Transfer of CMY-2 cephalosporinase from *Escherichia coli* to virulent *Klebsiella pneumoniae* causing a recurrent liver abscess

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**Running title:** Drug-resistant and virulent *K. pneumoniae*

**Key words:** Klebsiella pneumoniae; antimicrobial resistance; virulence; conjugation
A CMY-2-producing capsular type K2 *Klesbiella pneumoniae* strain (TVGHKP93) with multidrug-resistance was isolated from recurrent liver abscess in a patient who also carried CMY-2-producing *Escherichia coli* (TVGHEC01) in the stool. TVGHKP93 retained its high virulence compared with the isogenic strain (TVGHKP60) with wild-type resistance from the first liver abscess. Our conjugation experiment showed the successful transfer of the *bla*\textsubscript{CMY-2}-carrying plasmid from TVGHEC01 into TVGHKP60. The transconjugant showed both high virulence and multidrug-resistant phenotype as TVGHKP93.
Klebsiella pneumoniae liver abscess (KPLA) has been increasingly reported in Asia and is considered to be an endemic disease in Taiwan (1, 2). The capsular type of K. pneumoniae appears to be the major virulence factor (3, 4), and the K1 and K2 were the most prevalent types in KPLA (1). In KPLA, K. pneumoniae isolates almost demonstrate a unique antibiogram indicative of resistance to ampicillin only, and the multidrug-resistant isolates have rarely been reported (5, 6). Currently, several investigations provide evidence that KPLA is preceded by gastrointestinal colonization (7-10).

We identified an 84-year-old patient with diabetes who suffered from recurrent KPLA. The strain (TVGHKP60) isolated from the first abscess in November 2012 was susceptible to all antibiotics tested, except for ampicillin, consistent with the natural resistance of K. pneumoniae. The patient received intravenous ceftriaxone for 3 weeks and recovered well. After discharge, he received oral cefuroxime for another 2 weeks. However, the patient suffered from a recurrent liver abscess in January 2013 and the second K. pneumoniae strain with multidrug-resistance (TVGHKP93) was isolated. The patient received intravenous ciprofloxacin for 3 weeks and was discharged uneventfully. Interestingly, a multidrug-resistant Escherichia coli (TVGHEC01) was isolated from the patient’s stool during the recurrence episode. We further investigated the two K. pneumoniae strains and one E. coli strain from this case. The protocol was approved by the hospital’s institutional review board.

Bacterial identification and antimicrobial susceptibility were determined using a Vitek2 System (bioMérieux, Marcy l’Etoile, France). Antimicrobial susceptibility was interpreted according to the guidelines of the CLSI (11) and shown in Table 1. Pulsed-field gel electrophoresis DNA fingerprinting (12, 13) showed that the
wild-type TVGHKP60 strain was nearly identical (only one band difference) to the multidrug-resistant TVGHKP93 strain. Capsular genotyping, detection of \textit{rmpA/rmpA2}, molecular characterization of \(\beta\)-lactamases and colony mucoviscosity were performed as previously described (14-16). Multilocus sequence typing (MLST) was performed on the TVGHKP60 and TVGHKP93 strains and the results were analysed as previously described (17). The TVGHKP60 and TVGHKP93 strains both belonged to capsular type K2 and ST86. They showed hypermucoviscosity phenotypes and carried \textit{rmpA} and \textit{rmpA2} genes. Regarding the detection of \(\beta\)-lactamases, \textit{bla\textsubscript{SHV-1}} was detected in TVGHKP60, and \textit{bla\textsubscript{SHV-1}} and \textit{bla\textsubscript{CMY-2}} were detected in TVGHKP93. Interestingly, \textit{bla\textsubscript{CMY-2}} was also detected in the TVGHEC01. 

The \textit{K. pneumoniae} strains were cultured in LB broth at 37°C for 16 h to obtain bacterial growth curves as described previously (18). The isogenic \textit{bla\textsubscript{CMY-2}}-producing strain (TVGHKP93) and the original strain (TVGHKP60) showed no significant differences in their growth rates. The \textit{in vivo} virulence of the TVGHKP60 and TVGHKP93 strains was compared using a murine model of septicaemia generated by intraperitoneal injection. Male 6–8-week-old C57/B6 mice were observed for 1 week after intraperitoneal inoculation of 100 CFU of \textit{K. pneumoniae}. All animal care procedures and protocols were approved by the Institutional Animal Care and Use Committee of National Yang-Ming University. Upon intraperitoneal infection of mice, both strains showed hypervirulence with the lethal dose, 50\% (LD\textsubscript{50}) values of <100 CFU (Figure 1). The clinical \textit{K. pneumoniae} strain (capsular type K64) isolated from blood was used as the control and its LD\textsubscript{50} was \(10^7\) CFU.

Plasmids were extracted from TVGHEC01 for high-throughput sequencing using the Illumina/Solexa GAII sequencing platform. Coding sequences were predicted and annotated with NCBI-Protein Blast.
A circular map of a blaCMY-2-carrying plasmid, designated pCMY2, with 102,199 bp was obtained (GenBank accession no. LC019731). Nucleotide blast results revealed that pCMY2 exhibited a high level of similarity with a 108,660-bp *E. coli* plasmid, pC49-108 (GenBank accession no. KJ484638), which showed 94% coverage and shared 99% DNA sequence identity. Conjugation-related genes (associated with *tra* and *trb*) and type IV pili-associated genes were found in both plasmids. However, pC49-108 harboured the drug-resistance genes *addA5*, *dhfrA17*, and *blaCTX-M-1*, while pCMY2 carried an AmpC type β-lactamase, *blaCMY-2*, instead. The PCR results using four primer pairs located within the plasmid revealed that pCMY2 was present in both TVGHEC01 and TVGHKP93, but absent from TVGHKP60. It is reasonable to consider that horizontal transfer of a *blaCMY-2*-carrying resistance determinant from *E. coli* to *K. pneumoniae* developed in the patient’s bowel. Our attempts to mimic the natural transfer of the pCMY2 plasmid from TVGHEC01 into TVGHKP60 using conjugation experiments described previously (19) were successful. The conjugation frequency was determined to be 5.2 × 10⁻⁶. The MICs for for cefazolin, cefuroxime, cefoxitin, ceftriaxone, ceftazidime, and piperacillin/tazobactam increased in the transconjugant (TVGHKP60::*blaCMY-2*) and were the same as those of TVGHKP93 (Table 1). The *in vivo* virulence assessment also confirmed that *K. pneumoniae* TVGHKP60::*blaCMY-2* retained the hypervirulence characteristics (data not shown). These results implied that TVGHKP60 had acquired pCMY2 from TVGHEC01 in the patient’s gut, leading to the formation of the hypervirulent and multidrug-resistant TVGHKP93 in the recurrent liver abscess. Capsular type K2 and ST86 are considered to be hypervirulent clones that can
cause invasive diseases (20, 21). Our study firstly demonstrated that this virulent strain acquired the pCMY2 plasmid, but still retained its virulence. The selective pressure by cephalosporins may predispose to the possible plasmid transfer in this case. Although increased antimicrobial resistance is generally associated with decreased virulence and fitness, evidence has also shown the opposite, and it is increasingly evident that the relationship is often of greater benefit to the pathogen, resulting in a growing public health problem (22).

A recent study showed that multidrug-resistant and hypervirulent populations of *K. pneumoniae* were mostly non-overlapping, although two isolates with combined virulence and resistance features were detected (23). These results show that the threat of dual-risk *K. pneumoniae* strains, combining virulence and multidrug-resistance features, is becoming a reality. The multidrug-resistant and highly virulent TVGHKP93 derived from wild-type TVGHKP60 serves as a good example to verify the relationship between virulence and resistance in *K. pneumoniae*.

With the increasing rate of drug-resistant enterobacteriaceae colonizing the intestine, the possibility of interspecies transfer of drug-resistance determinants into highly virulent *K. pneumoniae* increases. The acquisition of an important mechanism of antibiotic-resistance, such as CMY-2, could suggest that virulent strains may be a potential cause of nosocomial infections in the future (24).

**Nucleotide sequence accession number.** The complete nucleotide sequence of pCMY2 was deposited as GenBank accession no. LC019731.

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**Competing interests:** None to declare.
References


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Figure legends

Figure 1.

In vivo virulence study. Mouse lethality data following challenge with the TVGHKP60 (filled circle) and TVGHKP93 (filled triangle) strains are presented.

Male C57BL/6 mice (n=6 from two independent experiments) were inoculated by intraperitoneal injection with 100 CFU of the TVGHKP60 and TVGHKP93 strains. Survival was assessed for 7 days following infection. The Kaplan–Meier method was used to evaluate the survival rate. Upon intraperitoneal infection, all mice from each group (TVGHKP60 versus TVGHKP93) were dead within 5 days (Log-rank test: P=0.6285).
Table 1. Antimicrobial susceptibility test data for the strains in this study.

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>K. pneumoniae (TVGHKP60)</th>
<th>K. pneumoniae (TVGHKP93)</th>
<th>E. coli (TVGHEC01)</th>
<th>K. pneumoniae transconjugant: TVGHKP60::bla&lt;sub&gt;CMY-2&lt;/sub&gt;</th>
</tr>
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<tbody>
<tr>
<td>Ampicillin</td>
<td>≥32</td>
<td>≥32</td>
<td>≥32</td>
<td>≥32</td>
</tr>
<tr>
<td>Cefazolin</td>
<td>≤4</td>
<td>≥64</td>
<td>≥64</td>
<td>≥64</td>
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<td>Cefuroxime</td>
<td>2</td>
<td>16</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>≤4</td>
<td>32</td>
<td>32</td>
<td>32</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>≤1</td>
<td>8</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>≤1</td>
<td>16</td>
<td>4</td>
<td>16</td>
</tr>
<tr>
<td>Cefepime</td>
<td>≤1</td>
<td>≤1</td>
<td>≤1</td>
<td>≤1</td>
</tr>
<tr>
<td>Piperacillin/Tazobactam</td>
<td>≤4</td>
<td>8</td>
<td>≤4</td>
<td>8</td>
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<tr>
<td>Gentamicin</td>
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<td>≤1</td>
<td>≤1</td>
<td>≤1</td>
</tr>
<tr>
<td>Amikacin</td>
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<td>≤2</td>
<td>≤2</td>
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<td>Ciprofloxacin</td>
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<td>Imipenem</td>
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<tr>
<td>Trimethoprim-sulfamethoxazole</td>
<td>0.19</td>
<td>0.38</td>
<td>0.094</td>
<td>0.38</td>
</tr>
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</table>

<sup>a</sup>The values were MIC-correlates determined by Vitek2 System, except for trimethoprim-sulfamethoxazole determined by E test.