Occurrence of False Positive Results for the Detection of Carbapenemases in Carbapenemase-Negative *Escherichia coli* and *Klebsiella pneumoniae* Isolates

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Abstract

Adequate detection of the production of carbapenemase in *Enterobacteriaceae* isolates is crucial for infection control measures and the appropriate choice of antimicrobial therapy. In this study, we investigated the frequency of false positive results for the detection of carbapenemases in carbapenemase-negative *Escherichia coli* and *Klebsiella pneumoniae* clinical isolates by the modified Hodge test (MHT). Three hundred and one *E. coli* and *K. pneumoniae* clinical isolates were investigated. All produced extended spectrum β-lactamases (ESBLs) but were susceptible to carbapenems. Antimicrobial susceptibility testing was performed by the disk diffusion and agar dilution methods. The MHT was performed using the standard inoculum of test organisms recommended by the CLSI. Genes that encoded ESBLs and carbapenemases were identified by PCR and DNA sequencing. Among the 301 clinical isolates, none of the isolates conformed to the criteria for carbapenemase screening recommended by the CLSI. The susceptibility rates for imipenem, meropenem, and ertapenem all were 100.0%, 100.0%, and 100.0%, respectively. Of the 301 *E. coli* and *K. pneumoniae* isolates, none produced carbapenemase. The MHT gave a positive result for 3.3% (10/301) of the isolates. False positive results can occur when the MHT is used to detect carbapenemase in ESBL-producing isolates and clinical laboratories must be aware of this fact.

Introduction

The production of extended-spectrum β-lactamases (ESBLs) is the most common mechanism of resistance to third-generation cephalosporins in *Enterobacteriaceae* bacteria, especially *Escherichia coli* and *Klebsiella pneumoniae*. Carbapenems are the drugs of last resort to treat severe infections caused by pathogens that produce ESBLs [1–2]. Although the rates of resistance to carbapenems are low, they treat severe infections caused by pathogens that produce ESBLs [1–2]. During the past decade the spread of antibiotic resistance in *Enterobacteriaceae* isolates, especially the increase in *K. pneumoniae* carbapenemase (KPC)-producing isolates, has become a major concern worldwide [4]. Infections due to KPC-producing isolates have become an important challenge in healthcare settings [5]. The rapid emergence of carbapenem-resistant *Enterobacteriaceae* isolates in our hospital was revealed by bacterial surveillance data. The rate of resistance to imipenem in *K. pneumoniae* rose from 1.3% in 2006 to 11.1% in 2009, whereas the rate in *E. coli* rose from 0.3% 2006 to 1.0% in 2009 (data not shown).

The production of carbapenemases, especially KPC-type carbapenemases, is the most common mechanism for carbapenem resistance in *Enterobacteriaceae* isolates [6–7]. However, the detection of carbapenemases can be difficult because, for some carbapenemase-producing *Enterobacteriaceae*, the minimum inhibitory concentration (MIC) of carbapenem is high but still within the susceptible range as defined by the Clinical and Laboratory Standards Institute (CLSI) criteria [4,8–9]. In 2009, the CLSI recommended the modified Hodge test (MHT) to screen for the production of carbapenemase in *Enterobacteriaceae* isolates with elevated MICs for carbapenems or reduced inhibition zones as measured by disc diffusion. The criteria were a diameter for the inhibition zones for meropenem and ertapenem of 19–21 mm and 16–21 mm, respectively, and MIC values of 2 μg/mL and 2–4 μg/mL, respectively (carbapenem breakpoints have been changed in M100-S20-U and M100-S21). According the new criteria, the initial screen test and the confirmatory test by MHT are no longer necessary for routine patient testing. Although the sensitivity and specificity of the MHT have been shown to exceed 90%, several studies have reported false positive or false negative results when this method was used to screen for carbapenemase in *Enterobacteriaceae* isolates [8,10]. In this study, we investigated the rate of false positives obtained when the MHT was used to test ESBL-producing and carbapenemase-negative *E. coli* and *K. pneumoniae* clinical isolates that did not conform to the CLSI criteria for carbapenemase screening.
Table 1. Antimicrobial activities of various antimicrobial agents against 301 E. coli and K. pneumoniae isolates.

| Drug                        | MIC (µg/ml) | S (%) | R (%) |
|-----------------------------|-------------|-------|-------|
| Imipenem                    | 0.06–0.25   | 100.0 | 0.0   |
| Meropenem                   | 0.06–0.25   | 100.0 | 0.0   |
| Ertapenem                   | 0.06–0.25   | 100.0 | 0.0   |
| Cefepime                    | 0.125–>128  | 30.9  | 48.2  |
| Ceftazidime                 | 0.25–>128   | 27.9  | 62.1  |
| Cefotaxime                  | 0.125–>128  | >128  | 99.7  |
| Ceftriaxone                 | 0.125–>128  | >128  | 99.7  |
| Cefoxitin                   | 1–>128      | 62.8  | 19.9  |
| Piperacillin-tazobactam     | 0.5–>128    | >128  | 0.0   |
| Aztreonam                   | 0.25–>128   | >128  | 8.6   |

Materials and Methods

Bacterial strains
Three hundred and one non-duplicate clinical isolates were analysed. The isolates included 153 E. coli isolates and 148 K. pneumoniae isolates and had been collected from January to December 2008 in Huashan Hospital, Fudan University, Shanghai, China. All the isolates produced ESBLs, as confirmed by the CLSI phenotypic confirmatory test initially, but were susceptible to carbapenems (zones of inhibition of ≥19 mm in diameter for ertapenem and ≥16 mm for meropenem, respectively) (9). As control, 18 carbapenem-resistant, KPC-2 type carbapenemase-producing K. pneumoniae clinical isolates were used in this study (11).

Antimicrobial susceptibility testing and the MHT

Antimicrobial susceptibility testing was performed using agar dilution methods recommended by the CLSI (9). Minimal inhibitory concentrations (MICs) of cefazolin, cefotixin, cefotaxime, ceftriaxone, aztreonam, ceftazidime, ceftiraxone, imipenem, meropenem, and piperacillin/tazobactam were determined in accordance with the CLSI criteria (9). The MHT was carried out on all isolates to detect carbapenemase using ertapenem and meropenem as described by the CLSI (9). A well characterized strain of E. coli (KPC-2 carbapenemase producer). B, 08-438 E.coli. C, 08-97 K. pneumoniae. D, E. coli ATCC 25922.

Table 2. Distribution of ESBL genes among the 301 E. coli and K. pneumoniae isolates.

| ESBL genes | Genotype   | Number | %     |
|------------|------------|--------|-------|
| SHV-type ESBLs | SHV-2, SHV-12, SHV-28, SHV-31 | 8 | 2.7   |
| CTX-M-type ESBLs | CTX-M-14, CTX-M-15, CTX-M-25 | 243* | 80.7  |
| TEM + CTX-type ESBLs | TEM-40, TEM-135, CTX-M-14, CTX-M-15 | 3 | 1.0   |
| SHV + CTX-M-type ESBLs | SHV-2, SHV-12, SHV-28, SHV-31, CTX-M-14, CTX-M-15, CTX-M-25 | 34 | 11.3  |
| Negative** |            | 13     | 4.3   |
| Total      |            | 301    | 100.0 |

*: 9.0% (22/243) isolates were producing both CTX-M-14 and CTX-M-15 type ESBL, and 1.2% (3/243) isolates were producing both CTX-M-14 and CTX-M-25 type ESBL.

**: We did not detect the TEM, SHV or CTX-M type ESBLs among these isolates.
Analysis of outer membrane proteins

The outer membrane proteins of all isolates with false-positive result on MHT were examined by sodium dodecyl sulfate polyacrylamide gel electrophoresis, as described previously [14].

Ethics Statement

As this study was focused on bacterial isolates collected from routine samples, approval from ethics committee at Fudan University was not necessary. Neither was there any need for informed consent procedures as no extra sampling was performed and no personal data was stored in relation to the isolates.

Results

Antimicrobial susceptibility testing

The results of the antimicrobial susceptibility testing indicated that, for the 301 isolates tested, the inhibition zones for meropenem and ertapenem all had a diameter greater than 22 mm, and the MIC values were all below 1 μg/mL. Therefore, none of the isolates conformed to the CLSI criteria for carbapenemase screening (namely, diameters for meropenem and ertapenem of 19–21 mm and 16–21 mm, respectively, and MIC values of 2 μg/mL and 2–4 μg/mL, respectively.). None of the isolates was found to be susceptible to cefotaxime and 99.7%, 62.1%, 48.2%, and 19.9% were resistant to cefotaxime, ceftazidime, cefepime, and cefoxitin, respectively. The susceptibility rates for imipenem, meropenem, and ertapenem were 100.0%, 100.0%, and 100.0%, respectively (Table 1).

PCR amplification of β-lactamase genes and the MHT

Of the 301 E. coli and K. pneumoniae isolates, none produced carbapenemase. Genes that encoded the CTX-M-type ESBLs were detected in 280 (93.0%) of the isolates, among which CTX-M-14, CTX-M-15, CTX-M-25, CTX-M-14 coupled with CTX-M-15, and CTX-M-14 coupled with CTX-M-25 type ESBL were found in 135 (48.2%), 112 (40.0%), 2 (0.7%), 28 (10.0%), and 3 (1.1%) of the isolates, respectively. Among 301 isolates, 18.3% were producing more than two types of ESBLs, such as TEM-type and CTX-M-type ESBLs, SHV-type and CTX-M-type ESBLs (Table 2). Although all the isolates were sensitive to carbapenems and carbapenemase-negative, the MHT yielded a positive result (Figure 1) for 3.3% (10/301) of the isolates and all the MHT-positive isolates (except one isolate was SHV-12 type ESBL producer) were CTX-M type ESBL producing (Table 3). Among 18 carbapenem-resistant K. pneumoniae clinical isolates, all were produced KPC-2 type carbapenemase and all were producing a positive result of MHT.

Carbapenem inactivation assay

A carbapenem inactivation assay performed on all 301 carbapenem-susceptible isolates indicated that alterations in shape of the zones of inhibition around the test organism were not observed, suggesting no carbapenemase was involved in hydrolysis of carbapenems in these isolates. However The assay of all 18 KPC-2 type carbapenemase-producing K. pneumoniae isolates were positive.

Analysis of outer membrane proteins

Analysis of the outer membrane porin proteins by sodium dodecyl sulfate polyacrylamide gel electrophoresis showed that all 10 isolates (except 08-438 E.coli) exhibited loss of one of porin protein compared with the sensitive strains (Figure 2 and Figure 3). This suggests that the outer membrane porin proteins might play an important role in resulting to the false-positive result.
Discussion

Given the increasing prevalence of carbapenemase-producing Enterobacteriaceae isolates worldwide [4], simple and accurate tests are needed to detect isolates that produce carbapenemase. For this purpose, the CLSI have recommended the MHT for the detection of carbapenemase in Enterobacteriaceae isolates. Although the MHT is a simple method for detecting carbapenemase-positive isolates, false positive results are obtained occasionally [8,10]. In June 2010, CLSI changed the carbapenem breakpoints and reviewed the recommendation on carbapenemase detection by MHT among Enterobacteriaceae (M100-S20-U) and informed that MHT would not be recommended, except for epidemiological or infection control purposes. In this study, we used carbapenem-susceptible E. coli and K. pneumoniae isolates that did not conform to the CLSI criteria for screening for the presence of carbapenemase. False positive results for the MHT were obtained among 3.3% of the isolates. We presume that the false positive results are probably due to lower-level hydrolysis of ertapenem by ESBLs, particularly those of the CTX-M type, because we found that 97.3% of the isolates in our study produced CTX-M-type ESBLs [10,16].

Although the false positive results were observed in our study, MHT is not recommended (and never was) to detect carbapenemase among isolates that yielded negative-result on screening test. Whether for epidemiological or infection control purposes, adequate detection of the production of carbapenemase in Enterobacteriaceae isolates is crucial for infection control measures and the appropriate choice of antimicrobial therapy [8]. To improve the detection of carbapenemase-producing Enterobacteriaceae in clinical microbiology laboratories, several phenotypic tests to detect KPCs have been developed [17–20]. In addition to the MHT, a second phenotypic method has been shown to be promising for the identification of KPCs. This method utilizes boronic acid and 3-aminophenyl boronic acid-based compounds, and has proved to be highly sensitive and specific for the detection of KPCs.

In summary, as reported, CTX-M-type ESBLs might be hydrolyzing the ertapenem used in the assay [16]. Clinical laboratories must be aware that false positive results may occur, especially in geographical areas where the incidence of isolates that produce CTX-M-type ESBLs is relatively high, such as the Asia-Pacific region [21]. Hence, further work is necessary to determine the frequency of false positive results when the MHT is used to detect carbapenemase production in Enterobacteriaceae isolates and more accurate methods should be developed for use in clinical microbiology laboratories.

Author Contributions
Conceived and designed the experiments: FH DZ YZ. Performed the experiments: PW SC FH. Analyzed the data: FH ZX. Contributed reagents/materials/analysis tools: YG. Wrote the paper: FH.

References

1. Anderson KF, Lowsley DR, Rasheed JK, Biddle J, Jensen B, et al. (2007) Evaluation of Methods to Identify the Klebsiella pneumoniae Carbapenemase in Enterobacteriaceae. J Clin Microbiol 45: 2723–2725.
2. Lee K, Lim YS, Yong D, Yum JH, Chong Y (2003) Evaluation of the Hodge Test and the Imipenem-EDTA Double-Disk Synergy Test for Differentiating Metallo-β-Lactamase-Producing Isolates of Pseudomonas spp. and Acinetobacter spp. J Clin Microbiol 41: 4623–29.
3. Bandman D, Salvari JK, Bratu S, Quale J (2005) Evaluation of Techniques for Detection of Carbapenem-Resistant Klebsiella pneumoniae in Stool Surveillance Cultures. J Clin Microbiol 43: 5639–5641.
4. Elizabeth BH, Vincent HT (2010) Detection and Treatment Options for Klebsiella pneumoniae Carbapenemases (KPCs): an Emerging Cause of Multidrug-resistant Infection. J Antimicrob Chemother 65: 1119–1125.
5. James OS, Maurice AL-VH (2010) Guideline for Phenotypic Screening and Confirmation of Carbapenemases in Enterobacteriaceae. Int J Antimicrob Agents 36: 205–210.
6. Lledo W, Hernandez M, Lopez E, Molinari OL, Soto RQ, et al. (2009) Guidance for Control of Infections with Carbapenem-resistant or Carbapenemase-producing Enterobacteriaceae Isolates in Acute Care Facilities. JAMA 301: 1979–1982.
7. Munoz-Price LS, Quinl JP (2009) The Spread of Klebsiella pneumoniae Carbapenemases: A Tale of Strains, Plasmids, and Transposons. Clin Infect Dis 49: 1739–1741.
8. Pasteran F, Mendez T, Guerriero L, Rapoport M, Corso A (2009) Sensitive Screening Tests for Suspected Class A Carbapenemase Production in Species of Enterobacteriaceae. J Clin Microbiol 47: 1631–1639.
9. Clinical and Laboratory Standards Institute (2010) Performance Standards for Antimicrobial Susceptibility Testing: Twentieth Informational Supplement, M100-S20. Clinical and Laboratory Standards Institute, Wayne, PA.
10. Cecilia GC, Renata GP, Adriana GN, Danilo EX, Ana CG (2010) Cloverleaf Test (modified Hodge test) for Detecting Carbapenemase Production in Klebsiella pneumoniae: be Aware of False Positive Results. J Antimicrob Chemother 65: 249–251.
11. Chen S, Hu F, Xu X, Liu Y, Wu W, et al. (2011) High Prevalence of KPC-2-Type Carbapenemase Coupled with CTX-M-Type Extended-Spectrum β-Lactamases in Carbapenem-Resistant Klebsiella pneumoniae in a Teaching Hospital in China. Antimicrob Agents Chemother 55: 2493–2494.
12. Yigit H, Queran AM, Anderson GJ, Sanchez AD, Biddle JW, et al. (2001) Novel carbapenem-hydrolyzing β-lactamase, KPC-1, from a carbapenem-resistant strain of Klebsiella pneumoniae. Antimicrob Agents Chemother 45: 1151–61.
13. Patrick RM, Ellen JB, James HJ, Marie LL, Michael AP (2007) Detection and Characterization of Antimicrobial Resistance Genes in Pathogenic Bacteria, p1250–1256. In: Kamile Rasheed J, Franklin Cokerill, Fred C. Tenerover, eds. Manual of Clinical Microbiology, 9th ed. American Society for Microbiology, Washington, D.C..
14. Hsaka M, Gotoh N, Nishino T (1995) Purification of a 54-kdhalokin protein [OprJ] produced in NixB mutants of Pseudomonas aeruginosa and production of a monoclonal antibody specific to OprJ. Antimicrob Agents Chemother 39: 1731–5.
15. Woodford N, Fagan EJ, Ellington, MJ (2006) Multiplex PCR for Rapid Detection of Genes Encoding CTX-M Extended-spectrum β-lactamases. J Antimicrob Chemother 57: 1151–61.
16. Girlich D, Poirel L, Nordmann P (2008) Do CTX-M-β-lactamase Hydrolyze Ertapenem? J Antimicrob Chemother 62: 1155–1156.
17. Tsaihs A, Kristo J, Poulou A, Markou F, Boromisidou A, et al. (2008) First Occurrence of KPC-2-possessing Klebsiella pneumoniae in a Greek Hospital and Recommendation for Detection with Boronic Acid Disc Tests. J Antimicrob Chemother 62: 1257–1260.
18. Tsakris A, Kristo I, Poulou A, Themeli-Digalaki K, Ikonomidou A, et al. (2009) Evaluation of Boronic Acid Disk Tests for Differentiating KPC-possessing Klebsiella pneumoniae Isolates in the Clinical Laboratory. J Clin Microbiol 47: 362–367.

19. Giske CG, Gezelius L, Samuelsen Ø, Warner M, Sundsfjord A, et al. (2011) A sensitive and specific phenotypic assay for detection of metallo-β-lactamases and KPC in Klebsiella pneumoniae with the use of meropenem disks supplemented with aminophenylboronic acid, dipicolinic acid and cloucillin. Clin Microbiol Infect 17: 552–556.

20. Tsakris A, Poulou A, Themeli-Digalaki K, Voulgari E, Pittaras T, et al. (2009) Use of Boronic Acid Disk Tests to Detect Extended-spectrum β-lactamases in Clinical Isolates of KPC Carbapenemase-possessing Enterobacteriaceae. J Clin Microbiol 47: 3420–3426.

21. Bell JM, Chiuaz M, Turnridge JD, Barton M, Walters IJ, et al. (2007) Prevalence and Significance of a Negative Extended-Spectrum β-Lactamase (ESBL) Confirmation Test Result after a Positive ESBL Screening Test Result for Isolates of Escherichia coli and Klebsiella pneumoniae: Results from the SENTRY Asia-Pacific Surveillance Program. J Clin Microbiol 45: 1470–1482.