resistance mechanisms than ESBL in CTX-M-15-producing *K. pneumoniae* strains.

In our study, the most common plasmid replicon type was IncFII (52.1%) in CTX-M-15-producing strains, which is consistent with previous studies in Japan and France[12,39] and suggested an association between the spread of CTX-M-15 among *K. pneumoniae* with FII plasmids. Furthermore, we confirmed the significantly different distribution of specific ESBL genes and plasmid types between HMV and non-HMV strains, indicating possibly different propagation paths of ESBL genes in both groups. Additional studies are required to clarify the correlation between ESBL genes and their propagation paths.

The well-known virulence factors of *K. pneumoniae* include capsular polysaccharides, lipopolysaccharide (LPS), adhesion, and iron uptake acquisition.[1,2] *rmpA* acts on capsular polysaccharide production to regulate mucoid phenotype and contributes to HMV and hyKp. Most of the HMV *K. pneumoniae* strains were reported to harbor *rmpA* in China (81.3%).[12,28] Another virulence gene, *FimH*, encodes fimbriae that facilitate bacterial adhesion to host cells and adhesion, which is important for biofilm formation on abiotic surfaces.[40] In addition, *uge* and *wabG* were associated with biosynthesis of LPS, avoiding complement-mediated killing.[1,2,10] In Indonesia, more than half of the HMV strains had *fimH* (60.0%), *uge* (80.0%), and *wabG* (86.7%),[3] and all of the HMV strains harbored *fimH* in Egypt.[27] Moreover, most of CR-hvKp isolates possessed *wabG* (100%) in China and *uge* (80.0%) in Argentina.[41,42] Iron uptake acquisition is also one of the most important virulence factors. Iron is a critical element for bacterial growth, and hvKp expresses siderophores for iron acquisition. HvKp isolates often produce aerobactin and salmochelin systems, in which *iucA* and *iroB* synthesize aerobactin and salmochelin siderophore, respectively. In addition, *peg-344* is a virulence biomarker in hvKP.[1] Most HMV strains in Japan were reported to possess virulence genes (*rmpA* 74.2%, *fimH* 100%, *uge* 100%, *wabG* 100%, *iucA* 96.8%, and *iroB* 87.1%).[12,40,43] However, information on differences in virulence genes between ESBL and non-ESBL-producing HMV strains remains little known. In Taiwan, 73.0% of ESBL-producing HMV strains possessed *rmpA*, which is significantly more than that of non-ESBL-producing HMV strains.[20] On the other hand, other studies in Japan reported high possession rate of *rmpA*, *icuA*, and *iroB* among non-ESBL-producing HMV strains.[19,43]

These genes were more frequently detected in HMV strains (*rmpA* 94.4%, *fimH* 70.4%, *uge* 96.3%, *wabG* 98.1%, *iucA* 79.6%, *iroB* 70.4%, and *peg-344* 70.4%) than non-HMV strains (*rmpA* 7.5%, *fimH* 9.4%, *uge* 20.8%, *wabG* 83.0%, *iucA* 3.8%, *iroB* 5.7%, *peg-344* 1.9%), indicating that HMV strains have higher pathogenicity than non-HMV strains. We found that HMV strains tend to acquire high resistance to antibiotics at the same time in this study. In addition, HMV has been reported not to necessarily contribute hvKp, and *rmpA*, *rmpA2*, *iucA*, *iroB*, and *peg-344* are virulence biomarkers in hvKP isolates.[44] Moreover, Kakuta et al. defined *K. pneumoniae* isolates carrying two or more of these genes as hvKp.[12] All 54 HMV strains had at least two of these biomarkers in this study, suggesting that HMV can be closely related to hvKp.

Capsule type is another important virulence factor. The capsule surrounding the surface of *K. pneumoniae* contributes to the virulence associated with the viscous phenotype.[40] So far, more than 77 capsule types have been defined in *Klebsiella* species.[45] K1, K2, K5, K20, K54, and K57 capsule serotypes are particularly related to hvKp.[44] In our study, K2 (40.2%) was the most common capsule type in HMV strains in comparison with non-HMV strains, which basically parallels several reports from China, Korea, and Indonesia on the K1 or K2 capsule serotype in HMV strains.[3,45—47] K2 capsule serotype was also reported as the predominant capsule serotype among both ESBL and non-ESBL-producing HMV strains in Japan.[19,31,43] K20 (20.4%) was the second most common capsule serotype. K20 (29.0%) was reported as one of the most common capsule serotypes in Japan,[12] indicating that the K20 capsule serotype may have been increasing in HMV strains in Japan. It is known that *magA* is the serotype K1 allele of the polymerase gene,[48] and all strains with *magA* were K1 capsule serotypes in this study.

Multilocus Sequence Typing (MLST) analysis has provided useful epidemiological information on *K. pneumoniae* isolates, dissemination of HMV and antibiotic resistance mechanisms.[49] Various relationships between MDR-Kp and sequence type (ST) have been reported. ST11 was common in MDR-Kp in China,[50] while ST15 and ST25 were most commonly detected in CTX-M-15-producing *K. pneumoniae* isolates in Japan.[12,34] Several relationships between capsule serotype and ST have also been reported. K1-ST23, K20-ST268, K57-ST412 strains were identified in Japan.[12,40] However, information on the correlation between virulence, antibiotic resistance, and ST has been limited, though Fan et al. reported a correlation between ST258 and virulence and antibiotic resistance.[50]

There are several limitations to our study. First, no patient demographic data were available for sex, age, antibiotic administration histories, and clinical characterization. Therefore, we focused on analysis of the *in vitro* data. Second, only representative types of ESBL genes, virulence genes, and plasmid replicons were investigated. Third, MLST analysis was not performed in HMV and non-HMV ESBL-producing *K. pneumoniae* strains in this study. Further investigation of antimicrobial susceptibilities and virulence in HMV ESBL-producing *K. pneumoniae* strains, including MLST, is needed.

In conclusion, this study advances our knowledge of the characteristics and spread of HMV and non-HMV ESBL-producing *K. pneumoniae* in Japan, demonstrating that HMV strains possessed more virulence genes with higher antibiotic resistance than non-HMV strains. CTX-M-15 was the most prevalent type of ESBL gene in *K. pneumoniae* in Japan but there was no significant difference between HMV and non-HMV strains, and the FII plasmid may play an important role in the spread of CTX-M-15 among *K. pneumoniae*. Additional studies are needed to thoroughly evaluate the clinical threat posed by HMV *K. pneumoniae* strains.

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Declaration of competing interest

The authors declare no conflict of interest. The funders had no role in the design of the study, in the collection, analyses, or interpretation of data, in the writing of the manuscript, or in the decision to publish the results.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmii.2022.08.010.