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Carbapenemase-Producing Carbapenem-Resistant *Enterobacteriaceae* from Bangkok, Thailand, and Their Detection by the Carba NP and Modified Carbapenem Inactivation Method Tests

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Aim: The purpose of the study was to determine the epidemiology of carbapenemase genes among carbapenem-resistant *Enterobacteriaceae* and evaluate the Carba NP and modified carbapenem inactivation method (mCIM) tests in their detection.

Materials and Methods: A total of 287 nonduplicated *Enterobacteriaceae* isolates, which were at least resistant to one of the carbapenems, were identified and detected for carbapenemase genes by multiplex PCR covering bla_{KPC}, bla_{NDM}, bla_{VIM}, bla_{IMP}, and $bla_{OXA\text{-}48\text{-}like}$. All positive genes were then sequenced. These isolates were phenotypically tested for the production of carbapenemases by mCIM and Carba NP tests to evaluate the efficacy of these methods.

Results: Seven species of carbapenem-resistant isolates mainly *Klebsiella pneumoniae*, *Escherichia coli*, and *Enterobacter cloacae* were detected. Of these isolates, three families of carbapenemase genes, including bla_{NDM} ($bla_{NDM\text{-}1}$, $_{-4}$, $_{-5}$, $_{-9}$), bla_{OXA} ($bla_{OXA\text{-}48}$, $_{-181}$, $_{-232}$), and $bla_{IMP\text{-}14}$, were found. Of these, 223 (77.70%) carried at least one of the carbapenemase genes. The bla_{NDM} was detected in 160/223 (71.75%) isolates, of which 153/160 (95.63%) were the $bla_{NDM\text{-}1}$. Three types of the $bla_{OXA\text{-}48\text{-}like}$ group, $bla_{OXA\text{-}48}$, $bla_{OXA\text{-}181}$, and $bla_{OXA\text{-}232}$, were found, 91/104 (87.5%) harbored the $bla_{OXA\text{-}232}$. In addition, 25.11% (56/223) of the carbapenemase-producing isolates harbored a combination of bla_{NDM} and $bla_{OXA\text{-}48\text{-}like}$. Phenotypic detection methods, mCIM and Carba NP, showed 100% sensitivity and specificity to bla_{NDM}, $bla_{IMP\text{-}14}$, and $bla_{OXA\text{-}48}$, while the mCIM was positive in all $bla_{OXA\text{-}181}$ and $bla_{OXA\text{-}232}$ isolates, only 12.5% (1/8) and 28.95% (11/38), respectively, were detected by the Carba NP test.

Conclusions: This study revealed a unique prevalence of carbapenemase genes in Bangkok, Thailand, as well as demonstrated the efficacy and limitation of phenotypic detection methods of carbapenemase in the area where $bla_{NDM\text{-}1}$ and $bla_{OXA\text{-}232}$ were predominant.

Keywords: CRE, OXA-232, OXA-181, mCIM, Carba NP test

Introduction

Carbapenems are a class of β-lactam antibiotics with a broad spectrum of antibacterial activity and are generally stable against most beta-lactamase enzymes produced by bacteria. Therefore, carbapenems have been considered as the last line of defense against bacterial infection caused by multiple drug-resistant organisms. However, the widespread emergence and spread of carbapenem-resistant *Enterobacteriaceae* (CRE) worldwide have posed problems in appropriate treatment of infections caused by CRE and have also had implications in effective infection control interventions.[1] Adding to the above problems, the resistant mechanisms of CRE somehow vary and are dynamic, leading to a

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certain degree of difficulty on detection. Although two groups of resistant mechanisms, non-carbapenemase (such as a combination of other beta-lactamase enzymes, including AmpC or extended-spectrum β-lactamase (ESBL) and loss of porins) and carbapenemase producing, are reported, the latter group is responsible for greater impact on the spreading of resistant strains. At present, more than a hundred carbapenemase genes are reported.[2] Several carbapenem-resistant (CR) genes such as bla_{KPC}, bla_{NDM}, and $bla_{OXA\text{-}48\text{-}like}$ are commonly found worldwide. However, the distribution of some CR genes, that is, bla_{IMP}, bla_{VIM}, bla_{SPM}, bla_{SIM}, and bla_{GIM} is reported predominantly in certain regions. In Thailand, only limited data regarding the distribution of CR genes have been documented.[3,4] Accurate, rapid, and uncomplicated methods for detection of carbapenemase are required to identify the resistant mechanism of the suspect organisms. Several phenotypic detection methods of carbapenemase-producing isolates have been developed and used routinely in clinical microbiology laboratories.[5–7] However, some methods are rather specific in certain resistant mechanisms, while other tests have a broader spectrum of detection. For example, aminophenyl boronic acid inhibition testing is reported to be specific for *Klebsiella pneumoniae* carbapenemase (KPC) detection when performed with imipenem or meropenem,[8] while EDTA or dipicolinic acid is used for inhibition of metallo-beta-lactamase.[9] At present, two phenotypic tests for the production of carbapenemases, Carba NP and modified carbapenem inactivation method (mCIM), are recommended by the Clinical Laboratory Standard Institute (CLSI).[10] Although the Carba NP test has been originally claimed to gain 100% sensitivity and specificity for detection of carbapenemases,[6] the limitation of this method on detection of carbapenemases such as OXA-48-like beta-lactamase has been reported.[11] In contrast, the mCIM has shown to be an excellent candidate for detection of carbapenemase.[12–14] However, since it is a newly recommended test, a comparison study on the efficacy of this method with others is rarely reported. Therefore, it was the aim of this study to establish the efficacy of the Carba NP test and mCIM for detection of carbapenemase-producing *Enterobacteriaceae* compared with genotypic tests, as well as to report the prevalence of CR genes in the region.

Materials and Methods

Bacterial isolates and antimicrobial susceptibility testing

Nonduplicated clinical strains of *Enterobacteriaceae* isolated from Microbiology laboratory, Department of Pathology, Faculty of Medicine Ramathibodi Hospital, Mahidol University, and hospitals in Bangkok, Thailand, between March 2012 and September 2016, which showed resistance to at least one of the tested carbapenems (ertapenem, imipenem, meropenem, and doripenem) according to the CLSI breakpoints,[10] were collected and identified using conventional biochemical tests and confirmed by MALDI-TOF mass spectrometry (Autoflex™; Bruker Daltonics, Germany). Antimicrobial susceptibility testing of these isolates was performed with the minimum inhibitory concentration method using a customized gram-negative panel (THAN1F) (Sensititre; Thermo Scientific). Each isolate was stored in skim milk at −80°C until used. Five positive controls strains for carbapenemase genes were *K. pneumoniae* ATCC BAA-1705 (KPC), *K. pneumoniae* ATCC BAA-2470 (NDM-1), *K. pneumoniae* RAKP0022 (OXA-48), *K. pneumoniae* RAKP0016 (IMP-14), and *K. pneumoniae* ATCC13440 (VIM-1), and two reference strains *K. pneumoniae* ATCC13883 and *K. pneumoniae* ATCC BAA-1706 were used as negative controls.

Genotypic detection and characterization of carbapenemase genes

The multiplex PCR was performed for 287 of CRE isolates. Total DNA was extracted from all strains by boiling for 20 min, followed by a 10-min centrifugation at 14,000 rpm. The supernatant was collected and used for PCR amplification. Five carbapenemase genes, including bla_{KPC}, bla_{NDM}, $bla_{OXA\text{-}48\text{-}like}$, bla_{IMP}, and bla_{VIM} genes, were amplified using the primers following Poirel *et al.*[15] Amplification was performed in the thermal cycler (Labcycler, SensoQuest GmbH., Germany) with an initial denaturation at 94°C for 10 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, extension at 72°C for 1 min, and a final elongation at 72°C for 10 min. Multiplex PCR products were stained with GelRed™ nucleic acid gel stain and analyzed in 2% (w/v) agarose gel (Promega, Madison) (100 V for 45 min). Finally, the bla_{NDM}, $bla_{OXA\text{-}48\text{-}like}$, and bla_{IMP}, which were shown positive by multiplex PCR, were sequenced by using the following primers:

forward primer pre-NDM-for (5′-CACCTCATGTTT GAATTCGCC-3′)[16]
reverse primer pre-NDM-rev (5′-CTCTGTCACATC GAAATCGC-3′)[16]
forward primer pre-OXA-for (5′-TTGGTGGCATCG ATTATCGG-3′)[17]
reverse primer pre-OXA-rev (5′-GAGCACTTCTTT TGTGATGGC-3′)[17]
forward primer pre-IMP-for (5′-ATCCAAGCAGCA AGCGCGTTA-3′)[18]
reverse primer pre-IMP-rev (5′-CGTGCTGCTGCAA CGACTTGT-3′)[18]

Phenotypic detection of carbapenemase

Detection of carbapenemase-producing *Enterobacteriaceae* by the Carba NP and mCIM tests was performed following the CLSI guideline.[11] However, in this study, the Carba NP test was performed in a 96-well microtiter plate and only 40 μL of bacterial extraction, solution A (2 mL of 0.5% phenol red in 16.6 mL of DW plus 180 μL of 10 mM zinc sulfate solution, pH 7.8) and solution B (solution A +6 mg/mL of imipenem), was used.

Results

Identification and distribution of clinically isolated CRE

Between March 2012 and September 2016, 287 nonduplicated isolates of *Enterobacteriaceae*, which showed resistance to at least one of the tested carbapenems, were collected and identified. Seven species of these isolates included *K. pneumoniae* (205, 71.43%), *Escherichia coli* (44, 15.33%), and *Enterobacter cloacae* (25, 8.71%). These three species covered ∼95.5%. Other four species included *Citrobacter freundii* (5, 1.74%), *Providencia rettgeri* (4, 1.39%), *Klebsiella* (*Enterobacte*r) *aerogenes* (3, 1.05%), and *Citrobacter koseri* (1, 0.35%).

Table 1. Distribution of Carbapenemase Genes Among Carbapenemase-Producing Carbapenem-Resistant *Enterobacteriaceae* Isolates by Multiplex PCR ($N=223$)

Organisms	bla_{NDM}, n *(%)*	$bla_{OXA\text{-}48\text{-}like}$, n *(%)*	bla_{IMP}, n *(%)*	bla_{VIM}	bla_{KPC}	bla_{NDM} *and* $bla_{OXA\text{-}48\text{-}like}$, n *(%)*	n *(%)*
Klebsiella pneumoniae	62 (27.80)	49 (21.97)	2 (0.90)	0	0	55 (24.66)	168 (75.34)
Escherichia coli	22 (9.87)	4 (1.79)	0	0	0	1 (0.45)	27 (12.11)
Enterobacter cloacae	9 (4.04)	3 (1.35)	5 (2.24)	0	0	0	17 (7.62)
Klebsiella (Enterobacter) aerogenes	1 (0.45)	0	0	0	0	0	1 (0.45)
Citrobacter freundii	5 (2.24)	0	0	0	0	0	5 (2.24)
Citrobacter koseri	1 (0.45)	0	0	0	0	0	1 (0.45)
Providencia rettgeri	4 (1.79)	0	0	0	0	0	4 (1.79)
Total	104 (46.64)	56 (25.11)	7 (3.14)	0	0	56 (25.11)	223 (100)

Detection and characterization of carbapenemase genes

All 287 isolates of *Enterobacteriaceae* were tested for five carbapenemase genes by multiplex PCR. Of 287 isolates, 223 (77.70%) showed carrying at least one of the carbapenemase genes and 64 (22.30%) isolates were negative tested by the multiplex PCR method. The bla_{NDM} and $bla_{\text{OXA-48-like}}$ genes were found as predominant genes in which 71.75% (160/223) and 50.22% (112/223), respectively, of the carbapenemase gene-positive isolates were found carried these genes. Although the $bla_{\text{OXA-48-like}}$ gene was the second most commonly detected gene in this study, only three species (*K. pneumoniae*, *E. coli*, and *E. cloacae*) were found carrying the gene. Interestingly, 56 (25.11%) isolates contained a combination of the bla_{NDM} and $bla_{\text{OXA-48-like}}$ genes (Table 1). In addition, a high percentage (32.73%, 55/168) of *K. pneumoniae* isolates carried both bla_{NDM} and $bla_{\text{OXA-48-like}}$, while only one isolate (3.70%, 1/27) of *E. coli* contained two resistant genes. The bla_{IMP} gene was a rare resistant gene found in two and five isolates of *K. pneumoniae* and *E. cloacae*, respectively. The bla_{KPC} and bla_{VIM} genes were not detected in all tested isolates.

All 223 isolates of CRE, in which the resistant genes were detected from the previous multiplex PCR methods, were amplified for sequencing of the carbapenemase genes. The number of isolates that carried these genes is shown in Table 2. Of the 160 bla_{NDM}-positive isolates, 153 (95.63%) were $bla_{\text{NDM-1}}$, while the other three bla_{NDM}, including $bla_{\text{NDM-4}}$, $bla_{\text{NDM-5}}$ and $bla_{\text{NDM-9}}$, detected three species, including *K. pneumoniae*, *E. coli*, and *C. freundii*. In addition, 56/153 (36.60%) of $bla_{\text{NDM-1}}$ contained isolates found in combination with the $bla_{\text{OXA-48-like}}$ gene. The $bla_{\text{OXA-48-like}}$ was detected in 112/223 (50.22%) isolates. Three types of the $bla_{\text{OXA-48-like}}$ group, $bla_{\text{OXA-48}}$, $bla_{\text{OXA-181}}$, and $bla_{\text{OXA-232}}$, were found. Among these, the $bla_{\text{OXA-232}}$ was found as majority (91/112, 81.25%), in which 53/91 (58.24%) were found in combination with $bla_{\text{NDM-1}}$. Only 10/112 (8.93%) isolates were $bla_{\text{OXA-48}}$, while 11 (9.82%) isolates were $bla_{\text{OXA-181}}$. The seven isolates of bla_{IMP} were $bla_{\text{IMP-14}}$, found in two and five isolates of *K. pneumoniae* and *E. cloacae*, respectively.

Detection of carbapenemase enzyme by the Carba NP test versus mCIM

To evaluate phenotypical tests for carbapenemase production, two methods the Carba NP test and mCIM were selected. All 287 isolates of carbapenem-resistant bacteria were tested. The result revealed that all 64 carbapenemase gene-negative isolates showed negative result in both the Carba NP test and mCIM. Of 223 carbapenemase gene-

Table 2. Identification of Carbapenemase Genes Among Carbapenemase-Producing Carbapenem-Resistant *Enterobacteriaceae* Isolates Containing Resistant Genes ($N=223$)

PCR	*SQ*	*KP,* n *(%)*	*EC,* n *(%)*	*ET,* n *(%)*	*OT,* n *(%)*	*Total,* n *(%)*
$bla_{\text{OXA-48}}$ (56)	$bla_{\text{OXA-48}}$	9 (4.04)	1 (0.45)	0	0	10 (4.48)
	$bla_{\text{OXA-181}}$	5 (2.24)	3 (1.35)	0	0	8 (3.59)
	$bla_{\text{OXA-232}}$	35 (15.70)	0	3 (1.35)	0	38 (17.04)
bla_{IMP} (7)	$bla_{\text{IMP-14}}$	2 (0.90)	0	5 (2.24)	0	7 (3.14)
bla_{NDM} (104)	$bla_{\text{NDM-1}}$	58 (26.01)	20 (8.97)	10 (4.48)	9 (4.04)	97 (43.50)
	$bla_{\text{NDM-4}}$	2 (0.90)	0	0	0	2 (0.90)
	$bla_{\text{NDM-5}}$	1 (0.45)	2 (0.90)	0	1 (0.45)	4 (1.79)
	$bla_{\text{NDM-9}}$	1 (0.45)	0	0	0	1 (0.45)
bla_{NDM} and $bla_{\text{OXA-48}}$ (56)	$bla_{\text{NDM-1}}$ & $bla_{\text{OXA-181}}$	2 (0.90)	1 (0.45)	0	0	3 (1.35)
	$bla_{\text{NDM-1}}$ & $bla_{\text{OXA-232}}$	53 (23.77)	0	0	0	53 (23.77)
	TOTAL	168 (75.34)	27 (12.11)	18 (8.07)	10 (4.48)	223 (100)

PCR, performed by simplex PCR; SQ, genotype by sequencing of PCR products; KP, *Klebsiella pneumoniae*; EC, *Escherichia coli*; ET, *Enterobacter* spp. (one isolate of *Klebsiella (Enterobacter) aerogenes* = $bla_{\text{NDM-1}}$, nine isolates of *Enterobacter cloacae*); OT, other organisms (four isolates of *Providencia rettgeri* = $bla_{\text{NDM-1}}$, one isolate of *Citrobacter koseri* = $bla_{\text{NDM-1}}$, four isolates of *Citrobacter freundii*, $bla_{\text{NDM-1}}$, and one isolate of *C. freundii* = $bla_{\text{NDM-5}}$).

positive isolates tested by the Carba NP test, only 189 (84.75%) were Carba NP positive and 34 (15.25%) isolates were Carba NP negative. Thirty-four isolates of positive carbapenemase gene, which were negative by the Carba NP test, included 29, 3, and 2 isolates of *K. pneumoniae*, *E. coli*, and *E. cloacae*, respectively. All these isolates were $bla_{\text{OXA-48-like}}$ gene positive. The data revealed that by using the Carba NP test alone, 34/56 (60.71%) of the $bla_{\text{OXA-48-like}}$ gene-positive isolates were undetectable. According to specific types of carbapenemase genes, the Carba NP test was positive in all bla_{NDM}, $bla_{\text{IMP-14}}$, and $bla_{\text{OXA-48}}$, but only 12.5% (1/8) and 28.95% (11/38) of $bla_{\text{OXA-181}}$ and $bla_{\text{OXA-232}}$, respectively, were detected by the Carba NP test. Of 223 isolates, none of the bla_{KPC} and bla_{VIM} was detected, and therefore, it was unable to demonstrate the capacity of the methods on detection of organisms that carried these genes. In this study, the Carba NP test demonstrated a sensitivity of 189 of 223 (84.75%) and a specificity of 189 of 189 (100%). The positive and negative predictive values of the test were 100% and 65.31%, respectively.

All 223 carbapenemase gene-positive isolates showed positive results when tested with mCIM. The mCIM showed 100% sensitivity and specificity in all carbapenemase genes detected by multiplex PCR in this study. This result reflected the greater efficacies of mCIM over the Carba NP test on detection of $bla_{\text{OXA-181}}$ and $bla_{\text{OXA-232}}$.

Discussion

Since the emergence of CRE some years ago, they have become one of the major causes of death among hospital-acquired infected patients. These organisms are also considered as public health threat worldwide.[19] Accurate and rapid identification of CRE either via phenotypic or genotypic approaches is crucial for individual treatment as well as infection control aspect. In this study, more than 95% of the CRE were found in three species, including *K. pneumoniae*, *E. coli*, and *E. cloacae*. However, *K. pneumoniae* accounted for the largest proportion of CRE.

In this study, we determined the prevalence of carbapenemase genes among seven species of *Enterobacteriaceae* using a conventional multiplex PCR. Five families (bla_{NDM}, bla_{KPC}, $bla_{\text{OXA-48}}$, bla_{IMP}, and bla_{VIM}) of carbapenemase genes were tested. Only three families bla_{NDM}, $bla_{\text{OXA-48}}$, and bla_{IMP} were found among the isolates. Even though the bla_{KPC} gene has been reported to spread rapidly in the last decade,[20] the data demonstrated that the prevalence of bla_{KPC} remains very low in Thailand.[4] Therefore, only the positive control strains of both genes were used to demonstrate the proper function of both techniques. The bla_{NDM} genes were found to be the most common carbapenemase genes, in which $\sim$72% of the tested isolates were detected and nearly all of the bla_{NDM}-positive isolates were $bla_{\text{NDM-1}}$. Three other bla_{NDM} genes, $bla_{\text{NDM-4}}$, $bla_{\text{NDM-5}}$, and $bla_{\text{NDM-9}}$, were also found. Despite several reports of these latter bla_{NDM} types in Europe and Asian countries,[21–25] this became the first official report of the non- $bla_{\text{NDM-1}}$ genes in Thailand. The second most common carbapenemase gene was the $bla_{\text{OXA-48-like}}$, in which three variants $bla_{\text{OXA-48}}$, $bla_{\text{OXA-181}}$, and $bla_{\text{OXA-232}}$ were found in this study. Interestingly, only less than 10% of the bla_{OXA} positive were identified as $bla_{\text{OXA-48}}$, while nearly 88% were $bla_{\text{OXA-232}}$. Although the $bla_{\text{OXA-181}}$ and $bla_{\text{OXA-232}}$ had been reported in Singapore and other Asian countries, the prevalence of these genes compared with the $bla_{\text{OXA-48}}$ is yet to be clearly documented.[26–32] Our results also demonstrated that 58.24% of the $bla_{\text{OXA-232}}$ isolates were found in combination with $bla_{\text{NDM-1}}$ gene. This finding became a solid data of high prevalence of the $bla_{\text{OXA-48-like}}$ in Thailand.

We demonstrated the use of two phenotypic procedures for detection of carbapenemase-producing isolates. From the outcome of our study, apart from the requirement of overnight incubation in the procedure, the mCIM has shown to be an excellent candidate for phenotypic detection of carbapenemase producers suitable in all levels of microbiology laboratory due to cost/effectiveness and simple technique requirement. Although the mCIM and Carba NP test showed excellent correlation with the bla_{NDM} genes with 100% agreement with the multiplex PCR result, our study demonstrated that the Carba NP test could detect $\sim$40% of CRE isolates that were multiplex PCR positive for $bla_{\text{OXA-48-like}}$ genes. However, when only the $bla_{\text{OXA-48}}$-positive isolates were analyzed, it showed 100% specificity for $bla_{\text{OXA-48}}$, which was similar to the original report by Nordmann.[6] Our data have clearly demonstrated that the Carba NP test has limitation in detection of the $bla_{\text{OXA-48-like}}$ genes, including $bla_{\text{OXA-181}}$ and $bla_{\text{OXA-232}}$ that, $\sim$90% and 70%, respectively, were undetectable. These results suggested that the efficacy of the Carba NP test very much relies on the prevalence of the $bla_{\text{OXA-48}}$ and $bla_{\text{OXA-48-like}}$ genes in the area. In contrast, both mCIM and the Carba NP test could detect $bla_{\text{NDM-1}}$ and its variants, including $bla_{\text{NDM-4}}$, $bla_{\text{NDM-5}}$, and $bla_{\text{NDM-9}}$. Similar results were also reported.[6] However, due to the limited numbers of bla_{NDM} variants, it is unable to demonstrate the specificity of these methods against those genes. Although both the Carba NP test and mCIM showed the positive results with the bla_{KPC} and $bla_{\text{IMP-14}}$, only eight isolates (one and seven isolates of bla_{KPC}-positive control strain and $bla_{\text{IMP-14}}$, respectively) were tested. Therefore, it was unable to establish the efficacy of the phenotypic tests against these genes.

It is worth notified here that 23.3% (64 isolates), which were non-carbapenemase-producing CRE, showed negative to all tested primers (data not shown). These carbapenem-resistant isolates may be associated with other resistant mechanisms particularly via a combination of ESBL or AmpC production and the loss or alteration of *OmpK35* and *OmpK36* as reported previously.[33]

In conclusion, our data suggested that carbapenem-resistant isolates in Thailand, particularly *K. pneumoniae*, have unique epidemiologic characters. Therefore, proper laboratory investigation techniques are required to provide precise information about carbapenem resistance prevalence in the area. In addition, to our knowledge, this is the first report of rare bla_{NDM} genes, $bla_{\text{NDM-4}}$, $bla_{\text{NDM-5}}$, and $bla_{\text{NDM-9}}$, as well as $bla_{\text{OXA-181}}$ and $bla_{\text{OXA-232}}$, in *K. pneumoniae* from Thailand.

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Disclosure Statement

No competing financial interests exist.

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