Emergence of a VIM-1 MBL and CTX-M-15 ESβL-producing *Klebsiella pneumoniae* clone from acute and rehabilitation hospitals in Italy

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SUMMARY

We report the emergence of VIM-1 MBL and CTX-M-15-producing *K. pneumoniae* isolates collected from patients at two acute care hospitals (I.R.C.C.S. "S. Matteo" and "Casa Sollievo della Sofferenza" Hospital) and a long-term rehabilitation facility in Northern Italy (I.R.C.C.S. "S. Maugeri").
Between February 2007 and October 2008, 30 *K. pneumoniae* strains showing decreased susceptibility to carbapenems were collected. PCR and sequencing experiments revealed the presence of $bla_{\text{VIM-1}}$ gene in 14/30 isolates. All the above isolates carried the $bla_{\text{SHV-5}}$ determinant as well; interestingly, 8/14 VIM positive isolates were also CTX-M-1-like producers. VIM-1 positive strains were present in all hospitals. PFGE genomic profiles of the 14/30 isolates showed that 2 different clones, A and B, were responsible for outbreaks.
The coexistence in the same bacterial cell of compatible plasmids carrying epidemiologically important emerging resistance genes, such as MBLs and CTX-Ms, is worrisome since it could predict the generation and spread of pan-resistant bacteria and the consequent treatment option limitations that can lead to significant morbidity and mortality.
Control measures should be applied to detect MBL-producing strains and to contrast the vertical and plasmidic diffusion of carbapenem-resistant *K. pneumoniae* in acute care and rehabilitation facilities.

KEY WORDS: K. pneumoniae, Metallo-β-lactamase, ESβL.

Received February 11, 2013 *Accepted* April 4, 2013

INTRODUCTION

Carbapenems, because of the stability to hydrolysis by most β-lactamases, currently represent the drugs of choice for treatment of serious infections caused by multidrug-resistant Gram-negative bacteria (Cagnacci *et al.,* 2008).
However, the emergence of acquired metallo-β-lactamases (MBLs) and other class A or class D β-lactamases affecting carbapenems, is a therapeutic challenge.
Among the Ambler class A carbapenemases, KPC-type are globally spread, with KPC-2 and KPC-3 variants and highly epidemic clonal complex 258 (512) (Migliavacca *et al.,* 2013) being nowadays the most widespread in *Klebsiella pneumoniae* in Europe.
Acquired MBLs, such as VIM, IMP, and NDM, are associated with different mobile elements, and the genes for VIM-type enzymes are carried as mobile gene cassettes inserted in class 1 integrons (Yan Wei and Jie Wang, 2013).
These enzymes confer high-level resistance to most β-lactams, with the exception of aztreonam, and are not inhibited by class A β-lactamase inhibitors.

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Even if VIM enzymes have been found mainly in non-fermenting Gram-negative bacteria, their detection in members of the *Enterobacteriaceae* family is recently increasing.
Carbapenem MICs are typically low and variable for MBL-producing *Enterobacteria*, thus complicating their phenotypic detection in routine tests, in addition to the presence of other β-lactamases.
Most VIM-producing *Enterobacteriaceae* isolates that have been reported until now are sporadic and clonally unrelated, although clonal epidemics have been also described and some countries, such as Greece, are close to an endemic situation (Giakkoupi *et al.,* 2003).
Among *Enterobacteria*, *K. pneumoniae* strains producing enzymes belonging to any of the three MBL families have already achieved international spread, though significant local differences do exist.
VIM-positive *K. pneumoniae* was first observed around 2001 to 2003 in Southern Europe and was introduced later to Northern Europe (Germany, France and the Scandinavian countries) and the United States, mostly through colonized patients transferred from high-prevalence areas. Isolation rates of VIM positive *K. pneumoniae* in Northern Europe and the United States remain low, though some infection clusters limited to single hospitals have been reported. In addition, sporadic cases have been recorded in Tunisia, South Korea, and Venezuela. Until recently, VIM-producing *K. pneumoniae* and other *Enterobacteria* were frequently isolated in Mediterranean countries, reaching epidemic proportions only in Greece (Tzouvelekis *et al.,* 2012).
In Italy MBL producing *K. pneumoniae* clinical isolates have been described in Genoa (Cagnacci *et al.,* 2008), Bolzano (Aschbacher *et al.*, 2008) and in Aquila (Perilli *et al.,* 2012).
A nationwide survey of ESβL-production among *Enterobacteriaceae*, carried out in 2003, showed that CTX-M-type enzymes have achieved a sizeable prevalence among ESβL producers in Italy, mostly in *E. coli* and, to a lesser extent, in *K. pneumoniae* (Mugnaioli *et al.,* 2006).
The rates of CTX-M production were found to be 54.8% and 12.3% among ESβL-producing isolates of *E. coli* and *K. pneumoniae*, respectively, with an absolute predominance of group 1 enzymes (mostly CTX-M-1 and CTX-M-15 and, less frequently, CTX-M-32) (Rossolini *et al.,* 2008).
We report the emergence of VIM-1 MBL and CTX-M-15 producing *K. pneumoniae* isolates collected from patients at two acute care hospitals (one located in Northern and one in Southern Italy) and a long term rehabilitation facility in Northern Italy.

MATERIALS AND METHODS

Between February 2007 and October 2008, 30 *K. pneumoniae* strains, showing decreased susceptibility to carbapenems by Vitek 2 system, were obtained from 24 patients at Fondazione I.R.C.C.S "S. Matteo" (Pavia), from 1 patient at Fondazione I.R.C.C.S. Maugeri and from 5 patients at Hospital "Casa Sollievo della Sofferenza" S. Giovanni Rotondo, Foggia. One single isolate from each patient was obtained.
Identification and susceptibility testing were conducted using the Vitek 2 automated system.
MICs of carbapenems were determined by macrodilution method; ESβL production and MICs values were confirmed using E-test method (AB Biodisk).
Susceptibility to non-β-lactam antibiotics (aminoglycosides, fluoroquinolones and cotrimoxazole) was assessed by the disk diffusion test and the results were interpreted according to the Clinical and Laboratory Standards Institute guidelines (CLSI, 2009).
The presence of ESβL (Sabia *et al.,* 2012) and MBL enzymes was screened by the double and by the imipenem-imipenem (IMI) plus EDTA disc synergy tests. Preliminary identification was performed by isoelectrofocusing, as described elsewhere. Crude sonic extracts from *E. coli* harboring TEM-1 (pI, 5.4), TEM-4 (pI, 5.9), TEM-3 (pI, 6.3), SHV-2 (pI, 7.6), CTX-M-9 (pI, 8.1), and SHV-12 (pI, 8.2) were used as controls.
Conjugal transfer of resistance determinants was performed in liquid medium using the *E. coli* K12 strain J62 (*pro-*, *his-*, *trp-*, *lac-*, *Sm* R) and J53-2 (*met-*, *pro-*, *rif* R) as recipients. The transconjugants were selected on McConkey agar containing imipenem (0,5 mg/l) plus streptomycin (1000 mg/l) or rifampicin (100 mg/l).
PCR amplification of bla_{SHV}, bla_{CTX-M}, bla_{VIM}, and bla_{IMP} genes was performed using primers and conditions described elsewhere (Dashti *et al.*, 2010; Pagani *et al.*, 2003; Rossolini *et al.*, 2008).

P. aeruginosa VR-143/97 (VIM-1 producer) and *P. aeruginosa* ATCC 27853 were used like positive and negative control, respectively.
Full-length sequencing was carried out using automated fluorescent dye-terminator sequencing on ABI PRISM 3100 genetic Analyzer. Pulsed-field gel electrophoresis profiles of genomic DNA were analyzed by means of the Gene Path procedure (Bio-Rad Laboratories, Richmond, Calif.) using the no. 2 pathogen group reagent kit and the restriction enzyme XbaI. DNA fragments were electrophoresed in 1% agarose gel in 0.5 X TBE buffer with the Gene Path system (Bio-Rad).
Clonal relationships based on PFGE patterns were interpreted according to the criteria proposed by Tenover *et al.*, 1995.
Plasmid DNA was extracted and purified by the alkaline lysis method.
The patients were from different wards of I.R.C.C.S "S. Matteo", from the Cardiology Rehabilithation ward of I.R.C.C.S. Maugeri and from Intensive Care Unit ward of Hospital "Casa Sollievo della Sofferenza" - S. Giovanni Rotondo, Foggia. The isolates were mainly obtained from sputum and urinary samples.

RESULTS

Susceptibility tests using the disk diffusion method showed that all isolates were resistant to all β-lactams and exhibited resistance to most non-β-lactam antimicrobials tested (including cotrimoxazole and ciprofloxacin).
All the 30 *K. pneumoniae* showed carbapenems MICs values ranging from 0.25 to 128 mg/L by macrodilution method and from 0.064 to 32 mg/L by E-test.
The IMI-EDTA disc synergy test yielded a positive result in 14/30 strains, characterized by IMI MICs ranging from 2 to 128 mg/L, suggesting the presence of a class B enzyme (MBL).
PCR and sequencing experiments revealed the presence of $bla_{\text{VIM-1}}$ gene in such 14/30 isolates. All the above isolates carried $bla_{\text{SHV-5}}$ determinant; interestingly, 8/14 VIM positive isolates were also CTX-M-1 like producers. VIM-1 positive strains were present in all hospitals. PFGE genomic profiles of the 14/30 isolates showed that 2 different clones, A and B, were responsible for outbreaks.
The clone A was spread in six different wards of the Pavia Hospital; the same clone was also detected in Maugeri Facility; the clone B persisted in the Intensive Care Unit of S. Giovanni Rotondo Hospital. The *K. pneumoniae* strains were compared by PFGE to VIM-1 producing *K. pneumoniae* collected from Bolzano Regional Hospital during the period 2005-2006. These isolates were unrelated with the strains included in this study.
The 14 $bla_{\text{VIM-1}}$ producing *K. pneumoniae* isolates positive to conjugal transfer were subjected to plasmid analysis. The clone A harboured different conjugative plasmids which varied in size between 80 and 90 kb; the clone B harboured a conjugative plasmid with identical size equal to 85 Kb.
PCR experiments on the transconjugants and sequencing of the respective products confirmed the existence of CTX-M-15 ESβL and VIM-1 MBL β-lactamase genes on different plasmids.

DISCUSSION

The coexistence of two enzymes, a MBL and a non-MBL extended-spectrum β-lactamase, in the same strain has been previously documented for *Enterobacteriaceae*, with both VIM-1 and a CTX-M-type β-lactamase (Scoulica *et al.,* 2004), VIM-1 and SHV-5 (Kassis-Chikhani *et al.,* 2006), SHV-12 and VIM-4 (Luzzaro *et al.,* 2004), VIM-2 and IBC-1 (Galani *et al.,* 2005), IMP-1 and CTXM-2 (Livermore *et al.,* 2000), VIM-12 and a CMY-type cephalosporinase (Pournaras *et al.,* 2005), and VIM-1 and CMY-13 (Miriagou *et al.,* 2004).
Although nowadays KPC carbapenemases are the dominant enzymes, these data focus on the emergence of *K. pneumoniae* strains producing both VIM-1 and CTX-M-15 transferable enzymes in Italy and highlight the importance of detecting the association with other resistance markers and their multidrug resistance patterns. The increase in MBLs among clinical isolates of *Enterobacteriaceae* is an important and emerging resistance concern.
Carbapenem MICs are typically low and variable for MBL-producing *Enterobacteria*. This circumstance, together with the presence of other β-lactamases, complicates their phenotypic detection in routine tests. Our data show different levels of carbapenem resistance with MICs of IMI higher than meropenem ones; moreover MICs of car-

bapenems obtained by E-test were lower than those evaluated by macrodilution method.

The coexistence in the same bacterial cell of compatible plasmids carrying epidemiologically important emerging resistance genes, such as MBLs and CTX-Ms, is worrisome since it could predict the generation and spread of pan-resistant bacteria and the consequent treatment option limitations that can lead to significant morbidity and mortality.

Control measures including screening with chromogenic media (Luzzaro *et al.*, Microbial. Drug Resistance, in press), tests based on chelating agents and molecular methods, should be applied to detect MBL producing strains and to contrast the vertical and plasmidic diffusion of carbapenem-resistant *K. pneumoniae* in acute care and rehabilitation facilities.

REFERENCES

Aschbacher R., Doumith M., Livermore D. M., Larcher C., Woodford N. (2008). Linkage of acquired quinolone resistance (qnrS1) and metallo-β-lactamase (blaVIM-1) genes in multiple species of *Enterobacteriaceae* from Bolzano, Italy. *J. Ant. Chemother.* **61**, 515-523.

Cagnacci S., Gualco L., Roveta S, Mannelli S., Borgianni L., et al. (2008). Bloodstream infections caused by multidrug-resistant *Klebsiella pneumoniae* producing the carbapenem-hydrolysing VIM-1 metallo-β-lactamase: first Italian outbreak. *J. Ant. Chemother.* **61**, 296-300.

Clinical and Laboratory Standards Institute antimicrobial susceptibility testing standards. (2009). 100, S23.

Galani I., Souli M., Chryssouli Z., Katsala D., H. Giamarellou. (2004). First identification of an *Escherichia coli* clinical isolate producing both metallo-β-lactamase VIM-2 and extended-spectrum β-lactamase IBC-1. *Clin. Microbiol. Infect.* **10**, 757-760.

Giakkoupi P., Xanthaki A., Kanelopoulou M. (2003). VIM-1 metallo-β-lactamase producing *Klebsiella pneumoniae* strains in Greek hospitals. *J. Clin. Microbiol.* **41**, 3893-3896.

Kassis-Chikhani N., Decre D., Gautier V., Burghoffer B., Saliba F., et al. (2006). First outbreak of multidrug-resistant *Klebsiella pneumoniae* carrying bla_{VIM-1} and bla_{SHV-5} in a French university hospital. *J. Antimicrob. Chemother.* **57**, 142-145.

Livermore D.M., Woodford N. (2000). Carbapenemases: a problem in waiting? *Curr. Opin. Microbiol.* **3**, 489-495.

Luzzaro F., Docquier J.-D., Colinon C., Endimiani A, Lombardi G., et al. (2004). Emergence in *Klebsiella pneumoniae* and *Enterobacter cloacae* clinical isolates of the VIM-4 metallo-β-lactamase encoded by a conjugative plasmid. *Antimicrob. Agents Chemother.* **48**, 648-650.

Migliavacca R., Nucleo E., Asticcioli S., Casari E., Bracco S., et al. (2013). Multifocal diffusion of a KPC-3 producing ST512 *K. pneumoniae* clone in Northern Italy. *New Microbiologica.* **36**, 109-110

Miriagou V., Tzouvelekis L. S., Villa L., Lebessi E., Vatopoulos A.C., et al. (2004). CMY-13, a novel inducible cephalosporinase encoded by an *Escherichia coli* plasmid. *Antimicrob. Agents Chemother*. **48**, 3172-3174.

Mugnaioli C., Luzzaro F., De Luca F., Brigante G., Perilli M., et al. (2006). CTX-M-type extended-spectrum beta-lactamases in Italy: molecular epidemiology of an emerging countrywide problem. *AAC.* **50**, 2700-2706.

Perilli M., Bottoni C., Grimaldi A., Segatore B., Celenza G., et al. (2013). Carbapenem-resistant *Klebsiella pneumoniae* harbouring bla_{KPC-3} and bla_{VIM-2} from central Italy. *Diagn. Microbiol. Infect Dis.* **75**, 218-221.

Pournaras S., Ikonomidis A., Tzouvelekis L. S, Tokatlidou D., Spanakis N., et al. (2005). VIM-12, a novel plasmid-mediated metallo-β-lactamase from *Klebsiella pneumoniae* that resembles a VIM-1/VIM-2 hybrid. *Antimicrob. Agents Chemother.* **49**, 5153-5156.

Rossolini G.M., D'Andrea M.M., Mugnaioli C. (2008). The spread of CTX-M-type extended-spectrum β-lactamases. *Clin. Microbiol. Infect.* **14** (Suppl. 1), 33-41.

Sabia C., Gargiulo R., Sarti M. (2012). Evaluation of a double synergy differential test (DSDT) for differential detection of ESβL and AmpC-type β-lactamases in *Escherichia coli*, *Klebsiella pneumoniae* and *Proteus mirabilis*. *New Microbiol.* **35**, 221-225.

Scoulica E.V., Neonakis I.K., Gikas A.I., Tselentis Y. J. (2004). Spread of bla_{VIM-1} producing *E. coli* in a university hospital in Greece. Genetic analysis of the integron carrying the bla_{VIM-1} metallo-beta-lactamase gene. *Diagn. Microbiol. Infect. Dis.* **48**, 167-172.

Tenover F.C., Arbeit R.D., Goering R.V., Mickelsen P.A., Murray B.E., et al. (1995). Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *J. Clin. Microbiol.* **33**, 2233-2239.

Tzouvelekis L.S., Markogiannakis A., Psichogiou M., Tassios P.T., Daikos G.L. (2012). Carbapenemases in *Klebsiella pneumoniae* and other *Enterobacteriaceae*: an Evolving Crisis of Global Dimensions. *Clinical Microbiology Reviews.* **25**, 682-707.

Wei Y., Wang J. (2013). First Detection of VIM-4 Metallo-β-Lactamase-Producing *Citrobacter freundii* in China. *Ann. Lab. Med.* **33**, 84-85.