**Klebsiella pneumoniae** Isolates from Meningitis: Epidemiology, Virulence and Antibiotic Resistance

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*Klebsiella pneumoniae* (KP) resistance to broad-spectrum cephalosporin (BSC) in meningitis is important because of limited therapeutic options. To investigate the antibiotic resistance, virulence and epidemiology of KP in meningitis, we conducted a retrospective study for 33 non-metastatic isolates, including primary meningitis (n = 20) and post-craniotomy meningitis (n = 13) collected from 1999 to 2013. BSC resistance was found in 9 (27.3%) isolates, all from post-craniotomy meningitis, harboring *bla*<sub>TEM</sub>-2, *bla*<sub>CXM</sub>-2, *bla*<sub>SHV</sub>-2, *bla*<sub>TEM</sub>-1B, *bla*<sub>CMY</sub>-2, *bla*<sub>CMY</sub>-1, *bla*<sub>DHA</sub>-1, *ampC* (n = 2), and *bla*<sub>IMP</sub>-LA (n = 1). Positive virulence factors were hypermucoviscosity (n = 22), larger bacterial size (n = 24), virulent capsule serotypes (n = 24, K2, 11, K1, 5; K57, 3; K5, 2; K20, 2 and K54, 1), *rmpA* (n = 23), *rmpA*<sup>+</sup> (n = 20), aerobactin gene (n = 22) and high-grade serum resistance (n = 23, 69.7%). Higher mouse lethality (LD<sub>50</sub> < 10<sup>6</sup>) was found in 16 isolates (48.5%). Post-craniotomy isolates were significantly less virulent than primary meningitis isolates, except for similar serum resistance capability. The pulsotype and sequence typing (ST) results were diverse. A minor cluster with pulsotype C and ST23 (n = 5) was identified in primary meningitis isolates. In conclusion, virulence factors and BSC resistance corresponded to about 70% and 30% of KP meningitis isolates respectively. BSC remains appropriate for treating primary meningitis, whereas meropenem is indicated for post-craniotomy meningitis empirically.

*Klebsiella pneumoniae* causes different types of community-acquired and healthcare-associated infections, including pneumonia, bloodstream infection, surgical site infections, liver abscess and meningitis. The clinical spectrum of *K. pneumoniae* meningitis can be categorized into 3 distinct forms: first, metastatic meningitis, particularly from the distant liver abscesses; second, post-craniotomy meningitis following neurosurgical procedures for brain lesions or head injury; and third, primary or spontaneous meningitis, usually among elderly patients or those with underlying immunocompromised conditions. In Taiwan, *K. pneumoniae* has remained the leading causative pathogen with prevalence rates about 25% to 40% among adult patients with bacterial meningitis. Besides, *K. pneumoniae* was responsible for 50% of severe meningitis in adult patients who required intensive care and up to 68% among a subpopulation of adult diabetic patients, with high mortality rates ranging from 48.5% to 66%.

The pathogenesis of *K. pneumoniae* for being capable of invasion into the central nervous system is not well elucidated. The meningeval metastatic isolates frequently arising from the liver abscess have been recognized as “hypervirulent” *K. pneumoniae* (hvKP), which is commonly hypermucoviscous on blood agar plates. Distinct from hvKP, less virulent “classic” *K. pneumoniae* (cKP) that does not exhibit the hypermucoviscosity (HV) phenotype usually causes various infections in hospitals. We previously reported that the *rmpA* (regulator of mucoid phenotype) gene was highly prevalent among both primary meningitis and liver abscess isolates. Nonetheless, the detailed profiles of virulence, virulence determinant factors and relationship with antibiotic resistance of *K. pneumoniae* from non-metastatic meningitis isolates have not been fully characterized. We hypothesized that...
post-craniotomy meningitis could be caused by cKP through disruption of the central nervous system barrier, whereas primary meningitis might commonly be caused by hvKP.

Therefore, we aimed to investigate the molecular epidemiology and representative virulence factors in terms of capsule sizes, capsule serotypes, HV phenotype, serum resistance, pathogenic genes, including plasmid-borne *rmpA*, *rmpA*2, aerobactin gene and chromosomal *rmpA* (c-*rmpA*), *kfu* (responsible for an iron uptake system) and *allS* (associated with allantoin metabolism), as well as mouse lethality capability for *K. pneumoniae* strains isolated from cerebrospinal fluid (CSF) among adult patients without liver or distant abscesses. Antibiotic resistance profiles and virulence factors between non-metastatic *K. pneumoniae* isolates of primary meningitis and post-craniotomy meningitis were compared.

**Materials and Methods**

**Bacterial isolates.** We previously collected 33 *K. pneumoniae* isolates from CSF of non-metastatic meningitis patients without liver or distant abscess, including isolates from Taipei Veterans General Hospital in Taipei City, northern Taiwan, 2003–2006 (*n* = 7); National Cheng-Kung University Hospital in Tainan City, southern Taiwan, 1999–2007 (*n* = 10); and Chi-Mei Medical Center in Tainan City, southern Taiwan, 2005–2013 (*n* = 16). The isolates included primary meningitis (*n* = 20) and post-craniotomy meningitis (*n* = 13). These isolates were obtained as part of routine care on clinical indication.

**Antimicrobial susceptibility testing.** The minimum inhibitory concentrations (MICs) of antimicrobial agents were determined by using the agar dilution method16. The tested compounds included ampicillin, aztreonam, cefotaxime, ceftazidime, meropenem, ciprofloxacin, colistin sulfate (Sigma Chemical Company, St. Louis, MO, USA) and tigecycline (Wyeth, Puerto Rico).

According to the British Society for Antimicrobial Chemotherapy Standing Committee for Antimicrobial Susceptibility Testing (Version 14.0, 2015), the susceptible MIC breakpoint for colistin against Enterobacteriaceae is ≤2 μg/mL and it is considered resistant if MIC > 2 μg/mL. The breakpoints for tigecycline against Enterobacteriaceae are ≤1 μg/mL as susceptible and >2 μg/mL as resistant.

**Phenotypic detection of extended-spectrum β-lactamase (ESBL).** ESBL double-disk synergy test was performed with disks containing ceftazidime or cefepime with clavulanic acid on Mueller-Hinton agar plates, and the results were interpreted as described previously17. Control experiments were assured by testing *E. coli* ATCC 25922 and *K. pneumoniae* 700603.

**DNA manipulation and PCR amplification.** Genomic DNA was extracted using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). Plasmid DNA was also extracted using QIaprep Spin Miniprep (Qiagen, Hilden, Germany). PCR amplifications were performed by using specific primers as previously described.

**PCR detection and sequencing of antibiotic resistance genes.** Plasmid DNA as templates, PCR was used to amplify the ESBL genes (*bla*TEM, *bla*SHV, *bla*CTX-M), *ampC* genes (*bla*DHA-1, and *bla*CMY-2) and to screen the representative carbapenemase gene (*bla*KPC-2) using specific primers as previously published18. Amplicons of β-lactamase genes were purified with PCR clean-up kits (Roche Diagnostics, GmbH, Penzberg, Germany) and were sequenced on an ABI PRISM 3730 sequencer analyzer (Applied Biosystems, Foster City, CA, USA).

**PCR detection of virulence factors.** Genomic DNA as templates, PCR was used to amplify the K capsule serotype-specific genes (including 6 liver abscess-associated capsule serotypes K1, K2, K5, K20, K54, and K57), *c-rmpA*, *kfu* and *allS* genes. Plasmid DNA as templates, PCR was used to amplify the *rmpA*, *rmpA*2 and aerobactin gene. Specific primers were used as previously described19–21.

**Phenotypic detection of virulence factors with HV phenotype and capsule size.** The HV phenotype was defined positive as a viscous string of bacteria. Grades 1 and 2 were regarded as low virulent, whereas primary meningitis might commonly be caused by hvKP.

**Phenotypic detection of virulence factor with serum resistance.** The susceptibility of the *K. pneumoniae* isolates to human serum was analyzed as described previously22. In brief, twenty-five microliters of the bacterial suspension (about 2 × 10⁸ CFU) were mixed with 75 μL of pooled normal human serum in microtiter plates and then incubated at 37 °C for a period of 3 h. The test was performed in triplicate and the number of recovered bacteria was determined and graded. Resistance grading was defined from grade 1 to grade 6, with grade 6 (viable counts at 1, 2 and 3 h >100% and increasing throughout the 3-h period) considered to be the most serum resistant. Grades 5 and 6 were regarded as high-grade serum resistance.

**Mouse lethality experiment.** Determination of the virulence of *K. pneumoniae* in mouse lethality tests and the median lethal dose (LD₅₀) was performed as described previously23. In brief, female BALB/c mice (6–7 weeks old) were obtained from the National Laboratory Animal Center (NLAC) (Tainan, Taiwan). Mice were maintained under standard conditions of temperature, light and feeding according to NLAC guidelines and the Chi Mei Medical Center Animal Care and Use Committee approved-protocols (Permit Number: 100120771). Each dose was injected intraperitoneally with 0.1 mL of bacterial suspension into four mice. After 14 days, calculations were based on the number of survivors. The degree of virulence was read as highly virulent for an LD₅₀ of <10⁸ CFU; moderate virulence for an LD₅₀ of 10⁴–10⁵ CFU; low virulence for an LD₅₀ of 10⁶–10⁷ CFU; and no virulence for an LD₅₀ of >10⁸ CFU.
Molecular genotyping of isolates
Pulsed-field gel electrophoresis (PFGE). PFGE, as a major strain typing method, was used to confirm genetic relatedness of isolates as described by Pfaller et al. Whole chromosomal DNA in agarose was digested with XbaI (Bio-Rad Laboratories, CA, USA), and the restriction fragments were separated in a CHEF Mapper XA System (Bio-Rad Laboratories, CA, USA). Cluster analysis was performed by the Dice similarity coefficients and unweighted-pair group matching algorithm (UPGMA) with a tolerance of 1.0% and 1.0% optimization using the BioNumerics program. All bands had to match exactly to classify isolates as indistinguishable. Isolates were designated nontypeable if repeated attempts to prepare DNA failed.

Multilocus sequence typing (MLST). MLST was performed with 7 housekeeping genes as previously described. Multiple sequences alignment analysis was conducted by using Institute Pasteur (http://bigdb.web.pasteur.fr/klebsiella/klebsiella.html).

Data collection. The imaging studies and following main data relevant to patient characteristics were collected: gender, age, comorbidities, use of ventilator, CSF profiles, and random blood sugar obtained on the day of CSF collection. Outcome was described as in-hospital mortality within the same hospital episode.

Statistical Analysis. The Fisher’s exact test was used for categorical variables and Student's t-test was used for continuous variables, where appropriate. 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).

Ethical approval. The study and waiver from the informed consent process were approved by the Institutional Review Board (IRB) of the Chi Mei Medical Center, Tainan city, Taiwan (IRB Serial number 10603-013).

Results
MIC. All 20 primary meningitis isolates were susceptible to cefotaxime and ceftazidime. Nine of 13 post-cranioectomy meningitis isolates were resistant to cefotaxime and ceftazidime, but were all susceptible to meropenem, colistin and tigecycline. These 9 isolates were of multi-resistant phenotype. In contrast, all the 20 primary meningitis isolates were only resistant to ampicillin (Table 1).

ESBL and AmpC phenotypes. Resistance to cefotaxime and ceftazidime was found in 9 isolates. Among them, ESBL phenotype was detected in 7 isolates and AmpC phenotype was suspicious for 2 isolates (KP-004 and KP-042). ESBL and AmpC β-lactamase genes. PCR amplifications for β-lactamase genes were performed for all 33 isolates. ESBL genes were detected in 7 isolates (6 with bladSHV-5 and 1 with bladTEM-1β). AmpC genes were detected in 4 isolates (2 with blaCMY-2, and 2 with bladOXA-10), including 2 isolates with dual ESBL and AmpC genes (1 with bladOXA-10 plus bladSHV-5 and 1 with bladTEM-1β plus bladCMY-2). KPC-2 gene was not identified. Overall, 9 of 13 (69.2%) post-cranioectomy meningitis isolates produced ESBL and/or AmpC β-lactamases (Table 2).

Hypermucoviscosity (HV) phenotype. The prevalence of HV phenotype was 66.7% (22/33) of K. pneumoniae CSF isolates in general, and was specifically in the subgroups of the isolates as follows: (1), 95.0% (19/20) of primary meningitis isolates; (2), 23.1% (3/13) of post-cranioectomy isolates and (3), 11.1% (1/9) of ESBL/AmpC-producing isolates (Table 2). Primary meningitis isolates were significantly more hypermucoviscous than post-cranioectomy meningitis isolates (p < 0.0001), in accordance with our hypothesis that hvKP was the main etiology of primary KP meningitis, while cKP was mainly responsible for post-cranioectomy meningitis (Table 3). The cKP group has higher prevalence of ESBL/AmpC production than hvKP group (9/11 vs. 0/22, p < 0.0001, Table 2).

Bacterial (capsule) size. By measuring the short transverse diameter, mean bacterial size of each bacterium ranged from 0.92 ± 0.16 μm to 10.73 ± 1.11 μm. All 20 isolates recovered from primary meningitis had mean bacterial sizes of more than 2 μm. All nine strains with a mean bacterial size of < 2 μm were post-cranioectomy meningitis isolates, which were significantly different to primary meningitis isolates (p < 0.0001). In addition, only two of the nine isolates with ESBL/AmpC β-lactamase production had a mean bacterial size more than 2 μm (Tables 2 and 3).

The HV phenotype was highly prevalent in the isolates with mean bacterial size of > 2 μm (91.7%, 22/24), whereas none of the nine isolates with mean capsule size of < 2 mm had the HV phenotype (p < 0.0001).

Capsule K genotypes. The common liver abscess-associated capsule serotypes were identified in 24 (72.7%) isolates by PCR methods, including capsule K2 (11 strains), K1 (5 strains), K57 (3 strains), 2 strains each for K5 and K20, and one K54 respectively. These virulent K serotypes were highly prevalent in primary meningitis isolates than in post-cranioectomy isolates (19/20 vs. 5/13, p = 0.0007). However, specific K serotype (such as K1 or K2) was not statistically different between the two groups (Table 3).

Meanwhile, the isolates with virulent K serotypes had higher prevalence of > 2 μm bacterial size (23/24 vs. 1/9, p < 0.0001), higher proportion of HV phenotype (22/24 vs 0/9, p < 0.0001), and lower prevalence of ESBL/AmpC production (2/24 vs. 7/9, p = 0.0003) than those with undefined capsule K serotypes (Table 2).

Prevalence of virulence-association genes. Among the isolates of K. pneumoniae recovered from CSF, the prevalence of rmpA was 69.7% (23/33); rmpA2, 60.6% (20/33); c-rmpA2, 6.1% (2/33); kfu, 30.3% (10/33); alls, 15.6% (5/33); and aerobactin gene, 66.7% (22/33). In general, the rmpA, rmpA2 and aerobactin gene were highly prevalent in primary meningitis isolate than in post-cranioectomy isolates (Table 3).
The HV phenotype was highly prevalent in those with rmpA-positive vs. rmpA-negative isolates (22/23 vs. 0/10, p < 0.0001), with rmpA2-positive vs. rmpA2-negative isolates (19/20 vs. 3/13, p < 0.0001) and with aerobactin-positive vs. aerobactin-negative isolates (21/22 vs. 1/11, p < 0.0001). In similar, the isolates positive for rmpA, rmpA2 and aerobactin gene had larger bacterial size (≥2 μm) and were highly prevalent in the isolates with virulent K serotypes (such as K1, K2, K5, K20, K54 and K57).

Serum resistance. In general, the distribution of isolates in the grading of serum resistance were 23 strains in grades 5 and 6; grade 4 (1 strain), grade 3 (3 strains); and grade 2 (6 strains) (Table 3). Unexpectedly, the prevalence of high serum resistance (grades 5 and 6) could not reach statistically significant difference between primary meningitis isolates and post-craniotomy isolates (16/20 vs. 7/13, p = 0.1393), which was distinct from the above-mentioned virulence factors between groups (Table 3).

However, the 23 isolates with high serum resistance (grades 5–6) were still significantly associated with virulence K serotypes (n = 20, p = 0.0105), HV phenotype (n = 18, p = 0.0494), rmpA (n = 19, p = 0.035), rmpA2

### Table 1. MIC profiles of cerebrospinal fluid Klebsiella pneumoniae isolates from post-craniotomy and primary meningitis.

| MIC (µg/mL) of antibiotic compounds | Post-craniotomy meningitis (n = 13) | Primary meningitis (n = 20) | Total (n = 33) |
|---|---|---|---|
| Ampicillin | >16 | >16 | >16 |
| MIC<sub>50</sub> | >16 | >16 | >16 |
| MIC<sub>90</sub> | >16 | >16 | >16 |
| MIC range | >16 | >16 | >16 |
| Aztreonam | >16 | ≤1 | ≤1 |
| MIC<sub>50</sub> | >16 | ≤1 | ≤1 |
| MIC<sub>90</sub> | ≤1–>16 | ≤1 | ≤1–>16 |
| MIC range | ≤1–>16 | ≤1 | ≤1–>16 |
| Cefotaxime | >32 | ≤1 | ≤1 |
| MIC<sub>50</sub> | >32 | ≤1 | ≤1 |
| MIC<sub>90</sub> | ≤1–>32 | ≤1–2 | ≤1–>32 |
| MIC range | ≤1–>32 | ≤1–2 | ≤1–>32 |
| Ceftazidime | >16 | ≤1 | ≤1 |
| MIC<sub>50</sub> | >16 | ≤1 | ≤1 |
| MIC<sub>90</sub> | ≤1–>16 | ≤1 | ≤1–>16 |
| MIC range | ≤1–>16 | ≤1 | ≤1–>16 |
| Cefepime | 2 | ≤1 | ≤1 |
| MIC<sub>50</sub> | 2 | ≤1 | ≤1 |
| MIC<sub>90</sub> | 16 | ≤1 | 8 |
| MIC range | ≤1–>16 | ≤1 | ≤1–>16 |
| Piperacillin-tazobactam | >64 | ≤4 | ≤4 |
| MIC<sub>50</sub> | >64 | ≤4 | ≤4 |
| MIC<sub>90</sub> | 8 | >64 | >64 |
| MIC range | ≤4–>64 | ≤4–16 | ≤4–>64 |
| Meropenem | ≤0.25 | ≤0.25 | ≤0.25 |
| MIC<sub>50</sub> | ≤0.25 | ≤0.25 | ≤0.25 |
| MIC<sub>90</sub> | ≤0.25 | ≤0.25 | ≤0.25 |
| MIC range | ≤0.25 | ≤0.25 | ≤0.25 |
| Ciprofloxacin | 2 | ≤0.06 | ≤0.06 |
| MIC<sub>50</sub> | 2 | ≤0.06 | ≤0.06 |
| MIC<sub>90</sub> | >2 | 0.12 | 2 |
| MIC range | ≤0.06–>2 | ≤0.06–0.25 | ≤0.06–>2 |
| Colistin | 1 | 1 | 1 |
| MIC<sub>50</sub> | 2 | 1 | 1 |
| MIC<sub>90</sub> | ≤0.5–2 | ≤0.5–1 | ≤0.5–2 |
| MIC range | ≤0.5–2 | ≤0.5–1 | ≤0.5–2 |
| Tigecycline | 0.5 | 0.5 | 0.5 |
| MIC<sub>50</sub> | 1 | 1 | 1 |
| MIC<sub>90</sub> | ≤0.25–2 | ≤0.25–2 | ≤0.25–2 |
| MIC range | ≤0.25–2 | ≤0.25–2 | ≤0.25–2 |
Table 2. Epidemiology and virulence profiles of Klebsiella pneumoniae isolates from CSF of post-craniotomy and primary meningitis. NOTE: CSF, cerebrospinal fluid; PFGE, Pulsed-field gel electrophoresis; MLST, multilocus sequence typing (*one nucleotide change); SR, serum resistance (G grade); ESBL, extended-spectrum β-lactamase; HV, hypermucoviscosity phenotype. x, unidentified, non-virulent capsule K types (i.e., not for K1, K2, K5, K20, K54, K57 types). *From Taipei Veterans General Hospital in Taipei City, northern Taiwan, 2003–2006 (n = 7); †from Chi-Mei Medical Center in Tainan City, southern Taiwan, 2005–2013 (n = 16); ‡from National Cheng-Kung University Hospital in Tainan City, southern Taiwan, 1999–2007 (n = 10).

| CSF Strains | PFGE | MLST | ESBL | AmpC | Virulence factors | Bacterial size (μm) | HV (mm) | Capsule | rmpA | rmpA2 | c-rmpA | Kfu | allS | aerobactin | SR | LD<sub>50</sub> (log) |
|-------------|------|------|------|------|-------------------|-------------------|--------|---------|------|------|------|-----|------|-----------|-----|----------------|
| Primary meningitis (n = 13) | | | | | | | | | | | | | | | | | | |
| KP-004<sup>a</sup> | A | 17 | – | CMV-2 | 1.11 ± 0.21 | <0.5 | x | – | – | – | – | – | – | – | G2 | >6.50 |
| KP1050<sup>b</sup> | O | 107 | SHV-5 | – | 1.42 ± 0.16 | <0.5 | x | – | – | + | – | + | – | – | G6 | >6.84 |
| KP1051<sup>c</sup> | H1 | 17 | SHV-5 | – | 1.44 ± 0.11 | <0.5 | x | – | – | – | – | – | – | – | G2 | >6.72 |
| KP1052<sup>c</sup> | N1 | 15 | SHV-5 | DHA-1 | 1.72 ± 0.07 | <0.5 | x | – | – | – | – | – | – | – | G6 | >6.42 |
| KP1053<sup>c</sup> | G | 54 | SHV-5 | – | 1.28 ± 0.15 | <0.5 | x | – | – | – | – | – | – | – | G6 | >6.55 |
| KP-007<sup>a</sup> | S | 66 | – | TXY-11 | 0.42 ± 0.16 | <0.5 | x | – | – | – | – | – | – | – | G4 | >6.52 |
| KP-232<sup>c</sup> | E1 | 65 | SHV-5 | – | 4.62 ± 0.65 | >2 | 2 | + | + | + | – | – | + | – | G3 | >6.36 |
| KP-960<sup>‡</sup> | N2 | 15 | – | – | 3.95 ± 0.46 | >2 | 2 | + | + | + | – | – | – | – | G6 | >6.84 |

Primary meningitis (n = 20)

| CSF Strains | PFGE | MLST | ESBL | AmpC | Virulence factors | Bacterial size (μm) | HV (mm) | Capsule | rmpA | rmpA2 | c-rmpA | Kfu | allS | aerobactin | SR | LD<sub>50</sub> (log) |
|-------------|------|------|------|------|-------------------|-------------------|--------|---------|------|------|------|-----|------|-----------|-----|----------------|
| KP-002<sup>a</sup> | C4 | 23 | – | – | 2.42 ± 0.33 | >2 | 1 | + | – | – | – | – | – | + | G5 | 4.83 |
| KP-003<sup>a</sup> | C5 | 23 | – | – | 10.73 ± 1.11 | >2 | 1 | + | + | – | + | + | + | + | G6 | 4.50 |
| KP-005<sup>a</sup> | M | 268 | – | – | 7.24 ± 0.76 | >2 | 20 | + | + | – | – | – | – | – | G5 | >6.49 |
| KP-006<sup>a</sup> | Q | 65 | – | – | 3.67 ± 0.42 | 0.5–1 | 2 | + | + | – | + | + | + | + | G4 | 4.41 |
| KP-064<sup>a</sup> | R1 | 218 | – | – | 3.74 ± 0.49 | >2 | 57 | + | + | – | – | – | – | – | G2 | >6.45 |
| KP-088<sup>a</sup> | P2 | 373 | – | – | 4.77 ± 0.46 | >2 | 2 | + | + | – | – | – | – | – | G6 | 4.19 |
| KP-777<sup>a</sup> | C2 | 23 | – | – | 7.75 ± 1.37 | >2 | 1 | + | + | + | + | + | + | + | G5 | >6.45 |
| KP-600<sup>a</sup> | C1 | 33 | – | – | 2.33 ± 0.24 | <0.5 | x | – | – | – | – | – | – | – | G2 | >6.45 |
| KP-777<sup>a</sup> | C2 | 23<sup>‡</sup> | – | – | 5.30 ± 0.71 | >2 | 1 | + | + | + | + | + | + | + | G4 | 4.98 |
| KP-164<sup>a</sup> | R1 | 218 | – | – | 3.04 ± 0.48 | >2 | 57 | + | + | – | – | – | – | – | G3 | >6.15 |
| KP-552<sup>a</sup> | B | 23 | – | – | 7.05 ± 0.72 | >2 | 1 | + | + | + | + | + | + | + | G5 | 5.35 |
| KP-780<sup>a</sup> | P1 | 373 | – | – | 6.92 ± 0.53 | >2 | 2 | + | + | – | – | – | + | + | G6 | >6.44 |
| KP-783<sup>a</sup> | P1 | 373 | – | – | 3.06 ± 0.43 | >2 | 2 | + | + | – | – | – | – | + | G6 | 5.4 |
| KP-957<sup>a</sup> | K | 65 | – | – | 4.56 ± 0.56 | >2 | 2 | + | + | – | – | – | – | – | G6 | 3.94 |
| KP-958<sup>a</sup> | I2 | 86<sup>†</sup> | – | – | 3.45 ± 0.49 | >2 | 2 | + | + | – | – | – | – | – | G5 | 1.56 |
| KP-959<sup>a</sup> | unidentified | 1049 | – | – | 4.71 ± 0.89 | >2 | 5 | + | + | – | – | – | – | – | G6 | >6.50 |
| KP-961<sup>a</sup> | I2 | 373 | – | – | 4.58 ± 0.39 | >2 | 2 | + | + | – | – | – | – | – | G6 | 3.77 |
| KP-962<sup>a</sup> | F | 1544 | – | – | 5.09 ± 1.06 | 0.5–1 | 20 | + | + | – | – | – | – | – | G6 | 5.14 |
| KP-963<sup>a</sup> | H2 | 29 | – | – | 4.51 ± 0.53 | >2 | 54 | + | + | – | – | – | – | – | G6 | 5.41 |
| KP-966<sup>a</sup> | L | 592 | – | – | 2.75 ± 0.45 | 0.5–1 | 57 | + | – | – | + | + | – | – | G2 | >6.59 |

(n = 19, p = 0.0007) and aerobactin gene (n = 19, p = 0.0059), in comparison to isolates with lower grade serum resistance (grades 1–4).

Mouse lethality experiments. LD<sub>50</sub> > 10<sup>6</sup> (low virulence) was found in 10 of 13 post-craniotomy isolates, but in 7 of 20 primary meningitis isolates (p = 0.0324). The prevalence of virulence factors were significantly higher in isolates with LD<sub>50</sub> < 10<sup>6</sup> (high virulence) than those with low virulence (LD<sub>50</sub> > 10<sup>6</sup>), including mean bacterial size >2 μm (16/16 vs. 8/17, p = 0.0009), HV phenotype (16/16 vs. 6/17, p < 0.0001), rmpA (16/16 vs. 7/17, p = 0.0003), rmpA2 (14/16 vs. 6/17, p = 0.0039), aerobactin gene (16/16 vs. 6/17, p < 0.0001), virulent K serotypes (16/16 vs. 8/17, p = 0.0009) and high-grade serum resistance (15/16 vs. 8/17, p = 0.0066).

PFGE. Two isolates were nontypeable. The remaining 31 isolates were classified into 19 major pulsotypes (A–S types) by using the Dice coefficient and UPGMA algorithm (Fig. 1). Among them, 8 pulsotypes with a similarity coefficient of more than 80% included 5 isolates in pulsotype C (C1-CS), 3 isolates in pulsotype P (P1-P2) and 2
isolates each in pulsotype E (E1-E2), pulsotype H (H1-H2), pulsotype I (I1-I2), pulsotype J (J1-J2), pulsotype N (N1-N2) and pulsotype R (R1-R2). In general, no major single clone (indistinguishable or >95% similarity) was broadly disseminated among the Klebsiella pneumoniae strains that caused meningitis in Taiwan.

MLST. From all isolates performing MLST, 6 strains were ST23 (including one ST23-like variant), 5 strains were ST373, 4 strains were ST65, 2 strains were ST86 (including one ST86-like variant), ST15, ST17 and ST218 respectively. Others were one each for ST29, ST54, ST107, ST185, ST268, ST592, ST661, ST1049, ST1544 and unidentified ST (strain KP-023).

The association between genotyping, sequence type and K capsule serotypes of Klebsiella pneumoniae meningitis isolates were summarized in Fig. 1. Notably, the 5 primary meningitis isolates of pulsotype C (C1-C5) were capsule serotype K1, including ST23 in 4 strains and ST 23-like in 1 strain. Five ST373 strains belonged to pulsotypes J and P. Isolates of ST373, ST65 and ST86 belonged to capsule serotype K2. Two isolates of ST218 and 1 isolate of ST29 belonged to capsule serotype K57 and K54 respectively.

Demographic data and clinical outcomes. The 33 K. pneumoniae isolates from CSF basically of primary meningitis or post-craniotomy meningitis were collected from 1999 to 2013. However, detailed clinical data could be retrieved for the 12 patients from 2009 to 2013 (Table 4). The reasons of craniotomy were mainly to remove hematoma of various causes. Five of six patients in this group underwent craniectomy and two patients developed brain abscess (Fig. 2). The main comorbidities of patients with primary meningitis were diabetes mellitus and alcoholic liver cirrhosis (Table 4). Two patients in this group developed brain abscess and two patients developed ventriculitis (Fig. 2). Only one patient died during the hospital episode. There were no statistically significant differences in the demographic data, CSF profiles and clinical outcomes between the two groups of K. pneumoniae meningitis.

Discussion

The study investigated the antibiotic resistance profiles, β-lactamase genes, various hypervirulence determinants, mouse lethality, as well as the epidemiology of genomic macrorestriction patterns and sequence types of K. pneumoniae isolates each in pulsotype E (E1-E2), pulsotype H (H1-H2), pulsotype I (I1-I2), pulsotype J (J1-J2), pulsotype N (N1-N2) and pulsotype R (R1-R2). In general, no major single clone (indistinguishable or >95% similarity) was broadly disseminated among the Klebsiella pneumoniae strains that caused meningitis in Taiwan.

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Figure 1. Fingerprinting profiles (A to S) of XbaI-digested genomic DNA using the Dice coefficient and UPGMA for *Klebsiella pneumoniae* cerebrospinal fluid isolates collected in Taiwan. Eight pulsotypes (C, E, H, I, J, N, P, and R) show a Dice similarity coefficient of more than 80%. Corresponding MLST (multilocus sequence typing) and capsule K serotype of each isolate were presented. *One nucleotide change; x, unidentified, non-virulent capsule K types, i.e., not for K1, K2, K5, K20, K54, K57 types.*

| Strain* | Age (yr) | Sex | Comorbidity | Ventilator use | Sugarb (mg/dl) | RBC (per μl) | WBC (per μl) | Neutrophil (%) | Lymphocyte (%) | Sugar (mg/dl) | Protein (mg/dL) | Outcome |
|---------|----------|-----|-------------|---------------|----------------|-------------|--------------|--------------|---------------|--------------|---------------|---------|
| Post-craniotomy meningitis | | | | | | | | | | | | |
| KP0105 | 56 | M | Head injury | Nil | 114 | 18 | 72 | 28 | 54 | 108.0 | Survived |
| KP0106 | 23 | M | SAH | Nil | 1030 | 133 | 60 | 40 | 57 | 57.5 | Survived |
| KP0107 | 59 | M | ICH | Yes | 1250 | 2016 | 92 | 8 | 23 | 473.3 | Survived |
| KP0108 | 61 | M | AVM rupture | Yes | 200 | 75900 | 100 | 0 | <1 | 592 | Survived |
| KP0109 | 51 | F | AVM rupture | Nil | 110 | 1177 | 72 | 28 | <1 | 206.7 | Survived |
| KP960 | 56 | M | Head injury | Nil | 0 | 8 | 50 | 50 | 1 | 412.0 | Survived |
| Primary meningitis | | | | | | | | | | | | |
| KP958 | 48 | M | DM, ALC | Nil | 342 | 760 | 1632 | 97 | 3 | 118 | >500 | Survived |
| KP959 | 62 | M | ALC | Yes | Nil | 24500 | 94600 | 65 | 35 | 1 | >500 | Died |
| KP961 | 55 | M | DM | Nil | 489 | 576 | 12672 | 97 | 3 | 219 | 325 | Survived |
| KP962 | 68 | F | DM | Nil | 405 | 30 | 372 | 71 | 29 | 205 | 173.3 | Survived |
| KP963 | 50 | M | DM | Nil | 430 | 190 | 5712 | 97 | 3 | 241 | >500 | Survived |
| KP966 | 62 | M | ALC | Yes | 157 | 27000 | 62700 | 72 | 28 | <1 | >500 | Survived |
| p | 0.390 | 1.000 | NA | 1.000 | NA | 0.176 | 0.522 | 0.345 | 0.345 | NA | NA | 1.000 |

Table 4. Clinical profiles and outcome of *Klebsiella pneumoniae* isolates from CSF of post-craniotomy and primary meningitis. Note CSF, cerebrospinal fluid; M, male; F, female; SAH, subarachnoid hemorrhage related to a brain aneurysm; ICH, intracerebral hemorrhage; AVM, arteriovenous malformation; DM, diabetes mellitus; ALC, alcoholic liver cirrhosis; RBC, red blood cell count (normal, <5/μl); WBC, white blood cell count (normal, <5/μl); NA, not applicable; *from Chi-Mei Medical Center in Tainan City, southern Taiwan, 2009–2013 (n = 12); †random blood sugar obtained on the day of CSF collection.
33 *K. pneumoniae* isolates that were capable of invading the central nervous system to cause primary meningitis (*n* = 20) or post-craniootomy meningitis (*n* = 13). Generally consistent with our hypothesis, primary meningitis isolates were more susceptible to third- and fourth-generation cephaplorins, were more hypervirulent (hvKP) and had higher mouse lethality capability than post-craniootomy meningitis isolates (mostly cKP). Exceptionally, serum resistance did not reach statistically significant difference between groups, suggesting that high-grade serum resistance (*n* = 23) is the major common virulence determinant of *K. pneumoniae* isolates with capability to invade the central nervous system, regardless of primary or post-craniootomy meningitis.

The post-craniootomy isolates had significantly higher carriage rate of ESBL/AmpC genes (69.2%) than primary meningitis isolates did (0%), suggesting that it is appropriate for meropenem but not for broad-spectrum cephaplorin in the empirical therapy of post-craniootomy meningitis. Chang *et al.* reported data of 49 adult *K. pneumoniae* meningitis cases (36 spontaneous meningitis and 13 post-neurosurgical meningitis), collected over a period of 11 years (2000–2010) in Taiwan28. Data on antibiotic resistance of these isolates also showed none of 36 spontaneous meningitis isolates but only 2 of 13 (15.4%) post-neurosurgical meningitis isolates were resistant to third- and fourth-generation cephaplorins and one of the strains was ESBL-producing28. Thus, it is important to monitor the trend of antibiotic resistance in *K. pneumoniae* CSF strains for the empirical antibiotic strategy of meningitis.

With regard to specific virulence factors, primary meningitis isolates were more virulent than post-craniootomy isolates in term of the expression of larger bacterial size, HV phenotype, *rmpA*, *aerobactin* gene, virulent capsule K serotypes (K1, K2, K3, K20, K54, and K57), and high mouse lethality (LD<sub>50</sub> < 10<sup>7</sup>). Capsular K2 serotype was the most common serotype observed (33.3%). Most hvKP isolates were significantly associated with the above-mentioned virulence factors and exhibited high-grade serum resistance as well as high mouse lethality. Furthermore, aerobactin is one of the siderophore systems, which mediate acquisition of iron to help virulent bacteria to overcome iron starvation, while bacteria invade and proliferate in the human systems14, 24, 25. In general, the current data support our initial hypothesis. The presence of these virulent genes and characters of primary meningitis hvKP isolates might assist in their capability to invade CSF space through intact central nervous system barrier to cause infection, whereas the less virulent cKP might still be able to cross the CSF space through disruption of central nervous system barrier, probably with the aid of the mechanism of serum resistance. We found that high-grade serum resistance occurred in 7 of 13 (53.8%) post-craniootomy isolates and in 16 of 20 (80%) in primary meningitis isolates, without reaching significant difference (*p* = 0.139). We acknowledge that high serum resistance is the major common virulence determinant of hvKP and cKP isolates contributing to invade the central nervous system, regardless of primary or post-craniootomy status.

With regard to molecular epidemiology, no major genomic clone or sequence type broadly existed among the 33 *K. pneumoniae* CSF isolates. However, there were 5 primary meningitis isolates of pulsotype C (C1-C5) belonging to ST 23 or ST23-like sequence type. The ST23 has been recognized as the most prevalent MLST type of capsule serotype K1 *K. pneumoniae* isolates from liver abscess in Taiwan29. The ST65, ST86, ST373 and ST375 (not detected in the study) have been the major clones associated with K2 *K. pneumoniae* liver abscesses in Taiwan28, 30. In addition, ST65, ST65-like and ST86-like MLST types were most predominant among K2 serotype isolates of the community-acquired infection cases from Singapore, Hong Kong and China31, 32.

ST218 has been reported in capsule serotype K57 liver abscess isolates in Taiwan29. ST29 K54 ESBL-producing *K. pneumoniae* with HV phenotype was detected in Hunan, China31. We previously reported on a mycotic aneurysm caused by hypermucoviscous ST29 K54 *K. pneumoniae* in Taiwan34. Furthermore, the ST29 was the predominant ST of carbapenem-resistant *K. pneumoniae* isolates in Saudi Arabia35. The ST15, ST17, ST54, and ST107 isolates producing ESBL, DHA-1, KPC-2 or NDM-Imetallo-β-lactamase have been found in Taiwan, China and

Figure 2. Brain imaging studies of patients with *Klebsiella pneumoniae* meningitis from post-craniootomy meningitis groups (no. 1–6) and primary meningitis group (no. 7–12). No. 1, strain KP1050, hydrocephalus (computed tomography, CT); No. 2, strain KP1051, brain edema with compression effect (CT); No. 3, strain KP1052, brain abscess (CT); No. 4, strain KP1053, brain abscess (CT); No. 5, strain KP1054, subdural effusion (CT); No. 6, strain KP-960, subdural effusion (CT); No. 7, strain KP-958, ventriculitis (Magnetic Resonance Imaging, MRI); No. 8, strain KP-959, brain abscess (CT); No. 9, strain KP-961, hydrocephalus; No. 10, KP-962, essentially intact in brain parenchyma (CT); No. 11, strain KP-963, ventriculitis (MRI) and No. 12, strain KP-966, brain abscess (CT).
other various geographic areas in the world. Importantly, 5 of 9 ESBL/AmpC producers in our current study belonged to these international clones, which often exhibited high-grade serum resistance. In addition to our recent report on the bacteremic isolates, we continuously highlight the importance of monitoring the emergence of hypervirulence in the ESBL/AmpC-producing K. pneumoniae, particularly in the CSF isolates.

Although the K. pneumoniae isolates had different virulence mechanisms between the groups of primary and post-craniotomy meningitis, they did not result in significantly different clinical features and outcomes among limited patients between groups. The reasons could be explained by the effectiveness of BSC and meropenem against the isolates causing primary and post-craniotomy meningitis respectively. However, continuous monitoring for the emerging resistance profiles of K. pneumoniae between different groups of meningitis is clinically important.

Conclusion
Primary K. pneumoniae meningitis isolates had hypervirulence profiles of virulent capsule K serotypes, larger capsule size, HV phenotype, rmpA, rmpA2, aerobactin gene, high serum resistance and high capability of mouse lethality. Post-craniotomy K. pneumoniae meningitis isolates had relatively low virulence profiles and exhibited low mouse lethality, but still had high serum resistance which supported their capabilities to invade the central nervous system. The MLST international clones (ST15, ST17, ST54, and ST107) have been found in the post-craniotomy meningitis isolates in Taiwan, which could produce ESBL/AmpC β-lactamases. Therefore, physicians should be aware the emerging trend of antibiotic resistance in the empirical treatment for K. pneumoniae, particularly in post-craniotomy meningitis.

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**Author Contributions**

W.-L.Y. contributed to study design, data interpretation and reviewing the manuscript. Y.-H.K. and M.-F.L. contributed to manuscript drafting. C.-C.Y. and H.-J.T. contributed to data acquisition, experiments and analysis. Y.-C.C. contributed to the supervision of this investigation. All of the authors were involved in writing the manuscript, critically revising it for intellectual content and approving the final version submitted for publication.

**Additional Information**

Competing Interests: The authors declare that they have no competing interests.

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