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Acquired AmpC type beta-lactamases: an emerging problem in Italian long-term care and rehabilitation facilities

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SUMMARY

We report the multiple detection of *Proteus mirabilis* isolates, from 4 different long-term care and rehabilitation facilities (LTCRFs) of Northern Italy, resistant to expanded-spectrum cephalosporins and cephamycins and producing an acquired ampC-like β-lactamase, named CMY-16. Genotyping by PFGE showed that isolates were clonally related to each other, although not identical. In all isolates the bla_{CMY-16} gene was not transferable by conjugation and was found to be carried on the chromosome. These results revealed multifocal spreading of a CMY-16 producing *P. mirabilis* clone in Northern Italy and emphasize the emergence of similar acquired resistance determinants in the LTCRFs setting.

KEY WORDS: Proteus mirabilis, CMY-16, Long-term care and rehabilitation facilities

AmpC-type β-lactamases (CBLs) are a large group of enzymes of broad substrate specificity. They confer resistance to 7 α-methoxy-cephalosporins such as cefoxitin or cefotetan and are usually not affected by commercially available β-lactamase inhibitors. In some strains, the expression of CBLs together with the loss of outer membrane porins, have been demonstrated to provide resistance to all β-lactams including carbapenems (Alvarez *et al.,* 2004; Naas *et al.,* 1999; Philippon *et al.,* 2002).

Unfortunately, inhibitors (BRL 42715, Ro 47-8284, Ro 48-1220, Ro 48-1256) that are active against AmpC enzymes are not readily available, but cloxacillin (Pitout *et al.,* 1998) or cefoxitin (Papanicolaou *et al.*, 1990) have been used to selectively block AmpC activity after isoelectric focusing. Lack of activity inhibition against oxyimino β-lactams or cephamycins by clavulanate is indirect evidence for the presence of an AmpC enzyme, but some AmpC enzymes are unusually susceptible to inhibition by tazobactam (Philippon *et al.,* 2002).

A number of these enzymes are encoded by chromosomal genes resident in some Gram-negative pathogens (e. g. *Pseudomonas aeruginosa*, *Acinetobacter* spp. and several members of the family *Enterobacteriaceae*), while others are encoded by genes associated to mobile DNA elements that can be acquired by horizontal gene transfer (Livermore, 1995).

These acquired CBLs are usually plasmid-mediated and belong to at least five different lineages. They are currently emerging worldwide in various species of *Enterobacteriaceae,* such as *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Escherichia coli*, *Salmonella* spp. and *Proteus mirabilis* (Alvarez *et al.,* 2004; Literacka *et al.,* 2004; Miriagou *et al.,* 2004; Mulvey *et al.,* 2005; Nakano *et al.,* 2004; Navarro *et al.,* 2001; Philippon *et al.,* 2002; Yong *et al.,* 2005; D'Andrea *et al.,* 2006).

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Acquired CBLs have been reported in *P. mirabilis* from several geographic areas (France, Tunisia, Poland, Korea and Italy) and include enzymes of the ACC, CMY/LAT and DHA lineages, even if the finding of a CMY/LAT type CBL**,** derived from the chromosomal AmpC of *C. freundii*, is most commonly reported.

The use of cephamycins and β-lactam-inhibitor combinations to counter the threat of extended-spectrum beta-lactamases (ESβLs) mediated resistance determined a shift toward non-ESβL phenotypes in species without inducible chromosomal AmpC. Although prevalence of CBLs remains overall lower than that of acquired class A extended-spectrum β-lactamases, they have become an important cause of resistance to expanded-spectrum β-lactams in some settings (Alvarez *et al.,* 2004; Literacka *et al.,* 2004; Mulvey *et al.,* 2005). The aim of our study was to characterize cefoxitin resistant *P. mirabilis* isolated from long-term care and rehabilitation facilities (LTCRFs) of Northern Italy where detection of CMY-16 producers in tertiary care hospitals have been previously reported (D'Andrea *et al.,* 2006). During a three year period (May 2003-June 2006), 217 non-repetitive *P. mirabilis* isolates intermediate/resistant to cefotaxime, were collected from inpatients in four long-term care and rehabilitation facilities of Northern Italy (LTCRF S. Margherita, Pavia, LTCRF S. Maugeri, Pavia, LTCRF Redaelli, Milano and LTCRF Melegnano, Milano). The strains were all recovered from urinary tract of catheterized patients admitted to geriatric wards. Identification of isolates was carried out using the Phoenix automated system (BD Diagnostic Systems).

ESβL was detected by a CLSI diffusion test (M100-S17) and by a double-disk synergy test between clavulanate or tazobactam and cefotaxime (CTX), cefepime (FEP), ceftazidime (CAZ) and aztreonam (ATM).

Analytical isoelectrofocusing (IEF) of crude bacterial lysates for detection of β-lactamases was carried out on polyacrylamide gels containing Pharmalite ampholines (pH 3.5- 10, plus pH 4-6). Beta-lactamase bands were visualized using nitrocefin.

The activity against β-lactam substrates of β-lactamase bands separated by IEF was assayed by a substrate overlaying procedure using an antibiotic concentration of 1 mg/L in the medium overlay and *Escherichia coli* ATCC 25922 as an indicator strain. Substrate hydrolysis was revealed by the occurrence of bacterial growth above the enzyme bands. Conjugation experiments were 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 and Mueller-Hinton agar containing cefotaxime (8 mg/l) plus streptomycin (1000 mg/l) or rifampicin (100 mg/l). The nature of the resistance genes were studied by molecular techniques such as PCR and sequencing. Clonal relationships between the strains was investigated using Pulsed-Field Gel Electrophoresis, performed with the restriction enzyme SfiI (Bio-Rad). Nucleotide sequences were determined on both strands directly on PCR amplification products at an external sequencing facility (Macrogen Inc., Seoul, South Korea).

Southern-blot hybridization of total DNA preparation, digested with I-CeuI (New England Biolabs, Hertfordshire, United Kingdom) and electrophorized by PFGE, was performed to determine the gene location, (Tsao *et al.,* 1983). The bla_{CMY-2} gene generated with primers CMY/F 5'-GGGCCCGGACACCYTTTTGC-3' and CMY/R 5'-TTCCAATGAGCTCAGGATTTTATAC-3' (D'Andrea *et al.*, 2006) was used as a radioactive probe.

197/217 (90.78%) strains were ESβL producers, while 20/217 (9.21%) strains showed an AmpC phenotype: they were all susceptible to piperacillin-tazobactam, cefepime, aztreonam and carbapenems. The double-disk synergy test, carried out on these last strains, showed a synergy between tazobactam or clavulanate and cefepime, but did not reveal detectable synergy between clavulanate and cefotaxime, ceftazidime, or aztreonam.

All 20 isolates showed two β-lactamase bands by IEF with pIs >8.4 and 5.4, respectively.

Band characterized by a pI >8.4 was active on cefotaxime, ceftazidime, cefepime, aztreonam and cefoxitin, suggesting the presence of an acquired CBL, while the pI 5.4 enzyme did not show activity with any of the above compounds. This band was consistent with a TEM-type enzyme.

PCR and sequencing techniques confirmed the presence of the resistance gene bla_{TEM-1b} in all isolates. Molecular characterization by PCR revealed an acquired CBL gene of the CMY-LAT lineage.

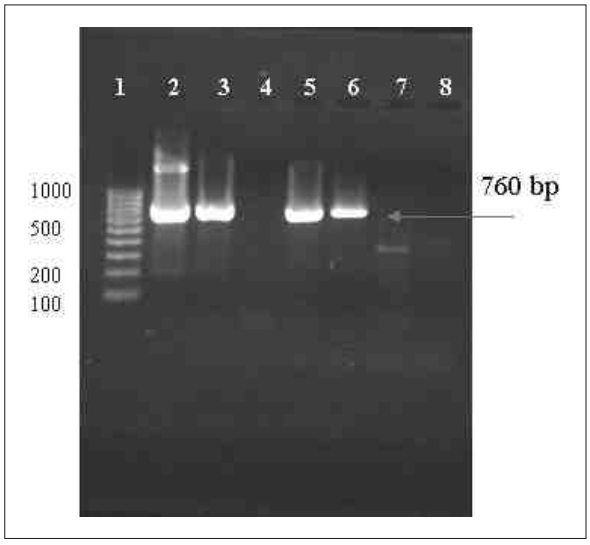

FIGURE 1 - *PCR products, obtained using primers AmpC/II_Fw and AmpC/II_Rev (D'Andrea et al., 2006), of three strains of P. mirabilis chosen as representative of different hospitals. Lane 1: marker 100 bp. Lane 2: Positive control (E.coli 26 SM[a]); Lane 3, 5, 6: P. mirabilis AmpC producers (Pm 20 RED[b], Pm 75 MG[c], Pm 3 MELE[d]). Lane 4: negative sample. Lane 7, 8: negative controls.*
[a]SM: S. Matteo Hospital, Pavia; [b]RED: LTCRF Redaelli; [c]MG: LTCRF S. Maugeri; [d]MELE: LTCRF Melegnano.

The size of the amplicon was 760 bp (Figure 1) and the sequencing results revealed that the determinant was bla_{CMY-16}. CMY-16 has a single amino acid substitution, A171S or N363S, respectively, according to the numbering scheme used by Decre *et al.*, if compared with either CMY-4 (31) or CMY-12 (8), while, when compared with CMY-2 (CAA62957.1), it has two amino acid substitutions: A171S and W221R.

At the nucleotide sequence level, bla_{CMY-16} exhibits two point mutations compared with bla_{CMY-4} (T511G and G1140A), bla_{CMY-12} (G1088A and G1140A), or bla_{CMY-2} (T511G and C661T).

Results of conjugation experiments showed that the bla_{CMY-16} determinant was not transferable, suggesting that the gene was inserted in the chromosome. In addition southern blot results showed that the genetic environment was likely to be conserved in all isolates. PFGE profiles of genomic DNAs digested with SfiI were identical or clearly related (Figure 2), suggesting that all isolates were clonally related.

The incidence of bla_{CMY-16} gene within the four LTCRFs was as follows: 15.6% (10/64) at LTCRF S. Margherita; 13.3% (2/15) at LTCRF S. Maugeri; 4.8% (6/125) at LTCRF Redaelli; 15.4% (2/13) at LTCRF Melegnano respectively. This report focuses on the increasing diffusion of *P. mirabilis* clone expressing an AmpC-type variant; the clinical strains investigated in this work were all clonally related, suggesting a worrisome vertical spread. The emergence of these enzymes in *P. mirabilis* has been reported in some areas (Bidet *et al.,* 2005; Decre *et al.,* 2002; Girlich *et al.,* 2000; Literacka, *et al.,* 2004; Philippon *et al.,* 2002; Rhimi-Mahjoubi *et al.,* 2002; Yong *et al.,* 2005), though acquired AmpC-type β-lactamases are overall less common than class A ESβLs. These data emphasize that the resistance of *P. mirabilis* to expanded-spectrum cephalosporins represents a growing relevant problem of clinical and epidemiological

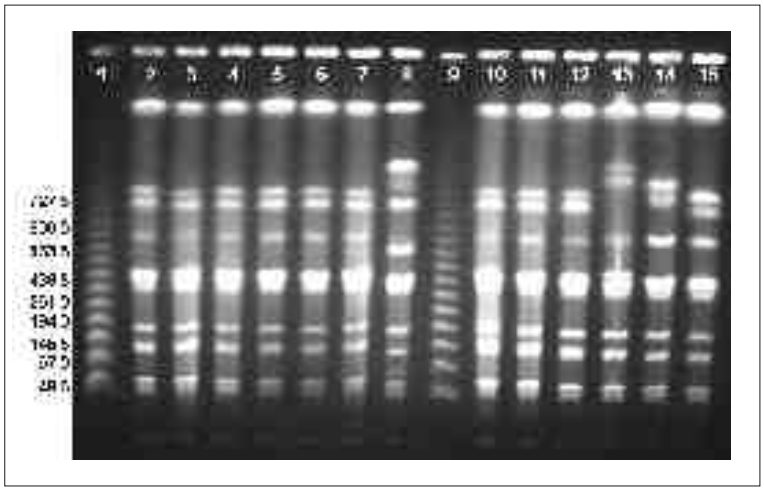

FIGURE 2 - *PFGE profiles of the SfiI-digested genomic DNAs of the AmpC-producing P. mirabilis isolates and reference strains. Lane 1 and 9: λ ladder. Lane 2, Pm (II). Lane 3, Pm 48 SMT[a]. Lane 4: Pm 50 SMT[a]. Lane 5: Pm 63 SMT. Lane 6, Pm 77 SMT. Lane 7, Pm, 78 SMT[a]. Lane 8: Pm 75 MG[b]. Lane 9, Pm 11 SMT. Lane 10, Pm 99 SMT. Lane 11, Pm 110 SMT. Lane 12, Pm 111 SMT[a]. Lane 13, Pm 76 MG[b]. Lane 14, Pm 3 MELE[c]. Lane 15, Pm 20 RED[d].*
[a]SMT: LTCRF Santa Margherita; [b]MG: LTCRF S. Maugeri; [c] MELE: LTCRF Melegnano; [d]RED: LTCRF Redaelli.

impact, not only in acute hospital settings, but also in LTCRFs. In particular acquired CBLs of the CMY-LAT lineage, which are the most common acquired CBLs, can also be encountered in different nosocomial settings in Northern Italy. Their prevalence can be underestimated, since the ESβLs screen methods available for clinical laboratories use classical β-lactamase-inhibitors ineffective on most CBLs except on ESβLs of CMY-LAT lineage.

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