The prevalence of carbapenemase genes and plasmid-mediated quinolone resistance determinants in carbapenem-resistant Enterobacteriaceae from five teaching hospitals in central China

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SUMMARY

We investigated the prevalence of β-lactamase genes and plasmid-mediated quinolone resistance (PMQR) determinants in 51 carbapenem-resistant Enterobacteriaceae (CRE) from five teaching hospitals in central China. The prevalence of carbapenem resistance in Enterobacteriaceae was 1·0% (51/5012). Of 51 CRE, 31 (60·8%) isolates were positive for one tested carbapenemase gene, while 10 (19·6%) were simultaneously positive for two tested carbapenemase genes. The positive rates of \( \text{bla}_{\text{KPC-2}} \), \( \text{bla}_{\text{NDM-1}} \), \( \text{bla}_{\text{IMP-4}} \), \( \text{bla}_{\text{IMP-26}} \) and \( \text{bla}_{\text{IMP-8}} \) were 54·9%, 17·6%, 11·8%, 11·8% and 3·9%, respectively. Of 10 CRE with two carbapenemase genes, three, five, one and one were positive for \( \text{bla}_{\text{KPC-2}} \) and \( \text{bla}_{\text{IMP-4}} \), \( \text{bla}_{\text{KPC-2}} \) and \( \text{bla}_{\text{IMP-26}} \), \( \text{bla}_{\text{KPC-2}} \) and \( \text{bla}_{\text{IMP-8}} \), and \( \text{bla}_{\text{KPC-2}} \) and \( \text{bla}_{\text{NDM-1}} \), respectively. Eight of nine \( \text{bla}_{\text{NDM-1}} \)-positive isolates lacked carbapenemases by the modified Hodge test, while 27/28 isolates harbouring \( \text{bla}_{\text{KPC-2}} \) were positive for carbapenemases determined by this test; 41·2% of the CRE-positive isolates also harboured ESBL genes in various combinations (three and two positive for \( \text{bla}_{\text{KPC-2}} \) also carried \( \text{bla}_{\text{DHA-1}} \) and \( \text{bla}_{\text{CMY-2}} \)). The positive rates of \( \text{qnrS1} \), \( \text{qnrA1} \), \( \text{qnrB} \) and \( \text{aac(6\')-Ib-cr} \) in CRE were 25·5%, 9·8%, 23·5% and 15·7%, respectively. In particular, 7/9 isolates harbouring \( \text{bla}_{\text{KPC-2}} \) were positive for these quinolone resistance genes, of which five carried \( \text{qnrS1} \) and two carried \( \text{qnrS1} \) and \( \text{qnrB4} \). All but two of 29 \textit{Klebsiella pneumoniae} isolates were grouped into 20 clonal clusters by PFGE, with the predominant cluster accounting for four \( \text{bla}_{\text{KPC-2}} \)-positive isolates distributed in the same hospital. We conclude that there is a high prevalence of \( \text{bla}_{\text{NDM-1}} \) and PMQR determinants in CRE isolates in central China. Multiple resistance determinants in various combinations co-exist in these strains and we report for the first time the co-existence of \( \text{bla}_{\text{KPC-2}} \) and \( \text{bla}_{\text{IMP-26}} \) in a strain of \textit{Klebsiella oxytoca}.

Key words: Carbapenemases, Enterobacteriaceae, quinolone resistance determinants.

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Carbapenemase genes in Enterobacteriaceae 1973

first documentation of co-existence for bla_{KPC-2} and bla_{IMP-26} in a single CRE isolate.

MATERIALS AND METHODS
Isolation and identification of isolates
From January 2011 to September 2012, a total of 5012 isolates of Enterobacteriaceae were recovered from clinical specimens collected from hospitalized patients in five teaching hospitals, including four in Nanchang, central China and one in Jiujiang (170 km north of Nanchang). Fifty-one (1.0%) isolates with resistance to at least one of ertapenem, imipenem and meropenem were defined as CRE. These CRE isolates originated from participants at different locations as follows: the first, second, third and fourth affiliated hospitals of Nanchang University (respectively hospital A, 17 isolates; hospital B, 25 isolates; hospital C, three isolates; hospital D, 4 isolates), and the affiliated hospital of Jiujiang College in Jiujiang (hospital E, two isolates). Identification was performed using a Vitek-32 automated microbiology analyser (bioMérieux, France) according to the manufacturer’s instructions, as well as additional standard biochemical testing. Only bacterial isolates comprising $>10^7$ c.f.u./ml by semi-quantitative culture from sputum specimens were considered significant and analysed in this study.

Antimicrobial susceptibility
Antimicrobial susceptibilities were determined using Gram-negative susceptibility cards in the Vitek system (bioMérieux) and by disc diffusion in accordance with the guidelines recommended by Clinical and Laboratory Standards Institute (CLSI) [21]. The antimicrobials were ampicillin, piperacillin, piperacillin/tazobactam, cefotaxime, cefazidime, ceftazidime, aztreonam, cefoxitin, imipenem, meropenem, trimethoprim/sulfamethoxazole, amikacin, gentamicin and levofloxacin. Results of susceptibility tests were interpreted according to the criteria recommended by CLSI [21]. Escherichia (Es.) coli ATCC 25922 was used as quality control strain.

Detection of antimicrobial resistance determinants
The modified Hodge test (MHT) was performed for detection of carbapenemases as described previously [21] and for ESBL production by the the CLSI-recommended confirmatory double-disk combination...
test [21]. Genes encoding carbapenemases, ESBL genes, plasmid-mediated AmpC and PMQR determinants were detected using polymerase chain reaction (PCR) and nucleotide sequencing employing previously published primers [22–24].

Pulsed-field gel electrophoresis (PFGE)

Genomic DNA from K. pneumoniae isolates was prepared for PFGE typing and cleaved with 40 U XbaI. Electrophoresis was performed on 1% agarose gels in 0.5 M Tris/borate/EDTA buffer on a CHEF-Mapper XA PFGE system (Bio-Rad, USA) for 24 h at 14°C, with run conditions of 6 V/cm, a pulse angle of 120° and pulse times from 5 s to 20 s. Bands were stained with ethidium bromide (0.5 μg/ml) prior to their identification under UV light. Comparison of the PFGE patterns was performed with Bionumerics software (Applied Maths, Belgium) using the Dice similarity coefficient. Clusters were defined as DNA patterns sharing >85% similarity.

RESULTS AND DISCUSSION

The 51 CRE identified were by species, K. pneumoniae (29), Enterobacter cloacae (8), K. oxytoca (6), Es. coli (4), K. ozaenae (2), Proteus mirabilis (1) and Citrobacter freudii (1). The positive rates of carbapenem resistance in various species of Enterobacteriaceae were as follows: K. pneumoniae, 2.8% (29/1023); E. cloacae, 1.5% (8/523); K. oxytoca, 1.4% (6/427), Es. coli, 0.2% (4/2253), K. ozaenae, 3.4% (2/58), P. mirabilis, 0.8% (1/128) and C. freundii, 1.3% (1/78). These organisms were recovered from sputum (31), urine (5), exudates (4), pus (5), blood (5) and one from a central venous catheter tip. All CRE isolates were resistant to ampicillin and pipercillin. The resistance rates to other antimicrobials were as follows: piperacillin/tazobactam (82.4%), ceftazidime (98.0%), cefotaxime (96.1%), cefepime (94.1%); aztreonam (94.1%), cefoxitin (90.2%), levofloxacin (76–5%), amikacin (43.1–1%), gentamicin (80–4%) and trimethoprim/sulfamethoxazole (76–5%). All but two of the 51 CRE isolates were resistant to multiple antimicrobials and (84.3%) were susceptible to at least one drug.

A total of 31 (60.8%) CRE isolates expressed carbapenemases based on the MHT and carriage of carbapenemase genes, including blanaNDM-1, was investigated further using PCR and DNA sequencing. Most (80.4%) isolates expressed carbapenemase genes and similar to previous reports on Enterobacteriaceae in China [25, 26]. 54.9% of the isolates in this study harboured blakPC-2. In China, blanNDM-1 expression has been identified mainly for Actinetobacter spp. [10–13] but the gene has been noted in Enterobacteriaceae in the country, albeit with a relatively low prevalence thus far [27]. We recently reported that two unrelated K. pneumoniae isolates harboured blanaNDM-1 [14, 28] and it was unexpected to find such a high prevalence (17.6%) of this gene in CRE in this study. The nine isolates expressing blanaNDM-1 included five K. pneumoniae, two K. ozaenae, one K. oxytoca and one Es. coli, indicating that the gene has been disseminated in Enterobacteriaceae in China. This should be a cause for concern for clinicians, microbiologists and administrators for infection control measures. Interestingly, all but one of the blanaNDM-1-positive isolates were negative for carbapenemases by the MHT, while 27/28 isolates harbouring blakPC-2 were positive by this method, indicating that the test is not suitable for the detection of NDM-1 production. The MHT has excellent sensitivity for detection of enterobacterial isolates producing KPC- and OXA-48-type carbapenemases, but has low sensitivity for NDM-1 producers [28], which is further supported by a previous study which reported negative or weakly positive MHT results for 11/15 NDM-1-producing strains [29]; therefore, it is important for clinical laboratories to develop sensitive methods for identifying such strains.

In addition to KPC-2 and NDM-1, IMP-type metallo-β-lactamases also contribute to carbapenem resistance in Enterobacteriaceae. In the present study, 14 CRE isolates expressed blaimp, including blaimp4 (6), blaimp26 (6) and blaimp8 (2) but all were negative for blimndm. Co-production of different carbapenemases was also found in clinically important organisms, which poses a challenge for infection control [7, 15–18]. We found that 10 isolates simultaneously harboured two carbapenemase genes in various combinations as follows: one isolate each of K. pneumoniae, E. cloacae and K. oxytoca had both blakPC-2 and blaimp4; three K. pneumoniae and two E. cloacae isolates had both blakPC-2 and blaimp26; one C. freundii isolate harboured both blakPC-2 and blaimp8; and a single K. oxytoca isolate harboured both blakPC-2 and blimndm. Co-existence of blakPC-2 and blaimp4 has previously been documented in K. pneumoniae [18, 30], and co-existence of blakPC-2 and blaimp8 was first identified in a K. oxytoca isolate from Shanghai, east China [31]. Recently, NDM-1
was also found to co-exist with other carbapenemases, including OXA-23, KPC-2, IMP-26 and OXA-181 [7, 15–17, 27]. To the best of our knowledge, the present study is the first to report co-existence of \textit{bla}KPC-2 and \textit{bla}IMP-26 in \textit{K. pneumoniae} and \textit{E. cloacae} isolates as well as the co-existence of \textit{bla}KPC-2 and \textit{bla}NDM-1 in \textit{K. oxytoca}. The 10 CRE isolates not expressing carbapenemase genes by PCR harboured at least one ESBL gene and/or plasmid-mediated AmpC gene. Carbapenem resistance in these isolates may therefore be associated with other carbapenemases not examined in this study, or other resistance mechanisms including loss of porins and efflux pumps.

Co-production of carbapenemases with other \textit{\beta}-lactamases results in resistance to nearly all clinically available \textit{\beta}-lactams. Since AmpCs and carbapenemases are not inhibited by clavulanic acid, co-production of ESBLs, AmpCs and carbapenemases can mask identification of ESBLs by the CLSI-recommended double-disk test. Detection of multiple \textit{\beta}-lactamases produced by Enterobacteriaceae in the clinical laboratory is therefore challenging. In the present study, although ESBL genes were expressed by 62.7% (32/51) of CRE isolates, only 29.4% (15/51) were found to produce ESBLs as determined by the CLSI-recommended double-disk test. Of 32 isolates with ESBL genes, 25 carried \textit{bla}CTX-M, including \textit{bla}CTX-M-3 (6), \textit{bla}CTX-M-14 (5), \textit{bla}CTX-M-15 (6), both \textit{bla}CTX-M-9 and \textit{bla}CTX-M-3 (4), \textit{bla}CTX-M-65 (2), \textit{bla}CTX-M-9 (1) and \textit{bla}CTX-M-84 (1). Thirty (58.8%) CRE isolates harboured \textit{bla}SHV and comprised 13 isolates with SHV-type ESBL genes, including \textit{bla}SHV-12 (10), \textit{bla}SHV-5 (1), \textit{bla}SHV-28 (1) and \textit{bla}SHV-36 (1). The remaining 17 \textit{bla}SHV-positive isolates carried SHV-type narrow spectrum \textit{\beta}-lactamase genes, including \textit{bla}SHV-1 (8) and \textit{bla}SHV-1 (9). \textit{bla}TEM was detected in 60.8% (31/51) CRE isolates, and all \textit{bla}TEM amplicons were identified as the narrow spectrum \textit{\beta}-lactamase gene, \textit{bla}TEM-1. Co-existence of \textit{bla}SHV- and \textit{bla}CTX-M-type ESBL genes was identified for six CRE isolates. Twenty-one (41.2%) of the CRE isolates with carbapenemase genes based on PCR also harboured ESBL genes in various combinations, and 21 (41.2%) also expressed AmpCs as determined by the three-dimension test. Thirteen (25.5%) isolates were positive for plasmid-mediated AmpC genes, including \textit{bla}DHA-1 (6), \textit{bla}CMY-2 (5), \textit{bla}MIR-3 (1) and \textit{bla}ACT-16 (1). Isolates expressing \textit{bla}KPC-2 simultaneously harboured \textit{bla}DHA-1 (3) and \textit{bla}CMY-2 (2). No plasmid-mediated AmpC genes were detected in \textit{bla}NDM-1-positive isolates.

Although PMQR determinants alone may not confer resistance to quinolones, they do supplement other quinolone resistance mechanisms. In China, PMQR...
determinants, especially \textit{aac-\((6')-Ib-cr\)}, have been found in Enterobacteriaceae clinical isolates [32]. In the present study, 32 (62.7\%) of 51 CRE isolates expressed PMQR determinants, including \textit{qnrS1} (11), \textit{qnrA1} (4), \textit{qnrB2} (2), \textit{qnrB4} (6), \textit{qnrB10} (1), both \textit{qnrS1} and \textit{qnrB4} (2), both \textit{qnrA1} and \textit{qnrB1} (1) and \textit{aac-\((6')-Ib-cr\)} (5). Two isolates with \textit{qnrS1} and one isolate with \textit{qnrB4} were positive for \textit{aac-\((6')-Ib-cr\)}. The positive rates for \textit{qnrS1}, \textit{qnrA1}, \textit{qnrB} and \textit{aac-\((6')-Ib-cr\)} were 25.5\% (13/51), 9.8\% (5/51), 23.5\% (12/51) and 15.7\% (8/51), respectively. Of 41 CRE isolates expressing carbapenemase genes, the prevalence of PMQR determinants was 65.8\% (27/41). Specifically, 77.8\% (7/9) of isolates harbouring \textit{bla}_{NDM-1} expressed PMQR genes, including \textit{qnrS1} (5), and both \textit{qnrS1} and \textit{qnrB4} (2).

In a previous study from China, \textit{qnr} genes were expressed by 67.5\% (27/40) of KPC-2-producing \textit{K. pneumoniae} isolates [19]. Co-existence of carbapenemase genes and PMQR determinants contributes to MDR. Of 29 \textit{K. pneumoniae} CRE isolates, 27 were successfully typed and grouped into 20 clonal clusters by PFGE (Fig. 1), the remaining two isolates were not typable despite repeated attempts. The predominant cluster included four \textit{bla}_{KPC-2}-positive isolates distributed within the same hospital (the second affiliated hospital of Nanchang University), suggesting dissemination of clonal carbapenemase-producing \textit{K. pneumoniae} in this facility. Four different clusters with two isolates each were also recovered from patients in this hospital. The remaining 15 profiles were represented by single isolates, and five isolates harbouring \textit{bla}_{NDM-1} had unique profiles indicating independent acquisition of these strains and the absence of cross-transmission between patients in the Nanchang area during the study period. In addition, strains of the same DNA profile were not isolated from patients receiving care in different hospitals, which underlined the fact that CRE did not spread between hospitals in the survey.

In conclusion, we report a high prevalence of \textit{bla}_{NDM-1} and PMQR determinants in CRE isolates from central China, as well as the co-existence in isolates of multiple resistance determinants in various combinations. Moreover, we provide the first reported co-existence of \textit{bla}_{KPC-2} and \textit{bla}_{IMP-26}. Co-existence of multiple resistance genes in CRE isolates contributes to MDR and poses formidable challenges for the treatment of clinically significant infections caused by these organisms. Effective surveillance and strict infection control strategies should be implemented to prevent nosocomial infections caused by these MDR pathogens in China.

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DECLARATION OF INTEREST

None.

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