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*Tn*21-Like Elements Disseminating Carbapenemase Genes in Italy: Report from the SENTRY Antimicrobial Surveillance Programme

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AMENDED ABSTRACT

Objective: To analyse the genetic context of metallo-ß-lactamase (MßL) containing integrons in *Pseudomonas aeruginosa* strains from geographically distinct regions of Italy.
Methods: 25 MßL expressing strains were collected from three geographically distinct hospitals in Italy. These were SENTRY sites #75 (Genoa-Northern Italy), #86 (Rome-central Italy) and #85 (Catania-Sicily). These strains were previously characterized with respect to their PFGE profile. The structure of the MßL containing integrons and the genetic context of these integrons was further analysed by a combination of PCR based approaches. These included (1) PCR amplification with primers anchored to 5' and 3' conserved sequences (CS). (2) PCR amplification using a combination of primers designed to 5' and 3' CS and the particular MßL gene. (3) PCR amplification using primers designed against *Tn*5051 *tnp*R and *tnp*A sequences. (4) Primers designed to amplify the insertion site of the integron. (5) Novel biotinylated primer based PCR to identify unknown sequences adjacent to known sequences. (6) Primers specific for the insertion element ISPa7 and insertion site of the ISPa7 element.
Results: All SENTRY site isolates contained class 1 integrons with a MßL gene in the first gene cassette position. Many of the strains also contained multiple class 1 integrons. All SENTRY site #75 isolates contained bla_{VIM-1} containing integrons harboured adjacent to a *tnp*R gene displaying 100% identity to the *tnp*R gene of the *Tn*21 like transposon *Tn*5051 previously identified harbouring bla_{IMP-13} in Italy and bla_{VIM-2} in Poland but lacked a *tnp*A gene. SENTRY site #86 isolates were of two different types. Type (A) consisted of a bla_{IMP-13} containing integron harboured by a complete *Tn*5051-type transposon and type (B) a bla_{VIM-1} containing integron which appeared to not be directly linked to a *Tn*5051 transposon even though the strain harbouring the integron contained a complete *tnp*R gene. This integron was also characterized by having the insertion sequence ISPa7 inserted downstream of the integrase gene as has been found recently in several other Italian MßL harbouring isolates (Genbank ID AJ50512, AJ5581665, Y18050). SENTRY site #85 isolates also contained two different types: Type (C) contained a bla_{VIM-1} integron identical to type (B). Type (D) also contained a bla_{VIM-1} integron but its integrase gene is truncated and its genetic context is yet to be confirmed. All isolates were also screened with primers specific for ISPa7 and primers anchored to ISPa7 and the integrase gene. Identical PCR products were amplified from all 25 MßL containing isolates.
Conclusions: All MßL containing isolates in this study contained a *tnp*R gene identical to *tnp*R of *Tn*5051 and several of the MßL containing integrons were harboured adjacent to this gene indicating that the *Tn*21-like transposon *Tn*5051 may be or may have been instrumental in their mobility. All strains also harboured the insertion element ISPa7 and this element was found in an identical position in all MßL containing isolates indicating that the integron that contains it is very widely dispersed throughout Italy in many different strains of *P. aeruginosa*. Many of the MßL containing isolates harboured multiple integrons generating enormous potential for transfer and dissemination of resistance genes.

INTRODUCTION

Pseudomonas aeruginosa is a leading cause of nosocomial acquired infections and intrinsically resistant to many antimicrobial agents possessing serious problems for therapeutic regimes. Carbapenems, such as imipenem and meropenem, are potent agents for the treatment of *P. aeruginosa* infections. Predictably, increasing usage of these compounds has resulted in the development of carbapenem-resistant *P. aeruginosa*. Low-level carbapenem resistance is often mediated by outer-membrane mutations in conjunction with the increased expression of one or more efflux pumps. High-level resistance to carbapenems (>32 mg/L) is still uncommon in *P. aeruginosa* and is usually indicative of metallo-ß-lactamase (MßL) production.

Four sub-classes of clinically relevant MßLs have been described: the IMP family, VIM family, SPM-1 and GIM-1. Whilst SPM-1 and GIM-1 appear to be restricted to Brazil and Germany, respectively, VIM-type MßLs are more widely disseminated and have been reported from North America, South America, Europe and SE Asia.

In this study, we characterized a number of MßL genes embedded in novel integrons from geographically different regions within Italy. We also present data on the nature of the transposition locus carrying the class 1 integrons. The carbapenem-resistant *P. aeruginosa* clinical isolates from Italy were submitted to the SENTRY Antimicrobial Surveillance Program in 1999-2001.

METHODS

Bacterial Strains. Among other selected pathogens, *P. aeruginosa* strains resistant to imipenem (MIC, ≥ 16 mg/L), meropenem (MIC, ≥ 16 mg/L), and ceftazidime (MIC, ≥ 32 mg/L) have been routinely screened for MßL genes as part of the SENTRY Program. Twenty-five isolates from medical centers located in Genoa (site 75), Rome (site 85) and Catania (site 86) met this criteria and were further evaluated in the present study. The main characteristics of these isolates are listed in Table 1.

PCR screening for integrons, bla_{VIM}/bla_{IMP} MßL genes *Tn*5051 and ISPa7 sequences. Primers were designed using the computer programme Primer designer version 1.01 (Scientific and Educational Software). PCR was performed as described previously PCR products were visualized by electrophoresis on 0.8% agarose gels in Tris Boric Acid/EDTA buffer (pH 7.0) and staining with 1% ethidium bromide. Primer positions are indicated in Figure 1.

DNA sequencing and sequence analysis. Sequencing was carried out on both DNA strands by the dideoxy-chain termination method with a Perkin Elmer Biosystems 377 DNA sequencer (Advanced Biotechnology Center, Imperial College London). Sequence analysis was performed using the Lasergene DNASTAR software package.

RESULTS

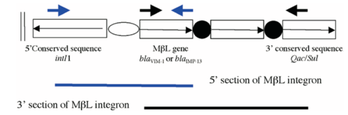
- PCR amplification with primers designed to the 5' and 3' conserved sequences of Class 1 integrons produce multiple products in many of the isolates evaluated (Figure 2).
- The integron structure was determined by amplification of the 5' and 3' sections of each integron separately, using PCR with primers anchored to the 5' integrase gene and the respective MßL gene, and the 3' Qac sequence and the MßL gene. The products were then sequenced directly and the two sequences joined to produce the full length integrons (Figure 3).
- PCR Amplification with *tnp*A and *tnp*R specific primers demonstrates that *Tn*5051 transposon sequences are widespread in Italian *P. aeruginosa* strains (Figure 4).
- Sequence analysis of insertion site of bla_{VIM-1} containing integron from Genoa and bla_{IMP-13} containing integron from Rome reveal that the integrons are inserted at different sites in the *tnp*M gene of *Tn*5051 separated by approximately 200bp (Figure 5).
- Investigation of the region upstream of the integrase gene in bla_{VIM-1} containing integrons of SENTRY Program isolates 86-10088, 85-2394 and 85-14297 reveals the presence of the insertion element ISPa7 (Figure 6).
- Screening of all MßL strains with ISPa7 specific primers and a combination of ISPa7 and integrase specific primers identified ISPa7 in all strains at the identical position (Figure 7).

Table 1. List of evaluated isolates by site of origin.

Hospital (no.)	Ribotype	VIM-type	IMP-type	ISPa7	*Tn*5051 *tnp*A	*Tn*5051 *tnp*R
75 (16)	1034-2	VIM-1	-	+	-	+
85 (2)	1034-2	VIM-1	-	+	-	+
(1)	45-6	VIM-1	-	+	-	+
(1)	60-2	VIM-1	-	+	-	+
86 (2)	1033-3	-	IMP-13	+	+	+
(1)	115-5	-	IMP-13	+	+	+
(1)	NTO	-	IMP-13	+	+	+
(1)	134-6	VIM-1	-	+	-	+

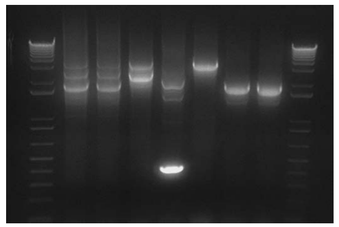
RESULTS

Figure 1: Positions of primers used to screen for integrons.



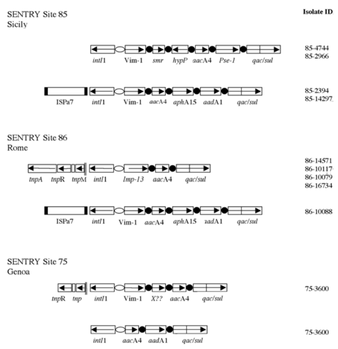
Legend: Bold arrows in the above figure denote PCR primer sites, open boxes genes with arrows inside them denoting the direction of transcription, filled circles 59 base elements.

Figure 2: Agarose gel of PCR products from *P. aeruginosa* MßL containing strains amplified with primers designed against Class 1 integron 5' and 3' conserved sequences.



Legend: Lanes 1&9 1kb plus DNA ladder, Lane 2&3 PCR products produced using template DNA of isolates 75-3600 and 75-3634 respectively. Lanes 4-8 PCR products produced using DNA templates of strains 85-4744, 85-2394, 86-10088, 86-10117 and 86-10079 respectively.

Figure 3: Schematic of integrons sequenced from Italian *P. aeruginosa* isolates harbouring MßL genes.



Legend: Open boxes represent the various genes in or associated with the various integrons and the arrows denote the direction of their transcription. Filled circles represent the 59 base elements associated with the resistance genes and open ellipses the *attI*1 site of each integron. Filled rectangles indicate the inverted repeats at the end of ISPa7 insertion sequences.

Figure 4: Diagram of PCR strategy to detect *Tn*5051 sequences in MßL containing strains.

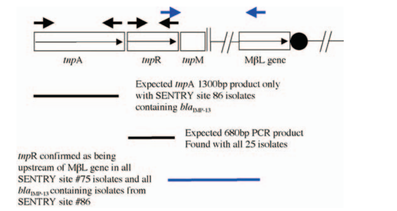


Figure 5: Schematic of insertion sites of Italian integrons into the *tnp*M gene of *Tn*5051.

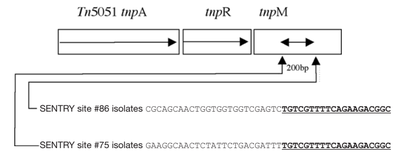
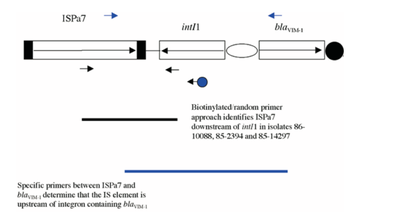
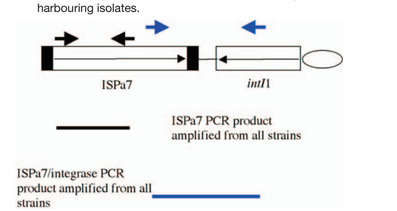


Figure 6: Schematic of PCR approach taken to identify the unknown sequence flanking the integrase gene.



Legend: Bold arrows denote primer binding sites and the blue filled circle represents the biotinylated primer.

Figure 7: Schematic of PCR approach and results that demonstrate that the insertion sequence ISPa7 is found in an identical position in all MßL harbouring isolates.



CONCLUSIONS

- Many Italian MßL-harbouring *P. aeruginosa* strains evaluated contained multiple integrons.
- The MßL containing integrons were amplified and sequenced and consisted of a diverse range of resistance gene cassettes. All isolates collected from Genoa (site 75) harboured an identical bla_{VIM-1} containing integron which is indicative of clonal dissemination within this medical center.
- We observed a great diversity among MßL harbouring integrons from isolates collected in different SENTRY Program sites except for an identical bla_{VIM-1} containing integron found in SENTRY sites 85 and 86 (Figure 3). This integron is also identical to previously sequenced bla_{VIM-1} harbouring integrons (e.g. Genbank AJ581664 Riccio *et al)*. This finding demonstrates that this particular integron has spread throughout Italy.
- The *tnp*R gene of *Tn*5051 was identified in all MßL containing isolates. However, only the bla_{IMP-13} containing isolates had a complete transposition locus. This finding indicates that *Tn*5051 may have been instrumental in their mobility.
- All strains also harboured the insertion element ISPa7 and this element was found in an identical position in all MßL containing isolates indicating that the integron that contains it is very widely dispersed throughout Italy in many different strains of *P. aeruginosa* and may also be involved in the mobility of the integron.
- The finding of numerous integrons and resistance gene cassettes in these isolates is a great cause for concern since these integrons provide a reservoir of resistance alleles facilitating their dissemination to other Gram-negative pathogens.

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