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Original Article

Multilocus Sequence Types and Virulence Determinants of Hypermucoviscosity-Positive *Klebsiella pneumoniae* Isolated from Community-Acquired Infection Cases in Harbin, North China

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SUMMARY: We investigated the molecular epidemiologic characteristics and virulence of hypermucoviscosity-positive *Klebsiella pneumoniae* in mainland China. We detected 16 hypermucoviscosity-positive strains in 65 total clinical isolates (24.62%). We found that 68.75% (11/16) of the positive strains had K2 genotype and carried the *rmpA* and *iucA* genes. Multilocus sequence typing revealed 5 sequence types (STs): ST65 [7], ST23 [4], ST86 [3], ST412 [1], ST375 [1], whereas the remaining 4 isolates were defined as other STs. The order of the median lethal dose values for the ST types was ST23 (2.19×10^3 CFU/mouse) < ST86 (1.70×10^4 CFU/mouse) < ST65 (5.05×10^7 CFU/mouse) < the other STs (1.90×10^8 CFU/mouse). In conclusion, the K2 with ST65 carrying *rmpA* and *iucA* was the most predominant among the hypermucoviscosity-positive *K. pneumoniae* strains obtained from community-acquired infection cases in Harbin, North China. Sequence types are a valuable tool to predict the risk of *K. pneumoniae* infection.

INTRODUCTION

Over the past 2 decades, hypervirulent (hypermucoviscosity-positive) *Klebsiella pneumoniae* (HvKP) has been reported worldwide as a cause of liver abscesses, endophthalmitis, meningitis, empyema (1). The mortality rate associated with HvKP has been significantly rising up to 31% (2). HvKP infections appeared to be more widespread in Asian countries like Taiwan (3,4), Japan (5), and Korea (6) but rare cases in Western Europe (7) and North America (8) have also been reported. In mainland China, very limited data are available regarding the prevalence and characteristics of HvKP isolated from community-acquired infection cases.

Multilocus sequence typing (MLST) has been used as a tool in phylogenetic studies on strains and in large-scale epidemiology. This approach is particularly helpful for the typing of bacterial pathogens and the identification of various clones that differ sharply by their features of virulence (9–11). In this study, we identified HvKP strains in sputum, throat swabs, blood, urine, pus, and stool specimens from inpatients with cases of community-acquired infection. We also determined the multilocus sequence types and virulence factors: the chromosomal and plasmid-encoded regular mucoid phenotypes (c-*rmpA*, *p-rmpA*/*p-rmpA*2), the mucoviscosity-associated gene A (*magA*), siderophore genes including aerobactin (*iucA*), yersiniabactin (*ybtS*), samochelin N (*iroN*), responsible for an iron uptake system (*kfu*) and associated with allantoin metabolism (*allS*) and murine lethality.

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MATERIALS AND METHODS

Collection of *K. pneumoniae* clinical isolates: From January 2011 to April 2012, 65 *K. pneumoniae* clinical isolates were collected from inpatients, in medical and surgical wards, from two tertiary hospitals in Harbin, Heilongjiang Province, North China. Strains were isolated from various clinical specimens: sputum (35.38%; 23/65), stool (32.31%; 21/65), urine (13.85%; 9/65), throat swab (10.77%; 7/65), blood (3.08%; 2/65), and liver abscess aspirate (4.62%; 3/65). Identification of the species was confirmed using the API 20E system (BioMérieux, Marcy l'Etiole, France).

Capsular phenotyping, K genotype detection, and assessment of virulence factors: String tests were performed as previously described (12). Capsular K genotypes, *crmpA*/*prmpA*/*prmpA*2, *magA*, siderophore genes (*iucA*, *iroN*, *ybtS*), *kfu*, and *allS* virulence genes were detected by PCR using specific primers previously employed (13–17).

MLST analysis: MLST was performed according to the method described by Diancourt et al. (18). Seven housekeeping genes (*gapA*, *mdh*, *gpi*, *rpoB*, *inf*, *phoE*, and *tonB*) were amplified, sequenced, and compared

with sequences available on the *K. pneumoniae* MLST database (http://www.pasteur.fr/recherche/genopole/PF8/mlst/Kpneumoniae.ht).

Measurement of the median lethal dose (LD_{50}) of *K. pneumoniae* clinical isolates in mice: Different dilutions of strains of *K. pneumoniae*, kept in a saline suspension (0.5 mL), were injected intraperitoneally into 6–7-week-old female BALB/c mice (5 mice/group). The number of colony-forming units (CFU/mL) of the injected samples was determined by incubating each *K. pneumoniae* suspension onto Luria-Bertani plates in duplicate at adequate dilutions. The LD_{50} was calculated using the IBM SPSS 13.0 software (version 13) to perform the mouse survival analysis over a 7-day period.

All animal experiments were performed in accordance with the guidelines of the Ethics Review Committee of Animal Experiments of the Harbin Medical University (Protocol number of Animal Experimentation Ethical Inspection: HMUIRB2013002).

Statistical analysis: The Bliss statistical analysis was used to calculate the LD_{50} values. The Mann-Whitney U test and the chi-square test were used in this study. A $p < 0.05$ was considered to be statistically significant.

RESULTS

Distribution of hypermucoviscosity strains: Among the 65 *K. pneumoniae* strains investigated, the prevalence rate of hypermucoviscosity-positive strains was 24.62% (16/65), which was lower than that of hypermucoviscosity-negative strains (75.38%, 49/65, Table 1).

MLST and capsular K genotyping: Twenty-two isolates were selected for this study. Nine different MLSTs were identified, including ST65, ST23, ST86, ST375, ST412, ST35, ST36, ST1107, and ST309. ST65 (n = 7), ST86 (n = 3), ST375 (n = 1), and ST1107 (n = 1) belong to the K2 genotype, whereas all the ST23 types (n = 4) belong to K1 and all ST412 types (n = 3) belong to K57. ST65, ST23, ST86, and ST375 were characterized by their distinctive K genotypes and were found in hypermucoviscosity-positive strains. Other STs (ST35, ST36, ST309, and ST1107), except ST412, were only found in hypermucoviscosity-negative strains (Table 1).

Determination of LD_{50} values of the HvKP strains:

Table 1. Molecular characterization of *K. pneumoniae* strains (N = 65)

Capsular K genotype (N)	MLST type	No of isolates (%)	
		Hv-positive	Hv-negative
K1 (4)	ST23	4 (25)	0
K2 (12)	ST65	7 (43.75)	0
	ST86	3 (18.75)	0
	ST375	1 (6.25)	0
	ST1107	0 (0)	1 (2.04)
K57 (3)	ST412	1 (6.25)	2 (4.1)
NA (46)	ST35	0 (0)	1 (2.04)
	ST36	0 (0)	1 (2.04)
	ST309	0 (0)	1 (2.04)
	NA	0 (0)	43 (87.76)
Total (65)		16 (24.62)	49 (75.38)

K, capsular K genotype; N, number of isolates; NA, not available; MLST, multilocus sequence type; Hv, hypermucoviscosity.

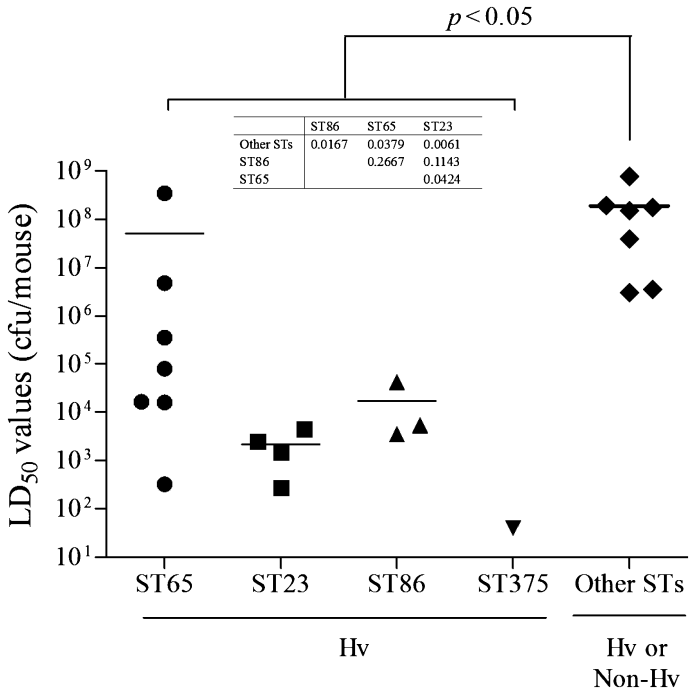

Fig. 1. LD_{50} values in mice. ST65, ST23, ST86 and ST375 and one ST412 isolates from hypermucoviscosity positive strains, other STs (ST1107, ST35, ST36, ST309 and two ST412) isolates from hypermucoviscosity negative strains. Suspensions (0.5 mL) of the isolates were injected i.p. into 6–7 week old female BALB/C mice (10 per group). LD_{50} values were calculated by SPSS software (version 13) on the basis of lethality over a 7-day period. $P < 0.05$ indicated significant statistical difference (Mann-Whitney U test). Hv, hypermucoviscosity; Non-Hv, Non- hypermucoviscosity.

The HvKP strains showed varying LD_{50} values (4.06×10^1–3.48×10^8 CFU/mouse); their mean (2.23×10^7 CFU/mouse) being lower than that of the hypermucoviscosity-negative strains (2.22×10^8 CFU/mouse, $p = 0.0029$, Fig. 1). The mean LD_{50} values of ST23 (2.19×10^3 CFU/mouse) and ST86 (1.70×10^4 CFU/mouse) isolates was significantly lower than that of other ST isolates (1.90×10^8 CFU/mouse). The LD_{50} values of the ST65 isolates were highly heterogeneous (3.22×10^2–3.48×10^8 CFU/mouse), with their mean (5.05×10^7 CFU/mouse) being significantly lower than that of other ST isolates, but higher than that of ST23 ($p < 0.05$).

Presence of virulence-associated genes in hypermucoviscosity-positive and -negative strains: The strains associated with the highest hypermucoviscosity trait carried the regular mucoid phenotype *c-rmpA* (93.75%, 15/16), the *p-rmpA*/*p-rmpA*2 genes (100%, 16/16), and siderophore genes (*iucA*: 100%, 16/16; *iroN*: 87.5%, 14/16; *ybtS*: 81.25%, 13/16; Table 2). The positivity rates of these genes in the hypermucoviscosity positive strains were significantly higher than in the hypermucoviscosity negative strains (*c-rmpA*: 10.20%, 5/49; *p-rmpA*: 2.04%, 1/49; *p-rmpA*2: 14.29%, 7/49; *iucA*: 28.58%, 14/49; *iroN*: 20.41%, 10/49; *ybtS*: 32.65%, 16/49; data not shown). All strains were found to lack the *allS* and *kfu* genes and *magA* was only found in the ST23 (K1) strains.

DISCUSSION

A new hypervirulent (hypermucoviscous) variant of *K. pneumoniae* has recently emerged. First described in the Asian Pacific Rim, it is now increasingly being recognized in western countries and its distribution shows Asian descent (1). Nevertheless, our results show that the rate of hypermucoviscosity strains in community-acquired cases was only 24.62% (16/65, Table 1), coinciding with reports from other parts of China (33.0%, 29/88) (19). However, these rates are lower than the prevalence rates of tissue abscess (51%) and liver abscess (85% and 98%) cases in Taiwan (13,20,21), but higher than the ones reported in Canada (8.1%) (22). These data suggest that hypermucoviscosity strains are not widely distributed among *K. pneumoniae* strains causing pneumonia, bacteraemia, diarrhoea and other community-acquired infections.

It is widely believed that the hypermucoviscosity trait provides a useful index for high virulence strains. However, the LD_{50} values of HvKP strains were highly variable in the present study, even those of the strains carrying the regulator of the mucoid phenotype gene (*c-rmpA*, *p-rmpA*/*p-rmpA*2) with an increased efficiency in the iron acquisition system (*iucA*, *iroN*, *ybtS*) showed similar virulence patterns. It is likely that other unknown traits might contribute to the level of virulence of HvKP strains. Further analysis showed that the *K. pneumoniae* STs demonstrated distinctive LD_{50} patterns. ST23 isolates showed relatively high virulence, whereas the LD_{50} values of ST65 isolates were highly variable. Only one of the ST65 isolates (Kp1) had low LD_{50} values (3.22×10^2 CFU/mL), whereas the others had medium (Kp2-Kp5) or high LD_{50} values (Kp6-Kp8) (11), showing that a robust percentage (42.86%) of ST65 isolates is highly virulent. A small number of ST375 and ST86 isolates might not be appropriate for

Table 2. Characterization of 22 *K. pneumoniae* strains

Strain name	MLST type	Sample source	String test	*crmpA*	*PrmpA*/ *prmpA2*	*iucA*	*iroN*	*ybtS*	LD_{50}[1)] CFU/mL
Kp1	ST65	Liver	+	+	+/+	+	+	+	3.22×10^2
Kp2	ST65	Stool	+	+	+/+	+	+	+	1.60×10^4
Kp3	ST65	Sputum	+	+	+/+	+	+	+	1.65×10^4
Kp4	ST65	Stool	+	+	+/+	+	+	+	7.95×10^4
Kp5	ST65	Stool	+	+	+/+	+	+	+	3.75×10^5
Kp6	ST65	Sputum	+	+	+/+	+	+	+	4.84×10^6
Kp7	ST65	Sputum	+	+	+/+	+	+	+	3.48×10^8
Kp8	ST86	Sputum	+	+	+/+	+	+	+	3.56×10^3
Kp9	ST86	Sputum	+	+	+/+	+	−	+	5.36×10^3
Kp10	ST86	Blood	+	+	+/+	+	+	−	4.21×10^4
Kp11	ST375	Sputum	+	+	+/+	+	+	−	4.06×10^1
Kp12	ST23	Liver	+	+	+/+	+	+	+	2.72×10^2
Kp13	ST23	Liver	+	+	+/+	+	+	+	1.49×10^3
Kp14	ST23	Sputum	+	+	+/+	+	+	+	2.50×10^3
Kp15	ST23	Sputum	+	+	+/+	+	+	+	4.50×10^3
Kp16	ST412	Sputum	+	−	+/+	+	−	−	3.11×10^6
Kp17	ST36	Sputum	−	+	−/+	−	+	+	3.55×10^6
Kp18	ST412	Sputum	−	+	−/+	−	−	−	3.95×10^7
Kp19	ST35	Blood	−	+	−/+	−	+	−	1.50×10^8
Kp20	ST309	Sputum	−	+	−/−	−	−	−	1.78×10^8
Kp21	ST1107	Sputum	−	−	−/−	−	−	−	1.92×10^8
Kp22	ST412	Sputum	−	−	+/+	−	+	−	7.62×10^8

1): median dose of lethality.
MLST, multilocous sequence type; *c-rmpA*, chromosomal regular mucoid phenotype; *p-rmpA*, plasmid regular mucoid phenotype; *iucA*, aerobactin A; *iroN*, samochelin N; *ybtS*, yersiniabactin S; +, positive; −, negative.

statistical comparison, although the LD_{50} values of ST86 (3.56×10^3–4.21×10^4 CFU/mL) and ST375 (4.06×10 CFU/mL) isolates indicated high virulence levels compared to other ST isolates (Fig. 1). Our mouse lethality analysis of *K. pneumoniae* isolates showed an ascending order of mean LD_{50} values: ST23 < ST86 < ST65 < other STs (ST412, ST35, ST36, ST309, and ST1107, Fig. 1). Our analysis revealed that different ST types could be used to predict the level of virulence in mice.

In HvKP strains, K1 is the most predominant capsular K serotype, followed by K2 (13). Our study demonstrated that K2 is the most common among HvKP strains (68.75%, 11/16), followed by K1 (25.00%, 4/16) and K57 (6.25%, 1/16), indicating that the distribution of the K genotype might vary from region to region. Peirano and colleagues (22) found K1 serotypes in hypermucoviscosity-negative strains in Canada, whereas other recent studies (16,23) also revealed that the common *K. pneumoniae* that possesses K1 and K2 serotypes is significantly less virulent in a mouse infection model, suggesting that K genotypes might not be directly associated with the hypermucoviscosity trait.

In conclusion, our data indicate that the virulence level and the presence of certain virulence factors are distributed in community-acquired *K. pneumoniae* strains in a sequence-type associated manner. The K2 in ST65 isolates carrying the *rmpA* and *iucA* genes was frequently identified among the clinical isolates of Harbin, North East China. Based upon our LD_{50} and MLST data, we suggest that sequence types are effective biomarkers to identify strains with particular virulence traits and elevated mouse lethality.

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Conflict of interest None to declare.

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