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High-level carbapenem-resistant OXA-48-producing *Klebsiella pneumoniae* with a novel OmpK36 variant and low-level, carbapenem-resistant, non-porin-deficient, OXA-181-producing *Escherichia coli* from Thailand

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ABSTRACT

Five $bla_{\text{OXA-48-like}}$-carrying Enterobacteriaceae isolates collected from two Thai patients in December 2012 were characterized. Three *Klebsiella pneumoniae* isolates giving two different pulsed-field gel electrophoresis patterns and sequence types (ST11 and ST37) from patient 1 harbored $bla_{\text{OXA-48}}$ locating on Tn*1999.2*, whereas two *Escherichia coli* isolates with the same pulsotype and ST5 from Patient 2 carried IS*Ecp1*-associated $bla_{\text{OXA-181}}$. One *K. pneumoniae* strain had $bla_{\text{SHV-12}}$, $bla_{\text{DHA-1}}$, *qnrB,* and *qnrS,* while another strain harbored $bla_{\text{CTX-M-15}}$, *qnrS* and *aac(6′)-Ib-cr*. The *E. coli* strain contained $bla_{\text{CTX-M-15}}$, $bla_{\text{CMY-2}}$, *qnrS,* and *aac(6′)-Ib-cr*. Interestingly, the OXA-48 producers with a novel OmpK36 variant by a substitution of Gly to Asp in the L3 loop-borne PEFXG motif exhibited high-level resistance to ertapenem, imipenem, and meropenem. In contrast, the OXA-181 producer with non-porin-deficient background showed low-level resistance to ertapenem only. Both patients died because of either septic shock or pneumonia. This study showed the impact of OXA-48-like carbapenemases in porin-defective clinical isolate background, which may lead to serious therapeutic problems in the near future.

1. Introduction

Carbapenem resistance in Enterobacteriaceae by carbapenemase production is a major concern worldwide since the carbapenems are the first treatment options for infections with multidrug-resistant, Gram-negative bacteria. Currently, the most prevalent carbapenemases among these bacteria include class A *Klebsiella pneumoniae* carbapenemase (KPC), class B New Delhi metallo-β-lactamase (NDM) and class D oxacillinase-48 (OXA-48). In addition, the combination of these plasmid-mediated carbapenemases and outer membrane protein loss and/or alteration can confer high-level resistance to carbapenems in Enterobacteriaceae clinical isolates, leading to difficult or limited treatment options (Nordmann et al., 2012).

The OXA-48 carbapenemase was initially identified in *K. pneumoniae* from Istanbul, Turkey in 2001. The $bla_{\text{OXA-48}}$ is originated from chromosome of *Shewanella* spp. and carried by the composite transposon Tn*1999* on IncL/M plasmid, which is a highly conjugative plasmid, resulting in its widespread (Poirel et al., 2012; Potron et al., 2011). Moreover, this gene was integrated into bacterial chromosome by the ability of the IS*1R* insertion sequence that disrupted in the Tn*1999* (Beyrouthy et al., 2014). The OXA-48 β-lactamase and its variants such as OXA-162, OXA-181, OXA-204, and OXA-232 are now widespread in *K. pneumoniae* and other genera of the family Enterobacteriaceae (Poirel et al., 2012). The first report of the OXA-48 group in Southeast Asian countries was OXA-181 produced by *K. pneumoniae* isolates from Singapore in 2012 (Koh et al., 2012). Until now, there has been only one report of an imported case of an OXA-48-producing *E. coli* isolate from Thailand to Norway (Samuelsen et al., 2013). In our hospital, the NDM-1- and IMP-14a-producing Enterobacteriaceae isolates were discovered in 2010 (Rimrang et al., 2012). Since then, Enterobacteriaceae is screened for carbapenemase production as recommended by the Clinical and Laboratory Standards Institute (CLSI) and confirmed for the presence of bla_{KPC}, bla_{NDM}, $bla_{\text{OXA-48}}$, bla_{IMP}, and bla_{VIM} by the multiplex PCR methods (CLSI, 2015; Poirel et al., 2011). Three *K. pneumoniae* isolates and two *E. coli* isolates collected

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from 2 patients in 2012 were found to carry $bla_{OXA-48-like}$ only. Therefore, we characterized these OXA-48-like producers from Thailand by both phenotypic and genotypic methods.

A part of this work was presented at the 25th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID), Copenhagen, Denmark, 2015.

2. Materials and methods

2.1. Clinical isolates

Five OXA-48-like-producing Enterobacteriaceae isolates as confirmed by the multiplex PCR method (Poirel et al., 2011) were collected from two patients in Srinagarind Hospital, Khon Kaen University, Thailand in December 2012. These included 3 *K. pneumoniae* isolates obtained from pleural fluid (isolates Kp-97-A and Kp-97-B) and sputum (isolate Kp-133) of the same patient and two *E. coli* isolates recovered from sputum of another patient. They were identified by conventional biochemical tests and kept in skimmed milk with 15% glycerol at −20 °C until analysis.

This study was conducted according to the guidelines of the Declaration of Helsinki and good clinical practices. The experimental protocol was approved by the Ethics Committee of Khon Kaen University (project number HE571290).

2.2. Antimicrobial susceptibility testing

The minimum inhibitory concentrations (MICs) of various antimicrobials for all isolates were determined by an agar dilution method using Mueller-Hinton agar (MHA) (Oxoid, Hampshire, England) (CLSI, 2015). These included amikacin (Siam Bheasach, Bangkok, Thailand), gentamicin (Sigma-Aldrich, St. Louis, MO, USA), ciprofloxacin (Sigma-Aldrich), cefotaxime (Sigma-Aldrich), ceftazidime (Sigma-Aldrich), ertapenem (MSD, Paris, France), imipenem (MSD, Whitehouse Station, NJ, USA), meropenem (Siam Bheasach), colistin (Sigma-Aldrich), fosfomycin (Meiji Seika Pharma, Tokyo, Japan), and tigecycline (Pfizer Inc., Philadelphia, PA, USA). MICs of colistin and tigecycline were interpreted using criteria of the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2015), whereas those of the remaining antimicrobials were interpreted according to the CLSI guidelines (CLSI, 2015). *E. coli* ATCC25922 was used as an antimicrobial-susceptible control strain.

2.3. Detection of various resistance determinants

All isolates were subjected to the modified Hodge test (MHT) (CLSI, 2015). PCR amplification of ESBL (bla_{SHV}, bla_{CTX-M}, and bla_{VEB}) (Chanawong et al., 2007), pAmpC (bla_{ACC}, bla_{ACT}, bla_{CMY}, bla_{DHA}, and bla_{FOX}) (Perez-Perez and Hanson, 2002), plasmid-mediated quinolone resistance (PMQR) [*qnrA*, *qnrB*, *qnrS*, *qnrD*, *qnrC*, *qepA*, and *aac(6′)-Ib*-] (Pasom et al., 2013) and porin genes [*ompK35*, *ompK36* (Kaczmarek et al., 2006), *ompC* (ompC-F: 5′-AAC TTA AAG TAC TGT CCC TCC TGG T-3′ and ompC-R: 5′-TGG TAA ACC AGA CCC AGA GC-3′) and *ompF* (ompF-F: 5′-AAG CGC AAT ATT CTG GCA GT-3′ and ompF-R: 5′-AAA CGA TAC CCA CAG CAA CG-3′) (this study)] were performed for all isolates. Either primers ompK35-InF (5′-GCA ATA TTC TGG CAG TGG TGA TC-3′) or IS1R (5′-TCT TCC GGA GCC TGT CAT AC-3′) (this study) vs. ompK35 reverse primer (Kaczmarek et al., 2006) were also used for amplification of *ompK35* and IS*1* insertion, respectively. PCR products of these genes were purified and sequenced using an automated sequencer (Applied Biosystems 373XL, Foster City, CA, USA).

2.4. Transposons associated with the $bla_{OXA-48-like}$

The genetic environments of the $bla_{OXA-48-like}$ from all isolates were determined by PCR techniques using specific primers for IS*1999*, IS*1R*, and *lysR* of the Tn*1999*-like transposon and IS*Ecp1* of the Tn*2013* transposon (Chanawong et al., 2007; Poirel et al., 2004; Potron et al., 2011).

2.5. Conjugation assay

Transfer of carbapenem resistance was performed by broth-mating method using streptomycin-resistant *E. coli* UB1637 as a recipient. Transconjugants were then selected on MacConkey agar (Oxoid) supplemented with 0.15 mg/L of ertapenem (MSD) and 1600 mg/L of streptomycin (M & H Manufacturing, Samutprakarn, Thailand).

2.6. Plasmid DNA analysis

Plasmids of donor isolates and their transconjugants were extracted by an alkaline lysis method of Bennett et al. (1986) and then separated on 0.8% agarose (Invitrogen, Barcelona, Spain). Plasmid sizes were estimated by comparing with plasmids of *E. coli* NCTC50192 and *E. coli* NCTC50193. The DNA fragments were transferred to nylon membranes (Roche Applied Sciences, Mannheim, Germany) by the method of Southern (1975), hybridized with either digoxigenin-labelled bla_{OXA-48}- or $bla_{OXA-181}$-specific probes and detected using the NBT/BCIP color detection kit (Roche Applied Sciences) according to the manufacturer's instruction. In addition, PCR-based replicon typing of the bla_{OXA-48}-carrying plasmids from the transconjugants was performed using specific primers of IncL/M and IncA/C (Carattoli et al., 2005).

2.7. Strain typing

Genetic relatedness among the OXA-48-like-producing isolates was determined by pulsed-field gel electrophoresis (PFGE) with *Xba*I restriction enzyme (New England Biolabs, Ipswich, MA, USA) according to the Centers for Disease Control and Prevention (CDC) PulseNet protocol with minor modifications (CDC, 2013). DNA fragments were separated in 1.2% agarose (SeaKem Gold agarose, Lonza, Rockland, ME, USA) at 12 °C by CHEF-DRII Pulsed-Field Electrophoresis System (Bio-Rad, Foster City, CA, USA) using 200 V for 18 h with pulse times ranging from 2.2 to 54.2 s. The Pulsenet universal strain *Salmonella enterica* serovar Braenderup H9812 was used as a DNA standard marker. The fingerprints were visually compared and interpreted according to the method of Tenover et al. (1995). Furthermore, MLST was carried out using the protocols of Diancourt et al. (2005) and Wirth et al. (2006) and the sequence types were designated using the Pasteur database (www.pasteur.fr/recherche/genopole/PF8/mlst) and the University of Warwick, UK scheme (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli) for *K. pneumoniae* and *E. coli* isolates respectively.

3. Results and discussion

Clinical history of both patients with the OXA-48-like-producing *K. pneumoniae* and *E. coli* isolates were as follows. **Patient 1:** A 60-year-old man with underlying of diabetes mellitus, hypertension, and old pulmonary tuberculosis was referred from other hospital with gastrointestinal bleeding and partial gut obstruction. He was underwent exploratory laparotomy and found upper jejunal mass. Segmental small bowel resection was performed. He initially received ceftriaxone and metronidazole and subsequently changed to piperacillin-tazobactam without clinical response. He developed postoperative complication with healthcare-associated pneumonia. Meropenem and vancomycin were given as empirical treatment. However, his condition was worsening and he died because of septic shock. The sputum and pleural effusion grew *K. pneumoniae* isolates reported as carbapenem-resistant Enterobacteriaceae (CRE) and *Acinetobacter baumannii* isolates. **Patient 2:** A 21-year-old female was admitted with history of fever, progressive enlargement of abdominal mass and weight loss for 2 months. She underwent exploratory laparotomy surgery and found large amounts of turbid ascites, severe adhesion and matted omentum,

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