Characterization of multidrug-resistant and extended-spectrum β -lactamase-producing *Klebsiella pneumoniae* strains from Malaysian hospitals

King Ting Lim,¹ Chew Chieng Yeo,² Rohani Md Yasin,³ Ganeswrie Balan⁴ and Kwai Lin Thong¹

Correspondence
Kwai Lin Thong
thongkl@um.edu.my

¹Institute of Biological Science, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia

²Faculty of Agriculture and Biotechnology, Universiti Darul Iman Malaysia, 20400 Kuala Terengganu, Malaysia

³Specialized Diagnostic Centre, Institute of Medical Research, Jalan Pahang, 50588 Kuala Lumpur, Malaysia

⁴Hospital Sultanah Aminah Johor Bharu, 80100 Johor Bharu, Malaysia

The emergence of multidrug-resistant (MDR) and extended-spectrum β -lactamase (ESBL)-producing *Klebsiella pneumoniae* poses a serious antibiotic management problem as resistance genes are easily transferred from one organism to another. Fifty-one strains of *K. pneumoniae* isolated from sporadic cases in various hospitals throughout Malaysia were analysed by antimicrobial susceptibility testing, PCR detection of ESBL-encoding genes and DNA fingerprinting. Although 27 of the 51 *K. pneumoniae* strains were MDR (i.e. resistant to three or more classes of antibiotics), the majority of the strains (98%) were sensitive to imipenem. PCR detection using ESBL gene-specific primers showed that 46 of the *K. pneumoniae* strains harboured *bla*_{SHV}, 19 harboured *bla*_{CTX-M}, 5 harboured *bla*_{OXA-1} and 4 harboured *bla*_{TEM-1}. Class 1 integron-encoded *intI1* integrase was detected in 21 of the 51 *K. pneumoniae* strains and amplification of the integron 5'CS region showed the presence of several known antibiotic resistance gene cassettes of various sizes. Results of conjugation and transformation experiments indicated that some of the ESBL-encoding genes (i.e. *bla*_{SHV}, *bla*_{CTX-M} and *bla*_{TEM-1}) were transmissible and were likely plasmid-encoded. DNA fingerprinting using PFGE and PCR-based methods indicated that the 51 *K. pneumoniae* strains were genetically diverse and heterogeneous.

Received 23 March 2009

Accepted 9 July 2009

INTRODUCTION

Klebsiella pneumoniae is an important bacterial pathogen associated with community and nosocomial infections especially in immunocompromised patients (Podschun & Ullmann, 1998). *K. pneumoniae* strains have the potential to cause severe morbidity and mortality, particularly in intensive care units and paediatric and surgical wards (Ben-Hamouda *et al.*, 2003).

Abbreviations: DDST, double-disc synergy test; ERIC-PCR, enterobacterial repetitive intergenic consensus PCR; ESBL, extended-spectrum β -lactamase; MDR, multidrug resistant; RAPD, random amplification of polymorphic DNA; REP-PCR, repetitive-sequence-based PCR.

A table listing the primers and PCR cycling parameters is available with the online version of this paper.

The emergence of extended-spectrum β -lactamase (ESBL) producers along with multidrug-resistant (MDR) isolates poses a serious problem in the hospital setting. Since the first report of ESBL-producing *Klebsiella* in 1983 from a patient in Germany, several outbreaks caused by this pathogen have been reported worldwide (Bradford, 2001; Ben-Hamouda *et al.*, 2003; Jarlier *et al.*, 1988; Livermore *et al.*, 2007; Romero *et al.*, 2007).

The widespread use of antibiotics coupled with the transmissibility of resistance determinants mediated by plasmids, transposons and gene cassettes in integrons are factors that contribute to the increase in antibiotic resistance in bacterial pathogens (Kang *et al.*, 2005). In Malaysia, Palasubramaniam *et al.* (2005) reported an association of ESBL SHV-5 *K. pneumoniae* with a nosocomial outbreak in a paediatric oncology unit in a Malaysian public hospital.

Rapid and discriminative subtyping methods are useful for determining the clonality of the strains in nosocomial outbreaks. Several methods are available for *K. pneumoniae* subtyping and these include biotyping, serotyping, arbitrarily primed PCR, ribotyping and PFGE (Ben-Hamouda *et al.*, 2003; Cartelle *et al.*, 2004; Singh *et al.*, 2006).

The objectives of this study were to determine the antimicrobial resistance profiles of *K. pneumoniae* strains isolated from various hospitals in Malaysia and to study their genetic diversity using PFGE as well as the PCR-based fingerprinting methods random amplification of polymorphic DNA (RAPD), repetitive-sequence-based (REP-PCR) and enterobacterial repetitive intergenic consensus PCR (ERIC-PCR). The presence of several ESBL-encoding genes and integrons was also determined by PCR and their transmissibility was examined by conjugation and transformation experiments.

METHODS

Bacterial strains. The bacterial strains selected in this study were 51 non-repeat *K. pneumoniae* strains from 51 different patients obtained from five public hospitals located in Peninsular Malaysia in the year 2004. The participating hospitals included Kota Bharu Hospital ($n=25$), Ipoh Hospital ($n=3$), Kuala Lumpur Hospital ($n=1$), Tuanku Ampuan Rahimah Hospital ($n=2$) and Sultanah Aminah Hospital ($n=20$). The strains were cultured from tracheal aspirates ($n=21$), urine ($n=2$), sputum ($n=1$), catheter tips ($n=2$), blood ($n=1$), pus ($n=1$) and swab samples ($n=3$); there were 20 unknown cultures. The 20 unknown cultures were tagged as such because the exact culture sites were not stated by the respective hospitals and should thus be noted as a limitation for this study. The strains were identified by standard biochemical methods by the microbiologists from the respective hospitals.

All strains were cultured in Luria–Bertani broth and stored in cryovials with 50 % glycerol (Invitrogen) at -20°C and -85°C . The purity of the strains was checked before commencement of analysis.

Antimicrobial susceptibility testing and screening for ESBLs.

The susceptibilities of the *K. pneumoniae* strains to 18 antimicrobial agents [ampicillin (10 μg), piperacillin (100 μg), amoxicillin–clavulanic acid (20 $\mu\text{g}/10\ \mu\text{g}$), ceftriaxone (30 μg), ceftazidime (30 μg), cefepime (30 μg), cefoperazone (75 μg), aztreonam (30 μg), imipenem (10 μg), amikacin (30 μg), streptomycin (10 μg), gentamicin (10 μg), kanamycin (30 μg), tetracycline (30 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), trimethoprim–sulfamethoxazole (75 μg) and nalidixic acid (30 μg) (Oxoid)] were determined by the disc diffusion method in accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2006).

All strains were screened for ESBL production using ceftriaxone, ceftazidime and aztreonam discs as recommended by the CLSI (2006). Double-disc synergy tests (DDSTs) were performed according to Jarlier *et al.* (1988) using ceftazidime, ceftriaxone, aztreonam, cefepime and amoxicillin–clavulanic discs. A phenotypic confirmatory test was performed with 30 μg ceftazidime [30/10 μg ceftazidime–clavulanic acid, 30/10 μg cefotaxime–clavulanic acid (Becton Dickinson)] and 30 μg cefotaxime (Oxoid) discs on Mueller–Hinton agar. The results were interpreted as described by the CLSI (2006). *Escherichia coli* strains ATCC 25922 and ATCC 35218, *K. pneumoniae* ATCC 700603 and *Pseudomonas aeruginosa* ATCC 27853 were used as

quality control strains for the antibiotic susceptibility test as recommended by the CLSI (2006).

PCR detection of ESBL-encoding *bla*_{TEM}, *bla*_{SHV}, *bla*_{OXA}, *bla*_{CTX-M}, *bla*_{DHA}, *bla*_{IMP}, *bla*_{VIM} and *bla*_{VEB} genes as well as integron-associated genes. A boiled suspension of bacterial cells and extracted plasmid DNA were used as DNA templates. Primers for the detection of ESBL-encoding genes (obtained from Operon Biotechnologies) were applied to the DNA template. Amplification was performed in a final volume of 25 μl containing 0.3 μM each primer, 35 μM each dNTP, 1.4 mM MgCl_2 and 0.5 U *Taq* DNA polymerase (Promega). The cycling parameters used were as previously described (Oliver *et al.*, 2002; Pagani *et al.*, 2003; Pai *et al.*, 2004; Jiang *et al.*, 2006; Giakkoupi *et al.*, 2003; Senda *et al.*, 1996; Machado *et al.*, 2005) (please refer to Supplementary Table S1 in JMM Online for a list of primers and cycling parameters). Primers 1R, 18F, 2F, 2R, 8R, 9F and 9R (Ensor *et al.*, 2007) were used for the further subgrouping of the *bla*_{CTX-M} gene into CTX-M groups 1, 2, 8/25 and 9 whereas primers CTX-M1R and CTX-M1F (Conceição *et al.*, 2005) were used to confirm the presence of *bla*_{CTX-M-15} by sequencing the resulting 876 bp amplicon. Detection of class 1, 2 and 3 integron-encoded integrases and associated gene cassettes was similarly carried out by PCR using established primers and amplification conditions listed in Supplementary Table S1. Amplified products were sequenced to validate their identity.

E. coli ATCC 352183 and *K. pneumoniae* ATCC 700603 were used as positive controls for *bla*_{TEM} and *bla*_{SHV}. However, no positive controls were available for the detection of *bla*_{OXA}, *bla*_{VEB}, *bla*_{DHA}, *bla*_{IMP}, *bla*_{VIM} and *bla*_{CTX-M} genes as well as for the further subgrouping of the CTX-M group. Therefore, negative results should be treated with caution.

Genotyping by RAPD, REP-PCR, ERIC-PCR and PFGE. RAPD analysis was performed using the primer OPAB11 (Latha *et al.*, 2004). The amplification reaction was carried out in a 25 μl volume containing 2.5 mM MgCl_2 , 50 μM each deoxynucleoside triphosphate, 0.3 μM of the selected primer, 5 μl crude DNA template and 1.5 U *Taq* DNA polymerase (Promega). ERIC- and REP-PCR analyses were performed using the ERIC-2 (de Bruijn, 1992) and REP (Navia *et al.*, 1999) primers, respectively. Amplification reactions were carried out in a 25 μl volume containing 2.5 mM MgCl_2 , 50 μM each deoxynucleoside triphosphate, 0.5 μM of the selected primer, 5 μl crude DNA template and 1.0 U *Taq* DNA polymerase (Promega). Following PCR, 10 μl aliquots of each sample were subjected to electrophoresis on 1.5 % agarose gels.

PFGE was performed according to previously published work (Thong *et al.*, 2007) with minor variations. Briefly, equal volumes of 1 % Seakem Gold agarose (Cambrex Bio Science) and standardized cell suspension ($\text{OD}_{610}=1.4$) were mixed to form agarose plugs. The bacteria were lysed within the plugs with cell lysis buffer [50 mM Tris, 50 mM EDTA (pH 8.0), 1 % Sarcosine, 1 mg proteinase K ml^{-1}] and incubated at 54°C for 3 h. The agarose-embedded chromosomal DNA was digested with 10 U *Xba*I followed by separation on a CHEF-DRIII system (Bio-Rad) in $0.5 \times$ Tris-borate-EDTA at 14°C for 22 h with pulse times of 1–40 s. Gels were photographed under UV light after staining with 0.5 μg ethidium bromide ml^{-1} .

Analysis of fingerprint profiles. The banding patterns generated by RAPD, ERIC-PCR, REP-PCR and PFGE were analysed with GelCompar II, version 2.5 (Applied Maths), and a cluster analysis based on the unweighted pair group method with arithmetic averages with a position tolerance of 0.15 was carried out. All PCR fingerprints and PFGE profiles were assigned an arbitrary designation and analysed by defining a similarity (Dice) coefficient, *F* (Thong *et al.*, 2007).

Transfer of resistance determinants and plasmid analysis.

Transfer of resistance genes by conjugation was assayed by mating experiments in Luria–Bertani broth using *E. coli* JM109 [*endA1*, *recA1*, *gyrA96*, *thi*, *hsdR17* (r_k^- , m_k^+), λ^- , *relA1*, *supE44*, $\Delta(lac-proAB)$, (*F'*, *traD36*, *proAB*, *lacI^qZAM15*)] (Promega), which is resistant to nalidixic acid, as the recipient strain. Transconjugants were selected on Luria–Bertani agar supplemented with ampicillin (100 $\mu\text{g ml}^{-1}$) plus nalidixic acid (100 $\mu\text{g ml}^{-1}$) (Sigma Aldrich).

For transformation experiments, plasmid DNA from the ESBL-producing *K. pneumoniae* strains obtained using the QIAprep Spin Miniprep kit (Qiagen) were electroporated into streptomycin-resistant *E. coli* DH10B (Invitrogen). Transformants were selected on Luria–Bertani agar plates supplemented with ampicillin (100 $\mu\text{g ml}^{-1}$).

For the estimation of plasmid sizes, plasmid DNA obtained from *K. pneumoniae* strains and *E. coli* transconjugant as well as transformants was digested with *EcoRI* or *SphI* (Promega). Digested plasmid DNA was separated on a 0.8 % agarose gel in 0.5 × Tris-borate-EDTA for 3 h at 70 V. A 1 kb DNA ladder (Promega) and λ DNA digested with *HindIII* (Promega) were used as DNA markers.

RESULTS AND DISCUSSION

Antimicrobial susceptibility

All 51 *K. pneumoniae* strains tested were sensitive to imipenem, except strain KB10, which showed intermediate resistance to imipenem. The majority (49 of 51, or 96 %) of the strains showed resistance to ampicillin. The resistance rates for the other antibiotics tested using the disc diffusion method are listed in Table 1. Previously published studies on Malaysian *K. pneumoniae* isolates (Loh *et al.*, 2007; Parasakthi *et al.*, 2000) did not include some of the antibiotics used in this study such as piperacillin, cefepime, aztreonam, tetracycline, streptomycin, kanamycin, chlor-

amphenicol and nalidixic acid. However, a similar trend of very high and very low resistance rates was observed for ampicillin and imipenem, respectively. This is also in concordance with the latest 2008 Malaysian National Surveillance on Antibiotic Resistance Report (available online at <http://www.imr.gov.my/report/nsar.htm>), which stated a very low imipenem resistance rate of 0.5 % and a very high ampicillin resistance rate of 98.8 %, which is not surprising as *Klebsiella* is known to be intrinsically resistant to ampicillin.

The resistance rates for gentamicin, amikacin and amoxicillin–clavulanic acid were, however, lower than those in the earlier studies on *K. pneumoniae* strains from Malaysia in 1999 (Parasakthi *et al.*, 2000) and 2000–2004 (Loh *et al.*, 2007). The strains used in this study were isolated from sporadic cases, in contrast to the strains used by Parasakthi *et al.* (2000), who investigated only nine outbreak strains isolated from a local University Hospital; all nine strains were reported to be resistant to gentamicin, amikacin and amoxicillin–clavulanic acid. Loh *et al.* (2007) reported the resistance rates from another public Malaysian hospital but over a 4-year period. They showed that the resistance rate for combined amoxicillin–clavulanic acid and ampicillin–sulbactam was 58.4 % whereas the resistance rate for aminoglycosides (combined gentamicin and amikacin) was 42.5 %. These figures are in sharp contrast to the rates of 10 %, 31 % and 12 % obtained for amoxicillin–clavulanic acid, gentamicin and amikacin, respectively, in this study.

The resistance rates of *K. pneumoniae* to the third-generation cephalosporins ceftazidime and ceftriaxone were lower than those in the study carried out in 1999 (Parasakthi *et al.*, 2000) but were higher than those in the study carried out for the years 2000–2004 (Loh *et al.*,

Table 1. Antimicrobial susceptibility profiles of all 51 *K. pneumoniae* isolates used in this study

Antimicrobial	Sensitive [<i>n</i> (%)]	Intermediate [<i>n</i> (%)]	Resistant [<i>n</i> (%)]
Amikacin	37 (73)	8 (15)	6 (12)
Ampicillin	0	2 (4)	49 (96)
Amoxicillin + clavulanic acid	27 (53)	19 (37)	5 (10)
Aztreonam	26 (51)	4 (8)	21 (41)
Cefepime	44 (86)	2 (4)	5 (10)
Cefoperazone	25 (49)	11 (22)	15 (29)
Ceftazidime	26 (51)	4 (8)	21 (41)
Ceftriaxone	28 (55)	5 (10)	18 (35)
Chloramphenicol	35 (69)	3 (6)	13 (25)
Ciprofloxacin	42 (82)	3 (6)	6 (12)
Gentamicin	35 (69)	0	16 (31)
Imipenem	50 (98)	1 (2)	0
Kanamycin	27 (53)	6 (12)	18 (35)
Nalidixic acid	27 (53)	6 (12)	18 (35)
Piperacillin	8 (15)	10 (20)	33 (65)
Tetracycline	36 (70)	4 (8)	11 (22)
Trimethoprim + sulfamethoxazole	35 (69)	1 (2)	15 (29)
Streptomycin	20 (39)	16 (31)	15 (29)

2007). Loh *et al.* (2007) reported fluctuating resistance rates of 18–21% to third-generation cephalosporins (combined ceftazidime, ceftriaxone and cefotaxime). The 2008 Malaysian National Surveillance on Antibiotic Resistance Report recorded the resistance rates to ceftazidime and ceftriaxone as 22.3% and 23.4%, respectively (<http://www.imr.gov.my/report/nsar.htm>). In contrast, our study showed resistance rates for ceftazidime and ceftriaxone of 41% and 35%, respectively.

The resistance rate of *K. pneumoniae* strains to ciprofloxacin in Malaysia is still relatively low (12%) when compared to Thailand, where a rate of 43% had been reported (Chaikittisuk & Munsrichoom, 2007). The resistance rates of the Malaysian *K. pneumoniae* strains to amikacin, chloramphenicol and trimethoprim-sulfamethoxazole were also lower than those of strains isolated from Thailand (resistance rates of 58%, 45% and 74%, respectively; Chaikittisuk & Munsrichoom, 2007).

Among the 51 *K. pneumoniae* strains, 27 were MDR (i.e. resistant to three or more classes of antimicrobial agents). The rate of MDR *K. pneumoniae* strains in Malaysia is relatively low (53%) when compared to the 94% MDR rate reported in India (Manchanda *et al.*, 2005).

Using the DDST, only 12 ESBL-producing strains were detected. However, based on the phenotypic confirmatory test, 24 strains were found to be ESBL producers. The phenotypic confirmatory test was found to be more efficient in determining ESBLs than the DDST. Although the DDST was considered a reliable method for ESBL detection (Tofteland *et al.*, 2007), it has been known to suffer from the non-standardization of the distance of disc placement for optimal sensitivity leading to variation from one laboratory to another (Bradford, 2001). Large discrepancies between the different ESBL detection methods were observed and could partly be due to problems in determining the zone of inhibition, especially for cephalosporins, where strains that were noted as negative nevertheless had zones of inhibition which fell outside the range determined by the CLSI. Tofteland *et al.* (2007) had previously reported discrepancies between different detection methods where two TEM-128 strains, which had a CTX-M9-like phenotype, were found to score negative using the ceftazidime combined disc method.

PCR detection of ESBL-encoding genes

Using specific primers, PCR was carried out on the genomic and plasmid DNA of the 51 *K. pneumoniae* strains for the following ESBL-encoding genes: *bla*_{TEM}, *bla*_{SHV}, *bla*_{OXA}, *bla*_{CTX-M}, *bla*_{VEB}, *bla*_{IMP}, *bla*_{VIM} and *bla*_{DHA}. The majority of the strains (46 of 51) showed positive amplification for *bla*_{SHV}. This was followed by *bla*_{CTX-M} (positive in 19 of 51 strains), *bla*_{OXA} (5 strains) and *bla*_{TEM} (4 strains). Palasubramaniam *et al.* (2005) had previously reported the prevalence of the *bla*_{SHV} gene in *K. pneumoniae* strains isolated from a Malaysian hospital.

Thus, *bla*_{SHV} appeared to be more common among Malaysian *K. pneumoniae* strains even though *bla*_{CTX-M} is now reported to be the most prevalent ESBL gene worldwide, replacing TEM and SHV β -lactamases in many European countries (Livermore *et al.*, 2007).

Sequencing of ten of the *bla*_{SHV} amplicons identified eight as SHV-11, one as SHV-12 and the remaining amplicon as SHV-1. Sequence analysis of all four *bla*_{TEM} amplicons identified them as TEM-1 whereas all five *bla*_{OXA} amplicons were identified as OXA-1. In the case of *bla*_{CTX-M}, sequences obtained identified 6 of the 19 amplified products as CTX-M-15. It was, however, not possible to group the remaining 13 *bla*_{CTX-M} amplicons obtained as the primers that were used did not amplify the entire *bla*_{CTX-M} reading frame. The subsequent use of primers 1R, 1,8F, 2F, 2R, 8R, 9F and 9R (Ensor *et al.*, 2007) to further subgroup the 13 *bla*_{CTX-M} genes could only indicate that they did not belong to groups 1, 2, 8/25 and 9.

Out of the 46 *bla*_{SHV}-positive strains, 16 also harboured *bla*_{CTX-M} and 2 were positive for *bla*_{OXA-1}. Two strains, KB16 and KB35, had three ESBL-encoding genes, i.e. *bla*_{SHV}, *bla*_{OXA-1} and *bla*_{CTX-M-15}. The presence of multiple ESBL-encoding genes in *K. pneumoniae* has been reported by Romero *et al.* (2007), and Livermore *et al.* (2007) had indicated that *bla*_{CTX-M-15} was genetically linked with *bla*_{OXA-1}.

No amplification product was obtained with primers that were specific for the *bla*_{VEB}, *bla*_{IMP} and *bla*_{DHA} genes. The majority of the ESBL-encoding genes were detected from plasmid DNA (i.e. 40 *bla*_{SHV}, 6 *bla*_{CTX-M} and 3 *bla*_{OXA}), inferring that most of the ESBL genes were plasmid-borne. PCR amplifications with extracted plasmid DNA as template were carried out in parallel with additional 16S rRNA primers to preclude chromosomal DNA contamination.

Using PCR, class 1 integron-encoded *intI1* integrases were found in 21 of the 51 *K. pneumoniae* strains whereas neither class 2 nor class 3 integron-encoded integrases were detected. In 12 of the 21 *intI1*-positive strains, the gene cassettes *aadB* (in 7 isolates), *aadA2-dhfrI* (in 2 isolates) and *aadA2-orfF-dhfrXII* (in 3 isolates) were amplified and identified by sequencing, with *aadB* encoding resistance to gentamicin, kanamycin and tobramycin, *aadA2* encoding resistance to streptomycin, and *dhfrXII* encoding resistance to trimethoprim (Daikos *et al.*, 2007).

Transfer of resistance determinants

Conjugation experiments were performed for four nalidixic acid-sensitive *K. pneumoniae* strains with *E. coli* JM109 as recipient. Plasmid transfer via conjugation with selection for the ESBL phenotype to *E. coli* JM109 was successful for only one out of four strains. The resulting single *E. coli* transconjugant showed resistance to aztreonam as well as gentamicin and was confirmed as an ESBL producer using the DDST. PCR of DNA extracted from the

Table 2. Transmissibility of antibiotic resistance determinants: antibiograms and the presence of ESBL-encoding genes in the donor *K. pneumoniae* strains and the resulting *E. coli* transconjugants and transformants

<i>K. pneumoniae</i> strain no.	Resistance profile* of <i>K. pneumoniae</i> donor	ESBL gene(s) detected in <i>K. pneumoniae</i>	Mode of transmission	Resistance profile* of <i>E. coli</i> transformant/transconjugant	ESBL gene(s) transferred
KB36	AMP, PIP, CAZ, CRO, CFP, ATM, KAN, GEN	<i>bla</i> _{SHV} , <i>bla</i> _{TEM}	Conjugation	AMP, ATM, GEN	<i>bla</i> _{SHV} , <i>bla</i> _{TEM}
KB1	AMP, PIP, TET, KAN, GEN, CIP, NAL	<i>bla</i> _{SHV} , <i>bla</i> _{CTX-M}	Transformation	AMP, PIP, TET	<i>bla</i> _{SHV} , <i>bla</i> _{CTX-M}
KB5	AMP, PIP, CAZ, ATM, TET, KAN, GEN, AMK	<i>bla</i> _{SHV}	Transformation	AMP, PIP, TET, CHL	<i>bla</i> _{SHV}
KB21	CRO, AMP, NAL, PIP, AMK, ATM, CFP, CAZ, KAN, GEN, TET, CHL, SXT, NAL	<i>bla</i> _{SHV}	Transformation	AMP, PIP, GEN, TET, CHL	<i>bla</i> _{SHV}
KB22	AMP, PIP, CAZ, CRO, TET, STR, KAN, GEN, CIP, CHL, SXT, NAL	<i>bla</i> _{SHV}	Transformation	AMP, PIP, TET, GEN, CIP, NAL	<i>bla</i> _{SHV}
KB24	AMP, PIP, CAZ, CRO, CFP, ATM, TET, KAN, GEN, AMK, CHL, SXT, NAL	<i>bla</i> _{TEM}	Transformation	AMP, PIP, CHL	<i>bla</i> _{TEM}
KB35	AMP, PIP, CAZ, CRO, FEP, CFP, ATM, STR, GEN, CHL, NAL	<i>bla</i> _{SHV} , <i>bla</i> _{CTX-M} , <i>bla</i> _{OXA}	Transformation	AMP, PIP, CHL	<i>bla</i> _{SHV}
KB45	AMP, PIP, CAZ, CRO, FEP, STR, KAN, GEN, CHL, SXT, NAL, ATM	<i>bla</i> _{SHV} , <i>bla</i> _{CTX-M}	Transformation	AMP, PIP, CHL, SXT, NAL	<i>bla</i> _{SHV} , <i>bla</i> _{CTX-M}
KB50	AMP, PIP, TET, STR, GEN, CIP, SXT, NAL	<i>bla</i> _{SHV}	Transformation	AMP, PIP, TET, SXT	<i>bla</i> _{SHV}

*AMC, amoxicillin–clavulanic acid; AMK, amikacin; AMP, ampicillin; ATM, aztreonam; CAZ, ceftazidime; CFP, cefoperazone; CHL, chloramphenicol; CIP, ciprofloxacin; CRO, ceftriaxone; FEP, cefepime; GEN, gentamicin; KAN, kanamycin; NAL, nalidixic acid; PIP, piperacillin; STR, streptomycin; SXT, trimethoprim–sulfamethoxazole; TET, tetracycline.

E. coli transconjugant showed that *bla*_{TEM-1}, *bla*_{SHV} and *intI1* genes were successfully transferred to the transconjugant (Table 2).

Transformation studies were performed for ten *K. pneumoniae* strains that harboured plasmids. Plasmid transfer of the ESBL phenotype by electroporation to *E. coli* DH10B was successful in eight out of the ten strains. All resulting DH10B transformants were resistant to β -lactam antibiotics (ampicillin and piperacillin). The majority of the transformants (five out of eight) were also resistant to tetracycline and chloramphenicol, indicating that these resistance determinants were likely plasmid-encoded and were thus co-transferred (Table 2).

Most of the ESBL-encoding genes detected in the parental *K. pneumoniae* strains were successfully transferred into the recipient *E. coli* DH10B, except for the *bla*_{SHV} and *bla*_{OXA-1} genes in strain KB35.

Both conjugation and transformation experiments had indicated that some of the ESBL-encoding genes (in particular *bla*_{SHV}) were plasmid-encoded and transmissible. The results indicated the high mobility of the ESBL-encoding genes in *K. pneumoniae* in agreement with observations made by Chanawong *et al.* (2002).

Preliminary plasmid analysis results indicated that 11 of the *K. pneumoniae* strains may not harbour any plasmids. Plasmids of sizes ranging from 3 to 310 kb were, however, detected in the other 40 *K. pneumoniae* strains and efforts are on-going to further characterize these plasmids, in particular those from the MDR strains.

Genotyping by PCR and PFGE

The genetic relatedness of the 51 *K. pneumoniae* strains was determined by PFGE as well as the PCR-based RAPD, ERIC-PCR and REP-PCR. With RAPD using the OPAB11 primer, the 51 strains were subtyped into 49 unique profiles ($F=0.30-1.0$). Subtyping using ERIC- and REP-PCR resulted in 46 ($F=0.47-1.0$) and 50 ($F=0.50-1.0$) different profiles, respectively. Genomic DNA from 49 *K. pneumoniae* strains that was digested with *Xba*I and separated by PFGE resulted in 47 distinct pulsed-field profiles ($F=0.54-1.0$). Two other *K. pneumoniae* strains, KB42 and KB49, could not be typed by PFGE despite repeated attempts.

Thus, genotyping of the 51 *K. pneumoniae* clinical strains by ERIC-PCR, RAPD, REP-PCR and PFGE showed that they were genetically diverse and heterogeneous. This was expected as the *K. pneumoniae* strains in this study were isolated from different places and sources and probably belong to various serotypes. Ben-Hamouda *et al.* (2003) and Pai *et al.* (2004) also reported similar heterogeneity among *K. pneumoniae* clinical strains.

In general, no direct correlation between DNA profiles and antibiotic susceptibility patterns was observed. Strains with identical DNA profiles frequently belonged to different

antibiotypes. The Malaysian MDR *K. pneumoniae* clinical strains studied were genetically very diverse and heterogeneous suggesting that multiple subtypes of the species were involved in infection. Carbapenemase production was not evident in all strains studied. Although *bla*_{SHV} appeared to be the predominant ESBL-encoding gene detected in Malaysia, *bla*_{CTX-M} is gaining in importance. This study also showed the potential role of integrons and plasmids in the spread of ESBL-encoding genes and other antibiotic resistance genes in *K. pneumoniae*.

ACKNOWLEDGEMENTS

This work was partially funded by grants from the Malaysian Toray Science Foundation, the Malaysian Ministry of Science, Technology and Innovation (MOSTI) and the University of Malaya. We thank Nurahan Maning (Kota Bharu Hospital), Tan Eng Am (Ipoh Hospital) and Zubaidah Abdul Wahab (Kuala Lumpur Hospital) for kindly providing strains for this study. K. T. L. was supported by NSF from MOSTI.

REFERENCES

- Ben-Hamouda, T., Foulan, T., Ben-Cheikh-Masmoudi, A., Fendri, C., Belhadi, O. & Ben-Mahrez, K. (2003). Molecular epidemiology of an outbreak of multiresistant *Klebsiella pneumoniae* in a Tunisian neonatal ward. *J Med Microbiol* **52**, 427–433.
- Bradford, P. A. (2001). Extended-spectrum β -lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat. *Clin Microbiol Rev* **14**, 933–951.
- Cartelle, M., Tomas, M. D. M., Pertega, S., Beceiro, A., Dominguez, M. A., Velasco, D., Molina, F., Villanueva, R. & Bou, G. (2004). Risk factors for colonization and infection in a hospital outbreak caused by a strain of *Klebsiella pneumoniae* with reduced susceptibility to extended-spectrum cephalosporins. *J Clin Microbiol* **42**, 4242–4249.
- Chaikittisuk, N. & Munsrichoom, A. (2007). Extended-spectrum β -lactamase producing *Escherichia coli* and *Klebsiella pneumoniae* in children at Queen Sirikit National Institute of Child Health. *J Infect Dis Antimicrob Agents* **24**, 107–115.
- Chanawong, A., M'Zali, F. H., Heritage, J., Xiong, J. H. & Hawkey, P. M. (2002). Three cefotaximes, CTX-M-9, CTX-M-13, and CTX-M-14, among *Enterobacteriaceae* in the People's Republic of China. *Antimicrob Agents Chemother* **46**, 630–637.
- CLSI (2006). *Performance Standards for Antimicrobial Susceptibility Testing*, 16th Informational Supplement. Wayne, PA: Clinical and Laboratory Standards Institute.
- Conceição, T., Brizio, A., Duarte, A., Lito, L. M., Melo Cristino, J. & Salgado, M. J. (2005). First description of CTX-M-15-producing *Klebsiella pneumoniae* in Portugal. *Antimicrob Agents Chemother* **49**, 477–478.
- Daikos, G. L., Kosmidis, C., Tassios, P. T., Petrikos, G., Vasilakopoulou, A., Psychogiou, M., Stefanou, I., Avlami, A. & Katsilambros, N. (2007). *Enterobacteriaceae* bloodstream infections: presence of integrons, risk factors and outcome. *Antimicrob Agents Chemother* **51**, 2366–2372.
- de Bruijn, F. J. (1992). Use of repetitive (repetitive extragenic palindromic and enterobacterial repetitive intergeneric consensus) sequences and the polymerase chain reaction to fingerprint the genomes of *Rhizobium meliloti* isolates and other soil bacteria. *Appl Environ Microbiol* **58**, 2180–2187.

- Ensor, V. M., Livermore, D. M. & Hawkey, P. M. (2007). A novel reverse-line hybridization assay for identifying genotypes of CTX-M-type extended-spectrum β -lactamases. *J Antimicrob Chemother* **59**, 387–395.
- Giakkoupi, P., Xanthaki, A., Kanelopoulou, M., Vlahaki, A., Mirigou, V., Kontou, S., Papafraggas, E., Malamou-Lada, H., Tzouveleakis, L. S. & other authors (2003). VIM-1 metallo- β -lactamase-producing *Klebsiella pneumoniae* in Greek hospitals. *J Clin Microbiol* **41**, 3893–3896.
- Jarlier, V., Nicolas-Chanoine, M. H., Fournier, G. & Philippon, A. (1988). Extended-broad spectrum β -lactamases conferring transferable resistance to newer β -lactam agents in *Enterobacteriaceae*: hospital prevalence and susceptibility patterns. *Rev Infect Dis* **10**, 867–878.
- Jiang, X., Zhang, Z., Li, M., Zhou, D., Ruan, F. & Lu, R. (2006). Detection of extended-spectrum β -lactamases in clinical strains of *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* **50**, 2990–2995.
- Kang, H. Y., Young, S. J., Jae, Y. O., Sung, H. T., Chui, H. C., Dong, C. M., Won, K. L., Yoo, C. L., Sung, Y. S. & other authors (2005). Characterization of antimicrobial resistance and class 1 integrons found in *Escherichia coli* strains from humans and animals in Korea. *J Antimicrob Chemother* **55**, 639–644.
- Latha, R., Suryanarayanan, T. S. & Swaminathan, M. S. (2004). Genetic diversity in *Acremonium endophytes* isolated from warm-season grasses as revealed by RAPD markers. *J Plant Biochem Biotechnol* **13**, 39–42.
- Livermore, D. M., Canton, R., Gniadkowski, M., Nordmann, P., Rossolini, G. M., Arlet, G., Ayala, J., Coque, T. M., Zdanowicz, I. K. & other authors (2007). CTX-M: changing the face of ESBLs in Europe. *J Antimicrob Chemother* **59**, 165–174.
- Loh, L. C., Chin, H. K., Chong, Y. Y., Jeyaratnam, A., Raman, S., Vijayasingham, P., Thayaparan, T. & Kumar, S. (2007). *Klebsiella pneumoniae* respiratory strains from 2000 to 2004 in a Malaysia hospital: characteristics and relation to hospital antibiotics consumption. *Singapore Med J* **48**, 813–818.
- Machado, E., Canton, R., Banquero, F., Galan, J. C., Rollan, A., Peixe, L. & Coque, T. M. (2005). Integron content of extended-spectrum β -lactamases producing *Escherichia coli* strains over 12 years in a single hospital in Madrid, Spain. *Antimicrob Agents Chemother* **49**, 1823–1829.
- Manchanda, V., Goyal, S. R., Kumar, A. & Thukral, S. S. (2005). Phenotypic characteristics of clinical strains of *Klebsiella pneumoniae* and evaluation of available phenotypic techniques for detection of extended spectrum beta-lactamases. *Indian J Med Res* **122**, 330–337.
- Navia, M. M., Capitang, L., Ruiz, J., Vargas, M., Urassa, H., Schellemerberh, D., Gascon, J. & Vila, J. (1999). Typing and characterization of mechanisms of resistance of *Shigella* spp. isolated from feces of children under 5 years of age from Ifakara, Tanzania. *J Clin Microbiol* **37**, 3113–3117.
- Oliver, A., Weigel, L. M., Rasheed, J. K., McGowan, J. E., Jr, Raney, P. & Tenover, F. C. (2002). Mechanisms of decreased susceptibility of cefpodoxime in *Escherichia coli*. *Antimicrob Agents Chemother* **46**, 3829–3836.
- Pagani, L., Amico, E. D., Migliavacca, R., Andrea, M. M. D., Giacobbone, E., Amicosante, G., Romero, E. & Rossolini, G. M. (2003). Multiple CTX-M extended-spectrum β -lactamases in nosocomial strains of *Enterobacteriaceae* from a hospital in Northern Italy. *J Clin Microbiol* **41**, 4264–4269.
- Pai, H., Kang, C. I., Byeon, J. H., Lee, K. D., Park, W. B., Kim, H. B., Kim, E. C., Oh, M. D. & Kang, W. C. (2004). Epidemiology and clinical features of bloodstream infections caused by AmpC-type β -lactamase-producing *Klebsiella pneumoniae*. *Antimicrob Agents Chemother* **48**, 3720–3728.
- Palasubramaniam, S., Subramaniam, G., Muniandy, S. & Parasakthi, N. (2005). SHV-5 extended-spectrum beta-lactamase from *Klebsiella pneumoniae* associated with a nosocomial outbreak in a paediatric oncology unit in Malaysia. *Int J Infect Dis* **9**, 170–172.
- Parasakthi, N., Vadivelu, J., Ariffin, H., Iyer, L., Palasubramaniam, S. & Arasu, A. (2000). Epidemiology and molecular characterization of nosocomially transmitted multidrug-resistant *Klebsiella pneumoniae*. *Int J Infect Dis* **4**, 123–128.
- Podschun, R. & Ullmann, U. (1998). *Klebsiella* spp. as nosocomial pathogens: epidemiology, taxonomy, typing methods, and pathogenicity factors. *Clin Microbiol Rev* **11**, 589–603.
- Romero, E. D. V., Padila, T. P., Hernandez, A. H., Grande, R. P., Vazquez, M. F., Garcia, I. G., Rodriguriz, J. A. G. & Bellido, J. L. M. (2007). Prevalence of clinical strains of *Escherichia coli* and *Klebsiella* spp. producing multiple extended-spectrum β -lactamases. *Diagn Microbiol Infect Dis* **59**, 433–437.
- Senda, K., Arakawa, Y., Ichiyama, S., Nakashima, K., Ito, H., Ohsuka, S., Shimokata, K., Kato, N. & Ohta, M. (1996). PCR detection of metallo- β -lactamase gene (*bla_{IMP}*) in Gram-negative rods resistant to broad-spectrum β -lactams. *J Clin Microbiol* **34**, 2909–2913.
- Singh, A., Goering, R. V., Simjee, S., Foley, S. L. & Zervos, M. J. (2006). Application of molecular techniques to the study of hospital infection. *Clin Microbiol Rev* **19**, 512–530.
- Thong, K. L., Lai, K. S., Puthuchear, S. D., Koh, Y. T., Ahmad, N. & Yasin, R. M. (2007). Subtyping of *Salmonella enterica* serovar Muenchen by pulsed-field gel electrophoresis, plasmid profiling and antimicrobial susceptibility testing. *Malay J Sci* **26**, 1–13.
- Tofteland, S., Haldorsen, B., Dahl, K. H., Simonsen, G. S., Steinbakk, M., Walsh, T. R., Sundsfjord, A. & the Norwegian ESBL Study Group (2007). Effects of phenotype and genotype on methods for detection of extended-spectrum- β -lactamase-producing clinical strains of *Escherichia coli* and *Klebsiella pneumoniae* in Norway. *J Clin Microbiol* **45**, 199–205.