Molecular mechanisms of β-lactam resistance in carbapenemase-producing *Klebsiella pneumoniae* from Sri Lanka

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Received 4 April 2014
Accepted 22 May 2014

Carbapenemases are increasingly important antimicrobial resistance determinants. Little is known about the carbapenem resistance mechanisms in Sri Lanka. We examined 22 carbapenem-resistant *Klebsiella pneumoniae* from Sri Lanka to determine their β-lactam resistance mechanisms. The predominant resistance mechanisms we detected in this study were OXA-181, NDM-1 carbapenemases and extended-spectrum β-lactamase CTX-M-15. All isolates were then genotyped by pulsed-field gel electrophoresis, variable-number tandem repeat sequence analysis and multilocus sequence typing, and seven distinct genotypes were observed. Five OXA-181-positive *Klebsiella pneumoniae* isolates were genotypically related to an isolate of Indian origin. Multilocus sequence typing found that these related isolates belong to ST-14, which has been associated with dissemination of OXA-181 from the Indian subcontinent. Other genotypes we discovered were ST-147 and ST-340, also associated with intercontinental spread of carbapenemases of suspected subcontinental origin. The major porin genes *ompK35* and *ompK36* from these isolates had insertions, deletions and substitutions. Some of these were exclusive to strains within single pulsotypes. We detected one *ompK36* variant, ins AA134-135GD, in six ST-14 and six ST-147, *bla*OXA-181-positive isolates. This porin mutation was an independent predictor of high-level meropenem resistance in our entire Sri Lankan isolate collection (\(P=0.0030\)). Analysis of the Sri Lankan ST-14 and ST-147 ins AA134-135GD-positive isolates found ST-14 was more resistant to meropenem than other isolates (mean MIC: 32 ± 0 μg ml\(^{-1}\) and 20 ± 9.47 μg ml\(^{-1}\), respectively, \(P=0.0277\)). The likely international transmission of these carbapenem resistance determinants highlights the need for regional collaboration and prospective surveillance of carbapenem-resistant *Enterobacteriaceae*.

INTRODUCTION

Carbapenemase-producing *Enterobacteriaceae* (CPE) have disseminated worldwide in a remarkably short time (Castanheira et al., 2011; Poirel et al., 2010). Transferable carbapenem-hydrolysing enzymes emerged in the late 1980s (Watanabe et al., 1991), and are represented by Ambler classes A, B and D. Each Ambler class uses a distinct mechanism for hydrolysis of the β-lactam ring (Queenan & Bush, 2007). Newer carbapenemases have evolved since the first report of IMP, but not all of these possess sufficient hydrolytic activity to confer carbapenem resistance (Poirel et al., 2012; Queenan & Bush, 2007). Weak carbapenem-hydrolysing enzymes require diffusion limitation to augment their function and confer a resistant phenotype (Dournih et al., 2007). The regulatory and genetic mechanisms that reduce carbapenem permeability include decreased porin expression, porin deletion and mutations that constrict the porin eyelet (Nikaido, 2003).

Hospital patients with indwelling medical devices are at increased risk of infection with resistant bacteria (Ben-Ami et al., 2009; Muñoz et al., 2001; Toubes et al., 2003). The lack of suitable antimicrobial agents for CPE infections results in
mortality rates of $\geq 50\%$ (Cuzon et al., 2011). Travellers returning from endemic regions such as the Indian subcontinent help transmit these organisms between countries (Decre et al., 2010; Yong et al., 2009). Though CPE are widely recognized in India, much less is known about the epidemiology and molecular mechanisms of carbapenem resistance in neighbouring Sri Lanka. In the present study we investigated the predominant types of carbapenem resistance in Enterobacteriaceae from Sri Lankan teaching hospital patients.

METHODS

Collection. CPE were prospectively collected between January and April 2012 in a Sri Lankan teaching hospital. These bacteria were isolated from patients in neurological trauma wards and were recovered from 19 tracheal secretions and one blood culture, after they produced a reduced imipenem inhibition zone on preliminary susceptibility testing (Benenson et al., 2011). A modified Hodge test was performed on isolates considered resistant to imipenem (Lee et al., 2001).

Organism Identification. The clinical isolate collection was transported to Western Australia and subcultured on arrival in accordance with our Australian Quarantine and Inspection Service import licence. Bacterial isolates were identified by matrix-assisted laser desorption ionization-time of mass spectrometry (MALDI-TOF, Microflex running Biotype RT version 3.1, Bruker Daltonics). Twenty-two distinct isolates were recovered from the 20 patient specimens, and all were identified as Klebsiella pneumoniae.

Antibiogram. Phenotypic resistance was determined by agar dilution (Wiegand et al., 2008) using the following antimicrobial agents in Mueller–Hinton agar (PathWest Media): amoxicillin with and without clavulanate, norfloxacin, ciprofloxacin, nitrofurantoin, trimethoprim, sulphamethoxazole, ceflothin, cefazolin, meropenem, gentamicin, tobramycin, amikacin (Wiegand et al., 2008) and cefazolin. All isolates were resistant to more than one class of antimicrobial agents and all were resistant to penicillin and caspofungin. All isolates were sensitive to amikacin. All isolates were susceptible to colistin. The clinical isolate collection was trans-ferred in carbapenem-resistant K. pneumoniae this work we encountered an OXA-181-producing isolate (designated K1) as an epidemiological outlier.

Genotypic characterization. We ran in-house PCR-based assays targeting sequences within blaNDM-1, blakPCP, blaoPB, blaaMP, and blaoK32 genes to determine whether carbapenem resistance was mediated by carbapenemases. We used previously described assays to detect blakTX-M3, blakK32, (Fang et al., 2008), blaoAZV, blaoTEM (Dallenne et al., 2010), plasmid-borne AmpC enzymes (Pérez-Pérez & Hanson, 2002) blaaMP (Senda et al., 1996) blaoVIM (Tsakris et al., 2000) and ompK35, ompK36 (Landman et al., 2009) indicators. All products were sequenced and identified by National Center for Biotechnology Information BLAST.

Genotyping. We conducted DNA macrorestriction analysis of our isolate collection using XbaI as the restriction enzyme (Maslow et al., 1993), and variable-number tandem repeat (VNTR) (Turton et al., 2010). We analysed the results with proprietary software (BioNumerics version 6.5) using 80% as the cut-off threshold for clustering. Multilocus sequence typing was performed on all isolates following macro-restriction analysis (Diancourt et al., 2005). One year prior to this work we encountered an OXA-181-producing K. pneumoniae in a patient repatriated to Australia from the Indian subcontinent after intensive care unit admissions in Kathmandu and New Delhi, and included this isolate (designated K1) as an epidemiological outlier.

Bioinformatics. We assembled gene sequences, created alignments and reconstructed neighbour-joining trees with MauveWorkbench 6.9.1 (CLC bio). Neighbour-joining trees were reconstructed from in-silico translated sequence data using the Kimura Protein algorithm with 100 bootstrapping replicates.

Ethics. Approval for the study was granted by the Ethical Review Committee, Faculty of Medicine, University of Kelaniya, Ragama.

RESULTS

The isolates used in this study all came from patients in accident and trauma wards of a large teaching hospital and were confirmed by conventional bacteriological methods and MALDI-TOF mass spectrometry as K. pneumoniae. Apart from two isolates that were distinct strains, the isolates used in this study were from a series of different patients admitted to the acute wards of the hospital during a 3-month period. Three of the four isolates in a pulsed-field gel electrophoresis (PFGE) genotype cluster (KS9, 12, and 22) were admitted to the same emergency ward, and isolated from specimens collected at intervals of several weeks (10 January 2012, KS12; 4 February 2012, KS13; 1 March 2012, KS22 and 18 March 2012, KS9; at 7, 5 and 10 days after admission, respectively), indicating a degree of spatial and temporal clustering of closely related strains. However, VNTR genotype analysis indicated genotypic differences between these isolates and argues against a single environmental source.

All isolates were resistant to more than one class of antimicrobial agent, and all were resistant to penicillin/β-lactamase inhibitor combinations, first- and third-generation cephalosporins, cephamycins and monobactams. Twenty-one of the 22 strains analysed were resistant to meropenem. One isolate, KS1 was not resistant to meropenem according to the Clinical and Laboratory Standards Institute guidelines. Only one isolate (KS22) was sensitive to cefepime, and all isolates apart from KS1 and KS20 were sensitive to amikacin. All isolates were susceptible to colistin.

The blaNDM-1 and blaKPC-positive PCR assay control Ct values were 25.47 and 16.42, respectively. The $\beta$-l encountered in carbapenem-resistant K. pneumoniae Sri Lankan isolates included extended-spectrum $\beta$-l, AmpC, penicillinases and carbapenemases (Table 1). The K1 outlier isolate was genotypically close to five Sri Lankan isolates (Fig. 1).

We found substitution, insertion and deletion mutations in the Sri Lankan K. pneumoniae OmpK36 sequences. The mutations D48V, L58V, V207L, A221S, Q231S, ins AA234A, E237R, H240N and A285V were present in all tested Sri Lankan OmpK36 targets. A neighbour-joining tree showing the results of a Kimura protein analysis of OmpK36 amino acid sequences is shown in Fig. 2. A previously reported ins
AA134-135GD due to GGC GAC codon duplications was found in 12 (six ST-14- and six ST-147 blaOXA-181-positive isolates) of the 23 ompK36 genes tested, and was associated with a higher meropenem mean MIC (26 ± 8.94 µg ml⁻¹) than those lacking the insertion (12.07 ± 9.42 µg ml⁻¹; \( P = 0.0030 \)). AA134-135GD-positive isolates belonging to ST-14 were more resistant to meropenem (mean MIC: 26 ± 9.42 µg ml⁻¹; \( P = 0.0277 \)). Fewer mutations were observed in ompK35 sequences, with little variation from the reference sequences JQ781653 and GU945360. The epidemiological outlier K1 (ST-14) had a premature termination site at position 138.

**DISCUSSION**

The origin of both OXA-181 and NDM-1 carbapenemases is thought to be the Indian subcontinent (Castanheira et al., 2011). *K. pneumoniae* isolates expressing OXA-181 carry other carbapenemases including blaNDM-1 and blaVIM-5 in India (Castanheira et al., 2011; Potron et al., 2013). A subgroup of our Sri Lankan *K. pneumoniae* isolate collection is genotypically close but distinct from a *K. pneumoniae* clinical isolate ex-India, later identified as ST-14. Both Singapore and New Zealand have reported OXA-181 CPEs of likely Indian origin, *K. pneumoniae* multilocus sequence type 14 (Balm et al., 2013; Williamson et al., 2011). Other sequence types encountered in this study (ST-147 and ST-340) have recently been associated with the intercontinental dissemination of NDM-1 (Peirano et al., 2011). We recognize that, in the absence of independent corroboration, our data do not distinguish intercontinental transmission in one or other direction, and do not eliminate an external third source for CPE in either country.

Porin mutations that limit the diffusion of beta-lactams augment the protective effects of carbapenemases (Nikaido, 2003). We found insertions, deletions and substitutions present in genes encoding porins from Sri Lankan CPEs. Several substitutions were common to all ompK35 targets tested. Of interest, we found AA134-135GD in Sri Lankan CPEs in two multilocus sequence types. Isolates with ins AA134-135GD all exhibited a meropenem MIC ≥ 12 µg ml⁻¹ and were significantly more resistant than those without. Our findings agree with previous studies suggesting that an ins AA134-135GD mutation predicts high-level carbapenem resistance (Clancy et al., 2013). The insertion affects the third loop of OmpK36, resulting in an occluded porin eyelet (Alberti et al., 1995). Other insertions in this region were also associated with reduced carbapenem permeability, independent of porin expression levels (Clancy et al., 2013; García-Fernández et al., 2010). Mutations occurred with greater frequency in some positions of the OmpK36 peptide sequences. Phylogenetic analysis of the OmpK36 porin mutations produced a neighbour-joining tree resembling the DNA

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**Table 1.** \( \beta \)-Lactamase sequences encountered in Sri Lankan isolates, including extended-spectrum \( \beta \)-lactamases, AmpC, penicillinases and carbapenemases and corresponding E-strip MICs
Fig. 1. Genotypic relationships of carbapenem-resistant Sri Lankan *K. pneumoniae*. The dendrogram was generated from the PFGE profiles of each organism. An epidemiological outlier K1 (*), a known *bla*OXA-181-positive isolate found in a repatriated Western Australian intensive care patient from India, was included in the analysis. The number of repeats found at each VNTR locus, multilocus sequence type and isolate identification are included.

Fig. 2. Neighbour-joining tree of OmpK36 amino acid sequences. A Kimura protein algorithm with 100 bootstrapping replicates was used to generate the dendrogram. Of note, the neighbour-joining tree groupings resemble those of the PFGE phylodendrogram.

macrorestriction phylodendrogram, apart from the ST-15 isolates that grouped with either ST-340 or ST-14 isolates.

We encountered a surprising diversity of bacterial genotypes and antimicrobial resistance mechanisms in Sri Lankan *K. pneumoniae*, detecting *bla*NDM-1, *bla*OXA-181 and *bla*CTX-M-15 in this study. In the absence of systematic resistance surveillance data, it is unclear when, how and where carbapenem-resistant *Klebsiella* species first arrived in Sri Lanka. What is clear from this preliminary study is the importance of international collaboration in countering the increasing regional threat of carbapenem-resistant Enterobacteriaceae.
ACKNOWLEDGEMENTS

We thank Dr Philomena Chandrasiri of the National Hospital of Sri Lanka and Dr Malka Dassanayake of the Colombo North Teaching Hospital for providing access to clinical isolates. We would also like to thank our colleagues from PathWest Laboratory Medicine, in particular Dr Gerald Harnett, Dr Glenys Chidlow and Ms Julia Cattell for designing and validating the in-house PCR assays and Ms Lyn O’Reily for assistance with molecular typing.

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