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Molecular epidemiology of ESβL producing *P. mirabilis* strains from a long-term care and rehabilitation facility in Italy

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SUMMARY

We report the detection of multidrug resistant ESβL producing *Proteus mirabilis* isolates from a long-term care and rehabilitation facility (LTCRF) in Northern Italy.
53% of the collected *P. mirabilis* strains were ESβL producers. PCR and sequencing techniques confirmed the presence of the bla_{TEM-92} and bla_{CMY-16} resistance genes in 23/26 (88,5%) and 3/26 (11,5%) of the ESβL producers respectively.
PFGE showed that the TEM-92 β-lactamase producing isolates were not clonally related, indicating the presence of at least four different clonal lineages (A, B, C, D), whereas all the CMY-16 enzyme producers belonged in the same lineage. The bla_{TEM-92} and bla_{CMY-16} determinants were distributed in seven different wards, but in three of them they coexisted.
Our results show that the most patients are co-colonized by ESβLs producing *P. mirabilis* strains at the time of admission to an LTCRF. An effective strategy to curtail the spread of ESβLs mediated resistance in LTCRFs could be to activate sourveillance programs to monitor routinely the entry of resistant bacteria.

KEY WORDS: P. mirabilis, ESβL, Sourveillance

Proteus mirabilis is one of the most common gram-negative pathogens encountered in clinical specimens and can cause a variety of community- or hospital-acquired infections, including urinary tract, wound, and bloodstream infections. This organism is intrinsically resistant to nitrofurantoin and tetracycline, but it is naturally susceptible to β-lactams, aminoglycosides, fluoroquinolones, and trimethoprim-sulfamethoxazole (O'Hara *et al.*, 2000). However, drug resistance has been increasingly reported for this species, and the diffusion of resistance to oxyimino-cephalosporins due to the production of extended-spectrum β-lactamases (ESβLs) has become of great concern (Sturenburg *et al.*, 2003) Genes encoding for ESβLs are usually located in transferable plasmids and are generally mutants of the classical TEM-1/2 type β-lactamases (Bonnet *et al.*, 1999; Bradford., 2001). Moreover, co-resistance to aminoglycosides, fluoroquinolones and trimethoprim-sulfamethoxazole has frequently been reported among ESβLs-positive *P. mirabilis* strains (De Champs *et al.*, 2000; Luzzaro *et al.*, 2002; Winokur *et al.*, 2001).
The resistance to extended spectrum cephalosporins is increasing in this species,

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because of the production of ESβLs as TEM, CTX-M, PER and CBL type enzymes.

Over the last few years, ESβL producing *P. mirabilis* isolates have been recovered worldwide, with a relatively high prevalence in some settings, but the incidence and the spread of ESβL in *P. mirabilis* strains from long-term care facilities is currently unknown.

The ESβLs detected in *P. mirabilis* include several TEM-type derivatives, such as TEM-3, TEM-8, TEM-10, TEM-15, TEM-20, TEM-21, TEM-24, TEM-26, TEM-52, TEM-66, TEM-72 and TEM-92 (Bonnet *et al.*, 1999; Chanal *et al.*, 2000; De Champs *et al.*, 2001; Luzzaro *et al.*, 2002; Mariotte *et al.*, 1994; Palzkill *et al.*,1995; Perilli *et al.*, 2000; Pitout *et al.*, 1998), but also other enzymes of molecular class A such as PER-2 (Bauernfeind *et al.*, 1996) and CTX-M-2 (Bauernfeind *et al.*, 1996; Bonnet *et al.*, 2000; Tzouvelekis *et al.*, 2000).

TEM- 92 is a TEM-type ESBL recently detected in clinical isolates of *P. mirabilis* and *Providencia stuartii* from France (De Champs *et al.*, 2001). It contains the same aminoacid substitutions as TEM-52 (E104K M182T G238S) (Poyart *et al.*, 1998) plus a Q6K substitution in the signal peptide region (De Champs *et al.*, 2001). AmpC-like enzymes (molecular class C) have also been occasionally found in *P. mirabilis* isolates resistant to oxyimino-cephalosporins. (D'Andrea *et al.*, 2006). In this case, however, the resistance pattern typically differs from that due to ESβLs of class A for a decreased susceptibility to cephamycins as well, and for a poor susceptibility to β-lactamase inhibitors (Bret *et al.*, 1999; Coudron *et al.*, 2000; Verdet., 1999).

The aim of this study was to investigate the spread and the prevalence of ESβLs in *P. mirabilis* strains recovered from urinary tract of patients admitted to the geriatric wards of a LTCRF in Northern Italy and coming from acute-care hospitals of the same geographic area.

The strains analyzed in this study included 49 consecutive clinical isolates of *P. mirabilis* collected in the period November '04- June '05 from urinary samples of patients at the time of admission to S. Margherita LTCRF in Pavia (Northern Italy). Identification and susceptibility testing were performed by Vitek System (Bio-Mérieux). To confirm the production of ESβL the appropriate CLSI test was carry out according to the standard protocol (M100-S17). Analytical isoelectric focusing (IEF) of crude extracts, visualization of β-lactamase bands by nitrocefin, and detection of the activity of the β-lactamase bands by a substrate overlaying procedure, were assayed as previously reported (Pagani *et al.*, 2002).

Reference strains producing TEM-1, TEM-2, TEM-7, TEM-8, TEM-9, TEM-12, SHV-1, SHV-2, SHV-5, and MIR-1 were used as controls.

Conjugal transfer of resistance determinants was assayed in liquid medium with the *E. coli* K-12 strains J62 (*pro*$^-$, *his*$^-$, *trp*$^-$, *lac*$^-$, *Sm*^{R}) and J53-2 (met$^-$, *pro*$^-$, *rif* ^{R}) as recipients as previously described. The presence of genes bla_{TEM} and bla_{CMY} was demonstrated by PCR.

PCR amplification of bla_{TEM} alleles was carried out with primers TEM/f (5'- ATA AAA TTC TTG AAG ACG AA-3') and TEM/r (5'-ATATGAG-TAAGCTTGGTCTGACAG); the cycling conditions were as previously described (Pagani *et al.*, 2002).

PCR amplification of bla_{CMY} alleles was carried out with primers CII/f (5'- CAG GCY ATT CCG GGT ATG G-3') and CII/r (5'-GCC AGT TVA GCA TYT CCC-3') and the following cycling conditions: initial denaturation at 94°C for 5 min; denaturation at 94°C for 60 s, annealing at 50°C for 35s, and elongation at 72°C for 60 s, repeated for 35 cycles; final extension at 72°C for 10 min.

Sequencing was performed directly on PCR-generated amplicons, on both strands by the use of an automatic DNA sequencer.

PFGE patterns of genomic DNA were analyzed by the Bio-Rad Gene Path Procedure (Bio-Rad Laboratories, Richmond, Ca.). The DNA was cleaved overnight with the restriction endonuclease *Sfi*I. Clonal relationships, based on PFGE patterns, were interpreted according to the criteria proposed by Tenover *et al.* (1995).

All isolates were susceptible to piperacillin-tazobactam and carbapenems but resistant to fluoroquinolones, aminoglycosides, and trimethoprim-sulfamethoxazole. The CLSI test showed that 26/49 (53%) *P. mirabilis* strains were ESβL producers.

23/26 of the ESβL positive isolates showed, by IEF, a β-lactamase band characterized by a pI of 5.9. This band resulted especially active, in the bioassay, on cefotaxime, suggesting the production of the TEM-92 enzyme. PCR and sequencing confirmed the presence of the bla_{TEM-92} gene.

TABLE 1 - *The most important features of TEM and CMY producing* P. mirabilis *isolates.*

			CLSI Test (mm)					*MIC (μg/ml)*				
Strain	*Ward*	*Clone*	*CAZ*	*CAZ + clavulanate*	*CTX*	*CTX + clavulanate*	*FOX (mm)*	*CTX*	*CAZ*	*p.I.*	*bla TEM-92*	*bla CMY-16*
P.m. 1 SMT	Section C	A	23	38	17	38	16	>64	<8	5.9	+	-
P.m. 2 SMT	Section B	A1	22	30	18	30	19	>64	<8	5.9	+	-
P.m. 4 SMT	Geriatrics II	B	21	29	20	23	19	>64	<8	5.9	+	-
P.m. 14 SMT	Geriatrics I	C	21	31	22	28	25	<4	<8	5.9	+	-
P.m. 26 SMT	Section H	D	R	31	32	33	25	<4	<8	5.9	+	-
P.m. 33 SMT	Section A	A2	R	25	17	20	R	>64	<8	5.9	+	-
P.m. 34 SMT	Geriatrics III	A3	9	25	18	20	17	>64	<8	5.9	+	-
P.m. 48 SMT	Geriatrics III	E	15	10	10	9	13	>64	<8	8.4	-	+
P.m. 50 SMT	Geriatrics I	E	15	17	11	12	13	>64	<8	8.4.	-	+
P.m. 63 SMT	Geriatrics II	E	13	13	10	11	12	>64	>64	8.4	-	+

The results of the conjugation experiments showed that the TEM determinant was not transferable.

3/26 *P. mirabilis* strains were characterized by an uncertainly CLSI positive test. These strains resulted also resistant to cefoxitin, amoxi-clavulanate and oxyimino cephalosporin. The three isolates produced a β-lactamase characterized by pI >8.4 and able to hydrolyse cefotaxime, ceftazidime, cefepime and cefoxitin, suggesting the presence of an acquired CBL.

PCR and sequencing techniques confirmed the presence of the resistance gene $bla_{\text{CMY-16}}$ in these isolates. The three CMY-16 producing strains were from different wards of the S. Margherita LTCRF, but they were clonally related (data not shown) (Table 1).

The clonal relatedness of seven TEM-92 pro-

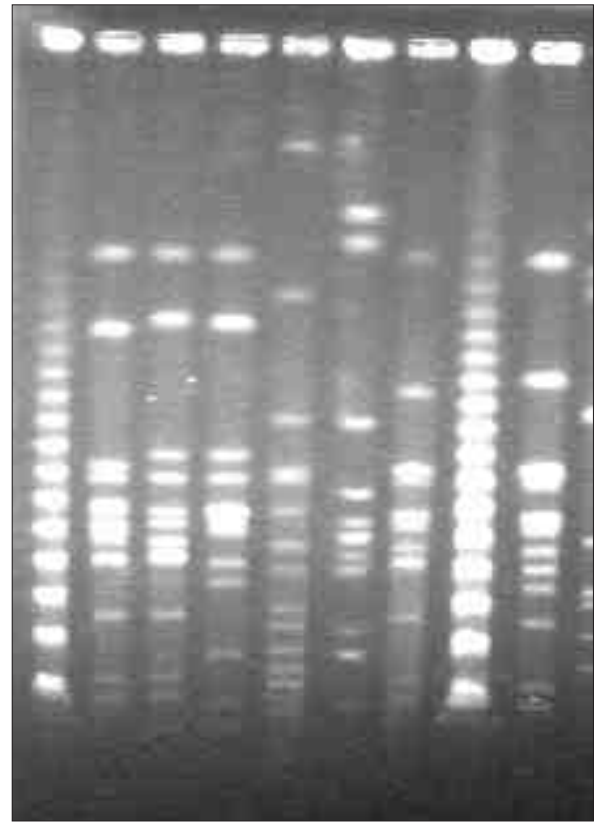

FIGURE 1 - *PFGE profiles of the* SfiI*-digested genomic DNAs of seven TEM-92 producing* P. mirabilis *isolates chosen as representative from each ward. Lane 1, 8: λladder. Lane 2: Pm 1 SMT[a]. Lane 3: Pm 2 SMT[a]. Lane 4: Pm 4 SMT[a]. Lane 5: Pm 14 SMT[a]. Lane 6: Pm 26 SMT[a]. Lane 7: Pm 33 SMT[a]. Lane 9: Pm 34 SMT[a].* [a]*SMT:* Santa Margherita *LTCRF.*

ducing isolates, one from each ward, chosen as representative were obtained by comparing PFGE genomic profiles after digestion with the *Sfi*I enzyme (Figure 1). The results indicate the presence of at least four different clonal lineages (A, B, C, D).

Genotyping results emphasize that the clone A was present within the S. Margherita LTCRF in 4 different wards: section A, section B, section C and geriatric III (Table 1). Interestingly all the above strains were from incoming patients from the same hospital. Lastly, the CMY-16 and TEM-92 resistant determinants coexisted in the same wards.

These data show that infections caused by multi drug-resistant bacteria among older adults in long- term care and rehabilitation facilities are a growing concern; the transfer of colonized/infected patients from acute-care facilities to LTCRF is probably the primary way of introducing resistant pathogens to this environment. Moreover, the results of this study revealed a high prevalence of TEM-92 producing strains among the *P. mirabilis* collected from colonized/infected patients at the time of admission to S. Margherita LTCRF in Pavia. This resistance determinant can spread vertically and rapidly within a LTCRF.

Rigorous preventive control measures should be applied to detect *reservoirs* of ESβL producing strains as soon as possible and to contrast their diffusion. An effective strategy to curtail the spread of ESβL mediated resistance in LTCRFs is to monitor the entry of resistant bacteria.

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