THE PREVALENCE OF CARBAPENEM RESISTANT *ENTEROBACTERIACEAE* IN THE LOWER NORTH REGION OF THAILAND

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ABSTRACT

The emergence and global spread of carbapenemase producers in *Enterobacteriaceae* has been reported and becoming a major concern in public health worldwide. The emerging of carbapenem-resistance *Enterobacteriaceae* (CRE) is one of the most important emerging antibiotic resistances in Thailand. In this present study, we determined the prevalence of CRE from 4 hospitals clinical microbiology laboratory includes, Budhachinnaraj hospital, Srisangworn hospital, Uthaithani hospital, Sawanpracharak hospital, entirely located in the lower north region of Thailand. The CRE prevalence was approximately 44.05% and all of CRE isolates were detected for carbapenemase production by phenotypic methods. The detection of carbapenemase production revealed that, the MHT was positive for 25%. Meanwhile, Carba NP was positive for 29.16%. However, this study focused on the simple phenotypic detection methods for carbapenemase detection. The preliminary information documented in this study may offer significant help in the control to reducing the spread of CRE and empirical antibiotic management.

Keywords: Carbapenem-resistant *Enterobacteriaceae* (CRE), Modified hodge test (MHT), Carbapenemase Nordmann-Poirel (Carba NP) test,

1. Introduction

Antimicrobial resistance is currently a major health concern in treating infectious diseases in Thailand over the past few decades. Carbapenems are a subgroup of β-lactam antibiotics, which frequently used as a last resort antibiotic active against various both gram negative and gram positive bacteria. The increasing of resistance to this class of β-lactam antibiotics is left with restricted therapeutic alternatives and related to the difficult-to-treat infection. The emergence of β-lactamases with direct carbapenem-hydrolyzing activity has contributed to an increased prevalence of CRE. Carbapenemases are members of the molecular class A, B, and D β-lactamases, known as carbapenem-hydrolyzing enzyme. These enzymes have ability to hydrolyze almost all β-lactam antibiotics including penicillins, cephalosporins, monobactams, carbapenems and most are resistant to β-lactamase inhibitors (Queenan & Bush, 2007).

The emergence and global spread of carbapenemase producers in *Enterobacteriaceae* has been reported worldwide and possibly carried this resistance either on chromosome or acquired via plasmid mediated resistance that spread rapidly between bacteria. For epidemiology of CRE in Thailand, The surveillance of CRE among clinical isolates of *Enterobacteriaceae* from the hospitals in Thailand during 2012-2013, the CRE prevalence in each regions were 21.9% in the north, 27.4% in the Northeastern, 24.5% in the Central, 9.3 % in Bangkok, 6.3 % in the East and 10.6% in the south of Thailand. Notably, NDM-1 has been mostly identified from isolates. Nevertheless, the increasing of CRE and the use of other antibiotic include β-lactam is ineffective (NARST, 2016). Therefore, it is important to detect for carbapenemase production in *Enterobacteriaceae* and determine the prevalence of CRE of clinically isolated from 4 clinical microbiology laboratories, located in lower north hospitals of Thailand by phenotypic methods. This study focused on the simple phenotypic detection methods for carbapenemase detection as well the information documented in this study may offer significant help in the control to reducing the spread of CRE and empirical antibiotic management.

2. Objectives of the study

The aims of this study are determine the prevalence of CRE by phenotypic method using disk diffusion methods and the prevalence of carbapenemase producing in CRE using modified hodge test (MHT) and carbapenemase Nordmann-Poirel (Carba NP) test.

3. Materials and methods

Bacterial strains and identification

A total of 168 clinical isolates of *Enterobacteriaceae* were obtained from 4 hospitals clinical microbiology laboratory includes, Budhachinnaraj hospital, Phitsanulok, Srisangworn hospital, Sukhothai, Uthai Thani hospital, Uthai Thani and Sawanpracharak hospital, Nakhon Sawan entirely located in the lower north region of Thailand. All isolates were preliminary determined for carbapenems susceptibility by standard disk diffusion method. Bacterial strains were collected, isolated in pure culture and stored in nutrient agar slant, then put in a suitable position in transport containers to laboratory. The samples were collected and obtained from hospitals during January 2015- June 2016. Bacterial samples were grown on MacConkey agar plate as isolation streak plate technique for identification process by using conventional methods.

Antimicrobials susceptibility

Antimicrobial susceptibility was tested using disk diffusion methods according to CLSI recommendations. In this study was evaluated susceptibility testing of carbapenems using the carbapenems disk including imipenem (IPM), meropenem (MEM), ertapenem (ETP) and doripenem (DOR) in each concentration at 10 µg using the current interpretive criteria according to CLSI guidelines. For quality control

Staphylococcus aureus ATCC 25923, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as quality control organisms (CLSI, 2014).

Detection of carbapenemase production

All carbapenems non-susceptible isolates were subjected to detection of carbapenemase production by phenotypic detection methods using two approaches, MHT and Carba NP test.

MHT

An indicator organism, *E. coli* ATCC 25922 was prepared a 0.5 McFarland standard then 1:10 dilution of inoculums suspension was diluted in sterilized NSS or broth. The inoculums will be inoculated on Mueller-Hinton agar plate as for the routine disk diffusion procedure and a 10 µg of ertapenem disk was placed in the center of the test area. All carbapenems non-susceptible isolates and QC organism was inoculated in a straight line out from the edge of the disk. QC organism of testing will be performed with each run (*Klebsiella pneumoniae* ATCC 1705 as positive control and *K. pneumoniae* ATCC 1706 as negative control) and incubated at 35±2 °C in ambient air for 16-20 hours by following the CLSI guideline M100-S26 (CLSI, 2016).

Carba NP

In this study, the Carba NP test protocol will be modified from CLSI guideline M100-S26 and Nordmann method**.** First step, the Carba NP test solution will be prepared using two separate solutions include solution A and solution B. After Carba NP solution was prepared, bacterial isolates were grown overnight on blood agar plate and used in a process of bacterial protein extraction. The enzymatic bacterial suspension in supernatant was used for the detection of carbapenemases production. 96-well microtiter plate was used according to Nordmann method [6]. Then incubated at 35±2 °C for up 2 hours. QC organism of testing was performed with each run, by using *K. pneumoniae* ATCC 1705 as positive control and *K. pneumoniae* ATCC 1706 as negative control (Nordmann & Dortet, 2012).

4. Results

A total of non-duplicated 168 obtained clinical *Enterobacteriaceae* isolates from 4 hospitals were cultured and identified by coventional methods. This identification of various species *of Enterobactericeae* family includes, 84 isolates of *E. coli* (50%), 59 isolates of *K. pneumoniae* (35.11%), 9 isolates of *Enterobacter cloacae* (5.36%), 2 isolates of *Citrobacter freundii* (1.19%), 1 isolate of *Citrobacter diversus* (0.56%), 2 isolates of *Enterobacter* spp. (1.19%), 4 isolates of *Proteus mirabilis* (2.38%), 3 isolates of *Morganella morganaii* (1.79%), 4 isolates of *Salmonella* spp. (2.38%). As shown in Table 1.

Table 1 A total of Bacterial identification in clinical isolates from 4 clinical microbiology laboratories located in the lower north region of Thailand.

Organisms	Hospitals				Total	%
	Budhachinnaraj hospital	Srisangworn hospital	Uthai Thani hospital	Sawanpracharak hospital		
E. coli	7	31	39	7	84	50.00
K. pneumoniae	25	10	15	9	59	35.11
E. cloacae	2	3	1	3	9	5.36
P. mirabilis	-	2	2	-	4	2.38
C. freundii	-	-	2	-	2	1.19
C. *diversus*	-	1	-	-	1	0.56
M. morganii	-	2	1	-	3	1.79
Enterobacter spp	-	1	1	-	2	1.19
Salmonella spp.	-	1	3	-	4	2.38
Total	34	51	64	19	168	

Within the overall identified of 168 *Enterobactericeae* isolates, 94 isolates (55.95%) were susceptible to all carbapenems (meropenem, ertapenem, doripenem and imipenem) by disk diffusion methods. For the other 74 non-susceptible isolates (44.05%), including 21 isolates of *E.coli* (12.5%), 41 isolates of *K.pneumoniae* (24.4%), 7 isolates of *E.cloacae* (4.16%), 2 isolates of *Enterobacter* spp. (1.19%), 1 isolate of *P.mirabilis* (0.6%), 2 isolates of *M. morganaii* (1.19%), were showed to be resistant to at least a carbapenem and identified to be CRE. A total of non-susceptible isolates, of 49 isolates (29.16%) were resistant to all carbapenems. Meanwhile, other 25 of non-susceptible isolates showed intermediated or resistance inhibition zone diameters to only an antibiotic or more than one antibiotic in carbapenems. As shown in Table 2.

Table 2 The susceptibility testing of carbapenems by disk diffusion method (168 isolates)

Organisms	Carbapenems susceptibility testing								Total			
	Budhachinnaraj hospital		Srisangworn hospital		Uthaithani hospital		Sawanpracharak hospital		Susceptible (S)		Non- Susceptible (NS)	
	S	NS	S	NS	S	NS	S	NS	isolate	%	isolate	%
E. coli	-	7	20	11	38	1	5	2	63	37.5	21	12.50
K. pneumoniae	1	24	5	5	10	5	2	7	18	10.71	41	24.40
E. cloacae	-	2	1	2	1	-	-	3	2	1.19	7	4.16
P. mirabilis	-	-	1	1	2	-	-	-	3	1.79	1	0.60
C. freundii	-	-	-	-	2	-	-	-	2	1.19	-	-
C. *diversus*	-	-	1	-	-	-	-	-	1	0.60	-	-
M. morganii	-	-	-	2	1	-	-	-	1	0.60	2	1.19
Enterobacter spp	-	-	-	1	-	1	-	-	-	-	2	1.19
Salmonella spp.	-	-	1	-	3	-	-	-	4	2.38	-	-
Total	1	33	29	22	57	7	7	12	**94**	**55.95**	**74**	**44.05**

The detection of carbapenemase production, 74 isolates of CRE (44.05%) were used as samples for carbapenemase detection. Of 2 isolates (1.19%) showed positive results for only MHT. Meanwhile, 9 isolates (5.36%) showed positive results for only Carba NP. The other isolates, 40 isolates (23.81%) showed positive result for both MHT and Carba NP as well as 23 isolate (13.69%) showed negative result for both tests. The results showed that, MHT was positive for 42 isolates (25%) includes, 7 isolates of *E.coli* (4.16%), 29 isolates of *K.pneumoniae* (17.26%), 6 isolates of *E.cloacae* (3.57%). Meanwhile, Carba NP was positive for 49 isolates (29.16%) includes, 10 isolates of *E.coli* (5.95%), 33 isolates of *K.pneumoniae* (19.64%), 6 isolates of *E.cloacae* (3.57%). As shown in Table 3 and Figure 1.

Table 3 The **Detection of carbapenemase production by MHT and Carba NP test**

Hospitals	Positive Carbapenemase production by			
	MHT		Carba NP	
	Isolates	%	Isolates	%
Budhachinnaraj hospital (34)	32	19.05	32	19.05
Srisangworn hospital (51)	1	0.60	2	1.19
Uthaithani hospital (64)	1	0.60	6	3.57
Sawanpracharak hospital (19)	8	4.76	9	5.36
Total (168)	42	25	49	29.16

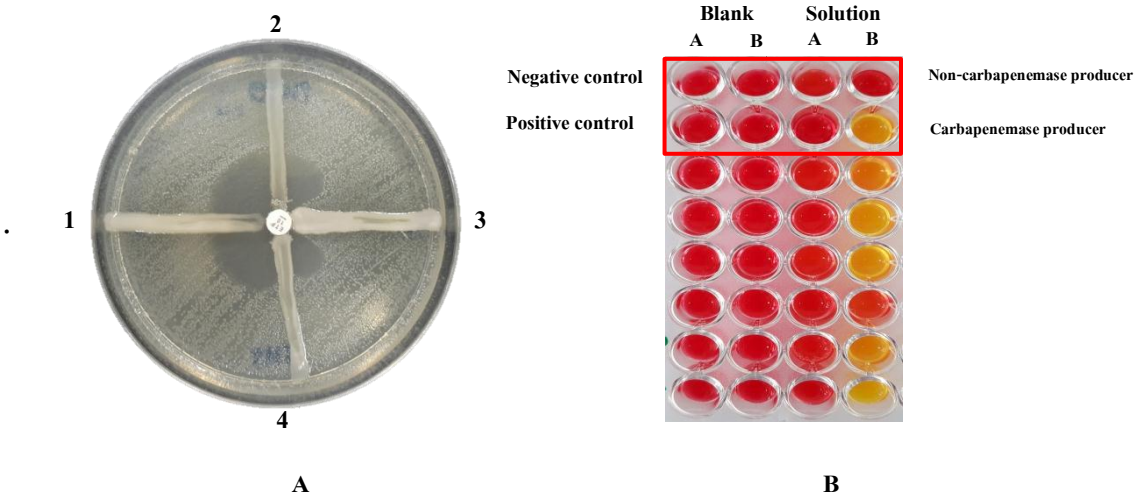

Figure 1 Representative a result of (A) MHT, 3 and 4 showed positive isolate with a clover leaf-like indentation of the *E. coli* ATCC 25922 strain growing along the test organism growth streak within the disk diffusion zone indicating production of carbapenemase, 1 and 2 showed negative test with no growth of the *E. coli* ATCC 25922 along the test organism growth streak within the disk diffusion. (B) Results of the Carba NP test, based on acidification of phenol red when imipenem is hydrolyzed, evidenced by the color change of the test solution from red to yellow.

5. Discussion

Multidrug resistance in *Enterobacteriaceae* are now spreading increasingly and becoming a major concern in public health worldwide. The emerging of CRE is one of the most important emerging antibiotic resistance traits correlate with the production of the carbapenem-hydrolysing β-lactamases. The emergence and global spread of carbapenemase producers in *Enterobacteriaceae* has been reported worldwide and possibly carried this resistance either on chromosome or acquired via plasmid mediated resistance that spread rapidly among *Enterobacteriaceae* family. The CRE have been emerged to different parts of the world and mostly identified belonging to main molecular classes includes class A, B and D β-lactamases. The situation of CRE in Asia, the overall regions were approximately 37.5% of their contributed epidemiology data, including the continuingly increasing of ESBL-producer and high-level carbapenem resistance among *Enterobacteriaceae* (Xu et al., 2015). For epidemiology of CRE in Thailand, The surveillance of CRE among clinical isolates of *Enterobacteriaceae* from the hospitals in Thailand during 2012-2013, the CRE prevalence in each regions were 21.9% in the north, 27.4% in the Northeastern, 24.5% in the Central, 9.3 % in Bangkok, 6.3 % in the East and 10.6% in the south of Thailand. Moreover, the previous study revealed the prevalence of CRE among 12,741 clinical isolates of *Enterobacteriaceae* at the largest University hospitals in Thailand was found in 181 isolates (1.4%) (Netikul & Kiratisin, 2015). The overall epidemiology data, given the fact that the prevalence of CRE was increasing during the past decade.

In this present study, we determined the prevalence of CRE from the lower north hospitals of Thailand. A total of 168 clinical isolates of *Enterobacteriaceae*, the CRE prevalence was approximately 44.05% among isolate in this study. All over of CRE isolates were detected for carbapenemase production, the results showed that, MHT was positive for 25%. Meanwhile, Carba NP was positive for 29.16%. Interestingly, the positive isolates for Carba NP were mostly *K. pneumoniae.* It is possible that among these isolates maybe positive for class B carbapenemase thus, the yielded low sensitivity and specificity of MHT can cause for a false-negative results. Accordingly, the prevalence of CRE among clinical isolates of *Enterobacteriaceae* from the hospitals in Thailand during 2012-2013, the CRE prevalence in the north was 27.4% with mostly NDM-1 has been identified from isolates (NARST, 2016). Therefore, the MHT result has a limited capability to detect of carbapenemase production and the results should be deliberately interpreted.

This uncorrelated result, referring to the different in terms of sensitivity and specificity of methods. Firstly, CLSI recommend the phenotypic detection of carbapenamase by MHT for the last several years. MHT is based on the inactivation of a carbapenem by carbapenemase-producing strains that enable a carbapenem-susceptible indicator strain to extend growth toward a carbapenem-containing disk, along the streak of inoculum of the tested strain**.** Although, MHT is simple and no require special reagent or media to perform. However, MHT has some limitation, the high levels of expression of AmpC coupled with decreased permeability may be elucidate as carbapenems hydrolyzing enzyme and give rise to false positive interpretation**.** Also, weakly positive and false negative results are occasionally found in MBLs, and especially NDM-1**.** Therefore, MHT has variable sensitivity and specificity and in addition, they are time consuming and required trained microbiologists for interpretation. From previous study, showed that MHT was not well correlated with the genotypic detection of CRE (Carvalhaes et al., 2009). The next development for carbapenemase detection later is Carba-NP test. This test is based on *in vitro* hydrolysis of the imipenem by a change in the pH value of the indicator from red to yellow/orange. This test is inexpensive, rapid and reproducible (Nordmann et al., 2012). Nevertheless, they may yield invalids results in some OXA-producing strain. Indicating that some have good specificity and sensitivity but none of them approaches 100%.

To resolve a defect of these phenotypic assay, a new specific phenotypic method was recently described, the carbapenem inactivation method (CIM) was usually described in 2015**.** Then, the CIM was developed to modified carbapenem inactivation method (mCIM) by CLSI working group**.** This test is based on the principle that when a 10 µg meropenem (MEM) disk is incubated for 4 h in an aqueous suspension of a carbapenemase-producing microorganism, the carbapenem in the disk is degraded by carbapenemase, in contrast, if the test microorganism does not produce carbapenemase, MEM retains its antimicrobial activity after incubation in the bacterial suspension. This test has high sensitivity and specificity for the detection of a variety of carbapenemase, with equal or better sensitivity for the detection of OXA-48-type carbapenemase in *Enterobacteriaceae***.** Nevertheless, the mCIM be not able to distinguish between serine-based carbapenemases and MBLs, therefore the

eCIM was established. The eCIM employs EDTA, in conjunction with the mCIM assay to differentiate serine from MBLs. However, mCIM with or without eCIM testing is not currently recommended for routine use (CLSI, 2018). The results of phenotypic detection can be preliminary guidelines for carbapenemase classification. However, as in all phenotypic assays, carbapenemase detection is requires genotypic assay for the precise identification of carbapenamase gene.

6. Conclusion

In conclusion, we determined the prevalence of CRE from 4 hospitals clinical microbiology laboratory includes, Budhachinnaraj hospital, Srisangworn hospital, Uthaithani hospital, Sawanpracharak hospital, entirely located in the lower north region of Thailand. The CRE prevalence was approximately 44.05% and all of CRE isolates were detected for carbapenemase production by phenotypic methods. The detection of carbapenemase production revealed that, the MHT was positive for 25%. Meanwhile, Carba NP was positive for 29.16%. However, this study focused on the simple phenotypic detection methods for carbapenemase detection. Therefore, further study is required molecular technique which, remain the gold standard for the precise identification of carbapenamase gene in reference laboratories. Nevertheless, this found is preliminary prevalence, the information documented in this study may offer significant help in the control to reducing the spread of CRE and empirical antibiotic management.

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