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ARTICLE

Correlation between *Klebsiella pneumoniae* carrying pLVPK-derived loci and abscess formation

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Abstract *Klebsiella pneumoniae*-caused liver abscess (KLA) is an emerging infectious disease. However, factors other than K1-specific loci that contribute to the pathogenesis of this disease have not been identified. pLVPK is a 219,385-bp plasmid of *K. pneumoniae* CG43, an invasive K2 strain associated with KLA. We aimed in this study to evaluate the involvement of pLVPK in *K. pneumoniae* virulence and its clinical significance in abscess formation. A pLVPK-cured CG43 was isolated and its virulence was examined in a mouse model. The prevalence of pLVPK-derived loci *terW*, *iutA*, *rmpA*, *silS*, and *repA* was investigated in 207 clinical isolates by screening with specific primers. Loss of pLVPK abolished the ability of *K. pneumoniae* to disseminate into extraintestinal sites and,

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consequently, attenuated abscess formation in mice. Primary *K. pneumoniae* abscess isolates (n=94) were more likely to be *terW*$^+$-*iutA*$^+$-*rmpA*$^+$-*silS*$^+$ than those related to non-abscess infections (n=113) (62% vs. 27%; p<0.0001). Logistic regression analysis indicated that the presence of the *terW-rmpA-iutA-silS* loci was a significant risk factor (odds ratio, 4.12; 95% confidence interval, 2.02–8.4; p< 0.0001) for abscess formation. pLVPK is a determinant for *K. pneumoniae* virulence and infection with strains carrying the pLVPK-derived *terW-rmpA-iutA-silS* loci may predispose patients to abscess formation.

Introduction

Klebsiella pneumoniae is a globally distributed pathogenic bacterium that is responsible for a broad spectrum of infections, including pneumonia, bacteremia, septicemia, purulent infections, urinary tract infections, and meningitis, that may be acquired in community or hospital settings [1]. Classically, pyogenic liver abscess (PLA) is a complication of intra-abdominal or biliary tract infections, resulting from mixed aerobic and anaerobic bacteria infections [2]. However, PLA that is predominantly mediated by primary infection with *K. pneumoniae* as a single pathogen has been reported in recent years [3–13]. In contrast with polymicrobial PLA, *K. pneumoniae*-caused liver abscess (KLA) is generically cryptogenic, without underlying hepatobiliary disorders, and is frequently complicated with septic metastatic lesions [11, 14–17]. By virtue of its primary and invasive natures, KLA is considered to be one of the most severe infections to be caused by *K. pneumoniae*. In addition to KLA, fulminant primary abscesses caused by *K. pneumoniae* have been described at non-hepatic sites, such as the lung, kidney, prostate, soft tissue, and bone, and can also result in severe morbidity [18].

Previous attempts to identify the bacterial attributes of KLA have reported several factors with statistical correlations to KLA isolates, including the K1 and K2 serotypes, K1-specific genes *magA* and *allS*, hypermucoviscosity (HV) phenotype, HV-regulatory gene *rmpA*, and the *Yersinia* HPI, *iucABCEiutA*, and *iroAiroNDCB* iron-acquisition systems [5, 13, 19–24]. Interestingly, sequence analysis has revealed that the KLA-associated genetic loci *iucABCEiutA*, *iroAiroNDCB*, and *rmpA* locate within pLVPK, a 219,385-bp plasmid of *K. pneumoniae* CG43 [25]. Animal experiments involving CG43, an invasive K2 strain associated with bacteremic liver abscess, have been reported [26]. Here, to examine the correlation between pLVPK presence and *K. pneumoniae* abscess formation, we examine the contribution of pLVPK to *K. pneumoniae* virulence and evaluate the prevalence of the pLVPK-derived *terW-iutA-rmpA-silS* loci in a collection of *K. pneumoniae* isolates recovered from patients with primary infections. In addition, we compare the association of those loci with pyogenic syndromes, focusing particularly on the formation of tissue abscesses in hepatic and non-hepatic sites.

Materials and methods

Bacterial isolates

We obtained 207 non-repetitive *K. pneumoniae* isolates that had been isolated from patients with primary *K. pneumoniae* infections at a referral medical center in central Taiwan during a 15-month period from April 2002. These isolates, which were confirmed as *K. pneumoniae* using the API 20E system (bioMérieux), were collected from various infection foci: 11.6% were from blood; 4% from liver aspirates; 0.4% from eye aspirates; 0.8% from cerebrospinal fluid; 26.2% from non-hepatic abscesses; 22.8% from sputum; 8.5% from wound pus; and 25.6% from other body fluids. Due to the difficulty in determining whether *K. pneumoniae* is the primary pathogen in a urinary tract infection, urine isolates were excluded. If cultures were concomitantly positive in more than one site, only that culture which was isolated from the primary infection focus was included. One isolate per patient was analyzed, and each isolate represented a single case. Isolates were cultured in Luria–Bertani (LB) broth and stored at −80°C until use.

Clinical definition

Medical records were reviewed and information related to infection foci, clinical manifestations, and underlying diseases was collected. The median age of the patients was 62 years, and the male-to-female ratio was 1.94. Of the patients, 45.9% had diabetes, which was defined as described [24]. Community acquisition and clinical syndromes were defined as described [7, 27]. A pyogenic *K. pneumoniae* infection was defined as abscess formation in a tissue that was primarily caused by *K. pneumoniae* without coexisting pathogens [18]. Clinical research was conducted according to the human experimentation guidelines of Chung-Shan Medical University.

Isolation of a pLVPK-cured derivative and determination of its virulence

pLVPK is the sole plasmid harbored by *K. pneumoniae* CG43. We eliminated pLVPK by using the sodium dodecyl sulfate (SDS) method described previously [28]. One thousand colonies were screened, and one of the four pLVPK-cured colonies that were identified by the loss of

the HV phenotype and were verified by Southern blot analysis with pLVPK-specific probes was selected for further studies. The virulence of the pLVPK-cured CG43 strain was determined in a mouse liver abscess model [26]. Briefly, 5×10^7 CFU bacterial culture of either the pLVPK-cured derivative or its parental strain was orally inoculated into 8-week-old BALB/c mice. Survival of the infected mice was monitored daily for 2 weeks. The mortality rate and the mean number of days to death were determined by the Kaplan–Meier method using Prism4 for Windows (GraphPad). Bacterial concentrations in various mouse tissues were determined by viable count measurement. All animal experiments were performed according to the animal experimentation guidelines of Chung-Shan Medical University.

Plasmid profiles, Southern hybridization, and PCR detection of pLVPK-related loci

Plasmids of clinical *K. pneumoniae* isolates were extracted with Wizard Plus SV Minipreps (Promega), separated on a 0.7% agarose gel, and then transferred onto a BrightStar-Plus nylon membrane (Ambion). After denaturation, neutralization, and UV cross-linking, the membrane was blotted with ULTRAhyb Hybridization Buffer (Ambion) overnight at 68°C against a *terW*-specific biotin-labeled probe which was prepared using a BrightStar Psoralen-Biotin kit (Ambion). After stringent washing, signals were detected with a BrightStar BioDetect kit (Ambion). pLVPK-derived genetic loci were detected by polymerase chain reaction (PCR) performed using genomic DNA as the template with specific primers for *terW* (atgcaattaaacacca gacag and ctcattctcttgagtgttttc), *iutA* (acctgggttatcgaaaacgc and gatgtcatagcctgattgc), *rmpA* (acgactttcaagagaaatga and catagatgtcataatcacac), *silS* (catagcaaaccttccaggc and atcgg cagagaaattggc), and *repA* (ggccaatgataacaatcag and gaat gaccagtacataatcc). An initial denaturation at 95°C for 10 min was followed by denaturation at 94°C for 45 s, annealing at 54°C for 45 s, and extension at 72°C for 1 min for 30 cycles. A *terW*$^+$-*iutA*$^+$-*rmpA*$^+$-*silS*$^+$ strain yielded four locus-specific amplicons of sizes 239, 1,115, 434, and 803 bp for *terW*, *iutA*, *rmpA*, and *silS*, respectively. All of the PCR products from at least ten clinical strains that were DNA sequenced revealed >98% homology with the published sequence [25]. CG43 was used as the positive control and pLVPK-cured CG43 was used as the negative control for the PCR experiments.

Determination of K genotype

Capsular antigens were determined by PCR detection of the K-serotype-specific *wzx* locus. Specific primers designed by previous investigators [29] were used for detecting K1 antigen with gtaggtattgcaagccatgc and gcccaggttaat gaatccgt, K2 antigen with ggagccatttgaattcggtg and tccctag cactggcttaagt, and K54 antigen with cattagctcagtggttggct and gcttgacaaacaccatagcag.

Statistical analysis

The χ^2 test and Fisher's exact test were used for the analysis of independence in contingency tables. Risk factors for abscess formation were analyzed with binary logistic regression using SPSS version 14.0.1. for Windows.

Results

Loss of pLVPK-attenuated *K. pneumoniae* virulence

A pLVPK-cured CG43 strain was isolated to characterize the role of pLVPK in the pathogenicity of *K. pneumoniae*. The phenotypic features encoded by pLVPK, including synthesis of aerobactin and display of the HV phenotype, were absent in the cured strain. However, in vitro growth and several capsule-related features, including the quantity of uronic acids, resistance to serum killing, and antiphagocytotic ability, were not significantly changed in the pLVPK-cured strain (Table 1). Despite its wild-type level of growth in vitro, the pLVPK-cured strain showed lethal dose 50 (LD_{50}) levels at least 1,000 times higher than that in the wild type strain in mouse peritonitis and KLA models (Table 1).

Previously, we utilized an oral inoculation method to establish a mouse KLA model that recapitulated the liver abscess characteristics [26]. In that model, *K. pneumoniae* CG43 established intestinal colonization within 12 h after oral inoculation, maintained its persistence throughout the entire course of infection, and disseminated into the spleen and liver by 24 and 36 h post-infection, respectively. All CG43-infected mice died within one week after oral infection with 5×10^7 CFU inoculums (Table 1). Compared to wild-type CG43, the pLVPK-cured strain showed significant attenuation of intestinal colonization and apparently lost its ability to disseminate to extraintestinal organs, including the spleen, liver, and blood (Fig. 1); moreover, it was avirulent to mice (oral LD_{50} $>10^9$ CFU; Table 1). These results indicated that, with no effect on bacterial growth capacity in vitro, pLVPK can significantly contribute to fulminant invasiveness and virulence of *K. pneumoniae* during KLA development in mice.

Prevalence of pLVPK-derived *terW-iutA-rmpA-silS* loci in *K. pneumoniae* abscess isolates

Compared to clinical ESBL (extended-spectrum beta-lactamase) strains, which usually maintain a number of

Table 1 Virulence characteristics of pLVPK–cured *Klebsiella pneumoniae*

Characteristics	Wild type	pLVPK–cured
Hypermucoviscosity[a]	Positive	Negative
Aerobactin synthesis[c]	Positive	Negative
Quantity of uronic acids[b]	17.0±2.2	15.3±0.9
Serum killing[d]	Resistant	Resistant
Antiphagocytosis[e]	Positive	Positive
Intraperitoneal LD_{50}[f]	<10 CFU	2×10^3 CFU
Oral LD_{50}[f]	2.6×10^6 CFU	$>10^9$ CFU
Mouse mortality (MDD)[g]	100% (4)	0% (>14)

[a] Hypermucoviscosity phenotype of colonies was determined using the string-forming test on blood agar as described previously [32]

[b] The uronic acid content of the capsular polysaccharides was determined as described previously [38] and was expressed as mean ± standard deviation ($\mu g/10^{10}$ CFU)

[c] Synthesis of aerobactin was assessed by cross–feeding with *Escherichia coli* LG1522 as the indicator [39][d] Bacterial resistance to serum killing was determined as described previously [38]. If the bacterial strain examined showed a reduction of 2 log in CFU after 30 min of incubation with non-immune human serum at 37°C, it was defined as serum-sensitive

[e] Phagocytosis of a particular strain by retinoic acid–induced U937 cells was determined as described previously [40]. If less than 1% of the bacteria of a particular strain were phagocytosed after 1 h incubation at 37°C, this strain was considered to be antiphagocytotic

[f] LD_{50} to BALB/c mice of a particular strain was determined by administering the strain intraperitoneally or orally as described previously [26]

[g] The mortality rate and mean number of days to death (MDD) of the BALB/c mice that were orally challenged with 5×10^7 CFU of pLVPK–cured or wild–type *K. pneumoniae* were determined as described previously [26]

resistance- (R-) plasmids ranging from 10 to 90 kb, the majority of our KLA isolates harbored a >200-kb plasmid (Fig. 2a). Southern hybridization results indicated that most of these large plasmids were homologous with pLVPK (Fig. 2b). To investigate the distribution of pLVPK in the *K. pneumoniae* isolates, the sequence deposited in GenBank (AY378100) was used, and a multiplex PCR assay was developed to detect the pLVPK-derived loci, including: *terW* (21,671–22,129), a tellurite resistance gene; *iutA* (61,276–63,477), which encodes a receptor for aerobactin–iron complexes; *silS* (175,080–176,555), a silver resistance gene; and *rmpA* (84,784–85,368), which encodes the HV

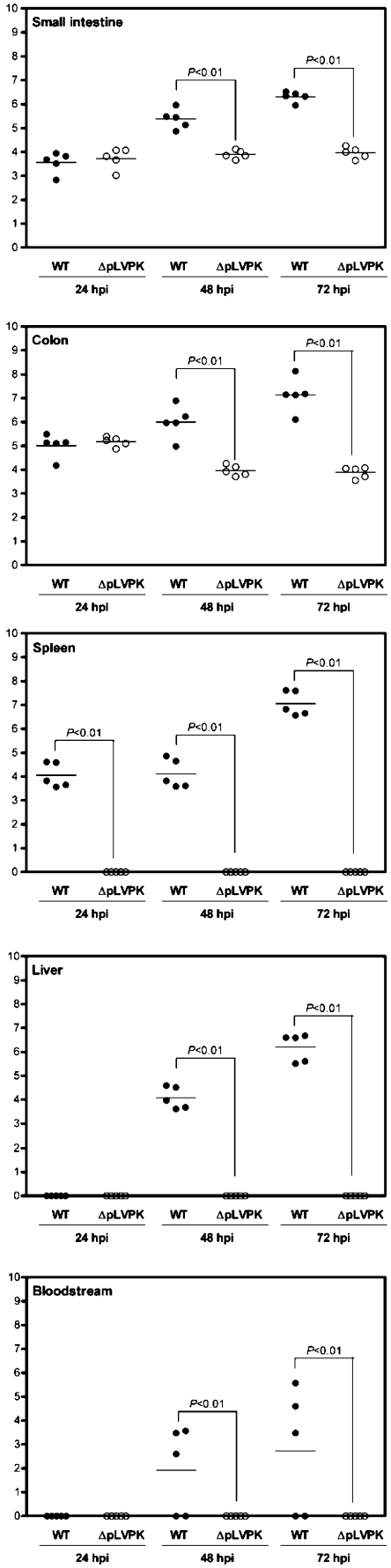

Fig. 1 Growth dynamics of pLVPK-cured *Klebsiella pneumoniae* in mice. Oral inoculation of equivalent doses (5×10^7 CFU) of wild-type (WT; *solid circles*) and pLVPK-cured *K. pneumoniae* (ΔpLVPK; *open circles*) in 8-week-old male BALB/c mice. Bacterial loads in tissue were determined at 24, 48, and 72 h post-infection (hpi). The number of $\log_{10}$ CFU was standardized per gram of wet organ weight. Samples that yielded no colonies (limit of detection is about 50 CFU) are plotted on the *x*-axis. The *bars* denote the geometric mean for each tissue from five mice at each time point. $p<0.01$ for comparison of the pLVPK-cured group with the wild-type group by Student's *t*-test

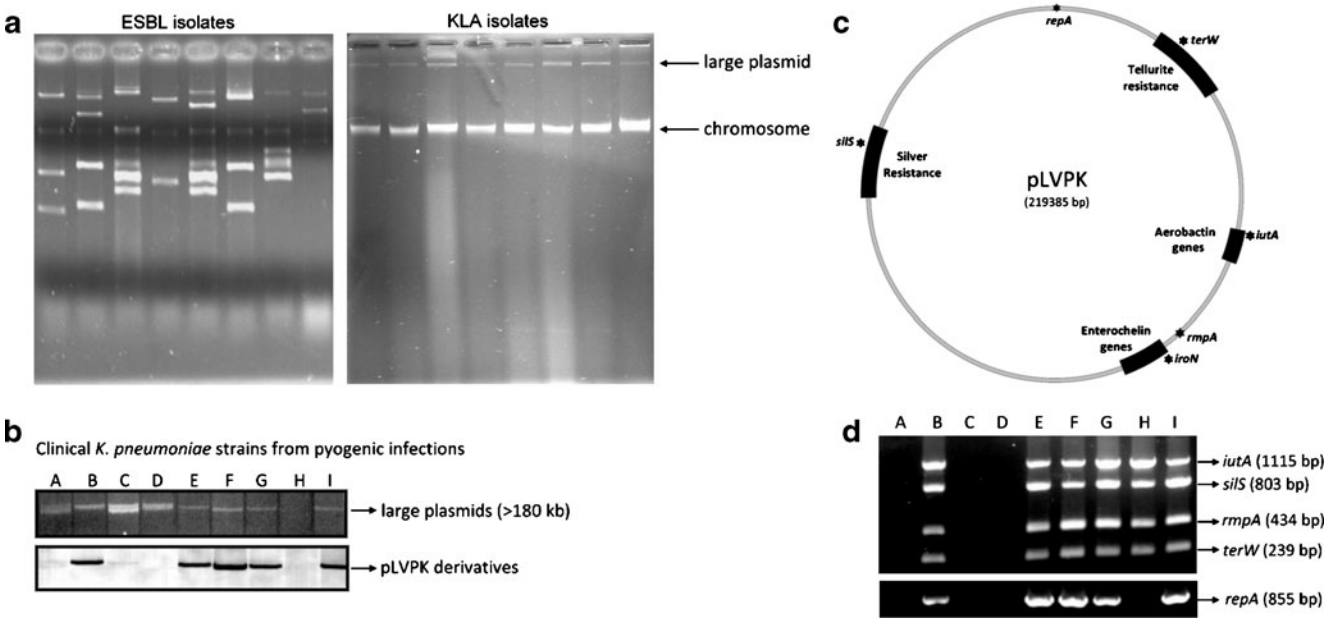

Fig. 2 Detection of pLVPK derivatives. **a** Plasmid profiles of clinical *K. pneumoniae* isolates that were ESBL strains (*left panel*) or were collected from KLA patients (*right panel*). **b** Southern hybridization with a *terW*-specific biotin-labeled probe. *A–H*: clinical isolates; *I*: CG43. **c** The map of pLVPK indicates the gene clusters encoding tellurite resistance, silver resistance, and the iron-chelating siderophores aerobactin and enterochelin. The *asterisks* indicate the genetic loci that were used for the detection of pLVPK derivatives in this study. **d** Multiplex PCR detection for *terW-silS-rmpA-iutA* loci. PCR was performed with specific primers as described in the Materials and methods section. The sizes of locus-specific amplicons are indicated in parentheses. *A–I*: isolates as indicated in **b**

phenotype activator (Fig. 2c). On the assumption that the pLVPK-derived plasmids share similar replication mechanisms, specific primers to detect the pLVPK *repA* gene (218,156–219,040) were designed. The *repA*$^+$ strains were considered to harbor their pLVPK derivatives in an extrachromosomal form.

To examine the correlation between pLVPK and *K. pneumoniae* abscess formations, we assessed the prevalence of *terW*$^+$-*iutA*$^+$-*rmpA*$^+$-*silS*$^+$ and *repA*$^+$ in a collection of 207 clinical isolates recovered from patients with primary *K. pneumoniae* infections. Of the 207 clinical isolates, 35 (16.9%) were regarded as KLA strains as they were obtained from tissue-invasive cases that presented with the formation of liver abscesses, while 59 (28.5%) were from cases associated with abscesses at non-hepatic sites, including lesions that occurred as empyema, endophthalmitis, necrotizing fasciitis, septic arthritis, along with lung, epidural, parotid, paraspinal, splenic, renal, prostate, muscle, and deep neck abscesses. The remaining 113 (54.6%) isolates were obtained from non-abscess-related cases, including pneumonia without abscess, primary peritonitis, cellulitis, biliary tract infection, primary bacteremia with no original infectious foci identifiable, and catheter-related infections. Based on the multiplex PCR results, 23 of the 35 KLA isolates (66%) were *terW*$^+$-*iutA*$^+$-*rmpA*$^+$-*silS*$^+$ and 16 (69.6%) of the *terW*$^+$-*iutA*$^+$-*rmpA*$^+$-*silS*$^+$ isolates were *repA*$^+$. As ascertained by Southern hybridization, the *terW*$^+$-*iutA*$^+$-*rmpA*$^+$-*silS*$^+$ but *repA*$^-$ KLA isolates had pLVPK genes in the chromosome. As the H strain shown in Fig. 2, most of the *terW*$^+$-*iutA*$^+$-*rmpA*$^+$-*silS*$^+$-*repA*$^-$ isolates had no pLVPK-derived plasmids, but they did reveal positive signals when multiplex PCR screened for the *terW-iutA-rmpA-silS* loci.

Among the 59 non-hepatic abscess isolates, the incidence of *terW*$^+$-*iutA*$^+$-*rmpA*$^+$-*silS*$^+$ was 61% (n=36). Overall, the *K. pneumoniae* isolates resulting in abscesses in the liver or other tissues exhibited a significantly higher incidence of *terW*$^+$-*iutA*$^+$-*rmpA*$^+$-*silS*$^+$ than that in the non-abscess-related isolates (62% vs. 27%; p<0.0001; Table 2). Furthermore, logistic regression analysis showed the presence of *terW-iutA-rmpA-silS* loci in *K. pneumoniae* to be a risk factor for abscess formation (odds ratio [OR], 4.66; 95% confidence interval [95%], 2.58–8.42; p=0.002), even after controlling for K genotype, diabetes mellitus, and community acquisition (OR, 4.12; 95% confidence interval, 2.02–8.4; p<0.0001; Table 3).

Presence of *terW-iutA-rmpA-silS* loci predisposes K1 and K54 strains to abscess formation

Compatible with the findings of other researchers [4, 12, 18, 24], our data shows that abscess formation tended to develop in diabetic patients (63% vs. 32%; p<0.001), in individuals who were infected with *K. pneumoniae* strains displaying the HV phenotype (51% vs. 27%; p<0.001), and in those infected in community settings (89% vs. 52%;

Table 2 Factors associated with abscess formation

	Pyogenic infection (n=94)			Non–abscess (n=113)	OR (95% CI)	p-value
	KLA (n=35)	Non–hepatic abscess (n=59)	Any abscess (n=94)			
repA$^+$ (n=75)	16 (46)	27 (46)	43 (46)	32 (28)	2.1 (1.2–3.8)	0.0133
terW$^+$ *iutA*$^+$ *rmpA*$^+$ *silS*$^+$ (n=88)	23 (66)	35 (59)	58 (62)	30 (27)	4.5 (2.5–8.0)	<0.0001
terW$^+$ *iutA*$^+$ *rmpA*$^+$ *silS*$^+$ *repA*$^+$ (n=41)	9 (26)	17 (29)	26 (28)	15 (13)	2.5 (1.2–5.1)	0.0137
terW$^-$ *iutA*$^-$ *rmpA*$^-$ *silS*$^-$ (n=20)	0 (0)	2 (3)	2 (2)	18 (16)	0.11 (0.03–0.51)	0.0007
K1	10 (29)	16 (27)	26 (28)	23 (20)	–	NS[a]
K2	8 (23)	12 (20)	20 (21)	14 (12)	–	NS[a]
K54	3 (9)	4 (7)	7 (7)	6 (5)	–	NS[a]
HV phenotype	19 (54)	29 (49)	48 (51)	30 (27)	2.9 (1.6–5.2)	0.0003
Diabetes	23 (66)	36 (61)	59 (63)	36 (32)	3.6 (2.0–6.4)	<0.0001
Community acquisition	35 (100)	49 (83)	84 (89)	59 (52)	7.7 (3.6–16.3)	<0.0001

Data are no. (%) of isolates. Statistical comparisons are between the any abscess group and the non-abscess group

[a] No statistical significance

p<0.001; Table 2). To further determine the genetic background of *terW*$^+$-*iutA*$^+$-*rmpA*$^+$-*silS*$^+$ *K. pneumoniae*, the capsular antigens of the 207 isolates were genotyped. In general, *terW*$^+$-*iutA*$^+$-*rmpA*$^+$-*silS*$^+$ isolates were of various K genotypes, with K1 and K2 being the most common (28 and 21%, respectively), followed by K54, which was the genotype in 7% of the abscess isolates. In comparisons with the incidences of K1, K2, and K54 in non-abscess-related isolates (20, 12, and 5%, respectively), no K genotype was found to be significantly correlated to abscess formation (Table 2). Nonetheless, after the *terW*$^+$-*iutA*$^+$-*rmpA*$^+$-*silS*$^+$ factor was included, the incidence of abscess formation in K1 isolates was significantly higher (67% vs. 36%; OR= 3.5; p=0.047; Fig. 3a). The enhancing effect of the *terW-iutA-rmpA-silS* loci on the incidence of abscess formation was most striking in the K54 isolates (88% vs. 0%; OR= 55; p=0.0047; Fig. 3a). Although the *terW*$^+$-*iutA*$^+$-*rmpA*$^+$-*silS*$^+$ isolates were broadly distributed among the K genotypes, the *repA*$^+$ incidences were significantly lower in the K1 (11%) and K54 (0%) strains than in the other K strains (range 61−89%; Fig. 3b), indicating that chromosome integration of pLVPK occurred in *K. pneumoniae* strains with particular genetic backgrounds.

Discussion

K. pneumoniae-caused liver abscess (KLA) has become a health problem in Taiwan with an annually increasing incidence [11, 30]. Despite the identification of an

Table 3 Logistic regression analysis of risk factors for abscess formation

Factor	No. of isolates (n=207)	Any abscess		Univariate analysis		Multivariate analysis	
		Yes (n=94)	No. (n=113)	OR (95% CI)	p-value	OR (95% CI)	p-value
terW$^+$ *iutA*$^+$ *rmpA*$^+$ *silS*$^+$	88	58 (62)	30 (27)	4.66 (2.58–8.42)	0.002[a]	4.12 (2.02–8.4)	<0.0001[a]
K1	49	26 (28)	23 (20)	–	0.22	–	0.327
K2	34	20 (21)	14 (12)	–	0.13	–	0.857
K54	13	7 (7)	6 (5)	–	0.53	–	0.682
HV phenotype	78	48 (51)	30 (27)	2.89 (1.61–5.16)	<0.0001[a]	2.51 (1.19–5.3)	0.016[a]
Bacteremia	72	35 (37)	37 (33)	–	0.5	–	0.827
Diabetes	95	59 (63)	36 (32)	3.61 (2.03–6.41)	<0.0001[a]	4.80 (2.34–8.87)	<0.0001[a]
Community acquisition	143	84 (89)	59 (52)	7.69 (3.62–16.32)	<0.0001[a]	7.13 (3.01–16.9)	<0.0001[a]

Data are no. (%) of isolates. Risk factors associated with abscess formation were analyzed with a binary logistic regression method of SPSS 14.0

[a] Statisticallysignificant

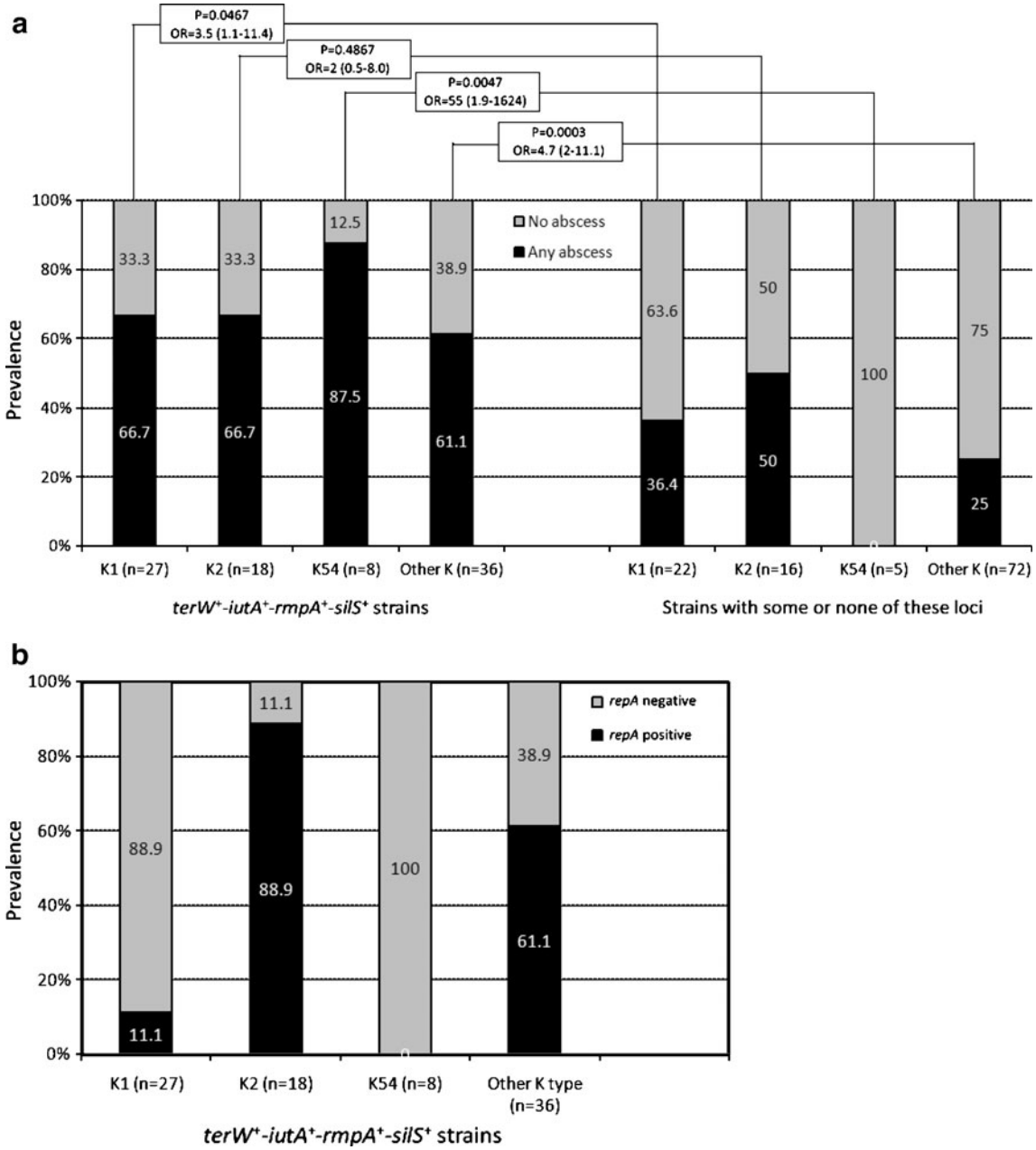

Fig. 3 *terW*$^+$-*iutA*$^+$-*rmpA*$^+$-*silS*$^+$ *K. pneumoniae*. **a** Incidence of abscess formation by *K. pneumoniae* isolates categorized by K genotype. Statistical comparisons by Fisher's exact test are between *terW*$^+$-*iutA*$^+$-*rmpA*$^+$-*silS*$^+$ isolates and isolates those with only some or none of these loci. **b** Positive rate of *repA* among *terW*$^+$-*silS*$^+$-*rmpA*$^+$-*iutA*$^+$ isolates categorized by K genotype

association between K1-specific genetic loci and KLA [23, 29, 31, 32], reports regarding KLA pathogenesis are limited. The large virulence plasmid pLVPK is a 219,385-bp plasmid of *K. pneumoniae* CG43 [25], a strain previously studied in animal liver abscess experiments [26]. In this study, a pLVPK-cured *K. pneumoniae* CG43 was generated and its virulence was examined in a KLA animal model. The incapability of the pLVPK-cured strain to cause liver abscess suggests that this inherent plasmid may contribute to the virulence of *K. pneumoniae*. As evident in the results from the plasmid profiling and Southern hybridization, many KLA isolates, in addition to strain CG43, also harbor plasmids that are larger than 200 kb, and most of these plasmids were derivatives of pLVPK. These observations suggest that the development of KLA might be attributed to the presence of pLVPK derivatives in clinical *K. pneumoniae* isolates. To explore that suggestion, we developed a multiplex PCR assay to investigate the prevalence of four pLVPK-derived genetic loci, *terW*, *iutA*, *rmpA*, and *silS* in 94 *K. pneumoniae*

clinical isolates recovered from patients with primary pyogenic infections and in 113 non-abscess-related *K. pneumoniae* isolates. A statistically significant correlation between the presence of pLVPK-derived *terW-iutA-rmpA-silS* loci and abscess formation was detected.

The requirement of pLVPK's presence for *K. pneumoniae* CG43 virulence supports our clinical finding that *terW-iutA-rmpA-silS* is an independent pathogenicity factor for abscess formation. In contrast to the fulminant invasiveness of CG43, the pLVPK-cured CG43 strain entirely lost its systemic dissemination ability, failed to cause liver abscess, and was, consequently, avirulent to BALB/c mice. That result was reminiscent of the findings of Nassif et al. [33, 34], in which the presence of a 180-kb plasmid was reported to contribute to K1 and K2 *K. pneumoniae* virulence. Due to the lack of a complete sequence for that 180-kb plasmid, the similarity between that plasmid and pLVPK could not be determined. However, based on the phenotypic features of HV and aerobactin synthesis, which were reported to be encoded by both pLVPK and the 180-kb plasmid, we reasoned that these two plasmids are related. Recently, as part of the *K. pneumoniae* NTUH-K2044 genome project, a pK2044 plasmid, which is 4,767 bp longer than pLVPK, was identified [35]. Similarities in genetic organization and the presence of sequence homology suggest that pLVPK and pK2044 evolved from a common ancestor. Given that pLVPK and pK2044 both harbor the *terW-iutA-rmpA-silS* loci, we consider the $terW^{+}$-$iutA^{+}$-$rmpA^{+}$-$silS^{+}$ isolate to be a *K. pneumoniae* strain that is carrying pLVPK derivatives.

Based on bioinformatic analysis, pLVPK carries virulence-associated genes, encoding capsular polysaccharide (CPS) synthesis regulators (*rmpA*/*rmpA2*), iron-acquisition systems (*iucABCDiutA*, *iroBCDN*, *fepBC*, and *fecIRA*), and gene clusters homologous with copper silver, lead, and tellurite-resistant genes [25]. The function of pLVPK was largely unknown due to the limited amount of published results, except for the HV phenotype (encoded by *rmpA*/*rmpA2*), aerobactin-chelating ability (by *iucABC-DiutA*), and tellurite resistance (by *terZABCDE*) [25]. Among these genetic loci, *rmpA* attracts the most attention due to its role in enhancing the display of HV phenotype. A number of studies have suggested that the HV phenotype, which was induced by the expression of *rmpA*, is a predominant factor in KLA [9, 18, 32, 36].

In addition, based on a clinical investigation of 151 *K. pneumoniae* bacteremia isolates collected from two medical centers in southern Taiwan, Yu et al. [18] demonstrated that strains which were positive for *rmpA* were significantly associated with pyogenic infections in hepatic and non-hepatic tissues. However, it is unlikely that *rmpA* singly determines the virulence of *K. pneumoniae* for KLA development, because when KLA isolates were analyzed with *rmpA* as the only factor, >40% of *rmpA*-positive isolates were obtained from non-abscess-related cases (47% [70/149] in our series and 42% [22/52] in the report by Yu et al. [18]). We believe that not only *rmpA* but also other genetic loci that may link to *rmpA* in pLVPK are involved in causing KLA. As evident in our previous study, KLA is the outcome of a series of events orchestrated by a panel of virulence genes [26]; thus, KLA development is more complex than that expected from a single factor outcome resulting from the display of HV phenotype by *rmpA*. Based on the finding that the coexistence of *rmpA* and other pLVPK-derived genes, including *terW*, *iutA*, and *silS*, was significantly predominant in *K. pneumoniae* strains associated with cases of tissue abscesses, this study indicates that derivatives of pLVPK, not *rmpA* per se, are prerequisites for *K. pneumoniae* virulence.

pLVPK has a single plasmid replication region of 1,756 bp (217,448–219,203 bp), which consists of the *repA* gene and interon-based replicons [25]. Based on the assumption that pLVPK derivatives have similar replication mechanisms, we suggest that the *repA* gene is detectable in strains that have pLVPK derivatives in an extra-chromosomal form. It is noteworthy that only 46% (41/89) of the $terW^{+}$-$iutA^{+}$-$rmpA^{+}$-$silS^{+}$ isolates were extra-chromosomal types. Given that a recent report indicated that the majority of pK2044 plasmids in the *K. pneumoniae* NTUH-K2044 genome have chromosome-integrated replicates [35], we reasoned that ectopic integration of pLVPK derivatives into chromosomes has occurred frequently in certain clinical isolates. That possibility was supported by the Southern hybridization results which showed that the positive signal of pLVPK-specific probes was located in the chromosomal region of the $terW^{+}$-$iutA^{+}$-$rmpA^{+}$-$silS^{+}$-$repA^{-}$ isolates. The *K. pneumoniae* NTUH-K2044 is a K1 strain, and we found that the chromosome-integrated form of pLVPK was more prevalent in the K1 and K54 strains than it was in other K isolates. It is conceivable that large-sized bacterial plasmids are difficult to maintain within their host cells. pLVPK has an F-plasmid-like partition system, with *parA/B* and *sopA/B* homologs [25]; therefore, its large elements may integrate into chromosomal DNA in the same manner as that reported for the IncP-2 plasmids CAM and OCT in *Pseudomonas*, where the large plasmid is predominantly influenced by the genetic mechanisms of its bacterial host [37]. Thus, it is foreseeable that the majority of K1 and K54 strains have their pLVPK elements as chromosomally integrated genome islands. The permanent recruitment of pLVPK elements into the genome of some *K. pneumoniae* strains might allow their host an adaptive fitness advantage in specific niches inside the human body, possibly through enhanced tissue invasiveness.

A myriad of epidemiological studies attempting to identify risk factors for KLA have been published and

most of the studies were conducted using bacteremia isolates. Although the invasiveness of KLA was emphasized by this approach, the selective criteria used for sample collection may have exaggerated the significance of certain factors. Given that *K. pneumoniae* infections occur at various sites in addition to the blood, and to avoid possible bias related to sample collection, we prospectively enrolled *K. pneumoniae* isolates from patients who were treated at a Taiwan referral medical center and diagnosed with a *K. pneumoniae* infection during a 15-month period from April 2002. Although we analyzed consecutive *K. pneumoniae* isolates from various foci, other than bacteremia isolates, our data showed that the HV phenotype, diabetic mellitus, and community acquisition to be significant risk factors for pyogenic tissue infections (Table 3), findings that are compatible with the results of other KLA studies in Taiwan [7, 11, 18, 21, 24]. Our data did not show significant associations between abscess formation and the K1 or K2 genotypes, even though these two were the most prevalent capsular genotypes among the KLA isolates studied; those results also agree with those reported previously [13, 21, 24]. However, the prevalence of K1 (28%; 26/94) among the abscess isolates was lower than that reported in other studies, which ranged from 38 to 63.4% [18, 24, 29, 32]. Although different sample collection methods might result in variations in K1 incidence rates, our results raise doubts about the idea that the K1 genotype per se is the only virulence determinant for KLA [29]. Instead, the significance of the pLVPK-derived loci *terW-iutA-rmpA-silS* in the risk of abscess formation caused by various K isolates suggests that horizontally acquired genetic determinants have conferred the ability to establish pyogenic infections on *K. pneumoniae* strains which may have different genetic backgrounds. We strongly suggest that the correlation of pLVPK-derived genetic loci with abscess formation be considered when interpreting epidemiological results. Although further elucidation of the molecular basis for the impact of the genes carried within pLVPK on the virulence of *K. pneumoniae* is needed, this study presents the first confirmation that genetic loci derived from virulence plasmids, other than a particular capsule type, are involved in pyogenic *K. pneumoniae* infections. Together with a thorough physical examination, our multiplex PCR assay for the detection of the *terW-iutA-rmpA-silS* loci may provide a useful risk-assessment tool to identify those infected patients who are more likely to develop tissue abscesses.

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