The Prevalence of Carbapenemase-Producing Enterobacteriaceae Isolated from Stool

ความชุกของเชื้อกลุ่ม Enterobacteriaceae ที่สร้างเอนไซม์ carbapenemases ที่แยกได้จากอุจจาระ

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ABSTRACT

Carbapenem resistance in Enterobacteriaceae has been increasingly reported worldwide. Carbapenemase production is the main mechanism of carbapenem resistance. In this study, we investigated the prevalence of carbapenemase-producing Enterobacteriaceae isolated from stool from patients in King Chulalongkorn Memorial Hospital during December 2012 to August 2013. Carbapenemase genes including bla_{IMP}, bla_{VIM}, bla_{GIM}, bla_{SIM}, bla_{SPM}, bla_{KPC}, bla_{OXA-48} and bla_{NDM} were detected by multiplex PCR. Carbapenemase activity was determined by modified Hodge test, boronic acid-based inhibition test and EDTA-meropenem combined-disk test. A total of 2,400 Enterobacteriaceae isolates were screened for carbapenemase producers by using MacConkey agar containing ertapenem, cloxacillin and $ZnSO_4$. Only 3 isolates (0.13 %) of *Klebsiella pneumoniae* were carbapenemase producers, which carried bla_{NDM} and had metallo-β-lactamase activity. The results showed that NDM (New Delhi Metallo-β-lactamase) was the most common carbapenemase among Enterobacteriaceae isolates.

บทคัดย่อ

การดื้อยากลุ่ม carbapenems ในเชื้อกลุ่ม Enterobacteriaceae มีรายงานเพิ่มสูงขึ้นในหลายประเทศทั่วโลก โดยการสร้างเอนไซม์ carbapenemases เป็นกลไกหลักที่เชื้อใช้ในการดื้อยากลุ่ม carbapenems ในการศึกษานี้ได้ทำการตรวจหาความชุกของเชื้อกลุ่ม Enterobacteriaceae ที่สร้างเอนไซม์ carbapenemases ที่แยกได้จากอุจจาระจากผู้ป่วยในโรงพยาบาลจุฬาลงกรณ์ ตั้งแต่เดือนธันวาคม 2555 ถึงเดือนสิงหาคม 2556 โดยทำการตรวจหายีนที่สร้างเอนไซม์ carbapenemases ได้แก่ ยีน *bla* ชนิด IMP, VIM, GIM, SIM, SPM, KPC, OXA-48 และ NDM ด้วยวิธี multiplex PCR ตรวจหาการทำงานของเอนไซม์ carbapenemases ด้วยวิธี modified Hodge test, boronic acid-based inhibition test และ EDTA-meropenem combined-disk test เชื้อกลุ่ม Enterobacteriaceae จำนวน 2,400 สายพันธุ์ ถูกนำมาตรวจคัดกรองเพื่อหาเชื้อที่สร้างเอนไซม์ carbapenemases โดยใช้ MacConkey agar ที่มี ertapenem, cloxacillin และ $ZnSO_4$ พบเชื้อ *Klebsiella pneumoniae* จำนวน 3 สายพันธุ์ (0.13 %) เท่านั้นที่สร้างเอนไซม์ carbapenemases ชนิด metallo-β-lactamases ซึ่งมียีน *bla* ชนิด NDM (New Delhi metallo-β-lactamase) จากผลการศึกษานี้แสดงให้เห็นว่าเอนไซม์ carbapenemases ชนิด NDM พบมากที่สุดในเชื้อกลุ่ม Enterobacteriaceae

Key Words: Enterobacteriaceae, Carbapenemases, Carbapenem resistance

คำสำคัญ: เชื้อกลุ่ม Enterobacteriaceae เอนไซม์ carbapenemases การดื้อยากลุ่ม carbapenems

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Introduction

Carbapenems are the last resort antimicrobial agents for treatment of infection caused by multidrug-resistant Enterobacteriaceae. The emergence of carbapenem-resistant Enterobacteriaceae has been increasingly reported worldwide. The major mechanism of carbapenem resistance is the production of carbapenemases. Carbapenemases belong to class A, B and D β-lactamases (Queenan and Bush, 2007). The class A β-lactamases can hydrolyze penicillins, cephalosporins, aztreonam and carbapenems and are inhibited by β-lactamase inhibitors such as clavulanic acid, boronic acid and tazobactam. Enzymes in class A include *Klebsiella pneumoniae* carbapenemase (KPC), *Bi*ceˆtre carbapenemase (BIC), Guiana extended spectrum (GES), *Serratia marcescens* enzyme (SME), Imipenem-hydrolyzing- β-lactamase (IMI) and non-metallocarbapenemase-A (NMC-A). The class B β-lactamases can hydrolyze all β-lactams except aztreonam and are inhibited by EDTA and dipicolinic acid (Mendes et al., 2006). These enzymes include active on imipenem (IMP), Verona integron-encoded metallo- β -lactamase (VIM), New Delhi metallo-β-lactamase (NDM), German imipenemase (GIM), Seoul imipenemase (SIM) and Sao Paulo metallo- β -lactamase (SPM). The spread of IMP and VIM have been reported worldwide and mostly identified in enterobacterial species and *Pseudomonas* spp. (Walsh TR, 2005). Recently, the NDM-1 was first reported in Enterobacteriaceae isolates from an Indian patient in Sweden (Yong et al., 2009). The NDM-1 carbapenemases were disseminated in India and Pakistan (Nordmann et al., 2011) and now have been reported in many geographic regions. Oxacillinases (OXA) carbapenemases belong to class D β-lactamases, which can hydrolyze almost all beta-lactams and are inhibited by NaCl (Poirel et al., 2010). The first report of OXA-48 was identified in *Klebsiella pneumoniae* from Turkey and has been spreading worldwide (Poirel et al., 2012). In 2009, screening of stool specimens is recommended by the Centers for Disease Control and Prevention to identify carriers of carbapenem-resistant Gram-negative rods and initiate appropriate infection control measures (Julie Blackburn, 2013). In this study, we investigated the prevalence of carbapenemase-producing Enterobacteriaceae isolated from stool from patients in King Chulalongkorn Memorial Hospital.

Materials and methods

Bacterial strains

A total of 2,400 Enterobacteriaceae recovered from stools from patients in King Chulalongkorn Memorial Hospital during December 2012 to August 2013, were screened for carbapenemase producers. All isolates were plated onto MacConkey agar (Oxoid Ltd., Basingstoke, Hampshire, England) containing ertapenem (0.25 µg/ml) (LABORATORIES MERCK SHARP & DOHME-CHIBRET, France), cloxacillin (250 µg/ml) (M&H Manufacturing, Thailand) and $ZnSO_4$ (70 µg/ml) (SIGMA-ALDRICH, St.Louis, MO, USA) as modified from previous report (Nordmann et al., 2012).

Antimicrobial susceptibility test and minimum inhibitory concentration (MIC) determination

The minimum inhibitory concentrations of imipenem (Merck Sharp & Dohme Corp., USA), meropenem (AstraZeneca UK Limited, United Kingdom), ertapenem (LABORATORIES MERCK

SHARP & DOHME-CHIBRET), cefotaxime (SIGMA-ALDRICH,co., St.Louis, MO, USA), ceftriaxone (Siam Bheasach Co.,Ltd., Thailand) and ceftazidime (SIGMA-ALDRICH, St.Louis, MO, USA) were determined by agar dilution method and interpreted according to the Clinical and Laboratory Standards Institute (CLSI) guidelines. The quality control strains were *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Enterococcus faecalis* ATCC 29212 and *Staphylococcus aureus* ATCC 29213. MICs of ≥ 2, ≥ 4, ≥ 4, ≥ 16, ≥ 4 and ≥ 4 µg/ml were classified as resistant to ertapenem, imipenem, meropenem, ceftazidime, ceftriaxone and cefotaxime, respectively.

Detection of carbapenemase genes

All isolates from MacConkey agar (Oxoid) containing ertapenem (LABORATORIES MERCK SHARP & DOHME-CHIBRET), cloxacillin (M&H Manufacturing) and $ZnSO_4$ (SIGMA-ALDRICH) were tested for the presence of carbapenemase genes. Plasmid DNA was extracted by Hi Yields™ Plasmid Mini Kit (RBC Bioscience's product, Taiwan). Carbapenemase genes including bla_{IMP}, bla_{VIM}, bla_{GIM}, bla_{SIM}, bla_{SPM}, bla_{NDM}, bla_{OXA-48} and bla_{KPC} were determined by 2 multiplex PCRs. The first multiplex PCR was used to detect the bla_{IMP}, bla_{VIM}, bla_{GIM}, bla_{SIM} and bla_{SPM} (Ellington et al., 2007). The second multiplex PCR was used to amplify the bla_{OXA-48}, bla_{KPC} and bla_{NDM} genes (Poirel et al., 2011). The PCR products were analyzed by electrophoresis on 1.2 % agarose gel.

Detection of carbapenemase producers

Phenotypic detections of carbapenemases were performed by modified Hodge test (MHT), boronic acid-based inhibition test and EDTA-meropenem combined-disk test. The MHT using ertapenem disk (10 µg) (Oxoid) was used to screen for carbapenemase production (CLSI 2013). The presence of a clover leaf-like indentation of the *E.coli* 25922 growing along the test organism growth streak within the disk diffusion zone was interpreted as a positive result for MHT. The class A carbapenemases were detected by the boronic acid-based inhibition test using meropenem disk (10 µg) (Oxoid) and meropenem containing 400 µg of phenylboronic acid (SIGMA-ALDRICH, St.Louis, MO, USA). The difference of inhibition zone ≥ 5 mm. was considered positive (Tsakris et al., 2010). The class B carbapenemases were detected by the EDTA-meropenem combined-disk test using meropenem disk (10 µg) and meropenem disk containing 292 µg of EDTA (Affymetrix, Netherlands). The interpretation of this test was similar to boronic acid-based inhibition test (Tsakris et al., 2010).

Results

Of the 2,400 Enterobacteriaceae isolates, 74 (3.1%) were positive on MacConkey agar containing ertapenem, cloxacillin and $ZnSO_4$. All 74 isolates were screened for carbapenemase genes by 2 multiplex PCRs (Figure 1).

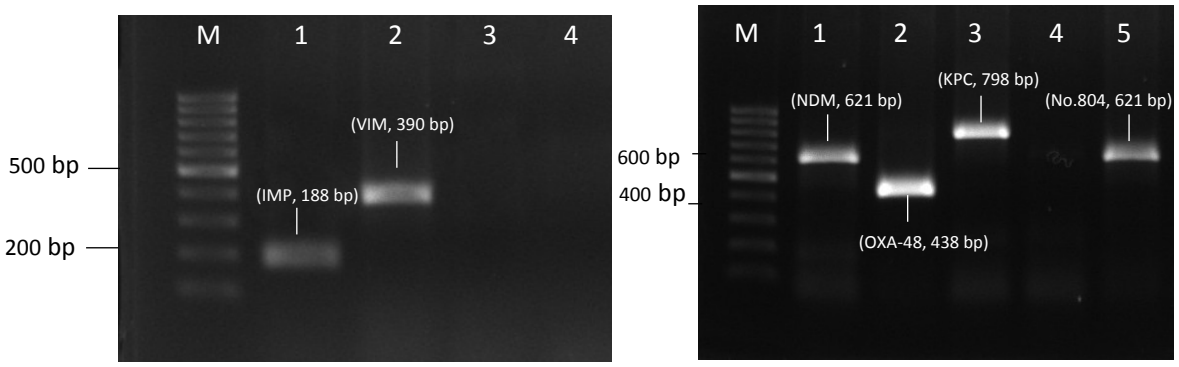

Figure 1 (A) Multiplex PCR analysis of the bla_{IMP}, bla_{VIM}, bla_{GIM}, bla_{SIM} and bla_{SPM}: M, 100-bp DNA ladder; Lane 1, positive control (*K.pneumoniae* harbouring bla_{IMP}); Lane 2, positive control (*P. aeruginosa* harbouring bla_{VIM}); Lane 3, *K.pneumoniae* isolate No.804; Lanes 4, negative control (sterile DDW). (B)Multiplex PCR analysis of the bla_{OXA-48}, bla_{KPC} and bla_{NDM}: M, 100-bp DNA ladder; Lane 1, positive control (*E.coli* harbouring bla_{NDM}); Lane 2, positive control (*K.pneumoniae* harbouring bla_{OXA-48}); Lanes 3, positive control (*K.pneumoniae* harbouring bla_{KPC}); Lane 4, negative control (sterile DDW); Lane 5, *K.pneumoniae* isolate No. 804 harbouring bla_{NDM}

Carbapenemase genes were detected in 3 Enterobacteriaceae isolates (0.125%), all of which were *K. pneumoniae*. These three isolates harbored bla_{NDM}, encoding the NDM-type metallo-β-lactamases. As expected, all isolates were positive for the MHT and EDTA-meropenem combined-disk test. Antimicrobial susceptibility results showed that all *K.pneumoniae* carrying the bla_{NDM} genes exhibited highly resistant to carbapenems and the third-generation cephalosporins. MICs of 6 antimicrobial agents, including ertapenem, meropenem and imipenem, cefotaxime, ceftriaxone and ceftazidime are shown in Table 1.

Table 1 Characteristics of clinical isolates carrying carbapenemase genes

No.	Organism	MIC (μg/ml)						MHT	Boronic acid-based	EDTA-meropenem	Carbapenemase
		IPM	MEM	ETP	CTX	CRO	CAZ		inhibition test	combined-disk test	gene
804	*Klebsiella pneumoniae*	16	32	64	>256	>256	>256	+	-	+	bla_{NDM}
992	*Klebsiella pneumoniae*	4	8	32	>256	>256	>256	+	-	+	bla_{NDM}
1596	*Klebsiella pneumoniae*	16	32	64	>256	>256	>256	+	-	+	bla_{NDM}

CAZ, ceftazidime; CRO, ceftriaxone; CTX, cefotaxime; ETP, ertapenem; IPM, imipenem; MEM, meropenem; MHT, modified hodge test.

Discussion

Various cabapenemases are increasingly reported in Enterobacteriaceae isolates. Carbapenemase genes are mostly plasmid-mediated and are spreading rapidly worldwide. The most prevalent carbapenemases in Enterobacteriaceae are the KPC-type class A β-lactamase and the zinc-dependent class B metallo-β-lactamase (MBLs), represented mainly by the VIM, IMP types (Tzouvelekis et al., 2012). In 1990s, the first report of IMP encoding *K. pneumoniae* was described in Japan. Currently, IMP has spread worldwide (Queenan and Bush, 2007). In 2008, a novel metallo-β-lactamase, NDM had been identified in a Swedish patient of Indian origin (Yong et al., 2009). These enzymes hydrolyze all β-lactam except aztreonam and are inhibited by EDTA. NDM carbapenemases were commonly found in India, Pakistan and the UK (Kumarasamy et al., 2010). In this study, carbapenemase-producing Enterobacteriaceae were screened by using MacConkey agar containing ertapenem, cloxacillin and $ZnSO_4$. $ZnSO_4$ enhances expression of MBLs and cloxacillin, an inhibitor of AmpC β-lactamases, can prevent growth of isolates expressing high levels of AmpC enzymes. This screening agar was modified from Supercarba medium (Nordmann et al., 2012) by using MacConkey agar instead of Drigalski agar. The low concentration of ertapenem (0.25 µg/ml) in the medium can facilitate the detection of OXA-48 carbapenemase producers with low-level resistance to carbapenems.

Of the 2,400 Enterobacteriaceae isolates, 3.1% were selected on this medium. PCR analysis showed that only 0.13% (3/2,400 isolates) carried bla_{NDM}, and had metallo-β-lactamase activities which were detected by phenotypic methods. Therefore, it is still needed to further improve the specificity of the medium for screening carbapenemase producers However, negative results from PCR cannot exclude other carbapenemase genes that did not include in multiplex PCRs.

Similar to our study, the prevalence of carbapenemase-producing Enterobacteriaceae from Thailand was previously reported to be 0.17 % (Rimrang et al., 2012). In Pakistan, the prevalence of faecal carriage of multidrug-resistant Enterobacteriaceae with the NDM-1 enzyme in Rawalpindi was high (18.5 %) (Perry et al., 2011). OXA-48 carbapenemase has been identified in *K.pneumoniae* isolates (Poirel et al., 2012) and rapidly spread worldwide including Asia, North America and Europe (Limbago et al., 2011). Recently, nosocomial outbreak of OXA-48-producing *Enterobacter cloacae* in the gut flora has been reported (Lise Cremet, 2012). The KPC enzymes were commonly found in the United States and then worldwide (Calfee, 2010). However, both OXA-48 and KPC were not detected in this study. Further studies should monitor carbapenemase-producing Enterobacteriaceae faecal carriage to control the spread of these organisms in hospital settings.

Conclusions

The prevalence of carbapenemase-producing Enterobacteriaceae from stool was 0.13 %. NDM was the most common carbapenemase among Enterobacteriaceae isolates. This is the first report of NDM-carrying *K. pneumoniae* isolates in King Chulalongkorn Memorial Hospital.

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References

Blackburn, J., Tsimiklis, C., Lavergne, V., Pilotte, J., Grenier, S., Gilbert, A., Lefebvre, B., Domingo, MC., Tremblay, C., and Bourgault, AM. 2013. Carbapenem Disks on MacConkey Agar in Screening Methods for Detection of Carbapenem-Resistant Gram-Negative Rods in Stools. Journal of Clinical Microbiology. 51(1): 331-333.

Calfee, DP. 2010. *Klebsiella pneumoniae* carbapenemase-producing enterobacteriaceae. Journal of Infusion Nursing. 33(3): 150-154.

Ellington, MJ., Kistler, J., Livermore, DM., and Woodford, N. 2007. Multiplex PCR for rapid detection of genes encoding acquired metallo-beta-lactamases. Journal of Antimicrobial Chemotherapy. 59(2): 321-322.

Kumarasamy, KK., Toleman, MA., Walsh, TR., Bagaria, J., Butt, F., Balakrishnan, R., Chaudhary, U., Doumith, M., Giske, CG., Irfan, S., Krishnan, P., Kumar, AV., Maharjan, S., Mushtaq, S., Noorie, T., Paterson, DL., Pearson, A., Perry, C., Pike, R., Rao, B., Ray, U., Sarma, JB., Sharma, M., Sheridan, E., Thirunarayan, MA., Turton, J., Upadhyay, S., Warner, M., Welfare, W., Livermore, DM., and Woodford, N. 2010. Emergence of a new antibiotic resistance mechanism in India, Pakistan, and the UK: A molecular, biological, and epidemiological study. Lancet Infectious Diseases. 10(9): 597-602.

Limbago, BM., Rasheed, JK., Anderson, KF., Zhu, W., Kitchel, B., Watz, N., Munro, S., Gans, H., Banaei, N., and Kallen, AJ. 2011. IMP-producing carbapenem-resistant *Klebsiella pneumoniae* in the United States. Journal of Clinical Microbiology. 49(12): 4239-4245.

Lise, C., Bourigault, C., Lepelletier, D., Guillouzouic, A., Juvin, ME., Reynaud, A., Corvec, S. and Caroff, N. 2012. Nosocomial outbreak of carbapenem-resistant *Enterobacter cloacae* highlighting the interspecies transferability of the bla_{OXA-48} gene in the gut flora. Journal of Antimicrobial Chemotherapy. 67(4): 1041-1043.

Mendes, RE., Castanheira, M., Campos Pignatari, AC., and Gales, AC. 2006. Metallo-β-lactamases. Jornal Brasileiro de Patologia e Medicina Laboratorial. 42(2): 103-113.

Nordmann, P., Girlich, D., and Poirel, L. 2012. Detection of carbapenemase producers in Enterobacteriaceae by use of a novel screening medium. Journal of Clinical Microbiology. 50(8): 2761-2766.

Nordmann, P., Naas, T., and Poirel, L. 2011. Global spread of Carbapenemase-producing Enterobacteriaceae. Emerging Infectious Diseases. 17(10): 1791-1798.

Perry, JD., Naqvi, SH., Mirza, IA., Alizai, SA., Hussain, A., Ghirardi, S., Orenga, S., Wilkinson, K., Woodford, N., Zhang, J., Livermore, DM., Abbasi, SA., and Raza, MW. 2011. Prevalence of faecal carriage of Enterobacteriaceae with NDM-1 carbapenemase at military hospitals in Pakistan, and evaluation of two chromogenic media. Journal of Antimicrobial Chemotherapy. 66(10): 2288-2294.

Poirel, L., Naas, T., and Nordmann, P. 2010. Diversity, epidemiology, and genetics of class D β-lactamases. Antimicrobial Agents and Chemotherapy. 54(1): 24-38.

Poirel, L., Potron, A., and Nordmann, P.. 2012. OXA-48-like carbapenemases: The phantom menace. Journal of Antimicrobial Chemotherapy. 67(7): 1597-1606.

Poirel, L., Walsh, TR., Cuvillier, V., and Nordmann, P. 2011. Multiplex PCR for detection of acquired carbapenemase genes. Diagnostic Microbiology and Infectious Disease. 70(1): 119-123.

Queenan, AM., and Bush, K.. 2007. Carbapenemases: The versatile β-lactamases. Clinical Microbiology Reviews. 20(3): 440-458.

Rimrang, B., Chanawong, A., Lulitanond, A., Wilailuckana, C., Charoensri, N., Sribenjalux, P., Phumsrikaew, W., Wonglakorn, L., Kerdsin, A., and Chetchotisakd, P. 2012. Emergence of NDM-1- and IMP-14a-producing Enterobacteriaceae in Thailand. Journal of Antimicrobial Chemotherapy. 67(11): 2626-2630.

Tsakris, A., Poulou, A., Pournaras, S., Voulgari, E., Vrioni, G., Themeli-Digalaki, K., Petropoulou, D., and Sofianou, D. 2010. A simple phenotypic method for the differentiation of metallo-β-lactamases and class A KPC carbapenemases in Enterobacteriaceae clinical isolates. Journal of Antimicrobial Chemotherapy. 65(8): 1664-1671.

Tzouvelekis, LS., Markogiannakis, A., Psichogiou, M., Tassios, PT., and Daikos, GL. 2012. Carbapenemases in *Klebsiella pneumoniae* and other Enterobacteriaceae: An evolving crisis of global dimensions. Clinical Microbiology Reviews. 25(4): 682-707.

Walsh, TR., Tolemen, M., Poirel, L., and Nordmann, P. 2005. Metallo-β-lactamase: the quiet before the storm? Clinical Microbiology Reviews. 18(2): 306-325.

Wayne, Performance Standards for Antimicrobial Susceptibility Testing. 2013. Clinical and Laboratory Standards Institute. Pennsylvania.

Yong, D., Toleman, MA., Giske, CG., Cho, HS., Sundman, K., Lee, K., and Walsh, TR. 2009. Characterization of a new metallo-β-lactamase gene, $bla_{\text{NDM-1}}$, and a novel erythromycin esterase gene carried on a unique genetic structure in Klebsiella pneumoniae sequence type 14 from India. Antimicrobial Agents and Chemotherapy. 53(12): 5046-5054.