Emergence of NDM-1- and IMP-14a-producing Enterobacteriaceae in Thailand

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Objectives: To detect carbapenemases in clinical isolates of Enterobacteriaceae collected from patients in a university hospital in Thailand between October 2010 and August 2011.

Methods: A total of 4818 Enterobacteriaceae isolates were screened for the presence of carbapenemases by ertapenem and imipenem disc diffusion tests. All positive screening isolates were subjected to modified Hodge test, phenylboronic acid– and EDTA–carbapenem combined disc tests and two multiplex PCRs of \textit{bla}_{IMP}, \textit{bla}_{VIM}, \textit{bla}_{SPM}, \textit{bla}_{SIM} and \textit{bla}_{GIM}, and of \textit{bla}_{KPC}, \textit{bla}_{NDM} and \textit{bla}_{OXA-48}. Carbapenemase-producing isolates were typed by PFGE and then characterized by antimicrobial susceptibility tests. Conjugation was performed using a broth culture mating method.

Results: Two isolates each of \textit{Escherichia coli}, \textit{Klebsiella pneumoniae} and \textit{Citrobacter freundii} produced NDM-1, whereas two other isolates of \textit{K. pneumoniae} produced IMP-14a. DNA fingerprints revealed that the metallo-\textit{b}-lactamase (MBL)-producing isolates were of different strains except for clonal strains of \textit{C. freundii}. In vitro transfer of carbapenem resistance was successful for the eight MBL-producing isolates. All MBL producers were susceptible to colistin and tigecycline. The six NDM-producing isolates were recovered from the urine of three patients, who had no history of travel outside Thailand. Interestingly, one patient had chronic urinary tract infections caused by a \textit{K. pneumoniae} strain and two strains of \textit{E. coli} producing NDM-1.

Conclusions: Surveillance of carbapenemases, particularly NDM-1, in Enterobacteriaceae is urgently needed to control and prevent the spread of these resistance determinants in our country.

Keywords: carbapenemases, metallo-\textit{b}-lactamases, \textit{Escherichia coli}, \textit{Klebsiella pneumoniae}, \textit{Citrobacter freundii}

Introduction

Carbapenem are recommended as the drugs of choice for the treatment of severe infections caused by multidrug-resistant Gram-negative bacilli including extended-spectrum \textit{b}-lactamase (ESBL)- and plasmid-mediated \textit{AmpC} \textit{b}-lactamase (\textit{pAmpC})-producing Enterobacteriaceae. Recently, carbapenem-non-susceptible Enterobacteriaceae by production of carbapenemases, particularly class \textit{A} \textit{Klebsiella pneumoniae} carbapenemase (KPC), class \textit{B} New Delhi metallo-\textit{b}-lactamase (NDM) and class \textit{D} OXA-48 carbapenemase, have emerged from many countries.¹,² The KPC was first reported in \textit{K. pneumoniae} from the USA in 1996 and was restricted within this country until the discovery of KPC-2 and KPC-3 from Israel in 2007.¹ Subsequently the spread of KPC among Enterobacteriaceae has been found in South America, Asia and Europe. On the other hand, a new subgroup of class \textit{B} or metallo-\textit{b}-lactamases (MBLs), NDM-1, was first identified in \textit{K. pneumoniae} and \textit{Escherichia coli} isolates from an Indian patient in Sweden in 2008.³ This enzyme has now been reported in members of family Enterobacteriaceae from many countries in Asia, North America, Europe and Australia.¹,² Besides the KPC and NDM, OXA-48, which was first reported in \textit{K. pneumoniae} from Turkey, has recently been discovered in Enterobacteriaceae from Europe, Asia and Africa.¹ Because of the high prevalence...
rates of ESBLs among Enterobacteriaceae and an increasing use of carbapenems in our hospital, surveillance of carbapenemases in these organisms may contribute useful information for clinicians to select appropriate antimicrobial therapy and for infection control to prevent the dissemination of these resistant strains in the hospital.

Materials and methods

Clinical isolates

A total of 4818 non-repetitive and consecutive clinical isolates of Enterobacteriaceae were collected from patients in Sirinagarind Hospital, Khon Kaen University, between October 2010 and August 2011. They were mostly obtained from urine (42.7%), and most isolates were E. coli (44.1%) and K. pneumoniae (25.3%). This study was conducted in accordance with the Declaration of Helsinki and good clinical practice, and was approved by the Ethics Committee of Khon Kaen University (project number HE551092).

Phenotypic tests for carbapenemase production

All isolates were screened for the presence of carbapenemases by a disc diffusion test using 10 μg ertapenem and 10 μg imipenem discs (Oxoid, Basingstoke, UK, or BBL, Becton Dickinson, NJ, USA). Isolates giving inhibition zone diameters of ≤21 mm to ertapenem disc or ≤20 mm to imipenem disc were suspected to produce carbapenemases. All positive screening isolates were subjected to the modified Hodge test (MHT) as recommended by CLSI and also detected for class A carbapenemase producers. Sequencing primers for the entire blaKPC,blaNDM,blaVIM,blaOXA, and 3blaIMP,blaGIM, and the other for blaOXA, blaNDM and blaOXA-48 were performed for all positive screening isolates.7,8 Pseudomonas aeruginosa carryingblaIMP-1, P. aeruginosa carrying blaVIM-2, K. pneumoniae ATCC BAA-1705 carryingblaKPC-1, K. pneumoniae carrying blaOXA-48 and Citrobacter freundii carryingblaGIM-1 were used as positive controls. Nucleotide sequences of the entire blaIMP and blaGIM were determined using amplification products. Sequencing primers for the entireblaIMP were IMP-F, IMP-R, 5′CS and 3′CS, whereas those for the entireblaNDM included NDM-F, NDM-R, IS125d-F (GGCGTAGATGGCTACACC), Ble-R (GATCAGTGACCGATCCTCA) and TrpF-R (AGCGTGGCTGCGACAGT) (Ellington et al.,7 Pairel et al.,8 Naas et al.9 and the present study).

PCR amplification of carbapenemase genes

Two sets of multiplex PCR techniques, one forblaIMP,blaVIM,blaOXA,blaKPC andblaNDM, and the other forblaOXA,blaNDM and blaOXA-48, were performed for all positive screening isolates.7,8 Pseudomonas aeruginosa carryingblaIMP-1, P. aeruginosa carrying blaVIM-2, K. pneumoniae ATCC BAA-1705 carryingblaKPC-1, K. pneumoniae carrying blaOXA-48 and Citrobacter freundii carryingblaGIM-1 were used as positive controls. Nucleotide sequences of the entireblaIMP and blaGIM were determined using amplification products. Sequencing primers for the entireblaIMP were IMP-F, IMP-R, 5′CS and 3′CS, whereas those for the entireblaNDM included NDM-F, NDM-R, IS125d-F (GGCGTAGATGGCTACACC), Ble-R (GATCAGTGACCGATCCTCA) and TrpF-R (AGCGTGGCTGCGACAGT) (Ellington et al.,7 Pairel et al.,8 Naas et al.9 and the present study).

Strain typing

DNA fingerprinting by PFGE was performed by the method of Arlet et al.10 Chromosomal DNA was digested by XbaI (Fermentas, Vilnius, Lithuania) for E. coli and K. pneumoniae DNA and BclI (Fermentas) for C. freundii DNA. DNA fragments were separated in 1% agarose (SeaKem Gold agarose, Lonza, Rockland, ME, USA) at 12°C by PFGE machine (CHEF Mapper, Bio-Rad, Foster City, CA, USA) using 200 V for 16 h with pulse times ranging from 10 to 25 s. Lambda ladder PFG marker (New England Biolabs, Hertfordshire, UK) was used as a DNA size marker. The fingerprints were visually compared and interpreted using criteria described by Tenover et al.11

Antimicrobial susceptibility testing

MICs of ertapenem (MSD, Paris, France), imipenem (MSD, Whitehouse Station, NJ, USA) and meropenem (Siem Bheasach, Bangkok, Thailand) and susceptibility testing to various antimicrobial agents (Oxoid or BBL) for the MBL-producing isolates were determined by an agar dilution method and disc diffusion test, respectively.4 E. coli ATCC 25922 was used as an antimicrobial-susceptible control.

Conjugation

Transfer of carbapenem resistance was carried out using a broth culture mating method using E. coli UB1637 (streptomycin resistant) as a recipient. A donor strain and a recipient strain were grown separately in Luria-Bertani broth (Hardy Diagnostics, Santa Maria, CA, USA) at 37°C for 4–5 h. The donor and recipient cultures were mixed together (1:25) and incubated at 37°C for 4–5 h. Transconjugants were selected on MacConkey agar (Oxoid) containing either 8 mg/L cefazidime (Sigma, St Louis, MO, USA) or 1 mg/L ertapenem plus 400 mg/L streptomycin (M & H Manufacturing, Samutprakarn, Thailand).3

Results and discussion

Of the 4818 Enterobacteriaceae isolates, 104 (2.2%) were screened positive by the ertapenem and/or imipenem disc diffusion tests. Among the 104 isolates, 27 were MHT positive, 36 were positive with the PBA–carbapenem disc test and 8 showed MBL activity. Subsequent multiplex PCR analysis revealed that two isolates each of E. coli, C. freundii and K. pneumoniae carriedblaNDM-like and two other isolates of K. pneumoniae harbouredblaIMP-like(Table 1). In this study, the eight MBL-producing isolates were positive with the ertapenem disc screening test, whereas the imipenem disc test could detect the six NDM-producing isolates only. This finding supports the CLSI guidelines that recommend the ertapenem disc for carbapenemase screening in Enterobacteriaceae. Of the eight MBL-producing isolates, two NDM producers were MHT negative, whereas MBL detection by the EDTA–carbapenem combined disc test was consistent with that of the PCR method. Therefore, the ertapenem disc screening test in combination with the EDTA–carbapenem combined disc test are recommended for routine laboratory detection of MBL.

Of the 104 positive screening isolates, neither blaOXA, norblaOXA-48 was found in any isolate. In addition, 31 of the 36 isolates of suspected class A carbapenemase producers were Enterobacter spp. Moreover, there were 21 isolates positive with the MHT, but negative with both multiplex–PCR methods. These isolates may give false positive results due to AmpC or ESBL production combined with porin loss as previously described. However, class A carbapenemases other than KPC could not be excluded for these isolates.

Nucleotide sequences of theblain two isolates from two isolates of K. pneumoniae were identical to that of theblain a previously described in P. aeruginosa from our hospital (A. Chanawong, S. Sopasri, A. Lulitanond, P. Sribenjalux and C. Wilailuckana; S. Sopasri, A. Lulitanond, P. Sribenjalux and C. Wilailuckana; GenBank accession numbers FJ267650 and FJ267651). Theblainthe IMP-14 and IMP-14a variants have been described in Thailand only.
Table 1. Carbapenemase screening test, MHT, EDTA-carbapenem combined disc test, and carbapenem MICs of the IMP-14a- and NDM-1-producing Enterobacteriaceae isolates and their transconjugants, and details of patients

<table>
<thead>
<tr>
<th>Patient number</th>
<th>Date of specimen collection</th>
<th>Specimen</th>
<th>Organism, isolate number and its transconjugant</th>
<th>Screening test (mm)</th>
<th>EDTA–combined disc test (mm)(^{a})</th>
<th>MIC (mg/L)</th>
<th>Underlying diseases</th>
<th>Antimicrobial therapy</th>
<th>Outcomes</th>
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<td>1</td>
<td>November 2010</td>
<td>urine</td>
<td>K. pneumoniae 22 T22</td>
<td>17 23 7</td>
<td>+ 2 0.25 0.5</td>
<td>symptomatic UTI</td>
<td>T cell lymphoma with neutropenia</td>
<td>IPM; VAN+COL+AMB</td>
<td>improved</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>bla IMP-14a</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>2</td>
<td>December 2010</td>
<td>sputum</td>
<td>K. pneumoniae 34 T34</td>
<td>19 28 6</td>
<td>+ 4 0.25 0.5</td>
<td>necrotizing enterocolitis, asymptomatic UTI</td>
<td>haemophagocytic lymphohistiocytosis</td>
<td>IMP+VAN</td>
<td>died from candidaemia</td>
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<tr>
<td>3</td>
<td>December 2010</td>
<td>urine</td>
<td>C. freundii 55 T55</td>
<td>9 18 18</td>
<td>+ 64 8 8</td>
<td>asymptomatic UTI</td>
<td>SLE with lupus nephritis cancer of cervix, gut obstruction, malnutrition</td>
<td>CAZ</td>
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<td>urine</td>
<td>K. pneumoniae 103 T103</td>
<td>12 16 16</td>
<td>+ 32 8 8</td>
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<td>T131</td>
<td>8 12 24</td>
<td>− 32 16 8</td>
<td>symptomatic UTI</td>
<td>OFX</td>
<td>improved</td>
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<td>metastatic adenocarcinoma</td>
<td>CRO; IPM</td>
<td>improved</td>
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<td>5</td>
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<td>C. freundii 119 T119</td>
<td>10 16 19</td>
<td>+ 64 8 8</td>
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<td>none</td>
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<td>8 13 32</td>
<td>+ 16 16 8</td>
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<td>K. pneumoniae 120 T120</td>
<td>10 17 19</td>
<td>− 16 4 4</td>
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<td></td>
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<td></td>
<td>E. coli UB1637</td>
<td>9 16 23</td>
<td>− 32 8 8</td>
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\(^{a}\)Increase in inhibition zone diameters of the ertapenem disc containing 292\(\mu\)g of EDTA compared with that of the ertapenem disc alone.

T, transconjugant; AMB, amphotericin B; CAZ, ceftazidime; COL, colistin; CRO, ceftriaxone; ETP, ertapenem; IPM, imipenem; MEM, meropenem; OFX, ofloxacin; UTI, urinary tract infection; VAN, vancomycin; SLE, systemic lupus erythematosus.
Nucleotide sequence analysis of \( \text{bla}_{\text{NDM-like}} \) from the six isolates revealed that they were \( \text{bla}_{\text{NDM-1}} \). Genetic sequences surrounding the \( \text{bla}_{\text{NDM-1}} \) were a part of IS\text{Ab}125 on the upstream region and \( \text{ble} \) (bleomycin resistance gene) and a part of \( \text{trpF} \) (phosphoribosylanthranilate isomerase gene) on the downstream region, similar to those reported from India, Hong Kong and Spain.\(^2\) Diversity of the genetic environment of \( \text{bla}_{\text{NDM-1}} \) has been reported.\(^2,3\)

Transfer of carbapenem resistance was successful in all MBL-producing isolates (Table 1). Either aminoglycoside or trimethoprim/sulfamethoxazole resistance was co-transferred to the \( \text{bla}_{\text{IMP-14a}} \)-carrying transconjugants. The carbapenem MICs for the NDM-1-producing isolates were higher than those of the IMP-14a producers, and so were their transconjugants (Table 1). All MBL-producing isolates were susceptible to colistin and tigecycline, but resistant to all \( \beta \)-lactams tested, except that five isolates were susceptible to aztreonam (data not shown). The NDM-1-producing isolates were 100\% susceptible to amikacin, 83.3\% to netilmicin, 50\% to gentamicin and <50\% to fluoroquinolones and trimethoprim/sulfamethoxazole. For the IMP-14a producers, one was susceptible to aminoglycosides and quinolones, while the other was resistant to these agents.

PFGE analysis revealed that the MBL-producing isolates were genetically different except that both isolates of \( \text{C. freundii} \) gave indistinguishable PFGE patterns (Figure 1). The six NDM-1-producing isolates were obtained from the urine of three patients over a long period, from December 2010 to August 2011 (Table 1). This suggested that a nosocomial outbreak did not take place even though patients 3 and 5 were hospitalized in the same ward. All patients had no history of travel outside Thailand. Interestingly, a \( \text{K. pneumoniae} \) strain and two strains of \( \text{E. coli} \) producing NDM-1 from the same patient (patient 4) were isolated from three urinary samples each collected within a 1–2 month interval. These findings demonstrated the \( \text{bla}_{\text{NDM-1}} \) carriage and the ease of transmission of this gene among various hosts. For the IMP-14a-producing isolates, they were recovered from urine and sputum of different patients admitted in different wards. All patients were improved after antimicrobial therapy except that one patient died from candidaemia.

In conclusion, this is the first known report of NDM-1 in Enterobacteriaceae from Thailand and of IMP-14a in \( \text{K. pneumoniae} \). Surveillance of carbapenemases, particularly NDM-1 among Enterobacteriaceae, is urgently needed for our country.

Acknowledgements
We are grateful to David Livermore (Antibiotic Resistance Monitoring & Reference Laboratory, HPA Microbiology Services–Colindale Unit, London, UK) and DMST Culture Collection (National Institute of Health, Thailand) for kindly providing the reference strains, and to staff of the Clinical Microbiology Laboratory, Srinagarind Hospital, and Mr Woravit Pasom for collecting the clinical isolates.

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Transparency declarations
None to declare.
References