Low prevalence of rmpA and high tendency of rmpA mutation correspond to low virulence of extended spectrum β-lactamase-producing klebsiella pneumoniae isolates

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Invasive syndrome caused by Klebsiella pneumoniae (KP), including liver abscess, is mainly caused by community-acquired strains with characteristics of positive hypermucoviscosity (HV) phenotype and regulator of mucoid phenotype A (rmpA) and transcriptional activator (rmpA2) genes. Extended-spectrum β-lactamase-producing KP (ESBL-KP) is commonly nosocomial and rarely HV-positive. We aimed to explore the reasons of the rarer prevalence of HV phenotype, rmpA and rmpA2 as well as the virulence phenotype among the ESBL-KP isolates from clinical specimens than those non-ESBL isolates. The β-lactamase genes, rmpA, rmpA2 and genes for K capsule serotype of 440 KP isolates were analyzed. The virulence of the isolates was characterized by the mouse lethality experiments. The prevalence rates of HV phenotype (~50% vs. < 10%) as well as rmpA and rmpA2 genes (~50–60% vs. < 20–30%) were significantly higher in non-ESBL than in the ESBL group (p < 0.0001). Expression of HV phenotype in the rmpA-positive KP isolates was significantly rarer in the ESBL group than in non-ESBL group (33.3% vs. 91.9%, p < 0.0001). The frameshift mutations of rmpA and/or rmpA2 corresponded to negative HV phenotype of KP isolates that harbored the rmpA and/or rmpA2, resulting in variable mouse lethality (LD50, ~10^3 - >5 x 10^7 CFU). The mutation rates might significantly differ among KP isolates from various sources. Virulence was dependent on rmpA-related HV phenotype. In conclusion, ESBL-KP isolates were less hypermucoviscous and less virulent than non-ESBL KP isolates, mostly due to concurrently lower carriage and higher mutation rates of the rmpA and rmpA2 genes.

Introduction

Klebsiella pneumoniae (KP) can cause various infections, like septicemia, pneumonia, urinary tract infection, meningitis, and tissue abscesses.1,2 In Taiwan, primary liver abscess is mainly caused by KP with diverse virulence determinants, like K1 or K2 capsular serotype, hypermucoviscosity (HV) phenotype, as well as the plasmid-borne regulator of mucoid phenotype A (rmpA) and rmpA2 (a transcriptional activator) genes.1-7 The HV phenotype was correlated with high serum resistance of KP.5 Isolates with rmpA and HV phenotype were highly virulent in the mouse lethality test.7

We previously discovered the prevalence rates of HV phenotype and rmpA of 151 blood KP isolates were 38% and 48%, respectively.5 Among them, 52 (90%) of 58 HV-positive isolates possessed rmpA, whereas, 20 (21.5%) of 93 HV-negative isolates carried rmpA. That is, 52 (72%) of 72 rmpA-positive isolates expressed HV phenotype. The association between rmpA and HV phenotype of KP was highly significant (p < 0.0001), however, we could not exactly explain the reasons why some (28%) rmpA-positive isolates did not exhibit the HV phenotype.1

Besides, Fang et al found that the rmpA was universally present in all tested strains, including invasive and non-invasive isolates.3 Furthermore, Yeh et al. reported non-virulence of some rmpA-positive KP isolates from Singapore.5 In similar, Lee et al. found 18% of 56 HV-negative KP isolates carrying rmpA or rmpA2.8 From the above observation, it seems that some KP isolates did not fully express the function of the rmpA gene. Therefore, the relationship between the rmpA and rmpA2 genes and the exhibition of the HV phenotype of KP has remained a mystery.

Extended-spectrum β-lactamase-producing KP (ESBL-KP) is mainly nosocomial and has rarely been found to be associated with the HV phenotype. In Korea, ESBL-KP comprised 33.8% of hospital-acquired bacteremia and only 8.4% of community-acquired bacteremia.9 Of 435 patients with community-onset KP bacteremia, 33 (7.6%) were infected with ESBL producers.10
In Taiwan, ESBL is rarely identified in community-acquired KP infection and is not associated with primary KP liver abscess.\(^1\) In addition, Lee et al. reported a negative association of the HV phenotype with ESBL-KP isolates (OR 0.042, \(p = 0.003\)).\(^3\) In China, Li et al. found a significantly lower proportion of ESBL-KP in the HV-positive isolates than in the HV-negative isolates (17% vs. 56%, \(P = 0.0006\)). Li et al. noticed an increasing trend of ESBL-KP among the hypermucoviscous isolates over time.\(^12\) In a small series from Algiers, the virulence profiles of 54 KP isolates have been well elucidated.\(^13\) The study comprised 74.1% isolates with ESBL-KP phenotype with ESBL-KP isolates (OR 0.042, \(p = 0.003\)). In addition, Lee et al. reported a negative association of the HV phenotype with ESBL-KP isolates (OR 0.042, \(p = 0.003\)).\(^3\) In China, Li et al. found a significantly lower proportion of ESBL-KP in the HV-positive isolates than in the HV-negative isolates (17% vs. 56%, \(P = 0.0006\)). Li et al. noticed an increasing trend of ESBL-KP among the hypermucoviscous isolates over time.\(^12\) The prevalence of the HV-related virulence determinants between the ESBL-KP blood isolates (2007–2010) and 48 non-ESBL KP blood isolates (2010) was not statistically significant. Again, the HV phenotype of ESBL-KP blood isolates (2007–2010) was more common than ESBL-KP non-blood isolates (2004–2005), but became significantly less common than non-ESBL KP blood isolates (2004–2005 and 2010, respectively, Table 1).

Moreover, 105 community-acquired blood KP isolates extracted from our previous study during 2003–2004,\(^1\) which were almost non-ESBL isolates, showed similar prevalence of HV phenotype to 2004–2005 non-ESBL KP blood isolates (48.6% vs. 55%, \(p = 0.403\)), but were more HV-positive than ESBL-KP blood isolates obtained from 2007–2010 (48.6% vs. 22.3%, \(p < 0.0001\), Table 1).

The prevalence of rmpA and rmpA2 genes among isolates

The rmpA was significantly lower in ESBL-KP isolates than that in non-ESBL KP isolates (21.1% vs. 58.6%, \(p < 0.0001\), Table 2). Specifically, the rmpA-positive sputum isolates were more common in non-ESBL than ESBL group (\(p = 0.001\)). Similar to HV phenotype, the rmpA prevalence of ESBL-KP blood isolates (2007–2010) was significantly lower than the non-ESBL blood isolates (2004–2005 and 2010, respectively). Different to higher HV phenotype of blood isolates in ESBL group and rarer HV phenotype of urine isolates in non-ESBL group (Table 1), however, rmpA prevalence was not statistically different among various isolates within each group (Table 2). For further external validation, the rmpA prevalence of the community-acquired KP blood isolates (2003–2004)\(^1\) was similar to non-ESBL blood isolates (2004–2005) but was higher than that of the ESBL-KP blood isolates (2007–2010, Table 2).

The association of HV phenotype with rmpA and/or rmpA2

Overall, 96 of 226 KP isolates (2004–2005) were HV-positive. Of these, only 6 (KP331, KP 347, KP350, KP476, KP 495, KP511) did not harbor rmpA and/or rmpA2, as demonstrated by the inability to amplify the corresponding gene sequences by PCR. Meanwhile 100 of 130 HV-negative isolates did not harbor rmpA and/or rmpA2 (\(p < 0.0001\)). Similarly, 37 of 166 ESBK-KP blood isolates (2007–2010) were HV-positive. Of these, 10 did not harbor rmpA and/or rmpA2. Meanwhile 95 of 129 HV-negative isolates did not harbor rmpA and/or rmpA2 (\(p < 0.0001\)).

The prevalence of HV phenotype among rmpA-positive KP isolates

The HV prevalence of rmpA-positive KP isolates was significantly lower in the ESBL group than that in the non-ESBL group (33.3% vs. 91.9%, \(p < 0.0001\), Table 3). Specifically, the
rmpA- associated HV phenotype of sputum isolates was significantly rarer in the ESBL-KP group than non-ESBL group \( (p < 0.0001) \). No statistical difference of the HV prevalence was detected in the rmpA-positive ESBL-KP blood isolates between 2 periods (2004–2005 vs 2007–2010), but it was significantly higher in the non-ESBL groups (2004–2005 and 2010, respectively). Among the 99 rmpA-positive non-ESBL KP isolates, the HV prevalence of urine isolates was significantly lower than the sputum and blood isolates. Among the 12 rmpA-positive ESBL-KP isolates, the difference in the HV prevalence among various isolates was not statistically different. The blood isolates might have higher rate of HV phenotype, but did not reach statistical significance \( (p = 0.067) \).

**Genetic mutation in rmpA- and/or rmpA2-positive KP isolates**

DNA sequencing of the rmpA and rmpA2 amplicons revealed the indel mutations (insertion or deletion of nucleotides) at poly (G) tract within rmpA gene as well as rmpA2 in all non-HV isolates but with rmpA and/or rmpA2 (Table 4; Fig. 1 and 2). Due to the triplet nature of gene expression by codons, a genetic mutation caused by indels of a number of nucleotides in a DNA sequence that is not divisible by 3 will result in a frameshift mutation and thereby a completely different translation from the original reading frame after the mutation to code for different amino acids. In similar, the truncated mutation occurring after a stop codon (TAA) in a reference rmpA2 sequence AB289643 will result in a truncated protein product (Fig. 2).

The frameshift mutations of rmpA and/or rmpA2 occurred in the HV-negative KP isolates that were PCR-positive for rmpA and/or rmpA2 genes (Table 4). In particular, among the 57 ESBL-KP isolates (2004–2005), K1 capsule serotype was found in 5 isolates, including 2 isolates with rmpA and rmpA2, 1 strain with rmpA but negative for rmpA2, and 2 isolates without rmpA and rmpA2. All 5 K1 isolates were HV-negative and the rmpA and rmpA2 were all mutated.

For HV phenotype-positive KP isolates, 10 isolates were randomly selected for sequencing analysis and the rmpA2 had not been mutated, but the rmpA2 had been mutated.

**Genotypes and transference of ESBL genes**

The 57 ESBL-KP isolates (2004 -2005) were further analyzed for the ESBL genes (Table 5). The identified ESBL types were predominately SHV-5, and then SHV-12 and CTX-M-3. Only
The isolates with HV phenotype exhibited the highest virulence for mouse lethalities (LD50, $>10^7$ CFU). The isolates without HV phenotype and negative for 

**Table 6.** Difference in distribution of rmpA and rmpA2 between K. pneumoniae isolates with and without ESBLs

| Isolates (n) | rmpA-positive | rmpA2-positive | $p$ for rmpA between comparators |
|--------------|---------------|----------------|----------------------------------|
| 2004 – 2005 (226)  | n = 111       | n = 100        | $p$ for rmpA between 2004–2005 isolates |
| ESBL-KP (57)       | 12 (21.1%)    | 12 (21.1%)     | < 0.0001 (vs. non-ESBL KP) |
| Sputum (24)        | 6 (25.0%)     | 3              | 0.743 (vs. non-sputum ESBL-KP) |
| Urine (18)         | 2 (11.1%)     | 3              | 0.303 (vs. non-urine ESBL-KP) |
| Blood (10)         | 4 (40.0%)     | 5              | 0.193 (vs. non-blood ESBL-KP) |
| Others *(5)            | 0              | 1              |                                    |
| Non-ESBL KP (169)  | 99 (58.6%)    | 88 (52.1%)     | 0.001* (vs. sputum ESBL-KP) |
| Sputum (105)       | 65 (61.9%)    | 62             |                                    |
| Blood (39)         | 23 (59.0%)    | 18             | 0.311 (vs. blood ESBL-KP) |
| Urine (8)          | 4 (50.0%)     | 1              | 0.051 (vs. urine ESBL-KP) |
| Abscess pus (6)    | 5 (83.3%)     | 5              | 0.707 (vs blood non-ESBL KP) |
| Wound (3)          | 0              | 0              | 0.709 (vs sputum non-ESBL KP) |
| CVCb tip (2)       | 0              | 0              | 0.235 (vs. other non-ESBL KP) |
| Others (6)         | 2 (33.3%)     | 2              |                                    |
| 2007–2010          | 49 (29.5%)    | 12 (7.2%)      |                                    |
| Blood ESBL-KP (166)|               | 0.116 (vs. non-blood ESBL-KP) |
| 2010               | 29 (60.4%)    | 27 (56.3%)     | 0.491 (vs. blood ESBL-KP) |
| Blood non-ESBL KP (48)|            | < 0.0001* (vs. non-ESBL KP) |
| 2003–2004 (from 2 medical centers)* | 59 (56.2%) | Not tested | 0.0005* (vs. blood non-ESBL KP) |
| Blood community-acquired KP (105)* | | | 0.0001* (vs. non-ESBL KP) |

**Discussion**

In the present study, the prevalence of the HV phenotype was significantly lower in ESBL-KP isolates (8.8%) than that in non-ESBL KP isolates (53.8%). Specifically, the prevalence of HV phenotype in blood ESBL-KP isolates (20–30%) was lower than that in blood non-ESBL isolates (50–55%), but was higher than non-blood ESBL-KP isolates (4.2%). These findings might be applicable to the clinical scenario that ESBL-KP sputum and urine isolates are usually colonization. Whereas among
Table 3. Proportion of hypermucoviscosity (HV) phenotype among rmpA-positive K. pneumoniae isolates with and without ESBLs from (implying expression rate or normal function of rmpA)

| rmpA-positive Isolates (n) | HV-positive | HV-negative | ρ |
|---------------------------|-------------|-------------|---|
| 2004 – 2005 (111)         | n = 95      | n = 16      | p for HV between 2004–2005 isolates |
| ESBL-KP (12)              | 4 (33.3%)   | 8 (66.7%)   | < 0.0001* (vs. non-ESBL KP) |
| Sputum (6)                | 1 (16.7%)   | 5           | 0.546 (vs. non-sputum ESBL-KP) |
| Urine (2)                 | 0           | 2           | 0.515 (vs. non-urine ESBL-KP) |
| Blood (4)                 | 3 (75.0%)   | 1           | 0.067 (vs. non-blood ESBL-KP) |
| Non-ESBL KP (99)          | 91 (91.9%)  | 8 (8.1%)    | < 0.0001* (vs. sputum ESBL-KP) |
| Sputum (65)               | 62 (95.4%)  | 3           | 0.279 (vs. blood ESBL-KP) |
| Blood (23)                | 22 (95.7%)  | 1           | 1.000 (vs. urine ESBL-KP) |
| Urine (4)                 | 1 (25.0%)   | 3           | 0.522 (vs. other non-ESBL KP) |
| Abscess pus (5)           | 4 (80.0%)   | 1           | p for HV (vs. 2004–2005 isolates) |
| Others a (2)              | 2 (100.0%)  | 0           | 0.054 (vs. non-blood ESBL-KP) |
| 2007 - 2010               |             |             | 0.617 (vs. blood ESBL-KP) |
| Blood ESBL-KP (49)        | 26 (53.1%)  | 23 (46.9%)  | < 0.0001* (vs. non-ESBL KP) |
| 2010                      |             |             | 0.0003* (vs. blood non-ESBL KP) |
| Blood non-ESBL BP (29)    | 25 (86.2%)  | 4 (13.8%)   | p for HV (vs. 2007–2010 isolates) |
| 2003–2004 (from 2 medical centers)b | 51 (86.4%) | 8 (13.6%) | 0.231 (vs. blood non-ESBL KP) |

a: bile (n = 1) and pericardial effusion (n = 1).
b: data extracted from reference 1 (Yu 2006) for external validation; the community-acquired KP isolates were almost non-ESBL KP (personal opinion).
*p < 0.05.

non-ESBL KP isolates, the prevalence of HV phenotype of urine isolates (12.5%) was lower than that of the sputum (59%) and blood isolates. In clinical application, therefore, non-ESBL KP sputum isolates may be more pathogenic than ESBL-KP sputum isolates.

The prevalence of rmpA was significantly lower in ESBL-KP isolates (21.1%) than that in non-ESBL KP isolates (58.6%). This difference in rmpA prevalence was also validated in blood isolates between ESBL-KP and non-ESBL groups (or community-acquired isolates). However, the rmpA prevalence did not significantly differ among various clinical isolates either in ESBL-KP or in non-ESBL KP group, respectively. Yet, the HV phenotype was rarer in non-blood isolates among ESBL group and in urine isolates among non-ESBL group, respectively. These findings imply different rmpA expression rates leading to different HV prevalence among various clinical isolates.

In general for KP isolates, the absence of rmpA and rmpA2 (rmpA systems) correlated to negative HV phenotype. Nevertheless, it is out of context that some strains (6.3%) of HV-positive isolates were negative for rmpA systems, implying the presence of mucoid factors other than rmpA systems. This finding is similar to our previous report that 10% of HV-positive blood KP isolates did not harbor rmpA.1 On the other hand, the association between HV phenotype and rmpA was significantly lower in the ESBL-KP isolates than non-ESBL KP isolates (implying rmpA expression rate, 33.3% vs. 91.9%, p < 0.0001). We proposed rmpA mutation responsive for the lower HV exhibition rate.

Overall, 64 (24.7%) of 259 HV-negative KP isolates were positive for rmpA system. Of these, all had frameshift mutation of rmpA systems. On the contrary, rmpA mutation was not found in the randomly selected HV-positive KP isolates. A frameshift mutation is not the same as a single-nucleotide polymorphism in which a nucleotide is replaced, rather than inserted or deleted. The earlier in the sequence the indel occurs, the more altered the protein. In general, the most common indels of rmpA mutants occurred at positions between 280–300 base areas (Fig. 1), about halfway on the rmpA complete codes (633 bases, GenBank accession number AB289642), indicating substantial alternations to the RmpA protein synthesis and leading to non-HV phenotype.

Therefore, the reasons of ESBL-KP isolates with low prevalence of HV phenotype were not only due to low prevalence of carrying rmpA systems, but also due to high prevalence of frameshift mutation in the rmpA systems. It could be speculated that ESBL-KP strains reduce genetic function of rmpA systems when they need to amplify ESBL genes under the antibiotic selective pressure. The mutation theory could also explain the discrepancy of HV phenotype exhibition in sputum isolates between ESBL-KP and non-ESBL KP (implying rmpA expression rate, 16.7% vs. 95.4%. p < 0.0001).
In fact, the spontaneous mutation of *rmpA* systems might not only be fortuitous. Lai et al. have found that some KP isolates produced frameshift mutation of *rmpA2*.\(^{14}\) In addition, Cheng et al. also conceived an idea that DNA slip-strand synthesis in *rmpA* or *rmpA2* may cause mutation and result in abnormal function of the 2 regulatory genes.\(^{15}\) Together with our current results support our hypothesis that mutation of *rmpA* systems contributes to the loss of the HV phenotype in the KP isolates positive for *rmp*\(\text{A}\) systems.

In general view of the current study, ESBL-KP or non-ESBL KP isolates positive for both *rmpA* and HV phenotype corresponded to high virulence to mouse lethality. In contrast, non-HV isolates with negative *rmpA* systems exhibited low virulence to lethality. Unexpectedly, a rare instance of HV-positive isolate without *rmpA* systems exhibited low virulence (eg., KP331, Table 6), indicating that virulence is dependent on *rmpA* systems-related HV phenotype rather than other mucoid factors-mediated HV phenotype.

The HV phenotype with high serum resistance has been recognized as a virulence factor for KP.\(^{1,2}\) Besides, the role of *rmpA* system in the HV-associated pathogenesis have previously been studied. However, different methods of the laboratory-constructed deletion of *rmpA* and/or A2 sometimes resulted in conflicting and confusing discrepancies, while interpreting the capability of the mutant strains in expressing the capsular polysaccharide production, hypermucoviscosity phenotype and virulence.\(^{4,14-17}\) For examples, Cheng et al. demonstrated *rmpA* as a major mucoid factor because introducing *rmpA*-carrying plasmids in to the *rmpA* or *rmpA2* mutants restored capsular polysaccharide production.\(^{15}\) Hsu et al. demonstrated that plasmid-borne *rmpA* enhanced capsular polysaccharide synthesis, capsule production and mucoviscosity.\(^{16}\) But the virulence of the *rmpA* mutant was not significantly reduced, with a LD\(_{50}\) of < 1 \(\times\) 10\(^2\) CFU.\(^{16}\) Thus other factors like capsular types than *rmpA*...
per se might play some roles in virulence. However, in our current study, even for isolates with the most virulent K1 or K2 capsular type, concurrent mutation of rmpA and rmpA2 could lose the HV phenotype and then reduce virulence, with a LD50 of >5 × 10⁶ CFU. We thought that the laboratory-constructed rmpA model might not fit to the true scenario of natural mutation in rmpA system.

In similar to the reports by Hsu et al. and our previous study, the LD50 of some HV-negative strains but with mutated rmpA systems ranged approximately between 10⁹ to 10⁸ CFU (eg., KP309, Table 6). It could be speculated that other virulence determinants on the same rmpA-encoded plasmid partly contributed to the virulence. The other virulence determinants than rmpA systems might be associated with the iron acquisition system, like aerobactin and kfu genes. A high proportion of ESBL-KP or multidrug-resistant KP isolates were found with virulence factors like adhesins, siderophores, serum resistance, and hemolysin. On the other hand, the LD50 of some HV-negative KP isolates with mutated rmpA systems were >5 × 10⁷ (eg., KP291 and KP420, Table 6). It could be suggestive of absence of other co-existing virulence determinants on the same plasmid.

Only 3 HV-positive isolates harboring blaSHV-5 were selected for conjugation experiments and the blaSHV-5 and rmpA should be located on different plasmids, based on the different transfer-ence ability of their harboring plasmids, which was evidenced by Southern blotting and PCR analysis for rmpA (non-transferable) and blaSHV-5 (self-transferable) in the parent isolates and their transconjugants.

**Conclusion**

To our best knowledge, this is the first large-scale report that provided the in vivo evidence for the naturally-occurring rmpA and/or rmpA2 frameshift mutation in the HV-negative KP isolates that harbor rmpA and/or rmpA2 genes. Overall, ESBL genes and rmpA were located on different plasmids. Compared to non-ESBL KP isolates, the ESBL-KP isolates had generally lower prevalence of HV phenotype, lower carriage rate of rmpA, lower expression rate of rmpA, and higher mutation rate of rmpA. Frameshift mutation of rmpA corresponded to diminished exhibition of HV phenotype among KP isolates. Virulence of either ESBL-KP or non-ESBL KP to mouse lethality is dependent on rmpA-related HV phenotype. The limitation of the study included small numbers of urine isolates overall.

**Materials and Methods**

**Clinical Isolates and Microbiological assays**

The KP was identified by Phoenix system (Becton Dickinson Company, Baltimore, MD, USA) and API 20E system (bioMerieux, Marcy l’Etoile, France). The hypermucoviscosity phenotype was defined positive as a viscous string of >5 mm of the colony on trypticase soy agar plate with 5% sheep blood (BD Diagnostics, MD, USA). ESBL production was screened and confirmed by the double disc test using cefotaxime and ceftazidime along with an amoxicillin-clavulanate disc, in accordance with Clinical and Laboratory Standards Institute (CLSI) standards. Quality control was performed by testing Escherichia coli American Type Culture Collection (ATCC) 25922, K. pneumoniae ATCC 700603 and Pseudomonas aeruginosa ATCC 27853.

**DNA manipulation, PCR amplification and sequencing**

The genome of NTUH-K2044 (http://genome.nhri.org.tw/ KP) carries 3 different copies of rmpA: 2 (open reading frames [ORFs] KPP020 and KPP302) are on the 224-kb large plasmid pK2044, and the other (ORF KP3619) is on the chromosome. In fact, the ORFs KPP020 and KPP302 were referred to rmpA and rmpA2, respectively, in previously published literature. Since the plasmid-borne rmpA copies (rmpA and rmpA2) have been well-known important virulent determinants, we focused on the analysis of rmpA.
(ORF KPP020) and rmpA2 (ORF KPP302) in the current study.

Genomic DNA was extracted using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) for screening the genes for K capsule serotype and plasmid DNA was also extracted by QIAprep Spin Miniprep (Qiagen, Hilden, Germany) for detection of rmpA (ORF KPP020) and rmpA2 genes (ORF KPP302). GoTaq® DNA Polymerase (Promega Corporation, Madison, WI, USA) containing bacterially derived Tag DNA polymerase, dNTPs and MgCl₂ in 2X Green GoTaq® Reaction Buffer (pH 8.5) was used for amplification of DNA templates by PCR.

The rmpA, rmpA2 and genes for K capsule genotypes were identified by PCR using specific primers as previous described and PCR products were confirmed by DNA sequencing. The primers for rmpA (forward, 5'-TAC ATA TGA AGG AGT AGT TAA T-3'; and reverse, 5'-GAG CCA TCT TTC ATG CTT CA-3') as well as forward, 5'-ACT GGG CTA CCT TTC ATG CTT CA-3'; and reverse, 5'-CTT GCA TGA GCC ATC TTT CA-3' were used to amplify rmpA (ORF KPP020) gene. The rmpA2-specific primers were: forward, 5'-TGT GCA ATA AGG ATG TTA CAT TAG T-3'; and reverse, 5'-TTT GAT GTG CAC CAT TTT TCA-3'. The primers for magA (forward, 5'-GGT GCT CTT TAC ATC ATT GC-3'; and reverse, 5'-GCA ATG GCC ATT TGC GTT AG-3') was used to amplify wezKpK1, a capsule serotype K1-antigen–specific polymerase. The primers for k2A (forward, 5'-CAA CCA TGG TGG TCG ATT AG-3'; and reverse, 5'-TGG TAG CCA TAT CCC TTT GG-3) was used specifically to identify isolates with a capsule serotype K2.

The rmpA and rmpA2 amplicons were recovered for further sequencing. All amplicons were purified with PCR clean up kits (Roche Diagnostics, GmbH, Penzberg, Germany) and were ligated into pGEM-T-Easy vector (Promega Co., Madison, Wisconsin, USA) for further sequencing on an ABI PRISM 3730 sequencer analyzer (Applied Biosystems, Foster City, CA, USA). Sequence analyses were performed online at the Basic Local Alignment Search Tool (BLAST) website. Multiple sequences alignment analysis was conducted by using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

PCR amplification for β-lactamase genes
Plasmid DNA was extracted as templates, PCR was used to amplify blaTEM, blaSHV, blaCTX-M and blaKPC using specific primers as previously published as follows: The blaTEM-specific primers were: TEM-forward (5'-ATAAAATCTTGGAAGACGAAA-3')

Table 5. Distribution of ESBL types and virulence factors of the 57 ESBL-KP isolates from 2004 to 2005

| ESBL type                  | Number (n = 57) | HV (n = 5) | rmpA (n = 12) | rmpA2 (n = 12) | K1 capsule (n = 5) | K2 capsule (n = 1) |
|----------------------------|----------------|------------|---------------|---------------|-------------------|-------------------|
| TEM-like                   | 2              | 0          | 0             | 0             | 0                 | 0                 |
| SHV-2a                     | 2              | 0          | 0             | 0             | 0                 | 0                 |
| SHV-2a + CTX-M-3 + TEM-1   | 1              | 0          | 0             | 0             | 0                 | 0                 |
| SHV-5 + CTX-M-3 + TEM-1    | 1              | 0          | 0             | 0             | 0                 | 0                 |
| SHV-5 + CTX-M-15 + TEM-1   | 1              | 0          | 0             | 0             | 0                 | 0                 |
| SHV-12                     | 7              | 0          | 2             | 3             | 0                 | 0                 |
| SHV-12 + CTX-M-15          | 1              | 0          | 0             | 0             | 0                 | 0                 |
| CTX-M-3 + SHV-11           | 1              | 0          | 0             | 0             | 0                 | 0                 |
| CTX-M-3                     | 3              | 0          | 1             | 0             | 0                 | 0                 |
| CTX-M-3 + TEM-1            | 1              | 0          | 0             | 0             | 0                 | 0                 |
| CTX-M-15 + SHV-11          | 1              | 0          | 0             | 0             | 0                 | 0                 |
| Undetermined               | 5              | 0          | 0             | 0             | 2                 | 0                 |

*HV: hypermucoviscosity phenotype.
Conjugation experiments, plasmid DNA analysis and Southern hybridization

Conjugation of plasmid was performed by using the sodium azide-resistant *E. coli* J53–2 as the recipient. The transconjugants were selected on Luria-Bertani agar plates supplemented by sodium azide (100 μg/mL) and cefotaxime (2 μg/mL). Plasmids in the transconjugants were extracted by QIAprep Spin Miniprep (Qiagen, Hilden, Germany) and then digested with restriction enzyme BamHI or Hind III (New England BioLabs, Ipswich, MA, USA), respectively. Restriction fragment length polymorphisms (RFLP) analysis was carried out by electrophoresis at 30 V for 2 h in 1% agarose gel. Moreover, Southern hybridization was carried out with a digoxigenin (DIG)-labeled probes for *bla*SHV and *rmpA* genes using a DIG system.

**Mouse lethality assay**

Determination of the virulence of KP in mouse lethality tests and the medium lethal dose (LD$_{50}$, expressed as colony-forming units) was performed as previously described.$^7$ In brief, a graded dose of 10$^2$ to 10$^7$ CFU of each strain in 10-fold serial dilutions in 0.1 mL of normal saline was injected intraperitoneally into mice (4 mice for each dose of inoculum). The number of survivors was monitored and calculated every day during 14 d. Twelve KP strains (4 for capsule serotypes K1, 3 for K2 and 5 for non-K1/K2) were selected for the experiments, including 6 ESBL isolates (3 with HV and 3 without HV phenotype) and 6 non-ESBL isolates (3 with HV and 3 without HV phenotype) for comparison.

**Ethical approval**

All female BALB/c mice (6 to 7 weeks old; weighing 20–25g) were purchased from the animal breeding center, National Laboratory Animal Center (NLAC, Taiwan) and maintained under standard conditions of temperature, light and feeding according
Table 6. The influence of rmpA and/or rmpA2 mutation on the virulence (LD_{50}) of KP isolates with various microbiobiologic characteristics

| Strain | Source | Capsule | ESBL | HV | PCR for rmpA | PCR for rmpA2 | Mutation of LD_{50} |
|--------|--------|---------|------|----|-------------|--------------|-------------------|
| KP379  | blood  | K2      | -    | +  | normal      | normal       | 1.8 x 10^2        |
| KP309  | urine  | K2      | -    | +  | normal      | normal       | 2.1 x 10^3        |
| KP291  | urine  | K2      | -    | +  | mutated     | mutated      | >5 x 10^7         |
| KP449  | sputum | K1      | -    | +  | normal      | mutated      | 8.1 x 10^2        |
| KP420  | sputum | K1      | SHV-5| +  | mutated     | mutated      | >5 x 10^7         |
| KP537  | urine  | K1      | SHV-5| +  | mutated     | mutated      | >5 x 10^7         |
| KP277  | urine  | Non-K1/K2| SHV-5| +  | mutated     | mutated      | >5 x 10^7         |
| KP491  | blood  | Non-K1/K2|     |    | mutated     | mutated      | 59.9             |
| KP493  | sputum | Non-K1/K2|     |    | normal      | mutated      | 1.2 x 10^2        |
| KP290  | blood  | Non-K1/K2| SHV-5| +  | normal      | normal       | 3.2 x 10^3        |
| KP570  | blood  | K1      | SHV-12| +  | normal      | normal       | >5 x 10^7         |
| KP331  | Urine  | Non-K1/K2|     |    | normal      | normal       | >5 x 10^7         |

Note: +: positive; --: negative.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Statistical analysis
The Chi-square or Fisher’s exact test was used in the analysis of categorical variables. A two-tailed p value < 0.05 was considered statistically significant. All statistics were performed using Stata version 12.1 (Stata Press, College Station, TX, USA).

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