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ORIGINAL ARTICLE

Characterization of the modified Hodge test-positive isolates of *Enterobacteriaceae* in Taiwan

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Background/Purpose: The modified Hodge test is a phenotypic test to detect KPC-type carbapenemase producers among *Enterobacteriaceae*, as recommended by the Clinical Laboratory Standards Institute. However, false positive results were reported. In this study, we aimed to large-scale investigate the characterization of the modified Hodge test-positive isolates of *Enterobacteriaceae* collected between 2006 and 2010 in Taiwan.
Methods: Fifty-six isolates, including 24 *Enterobacter cloacae*, 17 *Escherichia coli*, 10 *Klebsiella pneumoniae*, and 5 *Citrobacter freundii*, tested positive with the modified Hodge test. The *in vitro* activities of 10 antimicrobial agents were determined by the agar dilution method. Boronic acid combined-disk test was used to further confirm the KPC producers. Phenotype of ESBL, AmpC, class B carbapenemases, and profile of outer membrane proteins were investigated by the confirmatory test, boronic acid disk method, 2-mercaptopropionic acid double-disk method, and urea/sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), respectively. β-lactamase genes were examined by PCR and sequencing.
Results: These isolates were resistant to ceftazidime (100%), aztreonam (82.1%), ertapenem (64.3%), gentamicin (53.6%), ciprofloxacin (50%), levofloxacin (48.2%), cefepime (19.6%), imipenem (16.1%), meropenem (12.5%), and amikacin (8.9%). Phenotypic testing among isolates revealed the production of ESBLs, metallo-β-lactamases (MBLs), and AmpC in 10 (17.9%), 16 (28.6%), and 12 (44.4%) isolates, respectively. Carbapenemase and non-carbapenemase

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β-lactamase genes bla_{TEM-1}, bla_{SHV}, bla_{CTX-M}, bla_{IMP-8}, bla_{CMY-2}, and bla_{DHA-1} were found in 32 (57.1%), 19 (33.9%), 4 (7.1%), 16 (28.6%), 14 (25%), and 5 (8.9%) of the strains, respectively. No class A and D carbapenemase genes were detected. Outer membrane protein profile showed obviously decreased expression in 49 (87.5%) isolates with positive result of modified Hodge test.

Conclusions: Our data show that ESBLs, AmpC, and imipenemase-8 (IMP-8) carbapenemase coupled with decreased expression of outer membrane protein were prevalent in *Enterobacteriaceae* isolates testing positive for the modified Hodge test in Taiwan.

Introduction

Extended-spectrum β-lactamases (ESBLs) production in *Enterobacteriaceae* is a serious problem worldwide.[1] Carbapenems, such as ertapenem, imipenem, and meropenem, would be the main therapeutic option for treatment of serious infections or against highly resistant *Enterobacteriaceae* with ESBLs.[2,3] Recently, the rapid emergence and dissemination of carbapenem resistance in *Enterobacteriaceae* was reported.[4–6] These carbapenem-resistant strains are characterized by their resistance to almost all β-lactam antibiotics, including the third- and fourth-generation cephalosporins, as well as to fluoroquinolones and aminoglycosides.[4–6]

The mechanisms of carbapenem resistance in *Enterobacteriaceae* are through loss of porins in combination with the expression of AmpC enzymes or ESBLs or carbapenemase production.[7–10] Carbapenemases are divided into three classes: Ambler Class A (*Klebsiella pneumoniae* carbapenemase [KPC], *Serratia marcescens* enzyme (Sme), non-metallocarbapenemase-A (NMC-A), imipenemase (IMI), plasmidic extended-spectrum β-lactamases (PER), class A β-lactamase with carbapenemase activity from *Serratia fonticola* (SFC), class A β-lactamase from *Serratia fonticola* (SFO), and some allelic variants of Guiana extended-spectrum β-lactamase/integron-borne cephalosporinase (GES/IBC)), Class B (Australia integron-encoded metallo-β-lactamase (AIM), Germany integron-encoded metallo-β-lactamase (GIM), imipenemase (IMP), New Delhi metallo-β-lactamase (NDM-1), Seoul integron-encoded metallo-β-lactamase (SIM), São Paulo metallo-β-lactamase (SPM), Verona integron-encoded metallo-β-lactamase (VIM)), and class D (oxacillinase (OXA) and *Pseudomonas*-specific enzymes (PSE)).[11] The rapid emergence and spread of carbapenemase-producing strains is caused by epidemics of bacteria bearing plasmids coding for KPC, IMP, VIM, NDM-1, and OXA-48 enzymes.[11] Therefore, confirmation of carbapenemase production is very important to controlling the spread of carbapenemase-producing bacteria.

The modified Hodge test, which was recommended by the Clinical Laboratory Standards Institute (CLSI),[12] is a phenotypic screening test providing for a high level of sensitivity (> 90%) and specificity (> 90%) in detecting KPC-type carbapenemase producers among *Enterobacteriaceae*. However, false-positive results were reported in species of *Enterobacteriaceae*, including *Escherichia coli* and *K. pneumoniae* and intrinsic chromosomal AmpC β-lactamase of *Enterobacter cloacae* and *Serratia marcescens*, and these false positive results may occur due to minor carbapenem hydrolysis by AmpC enzymes, ESBLs, or loss of porins.[3,13–16]

A boronic acid-based method, using the 3-aminophenylboronic acid (APB)-imipenem combined disks, was reported to be promising in detection of the presence of Class A carbapenemases, and it had higher sensitivity and specificity than the modified Hodge test.[11,13–15,17]

In this study, we aimed to perform a large-scale investigation into the characterization of the modified Hodge test-positive isolates of *Enterobacteriaceae* collected from 2006 to 2010 in Taiwan. Our data revealed that ESBLs, AmpC enzymes, and IMP-8 carbapenemase coupled with decreased expression of outer membrane protein were prevalent in *Enterobacteriaceae* isolates testing positive by the modified Hodge test. Moreover, based on our knowledge, this is the first report of false detection of carbapenemase by the modified Hodge test in *Citrobacter freundii*.

Methods

Clinical isolates

Previous reports showed that Gram-negative bacilli producing KPC-type carbapenemase were resistant to cephalosporins, whereas their susceptibilities to carbapenems covered a wide range.[2,13,16] Therefore, our isolates resistant to third-generation cephalosporins or intermediate or resistant to carbapenems recovered from 2006 to 2010 were collected and screened for the KPC-type carbapenemase by the modified Hodge test. A total of 56 isolates of *Enterobacteriaceae* (24 *E. cloacae*, 17 *E. coli*, 10 *K. pneumoniae*, and five *C. freundii*) with positive modified Hodge test were obtained from two medical centers, the Department of Pathology at the National Cheng Kung University Hospital and the Department of Laboratory Medicine at the China Medical University Hospital in Taichung, Taiwan. All strains were nonduplicates and were identified by colony morphology, Gram stain, biochemical tests, or Vitek system (BioMérieux, Marcy l'Etoile, France).

Antimicrobial susceptibility testing

Minimal inhibitory concentrations (MICs) of amikacin, aztreonam, cefepime, ceftazidime, ciprofloxacin, ertapenem, imipenem, levofloxacin, and meropenem (Sigma

Chemical Co., St. Louis, MO USA) and gentamicin (Amresco Inc., Solon, OH USA) were determined in duplicate by the agar dilution method according to the recommendations of the CLSI.[12] Briefly, bacteria were suspended in saline to one-tenth the turbidity of the 0.5 McFarland standard, then inoculated directly onto antibiotic-containing Mueller-Hinton agar. After 16 to 20 hours of incubation, the MIC of each antibiotic was determined. *E. coli* ATCC 25922 was used as the quality control strain. The resistance breakpoints for these antimicrobial agents were determined according to the recommendations of the CLSI.[12]

Modified Hodge test

The modified Hodge test was performed as described by CLSI.[12] The enhanced growth of *E. coli* ATCC 25922 toward the ertapenem disk was interpreted as a positive result for carbapenemase production. *K. pneumoniae* ATCC BAA-1705 and ATCC BAA-1706 were used as the positive and negative quality control strains, respectively.

Boronic acid combined-disk test

Boronic acid compounds are effective as KPC inhibitors for the phenotypic detection of KPCs.[13–15,17] Two disks each containing ertapenem or imipenem (10 μg [BBL Microbiology Systems, Cockeysville, MD USA]) were placed on a Muller-Hinton agar plate inoculated with a tested strain and adjusted to a 0.5 McFarland standard. Ten microliters containing 400 μg of phenyl boronic acid (Sigma Chemical Co.) was added to one of the ertapenem or imipenem disks in each set. After incubation for 16 to 20 hours, enhancement of the zone of inhibition ($\geq$ 5 mm) in the area between the ertapenem or imipenem disks with (as compared with without) phenyl boronic acid was considered to be a positive result.[17] The boronic acid combined-disk test was performed in duplicate and *K. pneumoniae* ATCC BAA-1705 and ATCC BAA-1706 were used as the positive and negative quality control strains, respectively.

β-lactamases detection assays

Screening for ESBLs in *Enterobacteriaceae* was according to the recommendation from CLSI,[12] using cefotaxime (30 μg) and ceftazidime (30 μg) alone and combined with clavulanic acid (10 μg [BBL Microbiology Systems]). Isolates were screened for AmpC and Class B carbapenemases with the boronic acid disk method and the 2-mercaptopropionic acid double-disk method, respectively.[18,19]

Identification of carbapenemase activities in crude extracts of bacterial isolates was performed by a spectrophotometric assay using imipenem as the substrate.[9,13,20] Briefly, the bacterial cells from an overnight culture were diluted 1:20 into fresh medium and incubated for 2 hours. The bacterial cells were suspended in a solution of 10 mM HEPES (pH 7.5) and disrupted by sonication. The supernatants were obtained by centrifugation at 5000 × g for 10 to 15 minutes and 0.1 mM imipenem was added to measure carbapenemase activities by monitoring imipenem hydrolysis with a Beckman Coulter DU 800 UV/Vis Spectrophotometer (Beckman Coulter, Inc., Brea, CA USA) at 297 nm.

Outer membrane proteins analysis

Bacterial outer membrane proteins (OMPs) were isolated and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) through 10% SDS-PAGE with 4 M urea.[9] Purified OMPs were obtained by treatment of the cell envelopes with 2% sodium-*N*-lauryl sarcosinate (Sigma Chemical Co.). *E. coli* K12 was used as the control.

DNA isolation, polymerase chain reaction amplification, and direct sequencing

Total DNA of *Enterobacteriaceae* was extracted and suspended in 500 μL of 1 × Tris-ethylenediaminetetraacetic acid (EDTA) buffer. Cell suspensions were transferred to boiling water for 20 minutes. Cell debris was removed by centrifugation, and 2 μL of supernatant was used as a source of template DNA in a 50-μL polymerase chain reaction. Strains were analyzed for carbapenemase (bla_{KPC}, bla_{GES}, bla_{IMP}, bla_{OxA}, and bla_{PSE}) and β-lactamase genes (bla_{CMY}, $bla_{CTx\text{-}M}$, bla_{DHA}, bla_{SHV}, and bla_{TEM}), using the primers described previously.[6,9] The purified polymerase chain reaction products were directly sequenced using the automated ABI PRISM 3730 DNA sequencer (Applied Biosystems, Foster, CA USA).

Results

Antibiotic susceptibility of *Enterobacteriaceae* strains positive for the modified Hodge test

A total of 56 modified Hodge test positive isolates collected from 2006 to 2010, including 24 *E. cloacae*, 17 *E. coli*, 10 *K. pneumoniae*, and five *C. freundii*, were investigated. These isolates were resistant to ceftazidime (100%), aztreonam (82.1%), ertapenem (64.3%), gentamicin (53.6%), ciprofloxacin (50%), levofloxacin (48.2%), cefepime (19.6%), imipenem (16.1%), meropenem (12.5%), and amikacin (8.9%). The results are shown in Table 1.

Phenotypic and genotypic screening of modified Hodge test positive *Enterobacteriaceae* strains

The boronic acid combined-disk test was used to screen for the presence of Class A carbapenemases among strains with positive modified Hodge test results. There were four (7.1%) isolates, including two *E. cloacae*, one *E. coli*, and one *K. pneumoniae*, positive for Class A carbapenemases (enhancement of the inhibition diameter between 6–14 and 4–7 mm using ertapenem and imipenem disks, respectively). However, no imipenem hydrolysis was detected among these isolates through spectrophotometric assay. In addition, phenotypic testing among isolates with modified Hodge test positive results revealed the production of ESBLs, MBLs, and AmpC in 10 (17.9%), 16 (28.6%), and 12 (44.4%) isolates, respectively. Three *K. pneumoniae* isolates expressed a coexisting phenotype of ESBLs and AmpC.

Carbapenemase and noncarbapenemase β-lactamase genes were analyzed by polymerase chain reaction, which

Table 1 *In vitro* activity of 10 antimicrobial agents against 56 *Enterobacteriaceae* strains isolated from 2006–2010 that tested positive for the modified Hodge test

Antibiotic	μg/mL			Resistance (%)
	Range	MIC_{50}	MIC_{90}	
Enterobacter cloacae ($n = 24$)				
Amikacin	1–16	2	4	0
Gentamicin	1– > 256	2	256	25
Aztreonam	0.5– > 256	64	> 256	95.8
Ceftazidime	64– > 256	256	> 256	100
Cefepime	0.5–128	4	64	12.5
Ciprofloxacin	0.03–128	0.25	32	25
Levofloxacin	0.12–128	0.5	32	25
Ertapenem	0.5–128	2	4	75
Imipenem	0.25–8	0.5	2	8.3
Meropenem	0.06–4	0.12	1	4.2
Escherichia coli ($n = 17$)				
Amikacin	2– > 64	4	32	5.9
Gentamicin	2– > 256	256	> 256	70.6
Aztreonam	8–256	32	256	64.7
Ceftazidime	16– > 256	64	> 256	100
Cefepime	0.12– > 64	2	> 64	23.5
Ciprofloxacin	0.03–256	64	128	82.4
Levofloxacin	0.12–64	32	64	82.4
Ertapenem	0.12– > 256	0.5	128	41.2
Imipenem	0.12– > 32	0.25	4	17.6
Meropenem	0.03–16	0.06	4	11.7
Klebsiella pneumoniae ($n = 10$)				
Amikacin	2– > 64	2	> 64	40
Gentamicin	4– > 256	> 256	> 256	90
Aztreonam	2– > 256	64	256	90
Ceftazidime	256– > 256	>256	> 256	100
Cefepime	4– > 64	16	> 64	40
Ciprofloxacin	1– > 256	32	128	70
Levofloxacin	2–128	32	64	60
Ertapenem	0.5–256	2	256	90
Imipenem	0.5– > 32	1	32	40
Meropenem	0.06–16	1	8	40
Citrobacter freundii ($n = 5$)				
Amikacin	2–32			0
Gentamicin	2–>256			60
Aztreonam	2–64			60
Ceftazidime	64–256			100
Cefepime	0.25–8			0
Ciprofloxacin	0.03–16			20
Levofloxacin	0.12–16			20
Ertapenem	0.5–2			40
Imipenem	0.25–2			0
Meropenem	0.03–0.25			0

revealed the presence of bla_{TEM-1}, bla_{SHV}, bla_{CTx-M}, bla_{IMP-8}, bla_{CMY-2}, and bla_{DHA-1} were found in 32 (57.1%), 19 (33.9%), four (7.1%), 16 (28.6%), 14 (25%), and five (8.9%), respectively. The bla_{SHV-12} predominates (78.9%) among all of the tested bla_{SHV}-positive strains, and bla_{SHV-5} (10.5%) and bla_{SHV-11} (10.5%) were only detected in *K. pneumoniae*. The $bla_{CTx-M-14}$ (50%) and $bla_{CTx-M-22}$ (50%) were presented in one *E. coli* and three *K. pneumoniae*, respectively. No Class A and D carbapenemase genes (bla_{KPC}, bla_{GES}, bla_{PER}, bla_{OxA}, and bla_{PSE}) were detected among these isolates. Fourteen (25%) of the 56 isolates (13 *E. cloacae* and one *E. coli*) with positive modified Hodge tests yielded negative results in all of the genotypic tests for detecting AmpC, ESBLs, and carbapenemases.

Outer membrane protein profile of modified Hodge test positive *Enterobacteriaceae*

The outer membrane protein profile among 56 Modified Hodge test-positive isolates showed obviously decreased expression in 49 (87.5%) isolates. A total of 14 isolates with positive modified Hodge tests but negative to all of the genotypic tests represented loss or decreased OMPs expression. Among five *C. freundii*, analysis of outer-membrane proteins revealed that the levels of the 38 kDa protein were decreased compared with *C. freundii* ATCC 8090. This indicates that loss or decreased of outer membrane proteins combined with ESBLs, MBLs, and AmpC phenotypes are common in strains with positive result of modified Hodge test.

Discussion

This is the first large-scale study of investigate the characterization of the modified Hodge test-positive isolates of *Enterobacteriaceae*, and we found that strains carrying ESBLs, AmpC enzymes, and IMP-8 carbapenemase, as well as decreased of outer membrane proteins, were prevalent among *Enterobacteriaceae* isolates in Taiwan. The CLSI recommends detecting KPC-type carbapenemase producers in *Enterobacteriaceae* using the modified Hodge test.[12] However, false positive results were reported in Brazil.[3] They found that cefotaxime-M β-lactamase (CTX-M), particularly CTX-M-2, combined with porin loss in *K. pneumoniae*, was highly prevalent in strains with false detection of carbapenemase by the modified Hodge test.[3] Moreover, Pasteran and colleagues[13,14] also showed that false positive results were acquired from *K. pneumoniae*, *E. cloacae*, *E. coli*, and *S. marcescens* using the modified Hodge test in Argentina. Among them, false positive results were mainly due to strains harboring CTX-M-2, AmpC hyperproducers, and loss of porins.[13,14] Therefore, in different geographic areas, isolates without KPC production may, through various mechanisms, present false positive results when performing the modified Hodge test.

KPC-producing *Enterobacteriaceae* are widespread in Israel, Greece, South America, and the United States.[2] In China, particularly in the Zhejiang Province, KPC-2 was increasingly reported in *K. pneumoniae* and also in *C. freundii*, *E. coli*, and *S. marcescens*.[2] However, no KPC was described previously in Taiwan. Therefore, strains with positive results from the modified Hodge test need to be further investigated for the presence of Class A carbapenemase genes. Recently, the first arrival of KPC-2-producing *K. pneumoniae* was reported in Taiwan from a businessman working in the Zhejiang Province of China.[21]

To prevent spreading KPC-producing bacteria in Taiwan, continually monitoring KPC producers is required.

A boronic acid-based method using the APB-imipenem combined disks was reported to have 100% of sensitivity and specificity in detecting Class A carbapenemases in *Enterobacteriaceae*.[11,13] Recent study tested the effect of phenyl boronic acid on detection of KPC carbapenemase-producing *Enterobacteriaceae*.[17] The comparative study showed that the imipenem and meropenem disk with 400 μg phenyl boronic acid exhibits the 100% of sensitivity and 97.6% of specificity in the phenotypic detection of KPC producers.[17] Our four isolates, including two *E. cloacae*, one *E. coli*, and one *K. pneumoniae*, tested positive with the phenyl boronic acid-imipenem combined disks (enhancement of the inhibition diameters between 6—14 and 4—7 mm using ertapenem and imipenem disks, respectively). However, no imipenem hydrolysis or carbapenemase gene was detected through spectrophotometric assay and polymerase chain reaction, respectively, among these isolates. This indicates that few false positive results were obtained using carbapenem disks and 400 μg phenyl boronic acid as an inhibitor to detect Class A carbapenemase producers among *Enterobacteriaceae* in our isolates.

There were 14 (25%) isolates (13 *E. cloacae* and one *E. coli*), with ertapenem MICs between 0.25—64 μg/mL, which presented negative results for all of the genotypic tests for detecting AmpC, ESBLs, and Classes A, B, and D carbapenemases. There have been reported that intrinsic chromosomal AmpC β-lactamase expression of *E. cloacae* and *S. marcescens* may show positive results when doing the modified Hodge test.[13,14] Therefore, highly chromosomal AmpC production is also involved in acquiring positive results by the modified Hodge test in *Enterobacteriaceae* in Taiwan. Loss of outer membrane proteins has been reported in *E. coli*, *E. cloacae*, and *K. pneumoniae*, resulting in false detection of carbapenemase by the modified Hodge test.[3,13—16] Moreover, loss of OmpC/F and OmpK35/36 is commonly encountered in ertapenem-resistant *E. coli* and *K. pneumoniae* in Taiwan.[6,9,22] Based on the outer membrane protein profile analysis, our modified Hodge test-positive isolates showed obviously decreased expression of the outer membrane protein in 49 (87.5%) isolates. In addition, two studies has been reported that absence or decreased expression of two outer membrane proteins (the 41- and 38-kDa porins) may also contributed to carbapenem resistance in *C. freundii*.[23,24] Among our five chromosomal AmpC-producing *C. freundii* with low level resistance to ertapenem, all of them revealed that the levels of the 38-kDa protein were decreased compared with *C. freundii* ATCC 8090. This indicates that loss or decreased expression of outer membrane protein coupled with ESBLs, MBLs, and AmpC phenotypes contributed to the strains with positive results from the modified Hodge test.

In conclusion, our data revealed that ESBLs, AmpC enzymes, and IMP-8 carbapenemase coupled with the decreased expression of the outer membrane protein were prevalent in *Enterobacteriaceae* isolates with positive modified Hodge test results. Clinical microbiology laboratories should be careful to test for Class A carbapenemase genes to prevent incorrect reporting as KPC-type carbapenemase producers as a result of performing the modified Hodge test.

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