Journal of Microbiology, Immunology and Infection (2016) 49, 83—90

Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.e-jmii.com

ORIGINAL ARTICLE

Quantification and comparison of virulence and characteristics of different variants of carbapenemase-producing *Klebsiella pneumoniae* clinical isolates from Taiwan and the United States

Tsung-Ta Chiang [a], Ya-Sung Yang [a], Kuo-Ming Yeh [a], Sun-Kang Chiu [a], Ning-Chi Wang [a], Te-Yu Lin [a], Li-Yueh Huang [b], Feng-Yee Chang [a], L.K. Siu [a,b], Jung-Chung Lin [a], Jiun-Han Chen [c,*]

[a] *Division of Infectious Diseases and Tropical Medicine, Department of Internal Medicine, Tri-Service General Hospital, National Defense Medical Center, Taipei, Taiwan*
[b] *Division of Clinical Research, National Health Research Institutes, Taipei, Taiwan*
[c] *Department of Medical Laboratory Science and Biotechnology, Yuanpei University of Medical Technology, Hsinchu, Taiwan*

Received 30 April 2015; received in revised form 8 August 2015; accepted 31 August 2015
Available online 9 September 2015

KEYWORDS
Klebsiella pneumoniae;
KPC variants;
virulence

Abstract *Background/purpose:* The emergence of *Klebsiella pneumoniae* carbapenemase (KPC)-producing strains is a challenge for clinicians. The characteristics and virulence of variants of KPC-producing *K. pneumoniae* isolates were evaluated.
Methods: Five clinical isolates—three KPC subtypes from Taiwan (KPC2-TW, KPC3-TW, and KPC17-TW) and two clinical strains from the United States (US; KPC2-US, KPC3-US)—were included. Virulent traits and capsular serotypes were analyzed by Polymerase Chain Reaction (PCR). Serum killing, neutrophil phagocytosis, and mice lethargy studies were performed to evaluate virulence.
Results: Multilocus sequence typing (MLST) demonstrated that KPC2-TW and KPC17-TW belonged to sequence type (ST)11, and KPC2-US, KPC3-US, and KPC3-TW to ST258. KPC3-TW expressed capsular serotype K1, whereas the others were non-K1/K2/K5 isolates. MLST analysis indicated that ST11 strains were serum resistant, whereas ST258 isolates were serum sensitive.

* Corresponding author. Department of Medical Laboratory Science and Biotechnology, Yuanpei University of Medical Technology, Number 306, Yuanpei Street, Hsinchu 30015, Taiwan.
E-mail address: jhchen@mail.ypu.edu.tw (J.-H. Chen).

http://dx.doi.org/10.1016/j.jmii.2015.08.011

ST11 isolates exhibited significantly higher 15-minute phagocytic rates than ST258 isolates (70.28 ± 16.68% vs. 34.88 ± 10.52%, $p < 0.001$). The capsular serotype K1 strain was more resistant to neutrophil phagocytosis than non-K1/K2/K5 isolates (27.1 ± 10.23% vs. 54.46 ± 20.94%, $p = 0.050$). All KPC-producing strain variants from Taiwan and the US demonstrated less virulence in a mouse lethality study, where the LD_{50} ranged from approximately 10^6 colony-forming units (CFU) to $>10^7$ CFU. Immunological responses were not significantly correlated with KPC subtype; however, responses were associated with MLST and capsular serotype.

Conclusion: Production of KPC itself was not associated with increased virulence despite different variants of KPC. The ST11 KPC-producing strain was resistant to serum killing, whereas capsular ss K1 was associated with resistance to neutrophil phagocytosis.

Introduction

Klebsiella pneumoniae, particularly the hypervirulent strains found predominantly in the Asia Pacific region, is an important clinical pathogen that is highly associated with mortality and morbidity.[1–3] In recent years, the emergence of carbapenem-resistant *Enterobacteriaceae*, including carbapenem nonsusceptible *K. pneumoniae*, has made treating infected patients particularly challenging.[4,5] Since the first report of carbapenem-resistant *K. pneumoniae* (CRKP) in 1996, many studies have been conducted to evaluate the clinical impact of CRKP. The CRKP infection-related mortality rate is higher than those of extended-spectrum beta-lactamase (ESBL)-producing strains and wild-type susceptible *K. pneumoniae*.[6,7] Moreover, infection with carbapenem-resistant strains seems to be one of the risk factors for infection-related mortality.[8,9] The mechanisms underlying carbapenem resistance in CRKP bacteria include the production of carbapenem-hydrolyzing enzymes [such as *Klebsiella pneumoniae* carbapenemase (KPC), Verona integron-encoded metallo-β-lactamase, New Delhi metallo-β-lactamase, active on imipenem (IMP)-type carbapenemases, and oxacillin-hydrolyzing (OXA)-group β-lactamase] and the production of ESBL coupled with outer membrane porin loss.[10] Compared with other mechanisms of resistance, infections caused by KPC-producing strains result in increased mortality.[10] To date, 16 variants of KPC enzymes (KPC-2 to KPC-17) have been identified.[11] Each variant exhibits different characteristics, including hydrolytic ability and susceptibility to antimicrobials.[12] Further studies have been conducted to analyze the virulence of KPC-producing *K. pneumoniae*. Most of those studies have been performed *in vitro* and have revealed that the bla_{KPC-2} itself is not associated with the virulence of KPC-producing *K. pneumoniae*.[13–15] Thus, the increased mortality of infected patients might be due to the lack of effective treatment or might be caused by other comorbidities. However, virulence has been analyzed only in the bla_{KPC-2} and bla_{KPC-3} variants[13–15]; the association of virulence with other subtypes of KPC genes has not been evaluated. In this study, we evaluated and compared the virulence of different variants of KPC isolates from Taiwan and the United States (US) and determined the association of virulence with different subtypes of KPC genes.

Methods

Bacterial strains and labeling with fluorescein isothiocyanate

Three clinical KPC strain variants, KPC2-TW, KPC3-TW, and KPC17-TW, were isolated from Taiwan. In addition, two clinical strains, KPC2-US and KPC3-US, isolated from the US were also included for the purpose of comparison of geographic variance. The main characteristics of each strain are shown in Table 1.

The isolated *K. pneumoniae* were labeled with fluorescein isothiocyanate (FITC), as previously described, for subsequent phagocytosis studies.[16] The strains were isolated and incubated overnight at 37°C. The concentration was estimated using photospectrometry (Olympus, Tokyo, Japan) and confirmed by quantitative plate counting. The bacteria were heat-killed for 1 hour in a 70°C-water bath. Then, the bacteria were washed with phosphate-buffered saline (PBS) and labeled with FITC by incubation with 0.1 mg/mL FITC (Sigma Chemical Co., St. Louis, MO, US) in 0.10M $NaHCO_3$, pH 9.0, for 60 minutes at 25°C. The labeled bacteria were washed with PBS and centrifuged to remove unbound fluorochrome. The labeled bacteria were resuspended in PBS at a concentration of 2×10^8 cells/mL and stored at −70°C.

Serotyping and virulence gene detection by polymerase chain reaction

Serotyping [K1, K2, K5, and multilocus sequence typing (MLST)] was performed by polymerase chain reaction (PCR). Ten common virulent trait genes, including *allS*, *rmpA*, *mrkD*, *kfuBC*, *cf29a*, *fimH*, *uge*, *wabG*, and *ureA*[17,18] (primers data listed in Table 2), which are known to be associated with the virulence of *K. pneumoniae*, were also assayed.

Serum resistance assay

An overnight culture was diluted 1:100 and grown to the midexponential phase. An inoculum of 1×10^6 colony-forming units (CFUs) was mixed with nonimmune human

Table 1 Main characteristics of the strains used in this study.

Strains	Source	Capsular typing	MLST typing	Virulence traits									Reference
				allS	*rmpA*	*mrkD*	*kfuBC*	*cf29a*	*fimH*	*uge*	*wabG*	*ureA*	
KPC2 -TW	Clinical (TSGH, Taiwan)	Non-K1, K2, K5	ST11	+	−	+	−	−	+	+	+	+	This study
KPC3 -TW	Clinical (VGH, Taipei, Taiwan)	K1	ST258	+	−	+	−	−	+	+	+	+	This study
KPC17-TW	Clinical (NTUH, Taiwan)	Non-K1, K2, K5	ST11	−	−	+	−	−	+	+	+	+	This study
KPC2 -US	Clinical (New Jersey, USA)	Non-K1, K2, K5	ST258	+	−	+	−	−	+	+	+	+	[25]
KPC3 -US	Clinical (New Jersey, USA)	Non-K1, K2, K5	ST258	+	−	+	−	−	+	+	+	+	[25]

KPC = *Klebsiella pneumoniae* carbapenemase; MLST = multilocus sequence typing; NTUH = National Taiwan University Hospital; TSGH = Tri-Service General Hospital; VGH = Veterans General Hospital; − = negative result; + = positive result.

serum, donated by healthy volunteers, in a 1:3 volume ratio. The final mixture volume was 1 mL and was incubated at 37°C. Serial dilutions were plated on Mueller—Hinton agar for 0 hours, 0.5 hours, 1 hour, 2 hours, and 3 hours to obtain colony counts. The average percent survival was plotted against time to obtain a survival curve in response to serum treatment for each strain.[19] The response to serum killing was scored on a six-grade scale, as previously

Table 2 Primers used in this study.

Category	Genes	Primers	Primer sequence	Size (bp)	Annealing temperature (°C)	Reference
Capsular serotyping	K1	MagAF1	5′- GGTGCTCTTTACATCATTGC -3′	1283	50	[26,27]
		MagAR1*	5′- GCAATGGCCATTTGCGTTAG -3′			
	K2	Wzy-F1	5′- GACCCGATATTCATACTTGACAGAG -3′	641	50	[26]
		Wzy-R1	5′- CCTGAAGTAAAATCGTAAATAGATGGC -3′			
	K5	K5wzxF360	5′- TGGTAGTGATGCTCGCGA -3′	280	50	[26]
		K5wzxR639	5′- CCTGAACCCACCCCAATC -3′			
The activator of the allantoin regulon gene	*allS*	1416R	5′- CCGTTAGGCAATCCAGAC -3′	1090	48.9	[28]
		336F2	5′- TCTGATTTA(A/T)CCCACATT -3′			
Capsular polysaccharide biosynthesis gene	*rmpA*	rmpA-F	5′- ACTGGGCTACCTCTGCTTCA -3′	535	58.0	[17]
		rmpA-R	5′- CTTGCATGAGCCATCTTTCA -3′			
Iron uptake system gene	*kfuBC*	kfuB-F1179	5′- GAAGTGACGCTGTTTCTGGC -3′	797	55.5	[29]
		kfuC-R649	5′- TTTCGTGTGGCCAGTGACTC -3′			
Adhesion associated genes	*cf29a*	cf29a-F	5′- GACTCTGATTGCACTGGCTGTG -3′	826	51.4	[17]
		cf29a-R	5′- GTTATAAGTTACTGCCACGTTC -3′			
	fimH	fim-1	5′- GCTCTGGCCGATAC(C/T)AC(C/G)ACGG -3′	423	54.8	[17]
		fim-2	5′- GC(G/A) (A/T)A(G/A)TAACG(T/C) GCCTGGAACGG -3′			
	mrkD	mrkD-1	5′- TAT(T/C)G(G/T)CTTAATGGCGCTGG -3′	920	50.1	[17]
		mrkD-2	5′- TAATCGTACGTCAGGTTAAAGA(C/T)C -3′			
Uridine diphosphate galacturonate 4-epimerase gene	*uge*	uge-F	5′- GATCATCCGGTCTCCCTGTA -3′	534	52.7	[30]
		uge-R	5′- TCTTCACGCCTTCCTTCACT -3′			
Core LPS biosynthesis gene	*wabG*	wabG-F	5′- CGGACTGGCAGATCCATATC -3′	683	53.8	[17]
		wabG-R	5′- ACCATCGGCCATTTGATAGA -3′			
Urease synthesis associated gene	*ureA*	ureA-F	5′- GCTGACTTAAGAGAACGTTATG -3′	337	55.8	[17]
		ureA-R	5′- GATCATGGCGCTACCT(C/T)A -3′			

* This primer was designed by Fang et al.[27]
LPS = lipopolysaccharide.

described.[19]. Grades 1 and 2 indicate high sensitivity to serum killing, Grades 3 and 4 indicate intermediate sensitivity, and Grades 5 and 6 indicate serum resistance.

Human neutrophil phagocytosis assay

Neutrophils were isolated from three healthy volunteers, as previously described. Freshly drawn, heparinized blood was mixed with an equal volume of dextran—saline solution. After incubation at room temperature, the upper leukocyte-rich plasma was aspirated. The leukocyte-rich plasma was mixed with Ficoll—Hypaque in a 2:1 volume ratio, layered on a Ficoll—Hypaque density gradient, and centrifuged. The neutrophil/red blood cell (RBC) pellet was resuspended in cold 0.2% NaCl. Then, ice-cold 1.6% NaCl was added, samples were centrifuged, and the supernatant was discarded. The above two steps were repeated one or two times or until the cell pellets appeared free of RBC. The cell concentration was adjusted to 1×10^7 neutrophils/mL.

The phagocytosis assay was performed as previously described.[16,20] One hundred microliters of neutrophil suspension (approximately 1×10^6 neutrophils), 100 μL of freshly thawed ice-cold normal human serum, and 600 μL of PBS were added into polypropylene tubes. The tubes were prewarmed and incubated at 37°C with continuous agitation. Then, 200 μL stoked, FITC-labeled bacteria (approximately 4×10^7 CFU/mL) was added to each tube to a final volume of approximately 1 mL per tube. The tubes were incubated at 37°C with continuous agitation. At 0 minutes, 1 minute, 2 minutes, 4 minutes, 6 minutes, 8 minutes, 10 minutes, 15 minutes, 20 minutes, 25 minutes, 30 minutes, 45 minutes, and 60 minutes, tubes were placed immediately in an ice bath. Cells were then separated by centrifugation and resuspended in 1 mL of ice-cold (4°C) PBS. The suspension was placed in a new tube and mixed with ethidium bromide (EB) such that a final EB concentration of 50 μg/mL was obtained. An unintubated tube was reserved as a 0-minute control group.

A FACScan emitting an argon laser beam at 488 nm (Becton Dickinson Immunocytometry Systems, San Jose, CA, US) was used to detect FITC fluorescence to measure phagocytosis of *K. pneumoniae* by human neutrophils.[16,20] The instrument settings were as follows: the sideways scatter (SSC) threshold was 52. The detector was set at E00, 350, and 427 for forward scatter (FSC), SSC, and fluorescence 1 (FL 1-H, green), respectively. Fluorescence values were obtained after gating on the combination of all light scatters. Data were collected from a total of 10,000 cells and analyzed with the Cellquest version 1.0 software (Becton Dickinson Immunocytometry Systems). The distribution data collected by logarithmic amplifier were displayed as single histograms for FL1-H. After addition of EB, extracellular fluorescence was inhibited, and cells appeared red under microscopic examination. By contrast, FITC-stained bacteria being engulfed by neutrophils appeared green. The ingested percentage of bacteria was calculated.

Mice lethality study

To determine the LD_{50} in a murine model, 6- to 8-week-old, male, pathogen-free BALB/c mice (body weight 20—25 g) were obtained from the National Laboratory Animal Center. This study was conducted under the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All animal care procedures and protocols were approved by the Institutional Animal Care and Use Committee of the National Health Research Institute (IACUC-14-195). Tenfold serial dilutions of bacteria were prepared from a starting concentration of 10^7 CFU, and the adult BALB/c mice received intraperitoneal injections with three to four concentrations of each strain. Six mice were used as a sample population for each KPC variant strain. Inoculated mice were observed for 14 days for symptoms of infection. Survival of the inoculated mice was recorded daily.

Statistical analysis

The human neutrophil phagocytosis data have been expressed as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was performed to compare the differences in phagocytic rates from 0 minutes to 30 minutes between strains and groups. Student *t* test was used to assess the differences in phagocytosis at 30 minutes between groups. A p value ≤ 0.050 was considered statically significant.

Results

Detection of MLST, capsular type, and virulent genes

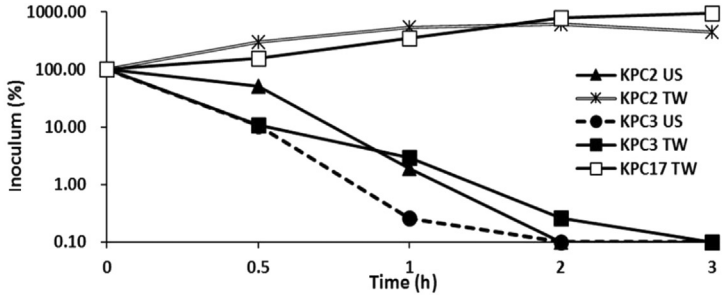
MLST analysis demonstrated that KPC2-TW and KPC17-TW belong to multilocus sequence type (ST) 11. KPC2-US, KPC3-US, and KPC3-TW were categorized as ST258. Screening for capsular typing revealed that KPC3-TW expresses serotype K1, whereas the others were all non-K1/K2/K5 isolates. Analysis of virulent gene factors is provided in Table 1. All strains expressed the following virulent trait genes: *mrkD*, *fimH*, *uge*, *wabG*, and *ureA*. In addition, with the exception of the KPC17-TW strain, all strains also expressed *allS*. All five strains were negative for the *kfuBC*, *cf29a*, and *rmpA* genes.

Serum killing assays

KPC17-TW and KPC2-TW, both ST11 strains, demonstrated serum resistance when incubated with normal human serum (952.19% and 445.31% viability, respectively; Figure 1). By contrast, the ST258 strains, KPC-2 US, KPC-3 TW, and KPC-3 US, were highly sensitive to serum killing (0%, 0%, and 0% viability, respectively; Figure 1). When KPC-producing isolates were subgrouped into ST11 and ST258 strains, the ST11 group was found to be more serum resistant than the ST258 group (Figure 1).

Phagocytosis reaction

Neutrophils from three healthy volunteers were isolated and incubated with each variant strain. The phagocytosis rates of these five strains were highly variable, where the



	0 h%	0.5 h %	1 h %	2 h %	3 h %
KPC2-TW (ST11)	100.00	300.54	541.90	612.97	445.31
KPC17-TW (ST11)	100.00	154.91	352.44	787.26	952.19
KPC2-US (ST258)	100.00	50.55	1.93	0.00	0.00
KPC3-TW (ST258)	100.00	11.00	2.95	0.26	0.00
KPC3-US (ST258)	100.00	10.57	0.26	0.00	0.00

Figure 1. Serum responses of different variants of *Klebsiella pneumoniae* carbapenemase(KPC)-producing *K. pneumoniae* isolates. The response to serum killing was scored on a six-grade scale. Grades 1 and 2 indicate high sensitivity to serum killing, Grades 3 and 4 indicate intermediate sensitivity, and Grades 5 and 6 indicate serum resistance.[19] KPC17-TW and KPC2-TW exhibited a Grade 6 and a Grade 5 response, respectively, whereas KPC2-US, KPC3-TW, and KPC3-US exhibited Grade 2 responses. KPC = *Klebsiellapneumoniae* carbapenemase; TW = Taiwan; US = United States.

mean 15-minute phagocytic rates (± SD) for KPC2-TW, KPC2-US, KPC3-TW, KPC3-US, and KPC17-TW were 60.46 ± 19.18%, 43.44 ± 8.02%, 27.41 ± 10.24%, 33.81 ± 8.84%, and 80.11 ± 6.22%, respectively (Figure 2). Overall, capsular serotype non-K1/K2/K5 KPC2-TW and KPC17-TW, both ST11 strains, were relatively more susceptible to phagocytosis (60.46 ± 19.18% and 80.11 ± 6.22%, respectively) than the other three strains (KPC2-US, KPC3-TW, and KPC3-US; 43.44 ± 8.02%, 27.41 ± 10.24%, and 33.81 ± 8.84%, respectively). When we further divided all variants into ST11 and ST258 strains according to the MLST analysis, the ST11 group exhibited a significantly higher 15-minute phagocytic rate than the ST258 group (70.28 ± 16.68% vs. 34.88 ± 10.52%, $p < 0.001$; Figure 3A). There were no significant differences in the 15-minute phagocytic rates between strains within the same sequence typing group (ST11: $p = 0.167$; ST258: $p = 0.175$; Figures 3B and 3C). There were also no significant differences in the 15-minute phagocytic rates between isolates from Taiwan and the US (55.99 ± 25.68% vs. 38.62 ± 9.23%, $p = 0.140$; Figure 3D). Subgrouping strains into KPC subtypes (KPC2, KPC3, and KPC17) revealed differences in the phagocytic rate ($p = 0.038$). However, there was no significant difference between any two of those strains, except KPC17 versus KPC3 (KPC2 vs. KPC3, $p = 0.166$; KPC2 vs. KPC17, $p = 0.110$; KPC17 vs. KPC3, $p = 0.022$; Figure 3E). In addition, the capsular serotype K1 KPC3-TW strain was relatively resistant to phagocytosis compared with the other non-K1/K2/K5 isolates (15-minute phagocytic rate, 27.1 ± 10.23% vs. 54.46 ± 20.94%, $p = 0.050$; Figure 3F).

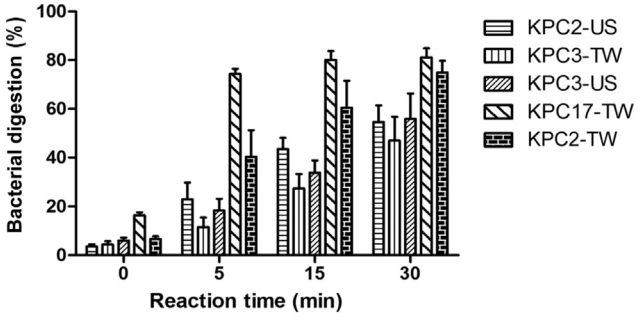


Figure 2. Neutrophil phagocytic rates of different variants of *Klebsiella pneumoniae* carbapenemase(KPC)-producing *K. pneumoniae* isolates. Data are presented as mean phagocytic rate ± standard deviation (SD) at 0 minutes, 5 minutes, 15 minutes, and 30 minutes. The phagocytic rates of these five strains were highly variable with mean 15-minute phagocytic rates (± standard deviation) for KPC2-TW, KPC2-US, KPC3-TW, KPC3-US, and KPC17-TW being 60.46 ± 19.18%, 43.44 ± 8.02%, 27.41 ± 10.24%, 33.81 ± 8.84%, and 80.11 ± 6.22%, respectively. KPC = *Klebsiella pneumoniae* carbapenemase; SD = standard deviation; TW = Taiwan; US = United States.

Mouse lethality study

All five isolates demonstrated relatively less virulence in the murine model, wherein the LD_{50} of all isolates ranged from 1.0091×10^6 CFU to $>10^7$ CFU.

Discussion

The emergence of CRKP, including KPC-producing *K. pneumoniae*, has proven to be a great challenge for physicians not only because of its multidrug resistance but also because of the higher mortality and morbidity rates of infected patients.[4,8,9] Previous studies evaluating the virulence and characteristics of KPC-producing *K. pneumoniae* concluded that expressing the bla_{KPC-2} gene itself is not associated with increased virulence.[13–15] In addition, different variants of KPC enzymes that exhibited different characteristics were also discovered.[12] Our study

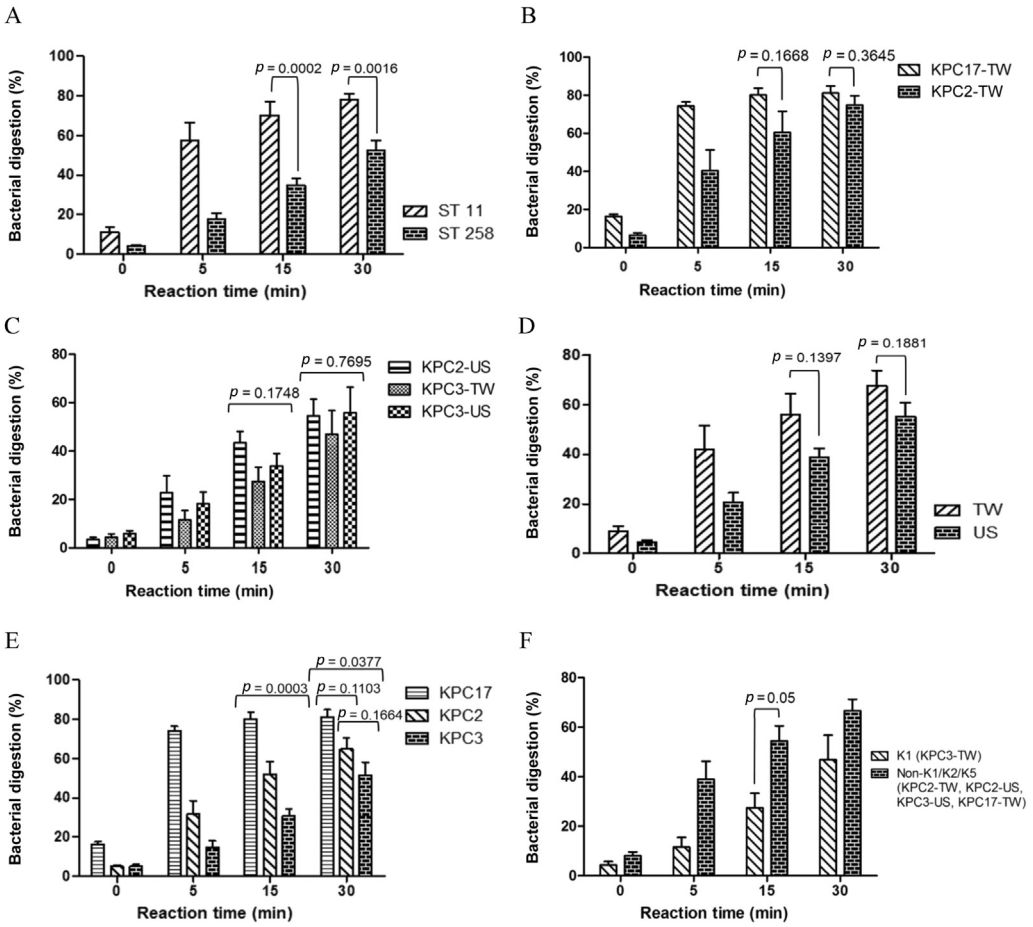


Figure 3. Phagocytic rates of five *Klebsiella pneumoniae* carbapenemase (KPC)-producing *K. pneumoniae* isolates. Data are presented as mean phagocytic rate ± SD at 0 minutes, 5 minutes, 15 minutes, and 30 minutes. (A) Comparison of neutrophil phagocytic rates of different MLST strains. ST11 strains include KPC2-TW and KPC17-TW; ST258 strains include KPC2-US, KPC3-US, and KPC3-TW. The ST11 strains exhibited a significantly higher 15-minute phagocytic rate than ST258 strains ($p < 0.001$). (B) Comparison of neutrophil phagocytic rates for all ST11 strains (KPC2-TW and KPC17-TW). There was no statistically significant difference in 15-minute ($p = 0.167$) or 30-minute phagocytic rates ($p = 0.365$). (C) Comparison of neutrophil phagocytic rates for all ST258 strains (KPC2-US, KPC3-US, and KPC3-TW). There was no statistically significant difference in 15-minute ($p = 0.175$) or 30-minute phagocytic rates ($p = 0.770$). (D) Comparison of neutrophil phagocytic rates for strains from Taiwan and the US. TW represents isolates from Taiwan. There were no statistically significant differences in the 15-minute phagocytic rates ($p = 0.140$). (E) Comparison of neutrophil phagocytic rates between strains expressing different variants of KPCs. Overall, the neutrophil phagocytic rates differed between these three groups ($p = 0.038$). However, there were no significant differences between any two of those strains, with the exception of KPC17 versus KPC3 (KPC2 vs. KPC3, $p = 0.166$; KPC2 vs. KPC17, $p = 0.110$; KPC17 vs. KPC3, $p = 0.022$). (F) Comparison of neutrophil phagocytic rates between strains with different capsular typing. The capsular type K1 strain was more resistant to neutrophil phagocytosis than non-K1/K2/K5 strains (15-minute phagocytic rate: 27.1 ± 10.23% vs. 54.46 ± 20.94%, $p = 0.050$). KPC = *Klebsiella pneumoniae* carbapenemase; MLST = multilocus sequence typing; SD = standard deviation; TW = Taiwan; US = United States.

demonstrated that all five KPC-producing *K. pneumoniae* isolates, expressing different variants of KPC enzymes, were less virulent, with the LD_{50} exceeding 10^6 CFU. We found that the bla_{KPC-2} expressing strains and the other variants we tested were not associated with the increased virulence of *K. pneumoniae*.

Although these strains were not particularly virulent, we still identified different immunological characteristics among different subtypes. There were no significant correlations between KPC variant subtypes and the response to serum killing. KPC3-TW and KPC3-US were both highly sensitive to serum killing; however, KPC2-TW and KPC2-US differed greatly, with the former being serum resistant and the latter being highly serum sensitive. There was also no significant association between the variant subtypes and the neutrophil phagocytic rate. By contrast, different MLST isolates may be associated with different serum responses and neutrophil phagocytic responses. The ST11 isolates were resistant to serum killing, whereas the ST258 isolates were highly sensitive. ST11 isolates had a significantly higher phagocytic rate than ST258 isolates. Previous studies have also demonstrated that different STs may be associated with different serum complement kill responses.[21]

Comparison between geographic regions revealed that isolates from the US all belonged to ST258, whereas those from Taiwan belonged predominantly to ST11, with the exception of KPC3-TW. Additional analysis of patient histories revealed that the patient infected with the KPC3-TW isolate had just returned from the US, where he had been hospitalized. The clinical isolate was collected at Veteran General Hospital in Taipei just a few days after the patient had returned to Taiwan. Based on these findings, we propose that the patient was likely infected with KPC3-TW in the US and only discovered this after returning to Taiwan. Thus, excluding this isolate, in this study, all clinical isolates from Taiwan belonged to the ST11 clonal complex, whereas the isolates from the US belonged to the ST258 complex. The geographic difference in predominant serotype was also consistent with the findings of previous studies.[22–24]

The capsular serotype K1 KPC3-TW was relatively more resistant to neutrophil phagocytosis than the other non-K1/K2/K5 serotype isolates (15-minute phagocytic rate, $27.1 \pm 10.23\%$ vs. $54.46 \pm 20.94\%$, $p = 0.050$). The capsular serotype may not affect the serum response. Both the capsular serotype K1 KPC3-TW and the non-K1/K2/K2 serotype KPC3-US, which belong to the same ST258 group and express the KPC3 enzyme, were highly sensitive to serum killing.

This study has some limitations. Only three variants and a total of five strains of KPC-producing *K. pneumoniae* clinical isolates were analyzed in this study. Thus, there are still many variants that have not been evaluated. Further studies to evaluate the virulence of all known variant subtypes, with more clinical isolates included, should be conducted to confirm the current results. Moreover, in this study, we only surveyed 10 common virulence traits to exclude other confounding factors that may be associated with the virulence of these strains. There are still many known and even unknown virulent factors that were not evaluated in our study.

In conclusion, we propose that neither the bla_{KPC-2} gene itself nor the genes encoding the other variant subtypes of KPC enzymes are associated with increased virulence. The higher mortality and morbidity rates of KPC-producing *K. pneumoniae* infected patients may be associated with other factors, such as lack of effective therapy, multiple comorbidities of the infected patients etc. In addition, different strain types identified by MLST may be associated with different immune responses. Further studies investigating additional clinical isolates and analyzing all variant subtypes of KPC enzymes should be conducted to evaluate the trend found in this study.

Conflicts of interest

All authors have no conflicts of interest to declare.

Acknowledgments

This study was supported by grants from the National Health Research Institutes, Tri-Service General Hospital (TSGH-C104-116, 117 & 118), and National Science Council (NSC 101-2314-B-016-022-MY3).

References

1. Siu LK, Huang DB, Chiang T. Plasmid transferability of KPC into a virulent K2 serotype *Klebsiella pneumoniae*. *BMC Infect Dis* 2014;**14**:176.
2. Wang JH, Liu YC, Lee SS, Yen MY, Chen YS, Wang JH, et al. Primary liver abscess due to *Klebsiella pneumoniae* in Taiwan. *Clin Infect Dis* 1998;**26**:1434–8.
3. Shon AS, Bajwa RP, Russo TA. Hypervirulent (hypermucoviscous) *Klebsiella pneumoniae*: a new and dangerous breed. *Virulence* 2013;**4**:107–18.
4. Huang SR, Liu MF, Lin CF, Shi ZY. Molecular surveillance and clinical outcomes of carbapenem-resistant *Escherichia coli* and *Klebsiella pneumoniae* infections. *J Microbiol Immunol Infect* 2014;**47**:187–96.
5. Perez F, Van Duin D. Carbapenem-resistant *enterobacteriaceae*: a menace to our most vulnerable patients. *Cleveland Clinic J Med* 2013;**80**:225–33.
6. Liu SW, Chang HJ, Chia JH, Kuo AJ, Wu TL, Lee MH. Outcomes and characteristics of ertapenem-nonsusceptible *Klebsiella pneumoniae* bacteremia at a university hospital in Northern Taiwan: a matched case-control study. *J Microbiol Immunol Infect* 2012;**45**:113–9.
7. MacKenzie FM, Forbes KJ, Dorai-John T, Amyes SG, Gould IM. Emergence of a carbapenem-resistant *Klebsiella pneumoniae*. *Lancet* 1997;**350**:783.
8. Ben-David D, Kordevani R, Keller N, Tal I, Marzel A, Gal-Mor O, et al. Outcome of carbapenem resistant *Klebsiella pneumoniae* bloodstream infections. *Clin Microbiol Infect* 2012;**18**:54–60.
9. Gasink LB, Edelstein PH, Lautenbach E, Synnestvedt M, Fishman NO. Risk factors and clinical impact of *Klebsiella pneumoniae* carbapenemase-producing *K. pneumoniae*. *Infect Control Hosp Epidemiol* 2009;**30**:1180–5.
10. Mouloudi E, Protonotariou E, Zagorianou A, Iosifidis E, Karapanagiotou A, Giasnetsova T, et al. Bloodstream infections caused by metallo-beta-lactamase/*Klebsiella pneumoniae* carbapenemase-producing *K. pneumoniae* among intensive care unit patients in Greece: risk factors for infection and impact of type of resistance on outcomes. *Infect Control Hosp Epidemiol* 2010;**31**:1250–6.
11. Wang D, Chen J, Yang L, Mou Y, Yang Y. Phenotypic and enzymatic comparative analysis of the KPC variants, KPC-2 and its recently discovered variant KPC-15. *PLoS ONE* 2014;**9**:e111491.
12. Wolter DJ, Kurpiel PM, Woodford N, Palepou MF, Goering RV, Hanson ND. Phenotypic and enzymatic comparative analysis of the novel KPC variant KPC-5 and its evolutionary variants, KPC-2 and KPC-4. *Antimicrob Agents Chemother* 2009;**53**:557–62.
13. Lavigne JP, Cuzon G, Combescure C, Bourg G, Sotto A, Nordmann P. Virulence of *Klebsiella pneumoniae* isolates harboring bla KPC-2 carbapenemase gene in a *Caenorhabditis elegans* model. *PLoS ONE* 2013;**8**:e67847.
14. McLaughlin MM, Advincula MR, Malczynski M, Barajas G, Qi C, Scheetz MH. Quantifying the clinical virulence of *Klebsiella pneumoniae* producing carbapenemase *Klebsiella pneumoniae* with a Galleria mellonella model and a pilot study to translate to patient outcomes. *BMC Infect Dis* 2014;**14**:31.
15. Siu LK, Lin JC, Gomez E, Eng R, Chiang T. Virulence and plasmid transferability of KPC *Klebsiella pneumoniae* at the Veterans Affairs Healthcare System of New Jersey. *Microb Drug Resist* 2012;**18**:380–4.
16. Heinzelmann M, Gardner SA, Mercer-Jones M, Roll AJ, Polk Jr HC. Quantification of phagocytosis in human neutrophils by flow cytometry. *Microbiol Immunol* 1999;**43**:505–12.

17. Brisse S, Fevre C, Passet V, Issenhuth-Jeanjean S, Tournebize R, Diancourt L, et al. Virulent clones of *Klebsiella pneumoniae*: identification and evolutionary scenario based on genomic and phenotypic characterization. *PLoS ONE* 2009;**4**:e4982.
18. Andrade LN, Vitali L, Gaspar GG, Bellissimo-Rodrigues F, Martinez R, Darini AL. Expansion and evolution of a virulent, extensively drug-resistant (polymyxin B-resistant), QnrS1-, CTX-M-2-, and KPC-2-producing *Klebsiella pneumoniae* ST11 international high-risk clone. *J Clin Microbiol* 2014;**52**:2530—5.
19. Podschun R, Sievers D, Fischer A, Ullmann U. Serotypes, hemagglutinins, siderophore synthesis, and serum resistance of *Klebsiella* isolates causing human urinary tract infections. *J Infect Dis* 1993;**168**:1415—21.
20. Lin JC, Chang FY, Fung CP, Xu JZ, Cheng HP, Wang JJ, et al. High prevalence of phagocytic-resistant capsular serotypes of *Klebsiella pneumoniae* in liver abscess. *Microbes Infect* 2004;**6**:1191—8.
21. Siu LK, Fung CP, Chang FY, Lee N, Yeh KM, Koh TH, et al. Molecular typing and virulence analysis of serotype K1 *Klebsiella pneumoniae* strains isolated from liver abscess patients and stool samples from noninfectious subjects in Hong Kong, Singapore, and Taiwan. *J Clin Microbiol* 2011;**49**:3761—5.
22. Lee CM, Liao CH, Lee WS, Liu YC, Mu JJ, Lee MC, et al. Outbreak of *Klebsiella pneumoniae* carbapenemase-2-producing *K. pneumoniae* sequence type 11 in Taiwan in 2011. *Antimicrob Agents Chemother* 2012;**56**:5016—22.
23. Chen L, Mathema B, Pitout JD, DeLeo FR, Kreiswirth BN. Epidemic *Klebsiella pneumoniae* ST258 is a hybrid strain. *MBio* 2014;**5**. e01355—14.
24. Jean SS, Lee WS, Hsueh PR. Nationwide spread of *Klebsiella pneumoniae* carbapenemase-2-producing *K. pneumoniae* sequence type 11 in Taiwan. *J Microbiol Immunol Infect* 2013;**46**:317—9.
25. Rivero A, Gomez E, Alland D, Huang DB, Chiang T. K2 serotype *Klebsiella pneumoniae* causing a liver abscess associated with infective endocarditis. *J Clin Microbiol* 2010;**48**:639—41.
26. Turton JF, Baklan H, Siu LK, Kaufmann ME, Pitt TL. Evaluation of a multiplex PCR for detection of serotypes K1, K2 and K5 in *Klebsiella* sp. and comparison of isolates within these serotypes. *FEMS Microbiol Lett* 2008;**284**:247—52.
27. Fang CT, Chuang YP, Shun CT, Chang SC, Wang JT. A novel virulence gene in *Klebsiella pneumoniae* strains causing primary liver abscess and septic metastatic complications. *J Exp Med* 2004;**199**:697—705.
28. Chou HC, Lee CZ, Ma LC, Fang CT, Chang SC, Wang JT. A novel virulence gene in *Klebsiella pneumoniae* strains causing primary liver abscess and septic metastatic complications. *J Exp Med* 2004;**199**:697—705.
29. Ma LC, Fang CT, Lee CZ, Shun CT, Wang JT. Genomic heterogeneity in *Klebsiella pneumoniae* strains is associated with primary pyogenic liver abscess and metastatic infection. *J Infect Dis* 2005;**192**:117—28.
30. Regue M, Hita B, Pique N, Izquierdo L, Merino S, Fresno S, et al. A gene, *uge*, is essential for *Klebsiella pneumoniae* virulence. *Infect Immun* 2004;**72**:54—61.