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Inter-Species Dissemination of an Integron Carrying *bla*VIM-1 Between *Pseudomonas aeruginosa* and *Enterobacter cloacae*

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SENTRY ANTIMICROBIAL SURVEILLANCE

ABSTRACT

Background: A class 1 integron carrying *bla*VIM-1, *aacA4*, *aph15* and *aadA1* have been reported in *P. aeruginosa* (PSA) isolates from five different Italian cities, three of them closely located to Genoa.
Methods: As part of the SENTRY Program, metallo-ß-lactamase (MßL) genes have been screened in Enterobacteriaceae with decreased susceptibility to carbapenems. PCR reactions were performed with primers to *bla*IMP and *bla*VIM families and *bla*SPM-1. Amplicons were sequenced in both strands. Integron structure was revealed with PCR-based strategies. Plasmid extraction was carried out and preparations were transformed into *E. coli* host.
Results: *E. cloacae* isolate 75-10344 was recovered from a blood culture in a hospital in Genoa, Italy, in 9/21/2004. PCR and sequencing results showed that this isolate carried *bla*VIM-1. Although PCR reactions failed to amplify the 3'CS of class 1 integron, genetic analysis showed that the MßL gene was located downstream of a class 1 integrase gene. *bla*VIM-1 was followed by *aacA4* that was upstream of *aph15*. Primers annealing in distinct regions of the 3'CS and increased PCR extension (up to 10 minutes) failed in amplifying *qacEΔ1/sul1*. Plasmid preparations showed the absence of plasmids and transformation experiments yielded no colonies.
Conclusions: The partial integron structure revealed so far in isolate 75-10344 is identical to integrons harboring *bla*VIM-1 carried by PSA isolates recovered from five different Italian cities, since 2001. Thus, PSA isolates are the likely source of the integron found in *E. cloacae* isolate 75-10433. However, our results suggest that a deletion of the *aadA1* and the 3'CS or even the insertion of a large genetic element may have occurred in the isolate 75-10344. Mobilization of MßL genes from PSA to members of Enterobacteriaceae family is very worrisome since it could jeopardize treatment of infections caused by ESBL- or AmpC-producing pathogens.

INTRODUCTION

In Italy, the presence of metallo-ß-lactamase (MßL) genes has been reported since 1999 when VIM-1 was first described. The gene encoding VIM-1, *bla*VIM-1, was initially characterized from a *Pseudomonas aeruginosa* isolate recovered in 1997 (Verona). *bla*VIM-1 was located as the first gene cassette of an integron named *In*70. Since then a diversity of integron-borne MßL genes, including, *bla*VIM-2, *bla*VIM-4, *bla*IMP-1, *bla*IMP-2, *bla*IMP-12 and *bla*IMP-13 have been reported from various Italian cities.

The integron *In*70 contains all key elements of a class 1 integron and carried, apart from *bla*VIM-1, three aminoglycoside resistance genes (*aacA4*, *aph15* and *aadA1*). *In*70 was initially reported in the northern region of Italy and was subsequently found among VIM-1-producing *P. aeruginosa* isolates from Pavia and Trieste (northern region), Rome (central region), and Sicily (southern region). In addition, *In*70 was discovered in an *Achromobacter xylosoxidans* isolate recovered from a urine specimen in Verona, Italy in 1998.

In this study, we report the first isolation of *In*70 in a VIM-1-producing *Enterobacter cloacae* strain from Genoa, Italy.

MATERIALS AND METHODS

Bacterial Strains. The SENTRY Antimicrobial Surveillance Program monitors the activity of more than 30 antimicrobial agents in medical centers located in North America, South America, Europe, Asia and Australian regions. Bacterial strains collected from selected sites of infection are susceptibility tested by reference broth microdilution methods according to the Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS) documents and interpretation criteria. Members of the Enterobacteriaceae family showing decreased susceptibility to imipenem and/or meropenem (MIC, ≥ 4 µg/ml) have been routinely screened for MßL genes.

Phenotypic detection of ß-lactamases. Production of MßLs was screened by the disk approximation test. Briefly, a 100 mm Mueller-Hinton agar plate was inoculated using a 0.5 McFarland suspension from fresh cultures. Imipenem, meropenem and ceftazidime disks were strategically aligned around disks containing either EDTA (750-µg) or thiolactic acid (360-g). The test was read after 18 - 20 hours of incubation at 35ºC. The appearance of either an elongated or a phantom zone between the carbapenems and/or ceftazidime and either disk containing an MßL inhibitor (EDTA or thiolactic acid) was considered a positive test. *Acinetobacter baumannii* 54/97 was used as a positive control. MßL Etest strips (AB BIODISK, Solna, Sweden) were used to confirm the disk approximation test results, each containing imipenem with and without EDTA.

MßL activity screening. Strain #75-10344 was grown in nutrient broth with ceftazidime (4 µg/ml), pelleted, re-suspended in 1 ml of 1mM TRIS, 100 mM $ZnCl_2$ solution and then ultrasonicated. The cell extract was centrifuged and 100 µl of the supernatant was used to carry out the spectrophotometer assay against imipenem at 299 nm. Inhibition was performed at room temperature, incubating the protein extract during 15 minutes with 25 mM of EDTA previously to the assay against imipenem.

PCR experiments. The investigation for *bla*IMP, *bla*VIM and *bla*SPM was initially performed by PCR amplification with the primers designed for the internal fragment of the MßL genes. The integron structure was revealed with a "walking" sequencing strategy, using the primers targeting the conserved segments of class 1 integrons and primers designed for the other gene cassettes found in the integron. The detection of the MßL related genes was carried out in 20 µl final volume using ABgene Taq DNA Polimerase (ABgene House, Surrey, United Kingdom). Primers were used at 10 mM concentration and 1µl of overnight bacterial culture at density OD_{260} 1 at 600 nm was used as a template. The cycling parameters were: 95ºC for 5 minutes followed by 30 cycles of 95ºC for 1 minute, annealing at 45ºC for 1 minute and extension 68ºC ranging from 1 to 4 minutes and ending with 5 minute incubation at 68ºC.

DNA sequencing and computer analysis. The amplicons obtained by PCR reactions were sequenced on both strands using ABIPrism 377 system. The nucleotide sequences were analyzed using Lasergene software package (DNASTAR, Madison, WI). Obtained sequences were compared to sequences available over the internet (http://www.ncbi.nlm.nih.gov/blast and http://www.ebi.ac.uk./fasta33/).

Plasmid content, conjugation and electroporation. Plasmid extraction from *E. cloacae* 75-10433 was undertaken with QIAprep Spin Mini prep kit (Quiagen, West Sussex, UK). The plasmid preparation obtained was electroporated into *E. coli* DH10B. Electroporation parameters were 2.5 kV, 25 µF and 400 Ω using the Bio-Rad Gene Pulser apparatus (Bio-Rad, Richmond, CA). Selection for transformants was performed on nutrient agar plates containing ceftazidime 4 µg/ml. Conjugation experiments were performed in liquid medium with rifampin-resistant derivatives of *E. coli* K-12 as the recipient. Transconjugant selection was performed on nutrient agar with ceftazidime (4 µg/ml) and rifampin (200 µg/ml).

RESULTS

- *Enterobacter cloacae* strain #75-10433 was recovered from a blood culture of a hospitalized patient in Genoa, Italy, in September 2004. It showed resistance to all ß-lactam agents tested, except for imipenem and meropenem (MIC, 8 µg/ml for both [intermediate]). In contrast, this isolate was susceptible to all fluoroquinolones tested and to polymyxin B (Table 1).

- Initial MßL screening experiments showed detectable carbapenem hydrolysis activity by the crude extract of ß-lactamases. Hydrolysis of imipenem was inhibited by EDTA.

- PCR amplification experiments detected a *bla*VIM-1-like gene in strain #75-10433. Analysis of the sequenced PCR products showed that *bla*VIM-1 was located in the first position of a class 1 integron, followed by *aacA4* and *aph15* gene cassettes (Figure 1).

- Additional analysis of the genetic context of the integron harboring *bla*VIM-1 showed 100% homology with integron *In*70, previously reported in *P. aeruginosa* isolates from five different Italian cities, three of them located near to Genoa (Figure 2). However, the *aadA1* (fourth position of *In*70, Figure 1) and the 3'-CS, usually found in the *In*70, were not detected.

Table 1. Antimicrobial susceptibility pattern of the *Enterobacter cloacae* strain #75-10433.

Antimicrobial agents	MIC (µg/ml)
Imipenem	8
Meropenem	8
Ceftriaxone	>32
Ceftazidime	>16
Cefepime	>16
Aztreonam	>16
Ampicillin/sulbactam	>32
Piperacillin	>128
Piperacillin/tazobactam	>64
Ciprofloxacin	0.25
Gatifloxacin	0.25
Levofloxacin	0.5
Moxifloxacin	0.25
Amikacin	2
Gentamicin	≤2
Tobramycin	4
Tetracycline	4
Trimethoprim/sulfamethoxazole	>2
Polymyxin B	0.5

- Reactions targeting the 3'-CS of class 1 integrons failed to yield PCR products even using longer extension period (up to 10 minutes) and primers targeting different regions of the *qacEΔ1/sul1* structure. These findings suggest a deletion of 3'-CS or the insertion of a large element within the *aph15* gene and the 3'-CS.

- Plasmid preparations of strain #75-10433 showed no plasmids. Transformation and conjugation experiments in *E. coli* host strain were unsuccessful, suggesting that this integron was chromosomally located.

Figure 1. Schematic representation of integron *In*70 found in VIM-1 producing *Pseudomonas aeruginosa* isolates from Italian cities and *In*70-like structure from the strain #75-10433. Genes are represented as open rectangles with arrows indicating the direction of their transcription. Filled circles represent the 59-be recombination sites and the open circles represent the attachment site *attI1*.

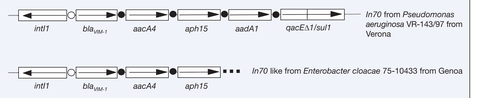

Figure 2. Locations of the Italian medical centers where the integron *In*70, carrying *bla*VIM-1, were detected. The green and yellow stars represent medical sites in which the *P. aeruginosa* and *A. xylosoxidans* isolates carrying *In*70 were found, respectively. The red star represents the Genoa site in which the *E. cloacae* isolate (75-10433) was recovered.

CONCLUSIONS

- Although *In*70 carrying *bla*VIM-1 have been reported in non-fermentative gram-negative bacterial (*P. aeruginosa* and *Achromobacter xylosoxidans*) isolates in various Italian regions, this is the first report of the presence of this integron in a member of the *Enterobacteriaceae* family. These findings indicate the occurrence of inter-species dissemination of *In*70, since *P. aeruginosa* isolates would be the likely source of the *In*70 found in the *E. cloacae* strain #75-10433.

- The results obtained in this study corroborate the evidence that integron-borne MßLs have a great potential of intra- and inter-species dissemination.

- The excision of the *aadA1* gene cassette and the possible deletion of the 3'-CS indicate that class 1 integron plays a major role in the genetic rearrangement and spread of resistance determinants among Gram-negative pathogens.

- The mobilization and further inter-species dissemination of integron-borne MßL genes pose a serious threat to the treatment of infections caused by Gram-negative bacilli in this region.

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