Journal of Antimicrobial Chemotherapy (2003) **51**, 397–400
DOI: 10.1093/jac/dkg047
Advance Access publication 6 January 2003

JAC

Brief reports

Characterization of a new integron containing VIM-2, a metallo-β-lactamase gene cassette, in a clinical isolate of *Enterobacter cloacae*

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Received 7 March 2002; returned 19 June 2002; revised 14 October 2002; accepted 18 October 2002

We report the first description of a new integron containing *bla*$_{VIM-2}$, a metallo-β-lactamase gene from *Enterobacter cloacae* KU680, which was isolated from peritoneal fluid of a liver cirrhosis patient in South Korea. Antibiotic susceptibility testing, and modified Hodge and EDTA-disc synergy tests, were carried out to screen for metallo-β-lactamase-producing strains. PCR and sequence analysis were used to identify and analyse the *bla*$_{VIM-2}$-containing integron. The isolate was resistant to most β-lactams, including imipenem, and demonstrated a positive modified Hodge and EDTA-disc synergy test, which are findings suggesting a metallo-β-lactamase. Preliminary PCR-based experiments detected the metallo-β-lactamase gene *bla*$_{VIM-2}$. Sequencing of the 4392 bp cloned PCR amplicon, containing the gene cassette *bla*$_{VIM-2}$, revealed the structure of the class 1 integron. The integron also contained additional insert gene cassettes, *aadA*, and unknown open reading frames '*orfII*' and '*orfIII*'. To the best of our knowledge, this is the first time that this metallo-β-lactamase gene has been detected in *E. cloacae*.

Introduction

Enterobacter cloacae is a well-recognized nosocomial pathogen that causes significant infections. This microorganism is intrinsically resistant to ampicillin and narrow-spectrum cephalosporins, due to a chromosomal cephalosporinase. Additional resistance to broad-spectrum cephalosporins and aztreonam is usually related to the mutational over production of the species-specific cephalosporinase, or production of plasmid-mediated extended-spectrum β-lactamases. But imipenem-resistant clinical isolates of *E. cloacae* are unusual and have been described in strains with porin alterations combined with hyperproduction of chromosomal cephalosporinase, and in strains producing class A carbapenem-hydrolysing non-metallo-β-lactamases, such as NmcA β-lactamase and IMI-1 β-lactamase.[1]

However, class B metallo-β-lactamases have been reported recently in several strains of Gram-negative bacilli.[2] These enzymes possess the broadest substrate hydrolysis range among β-lactamases of Gram-negative bacilli, including penicillins, cephalosporins, cephamycins and carbapenems, but not monobactams. Since the first finding of IMP-1 metallo-β-lactamase in a clinical isolate of *Pseudomonas aeruginosa* in Japan in 1988, IMP-2, VIM-1 and VIM-2 metallo-β-lactamases have been reported in various Gram-negative bacilli. The *bla*$_{VIM}$ and *bla*$_{IMP}$ genes occur in mobile gene cassettes inserted in the variable regions of integrons.[3] Gene cassettes are mobile elements, so that horizontal spread of resistance can be anticipated, in addition to clonal spread.

In 2000, an imipenem-resistant strain of *E. cloacae* was isolated in a tertiary care hospital in Busan, Korea, from the peritoneal fluid of a patient with liver cirrhosis, which carried

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a bla_{VIM-2}-containing integron. To the best of our knowledge, *E. cloacae* strains carrying metallo-β-lactamase genes have not been reported before. Therefore, we undertook this study to analyse the structure of the bla_{VIM-2}-containing integron.

Materials and methods

Bacterial strains and susceptibility testing

The *E. cloacae* KU680 isolate was identified by the conventional method, and with the API 20E system (bioMérieux Vitek, Marcy-l'Étoile, France). Antibiotic-containing discs (BBL, Cockeysville, MD, USA) were used for routine antibiograms by disc diffusion assay.[4] MICs of antimicrobial agents were determined by the agar dilution method.[5] *Escherichia coli* ATCC 25922 was used as MIC reference strain. Modified Hodge and EDTA-disc synergy tests were performed for the screening of metallo-β-lactamase-producing strains.[6]

Conjugation and isoelectric focusing of β-lactamase

The filter mating method was used to determine the transferability of the carbapenem resistance determinant, as previously described.[7] The isoelectric points of β-lactamases were determined by loading cell sonicates to precast pH 3 to 10 gels. The gel was overlaid with a filter paper soaked in 20 mM EDTA for 5 min, before the imipenem (0.5 mg/L)-containing Mueller–Hinton agar was added. In this manner, inhibition of imipenem-hydrolysing activities could be observed.

Molecular techniques

Searches for bla_{IMP-1} and bla_{VIM-2} genes in *E. cloacae* KU680 were performed by PCR amplification with the following sets of primers: for the bla_{IMP-1} gene, IMP-1F (5′-CATGGTTTGGTGGTTCTTGT-3′) and IMP-1R (5′-ATAATTTGGCGGACTTTGGC-3′); for the bla_{VIM-2} gene, VIM-2F (5′-ATGTTCAAACTTTTGAGTAAG-3′) and VIM-2R (5′-CTACTCAACGACTGAGCG-3′); for the class 1 integron, 5′CS-F (5′-CTTCTAGAAAACCGAGGATGC-3′) and 3′CS-R (5′-CTCTCAAGATTTTAATGCGGATG-3′). PCR amplification was carried out as previously described,[7] as was the preparation of recombinant plasmids containing PCR product, and transformation of them into *E. coli* DH5α.[7] Plasmids from successful clones were used to determine the sequence of the integron by the dideoxynucleotide-chain termination method, with an automatic DNA sequencer (ABI 3700, Perkin-Elmer, Foster City, CA, USA). The determination of the sequence was repeated with more than two clones from independent amplicons. Both strands were sequenced.

Nucleotide and amino acid sequence analysis

Nucleotide sequence analysis was performed with DNASIS for Windows (Hitachi Software Engineering America Ltd, San Bruno, CA, USA). Database similarity searches for nucleotide and deduced amino acid sequences were carried out at the NCBI website (http://www.ncbi.nlm.nih.gov).

Nucleotide sequence accession number

The nucleotide sequences of the bla_{VIM-2} gene of *E. cloacae* KU680 have been assigned to the GenBank nucleotide sequence database under accession number AF305559.

Results

Properties of E. cloacae KU680

Disc diffusion testing revealed that *E. cloacae* KU680 isolate was resistant to most β-lactams, including ampicillin, ampicillin–sulbactam, piperacillin, piperacillin–tazobactam, cefalothin, cefoxitin, cefotaxime, ceftazidime and aztreonam. The isolate was also resistant to tobramycin, intermediate to gentamicin, but susceptible to amikacin and ciprofloxacin. MICs of imipenem and meropenem for the isolate were 4 mg/L, and that of aztreonam was 64 mg/L. MICs of ampicillin, ampicillin–sulbactam, piperacillin, piperacillin–tazobactam, cefalothin, cefoxitin, cefotaxime and ceftazidime were >128 mg/L.

Isoelectric focusing of extract of the isolate showed two β-lactamase bands of pI ~5.3 and 9.0. The isolate showed positive modified Hodge and EDTA-disc synergy tests, and the only pI 5.3 band was no longer present when the gels were overlaid with EDTA, which are findings suggesting a metallo-β-lactamase. The band of pI ~9.0 was likely to be chromosomal AmpC cephalosporinase. Carbapenem resistance was not transferred by conjugation. A plasmid harbouring a carbapenem resistance determinant was not detected (data not shown). These results suggest that a metallo-β-lactamase gene may be located on the chromosome.

Sequence analysis of the bla_{VIM-2}-containing integron

Preliminary PCR-based experiments detected bla_{VIM-2} but failed to detect bla_{IMP-1}. Sequence analysis of the 4392 bp cloned PCR amplicon revealed the structure of the class 1 integron, such as the 5′-CS element containing an *IntI1* integrase gene with its own promotor region, an *attI1* recombination site, and the 3′-CS element containing *qacEΔ1*. The integron contained insert gene cassettes bla_{VIM-2}, *aadA1*, and unknown open reading frames (ORFs) '*orfII*' and '*orfIII*'. The bla_{VIM-2} gene was located immediately downstream of the *IntI1* integrase gene. The initiation codon of the bla_{VIM-2} gene was proceeded by a putative promoter region of P_c. The guanine at the second base in the –35 consensus of the promoter P_c was replaced by thymidine, and the guanine at the fourth base in the –10 consensus was replaced by adenine. The bla_{VIM-2} gene cassettes had a 59 bp element, and the

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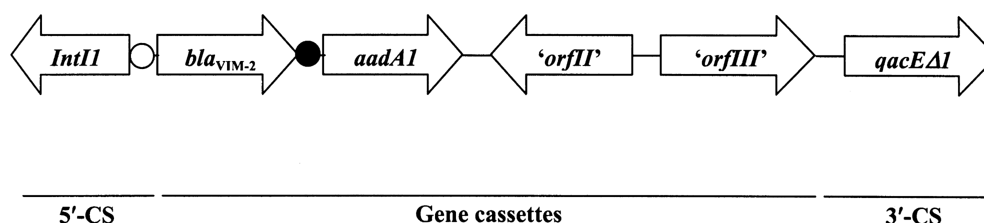

Figure 1. Schematic map of the class 1 integron that contains the bla_{VIM-2} gene cassette from the *E. cloacae* KU680 clinical isolate. The 5′-CS contains the *IntI1* integrase gene and the 3′-CS includes the disinfectant resistance determinant *qacEΔ1*. Inserted gene cassettes and their transcriptional orientation are indicated by arrows. The *attI1* recombination site is represented by a white circle and 59 bp element by a black circle.

qacEΔ1 gene also had a core site at its 5′ end. The sequences of *orfII* and *orfIII* did not show a typical cassette structure, i.e. possession of the consensus core sequence of GTTRRRY on the 5′ end (Figure 1).

Discussion

Since 1989, metallo-β-lactamases have spread among glucose non-fermenting Gram-negative bacilli, including *Pseudomonas aeruginosa*, *Pseudomonas putida*, *Alcaligenes* (*Achlomobacter*) *xylosoxidans*, *Acinetobacter baumannii* and *Acinetobacter* genomospecies 3.[7,8] However, these enzymes were rarely found in members of the family Enterobacteriaceae. The presence of the mobile bla_{VIM-2}-containing integron in *E. cloacae* suggests that the resistance can spread to other members of the family Enterobacteriaceae.

MICs of imipenem (4 mg/L) and meropenem (4 mg/L) for *E. cloacae* KU680 were relatively low compared with those for VIM-2-producing *P. aeruginosa* COL-1 (128 mg/L) and *Serratia marcescens* YMC 00/4/1591 (64 mg/L).[8,9] The difference in permeability coefficients of carbapenems between bacterial species may play a role in the low level of resistance to carbapenems.[10]

The P_c promoter for the bla_{VIM-2} gene is a strong promoter, due to the point mutations in the –35 and –10 consensus regions.[9] The P_2 promoter was not found upstream of the bla_{VIM-2} gene. P_2 expression may be responsible for up to 90% of bla_{VIM-2} transcription, as described for other integron-located genes.[9] Inactive P_2 promoter may also play a role in the low expression of resistance to carbapenems.

VIM-2 metallo-β-lactamase has no hydrolytic activity against aztreonam, but the MIC of aztreonam for *E. cloacae* KU680 was 64 mg/L, which is higher than the resistant breakpoint.[9] This result was possibly due to production of a chromosomal cephalosporinase (pI ~9.0). The isolate was found to be resistant to tobramycin and intermediate to gentamicin, but the new class 1 integron in this study contained only a streptomycin/spectinomycin adenyltransferase gene (*aadA1*) besides bla_{VIM-2} cassettes, suggesting that the gene(s) responsible for the aminoglycoside resistance resided in another location.

The structure of the new integron (*intI1 [bla_{VIM-2} aadA1 orfII orfIII] qacEΔ1*) was similar to that of an integron in *P. putida* YMC 97/8/322 (*intI1 [bla_{VIM-2} aacA7 aadA1] qacEΔ1*; GenBank accession number AF327064), that of an integron in *Acinetobacter* genomospecies 3 YMC 99/11/160 (*intI1 [bla_{VIM-2} aacA4 aadA1 orfII orfIII] qacEΔ1*; GenBank accession number AF369871), and that of an integron in *P. aeruginosa* YMC 95/1/704 (*intI1 [aacA4 bla_{VIM-2} orfI aadA1 orfII orfIII] qacEΔ1*; GenBank accession number AY029772), all of which were detected in South Korea. The nucleotide sequences of the two unknown ORFs (*orfII* and *orfIII*) were very similar to those of *P. aeruginosa* (99.8% and 99.7% identity), *Acinetobacter* genomospecies 3 (99.9% and 100% identity) and *S. marcescens* (both 99.7 % identity) strains previously isolated in Korea, which were arbitrarily named *orfII* and *orfIII*.[8] Since several bacterial species carrying bla_{VIM-2}-containing integrons (which show similarity in their possession of *orfII* and *orfIII* as well as bla_{VIM-2}) have been isolated in Korea, this may suggest the horizontal spread of bla_{VIM-2}. As a result of partial DNA sequencing we also found one *Morganella morganii* isolate containing an integron similar to that of *E. cloacae* KU680.

In conclusion, *E. cloacae* KU680, which carries a bla_{VIM-2} gene, represents an emerging threat. The bla_{VIM-2}-containing integrons are mobile, so horizontal spread of imipenem resistance to more frequent pathogens, such as *E. coli*, can be anticipated. The spread of bla_{VIM-2} could compromise the future usefulness of carbapenems for the treatment of infections caused by Gram-negative bacilli.

Acknowledgements

This work was supported by a research grant of the Korea Science and Engineering Foundation (1999-2-211-002-5).

References

1. Rasmussen, B. A., Bush, K., Keeney, D., Yang, Y., Hare, R., O'Gara, C. *et al.* (1996). Characterization of IMI-1 β-lactamase, a class A carbapenem-hydrolyzing enzyme from *Enterobacter cloacae. Antimicrobial Agents and Chemotherapy* **40**, 2080–6.

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2. Nordmann, P. & Poirel, L. (2002). Emerging carbapenemases in Gram-negative aerobes. *Clinical Microbiology and Infection* **8**, 321–31.

3. Poirel, L., Lambert, T., Türkoglü, S., Ronco, E., Gaillard, J.-L. & Nordmann, P. (2001). Characterization of class 1 integrons from *Pseudomonas aeruginosa* that contain the bla_{VIM-2} carbapenem-hydrolyzing β-lactamase gene and of two novel aminoglycoside resistance gene cassettes. *Antimicrobial Agents and Chemotherapy* **45**, 546–52.

4. National Committee for Clinical Laboratory Standards. (2001). *Performance Standards for Antimicrobial Susceptibility: Eleventh Informational Supplement M100-S11.* NCCLS, Wayne, PA, USA.

5. National Committee for Clinical Laboratory Standards. (2000). *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically—Fifth Edition: Approved Standard M7-A5.* NCCLS, Wayne, PA, USA.

6. Lee, K., Chong, Y., Shin, H. B., Kim, Y. A., Yong, D. & Yum, J. H. (2001). Modified Hodge and EDTA-disk synergy tests to screen metallo-β-lactamase-producing strains of *Pseudomonas aeruginosa* and *Acinetobacter* species. *Clinical Microbiology and Infection* **7**, 88–91.

7. Lee, K., Lim J. B., Yum J. H., Yong, D., Chong. Y., Kim, J. M. Y. *et al*. (2002). bla_{VIM-2} cassette-containing novel integrons in metallo-β-lactamase-producing *Pseudomonas aeruginosa* and *Pseudomonas putida* isolates disseminated in a Korean hospital. *Antimicrobial Agents and Chemotherapy* **46**, 1053–8.

8. Lee, K., Yum, J., Yong, D., Kim, J., Chong, Y. & Rossolini, G. (2001). Spreading of integron borne metallo-β-lactamase gene among Gram-negative bacilli in Korea. *Clinical Microbiology and Infection* **7**, *Suppl. 1*, 283.

9. Poirel, L., Naas, T., Nicolas, D., Collet, L., Bellais, S., Cavallo, J.-D. *et al*. (2000). Characterization of VIM-2, a carbapenem-hydrolyzing metallo-β-lactamase and its plasmid- and integron-borne gene from a *Pseudomonas aeruginosa* clinical isolate in France. *Antimicrobial Agents and Chemotherapy* **44**, 891–7.

10. Matsumura, N., Minamin, S., Watanabe, Y., Iyobe, S. & Mitsuhashi, S. (1999). Role of permeability in the activities of β-lactams against Gram-negative bacteria which produce a group 3 β-lactamase. *Antimicrobial Agents and Chemotherapy* **43**, 2084–6.

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