Molecular typing and resistance mechanisms of imipenem-non-susceptible *Klebsiella pneumoniae* in Taiwan: results from the Taiwan surveillance of antibiotic resistance (TSAR) study, 2002–2009

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We investigated the molecular mechanisms and clonality of imipenem-non-susceptible *Klebsiella pneumoniae* isolates collected during a Taiwan national surveillance programme, between 2002 and 2009. Genes for carbapenemases, plasmid-borne *ampC*-type genes and extended-spectrum *β*-lactamase (ESBL) genes were analysed by PCR. The major porin channels OmpK35 and OmpK36 were studied by SDS-PAGE. Molecular typing was performed with pulsed-field gel electrophoresis (PFGE) and multi-locus sequence typing (MLST). Our study revealed that all 29 of the isolates tested were ESBL producers. Of the *K. pneumoniae* isolates collected in Taiwan from 2002 to 2009, most (84.6%, 11/13) imipenem-resistant (MIC ≥ 2 mg l⁻¹) isolates carried the *bla*<sub>IMP</sub>-8 gene. Isolates with an imipenem MIC of 2 mg l⁻¹ produced ESBLs with or without DHA-1 in combination with OmpK35/36 loss. PFGE analysis revealed that six small clusters of isolates were clonally related. The MLST grouping results were in concordance with the PFGE results. The predominant sequence types (ST) were ST11, ST48 and ST101. Two novel STs, ST1033 and ST1034, were found. The dominant clone in Taiwan, ST11, has been reported worldwide to be associated with various resistance mechanisms.

INTRODUCTION

Carbapenems are important as a last-resort treatment for many multi-drug-resistant bacterial infections. Recently, carbapenem resistance in *K. pneumoniae* has caused increased concern about outbreaks of isolates with novel mechanisms of drug resistance, namely *Klebsiella pneumoniae* carbapenemase (KPC) and New Delhi metallo-*β*-lactamase (NDM)-1 (Gupta et al., 2011). However, various mechanisms, other than KPC and NDM, may result in carbapenem resistance. Carbapenem resistance can be mediated by production of carbapenemases or by the combination of outer membrane porin expression disruption and production of various *β*-lactamases (Doumith et al., 2009; Nordmann et al., 2009). Additionally, some clones of multidrug-resistant Gram-negative bacteria appear to be responsible for the dissemination of antibiotic resistance (Woodford et al., 2011). The high diversity of resistance mechanisms and clones (Kitchel et al., 2009; Potron et al., 2011; Qi et al., 2011; Seki et al., 2011; Shin et al., 2012; Warburg et al., 2012) of carbapenem-resistant *K. pneumoniae* makes it necessary to investigate resistance epidemiology in each country in order to combat the further spread of such resistance.

Carbapenem resistance among *K. pneumoniae* isolates is increasing in Taiwan and has challenged clinicians’ antimicrobial options (Chia et al., 2010). Previous hospital-based studies identified different mechanisms, including expression of the *bla*<sub>IMP</sub>-8 gene, outer-membrane protein (OMP) loss combined with *β*-lactamase production, and KPC, all of which cause carbapenem resistance in *K. pneumoniae* in Taiwan hospitals (Chia et al., 2010; Lauderdale et al., 2012; Yan et al., 2001). However, an overview of the clonality and resistance mechanisms of carbapenem-resistant *K. pneumoniae* is lacking. The aim of this study was to investigate the resistance mechanisms and clonality of imipenem-non-susceptible *K. pneumoniae* isolates, collected during a nationwide surveillance programme in Taiwan between 2002 and 2009.
METHODS

Bacterial strains. Twenty-nine imipenem-non-susceptible *K. pneumoniae* isolates were obtained. All isolates were from individual cases. These isolates were obtained from seven medical centres and six regional hospitals and were collected during a Taiwan Surveillance of Antibiotic Resistance (TSAR) programme (Ho et al., 1999), between January 2002 and December 2009, and a subproject of TSAR surveillance, which aimed to survey extended-spectrum β-lactamase (ESBL)-producing bacteria in 2002. Primary screening for carbapenem resistance was done by the individual participating hospitals. Further confirmation of imipenem susceptibility was performed at the National Health Research Institutes by using the criteria described below.

Susceptibility testing. Antimicrobial susceptibility was determined by the broth micro-dilution method according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2009a). The following antimicrobial agents were used: ampicillin, cefazolin, amoxicillin–clavulanic acid, cefotixin, cefotaxime, cefazidime, aztreonam, imipenem, amikacin, gentamicin, ciprofloxacin and trimethoprim–sulfamethoxazole. All drugs were diluted in Mueller–Hinton broth (Trek Diagnostic System) in serial twofold concentrations ranging from 0.025 to 64 mg l⁻¹. Two control strains, *Escherichia coli* ATCC 35218 and *E. coli* ATCC 25922, were included in each test run. Inoculated plates were incubated at 35 °C for 16–18 h. The MIC of each antimicrobial agent was defined as the lowest concentration that inhibited visible growth of the organism.

Detection of genes for carbapenemase, AmpC and ESBL. Carbapenemase genes (encoding class B families IMP, VIM, GIM, SPM and SIM; class A families NMC, IMI, SME, KPC and GES; and class D family OXA-48) (Queenan & Bush, 2007), plasmid-borne AmpC-like genes (encoding CMY, DHA and ACT) (Alvarez et al., 2004; Chia et al., 2010; Giakkoupi et al., 2006) and ESBL genes (encoding CTX-M, Eckert et al., 2004; TEM, Eckert et al., 2004; and SHV, Rasheed et al., 1997) were detected by PCR amplification. Primers for the class B carbapenemase NDM-1 were newly designed in this study (NDM-1F: 5′-TCTCGACATGCGCGGTTT-3′; NDM-1R: 5′-GAGATTGCGAGGCACTT-3′). Bacterial DNA was prepared by suspending one loop of freshly cultured cells in 500 μl sterile distilled water and heating the mixture at 95 °C for 10 min. The reaction was performed in a total volume of 50 μl. The amplification conditions were as follows: 95 °C for 5 min, followed by 35 cycles of 95 °C for 1 min, 54 °C for 1 min and 72 °C for 1 min, with a final stage at 72 °C for 10 min to complete the synthesis of DNA.

The amplicons were sequenced and the entire sequence of each gene was compared with sequences in the GenBank nucleotide database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequencing was conducted using corresponding primers specific for *bla*SHV, *bla*TEM, *bla*CMY, *bla*SHV-1 and *bla*ACT genes and an ABI Prism 377 automated sequencer (Perkin-Elmer).

Pulsed-field gel electrophoresis (PFGE). Total DNA was prepared and PFGE was performed as described previously (D’Agata et al., 2001). The restriction enzyme *XhoI* (New England Biolabs) was used at the temperature suggested by the manufacturer. Restriction fragments were separated by PFGE in a 1 % agarose gel (Bio-Rad) in 0.5 × TBE buffer (45 mM Tris, 45 mM boric acid, 1.0 mM EDTA; pH 8.0) for 22 h at 200 V and 14 °C and with ramp times of 2–40 s by using a CHEF Mapper apparatus (Bio-Rad). The gels were then stained with ethidium bromide and photographed under UV light. The Dice coefficient was used to calculate similarities and the unweighted pair-group method with arithmetic mean (UPGMA) was used for cluster analysis with BioNumerics software version 5.10 (Applied Maths).

**Isolation and analysis of OMPs** Bacterial OMPs were prepared as described previously (Hernández-Alles et al., 1999). The OMPs were then separated by 12 % SDS PAGE and visualized by using Coomassie blue staining (Bio-Rad). A reference strain, *K. pneumoniae* ATCC 13883, was included as a control.

Multi-locus sequence typing (MLST). MLST with seven housekeeping genes was performed on all 29 isolates according to the protocol described on the *K. pneumoniae* MLST website (http://www.pasteur.fr/recherche/genopole/PF8/mlst/Kpneumoniae.html). Allele sequences and sequence types (ST) were verified at the http://www.pasteur.fr/recherche/genopole/PF8/mlst/ website.

**RESULTS**

In 2010, the CLSI revised the carbapenem susceptibility breakpoints for *Enterobacteriaceae* (CLSI, 2010); the breakpoints were fourfold lower for imipenem and meropenem compared to the 2009 criteria (CLSI, 2009b). Among the 29 *K. pneumoniae* isolates tested, only three isolates, with imipenem MICs of 8 mg l⁻¹, were considered imipenem-non-susceptible according to the 2009 CLSI criteria (CLSI, 2009b). According to the 2010 criteria (CLSI, 2010), 13 (44.8 %) of the isolates were resistant to imipenem (MICs ≥ 4 mg l⁻¹), and 16 isolates exhibited intermediate susceptibility to imipenem (MICs of 2 mg l⁻¹) (Table 1). Four out of the 2515 (0.16 %) *K. pneumoniae* isolates collected during TSAR surveillance between 2002 and 2009 were originally identified as imipenem-non-susceptible. The other 25 isolates tested in this study were previously identified among 235 (10.6 %, 25/235) ESBL-producing *K. pneumoniae* isolates obtained from a subproject of TSAR surveillance in 2002, which set out specifically to collect ESBL-producing bacteria.

All 29 isolates were resistant to ampicillin, cefazolin, cefotaxime and ceftazidime. The rates of resistance to the other tested antibiotics decreased in the following order: trimethoprim–sulfamethoxazole (27/29 isolates, 93.1 %), gentamicin (26/29 isolates, 89.7 %), cefotixin (26/29 isolates, 89.7 %), ciprofloxacin (22/29 isolates, 75.9 %), aztreonam (22/29 isolates, 75.9 %) and amikacin (14/29 isolates, 48.3 %).

All of the isolates produced ESBLs. The *bla*CTX-M gene was detected in 28 isolates. Twenty-three isolates expressed CTX-M-3, four isolates expressed CTX-M-14 and one isolate expressed CTX-M-15. The *bla*SHV-12 gene was detected in 11 isolates. Some isolates harboured non-ESBLs that included SHV-1, SHV-11, LEN-1 and TEM-31. The carbapenemase gene *bla*IMP-8 was detected in 11 isolates. Regarding AmpC-type β-lactamases, the *bla*DHA-1 gene was detected in 11 isolates, the *bla*CMY-2 gene was detected in one isolate and the *bla*ACT-1 gene was not detected. No KPC- or NDM-type β-lactamases were detected. Twenty-two (75.9 %) isolates displayed OmpK35 loss, 10 (34.5 %) isolates displayed OmpK36 and eight isolates did not display either OmpK35 or OmpK36 expression (Table 1).

PFGE analysis revealed that six small clusters of isolates (14/29, 48.3 %) were clonally related (Fig. 1). Five of the six clusters contained isolates from specific hospitals, indicating
Table 1. Molecular characterization of the 29 *Klebsiella pneumoniae* isolates

IMP, imipenem; +, positive; -, negative/porin loss. Hospital regions are abbreviated followed in parentheses by their geographical area in Taiwan; N, north; M, middle; S, south.

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<th>Isolate</th>
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<th>Porin‡</th>
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that clonal spread occurred in each hospital. Pulsotype III contained isolates from two hospitals in northern Taiwan. The other 15 isolates that did not belong to the six clusters had distinct PFGE patterns. These data indicated that there had been outbreaks of imipenem–non-susceptible \textit{K. pneumoniae} in some hospitals. Using MLST, we found three major sequence types: ST11, ST48 and ST147; 11 isolates were assigned to \textit{K. pneumoniae} ST11, six isolates were assigned to ST48 and five isolates were assigned to ST147. Only one isolate of each of ST37, ST101, ST595 and ST873 were detected. Three isolates were found to belong to novel and unreported STs ST1033 (2-9-2-1-13-1-76; two isolates) and ST1034 (2-1-1-1-140-31-34; one isolate). The novel profiles and isolates have been defined in the \textit{K. pneumoniae} MLST database (available at http://www.pasteur.fr/recherche/genopole/PF8/mlst/). The MLST grouping results were in concordance with the results of PFGE analysis.

Among the 18 imipenem-non-susceptible \textit{K. pneumoniae} isolates caused by ESBL and/or AmpC-like production combined with porin loss, 16 (88.9 %) isolates lacked OmpK35 and 10 (55.6 %) isolates lacked OmpK36. No association was found between ST11 and OmpK36 loss among these Taiwan isolates (Table 1).

### DISCUSSION

Our study revealed that most imipenem-resistant (MIC >2 mg l$^{-1}$) isolates carried the \textit{bla}_{IMP-8} gene, and no isolate with an imipenem MIC of 2 mg l$^{-1}$ carried the \textit{bla}_{IMP-8} gene. Isolates with imipenem MICs of 2 mg l$^{-1}$ produced ESBLs with or without DHA-1 in combination with OmpK35/36 loss. All isolates in the study were ESBL producers. CTX-M-type $\beta$-lactamases were the most common ESBLs found in the \textit{K. pneumoniae} isolates tested.
OMP analysis revealed that most (75.9 %) isolates displayed OmpK35 loss and 10 (34.5 %) isolates exhibited OmpK36 loss.

PFGE analysis is a popular method for epidemiological typing of bacterial isolates. Though PFGE is more discriminatory than MLST in detecting relatively short-term evolutionary changes and in acute outbreak investigations, it often fails to identify long-term changes and more distant ancestral genetic similarities between isolates. Our results revealed ST11 to be the most common ST among the carbapenem-non-susceptible K. pneumoniae isolates tested in this study and this ST was identified in isolates from five hospitals from all geographical areas of Taiwan. The second and third most common STs were ST48 and ST147, respectively. Among these three STs, some isolates had the same pulsortypes, which indicates clonal dissemination. K. pneumoniae ST11 was first reported in France in 1997 and it has subsequently been detected across the world including in the USA, Israel, Sweden, Norway, Finland, the Netherlands, Hungry, Poland, the Czech Republic, Portugal, Spain, Italy, Brazil, China and South Korea (Andrade et al., 2011; Chudackova et al., 2010; Ko et al., 2010; Oteo et al., 2009; Qi et al., 2011; Rhee et al., 2010). Among the clinical isolates, ST11 was found in association with different ESBLs, primarily CTX-M-15, CTX-M-14 and SHV-5 (Hrabáková et al., 2009; Ko et al., 2010; Oteo et al., 2009). ST11 is the predominant clone of ESBL-producing K. pneumoniae in South Korea (Rhee et al., 2010) and it was also the most prevalent clone of ESBL-producing K. pneumoniae in nine Asian countries (Lee et al., 2011). Although ST11 was not considered the predominant clone of ESBL producers in Taiwan in a previous study that studied only 12 Taiwan isolates (Lee et al., 2011), ST11 was the predominant ST of the carbapenem-non-susceptible K. pneumoniae clones that harboured ESBLs in this study.

ST11 is a single-locus variant of ST258; they both belong to the clonal complex CC292 and are considered to be epidemic clones of multi-drug-resistant K. pneumoniae exhibiting worldwide spread (Woodford et al., 2011). ST258 is a major KPC-producing clone throughout the world (Cuzon et al., 2010). KPC-producing K. pneumoniae ST11 isolates have been found in China, Singapore, Taiwan and the UK (Balm et al., 2012; Lauderdale et al., 2012; Qi et al., 2011; Virgincar et al., 2011). Many VIM-producing K. pneumoniae in Hungary have also been found to belong to ST11 (Kristóf et al., 2010). ST11 is also a common ST for NDM-1-producing K. pneumoniae isolates (Giske et al., 2012). In the study, ST11 was associated with IMP-type metallo-β-lactamase expression, which has not been reported previously. Thus, ST11 may be a majority ST in Taiwan, leading to a high prevalence of multi-drug resistance by acquisition of different resistance genes. Previous studies have shown that carbapenem resistance could be the result of inactivation of the ompK36 gene by the insertion of the IS5 element in DHA-1-producing K. pneumoniae strains under in vivo selection pressures. This suggests that K. pneumoniae ST11 clones might be prone to the acquisition of porin gene alterations that result in carbapenem resistance (Shin et al., 2012) (Song et al., 2009); however, only five out of 11 K. pneumoniae ST11 isolates in the study expressed the blaCTX-M-1 gene. No association was found between ST11 and OmpK36 loss in these Taiwan isolates. Further investigation is required to identify the association between specific resistance mechanisms and STs, including whether the insertion of IS5 into the ompK36 gene is more common for ST11 or whether it may occur in various STs.

ST147 is an important clone of VIM-producing K. pneumoniae isolates in Hungary and Scandinavia (Damjanova et al., 2008; Papagiannitissis et al., 2011; Samuelsen et al., 2011), KPC-producing K. pneumoniae isolates in Greece and Canada (Giakkoupi et al., 2011; Peirano et al., 2011), and NDM-producing K. pneumoniae isolates in Canada, India, Sweden and the UK (Giske et al., 2012; Peirano et al., 2011). In addition, we also observed an association of ST147 with IMP-8-producing K. pneumoniae isolates in a Far East country.

In conclusion, OmpK35 and/or OmpK36 deficiency combined with AmpC and/or ESBL and the production of the metallo-β-lactamase IMP-8 were the major mechanisms for the development of imipenem-non-susceptibility in K. pneumoniae isolates (MIC ≥ 2 mg l−1). The dominant clone of the imipenem-non-susceptible K. pneumoniae isolates from Taiwan was ST11, an association that has been reported worldwide. The high prevalence of these STs illustrates their epidemic potential. As patients infected with carbapenem-resistant K. pneumoniae display a higher mortality rate than those infected with carbapenem-susceptible K. pneumoniae (Ben-David et al., 2012), continuous monitoring of carbapenem resistance, resistance mechanisms and potential epidemic clones is necessary.

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