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Dissemination of *bla*$_{VIM-1}$ Metallo-ß-lactamase Gene in Greece and France via the Transposon *Tn*21: Report from the SENTRY Surveillance Program

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AMENDED ABSTRACT

Background: Greece has a major problem with carbapenem resistant strains of *Pseudomonas aeruginosa* and *Acinetobacter baumannii* due to the widespread occurrence of strains harbouring the metallo-ß-lactamase (MßL) genes *bla*$_{VIM-1}$ and *bla*$_{VIM-2}$ on Class 1 integrons. However, little is known about the genetic mechanisms responsible for the spread of these alleles. Therefore, we have examined the genetic context of the integrons containing the MßL gene *bla*$_{VIM-1}$ in the Greek isolates *A. baumanii* 62-2633 and *P. aeruginosa* 62-5149 collected via the SENTRY program.
Methods: Adjacent sequences flanking the Class 1 integron were amplified by PCR using a novel degenerate primer approach. This consisted of PCR with nested primers anchored to the 5' end of the Class 1 integron and degenerate primers designed to randomly hybridize to upstream sequences. Sequencing was performed on both strands by the dideoxy-chain termination method and analyzed using DNAstar.
Results: Analysis of the sequence immediately upstream of the Class 1 integron in isolate 62-2633 revealed the *tnp*M and *tnp*R genes of transposon *Tn*21 (100% identity), the sequence was also identical to the sequence immediately flanking the VIM-2 containing integron in isolate 301-5433 collected in France. In addition to this the insertion site of the Class 1 integron harbouring the *bla*$_{VIM-1}$ MßL gene is identical to the insertion site of the Class 1 integron in the transposon *Tn*21. This insertion point does not truncate the transposon modulator gene *tnp*M. The sequence flanking the insertion site of the VIM-1 isolate in strain 62-5149 also isolated in Greece at the same SENTRY site was 100% identical to the *Tn*5051 transposon disseminating VIM-2 in Poland and IMP-13 in Italy. In these cases the *tnp*M gene is truncated with the insertions of the integrons in the *Tn*21-like transposon *Tn*5051 harbouring *bla*$_{VIM-2}$ in Poland and *bla*$_{IMP-13}$ in Italy.
Conclusions: The highly mobile transposon *Tn*21 is associated with SENTRY *A. baumanii* strain 62-2633 isolated from a Greek hospital. In comparison with the transposon *Tn*5051 associated with *bla*$_{VIM-1}$ in Greece, *bla*$_{VIM-2}$ in Poland and *bla*$_{IMP-13}$ in Italy, the transposon *Tn*21 associated with the *A. baumannii* strain of Greek origin is likely to be much more mobile. This is because the *tnp*M gene is not damaged in this isolate. The product of the *tnp*M gene is a transposition modulator, the expression of which increases transposition efficiency.

INTRODUCTION

Since the early 1990s when the metallo-ß-lactamase (MßL) IMP-1 was first described in Japan, new MßL genes have been reported all over the world in clinically important pathogens, such as *Pseudomonas* spp., *Acinetobacter* spp. and members of the *Enterobacteriacea* family. Three further sub-classes of clinically relevant MßLs have subsequently been described: the 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 appear to be widely disseminated and have been reported from North America, South America, Europe and Southeast Asia.

Recent studies in Greece have highlighted the fact that MßLs are widespread in that nation. MßL-producing isolates collected from Greece via the SENTRY Antimicrobial Surveillance Program were further studied to determine genetic structures that may be responsible for their spread.

METHODS

Bacterial Strains. A diversity of clinical isolates collected via the SENTRY Program were initially screened for presence of MßLs and subsequently for their general genetic loci. Among other selected pathogens, *P. aeruginosa* and *Acinetobacter* spp. strains resistant to imipenem (MIC, ≥ 16 µg/ml), meropenem (MIC, ≥ 16 µg/ml), and ceftazidime (MIC, ≥ 32 µg/ml) have been routinely screened for MßL genes. *P. aeruginosa* strain 62-5149 *and Acinetobacter baumannii* strain 62-2633 from Greece and *P. aeruginosa* strain 301-5433 (France) were the subjects of this study.

PCR screening for integrons and *bla*$_{VIM}$/*bla*$_{IMP}$ MßL genes. Primers were designed using the software 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.

DNA sequencing and sequence analysis. Gene 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.

Random flanking primer 2 step PCR. DNA sequences adjacent to the insertion site of Class 1 integrons in bacterial strains were amplified by a random primer PCR approach based on that of Sorenson, et al. (Figure 1). This consisted of two nested primers designed within the Class 1 integrase sequence approximately 400bp and 300bp downstream from the IRi inverted repeat of the Class 1 integron, respectively. The primer at position 400bp was biotinylated. Four different random primers were then designed with a novel sequence tag of 24bp followed by seven randomly assigned bases, four bases of two G and C residues in different combinations and a final T residue. Step 1 included 4 PCR reactions with the biotinylated primer at position 400bp and one of each of the random primers. The products of the PCR reaction were then incubated with Dynal streptavidin linked beads, washed and then denatured with 0.1M NaOH. The beads were then separated using a magnetic rack and the supernatant neutralised with 0.2M HCl and diluted prior to use as a template in the second step PCR. Step 2 consisted of amplification using a primer complimentary to the sequence tag of the random primer and anchored within the integron sequence with the nested primer at position 300bp. Individual products from step 2 PCR were isolated from gels after electrophoresis and sequenced with the primer at position 300bp.

RESULTS

- Integrons containing *bla*$_{VIM-1}$ from *A. baumannii* 62-2633 isolated in Greece and *bla*$_{VIM-2}$ from *P. aeruginosa* 301-5433 isolated in France were harboured by the transposon *Tn*21 (Figure 2A and 2B).
- The integron containing *bla*$_{VIM-1}$ in *P. aeruginosa* 62-5149 was located on the transposon *Tn*5051 (Figure 2C).
- Integrons harboured by *Tn*21 are inserted in an identical site, which is located immediately before the start codon of the *Tn*21 *tnp*M gene (Figure 3).
- The integron harboured by *Tn*5051 transposon in *P. aeruginosa* 62-5149 is inserted within the *tnp*M gene effectively truncating the gene (Figure 3).
- The *Tn*5051 transposon with the *bla*$_{VIM-1}$ containing integron in *P. aeruginosa* 62-5149 appears to be identical to the *Tn*5051 transposon responsible for dissemination of *bla*$_{VIM-2}$ in Poland and *bla*$_{IMP-13}$ in Italy (Figure 4).

Figure 1. Strategy for amplification of unknown DNA flanking the insertion site of Class 1 integrons-see methods.

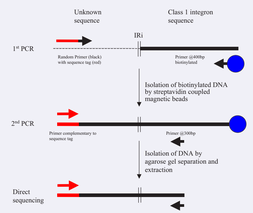

Figure 2A. Results of a FASTA search of Genbank for sequences displaying highest identity to the insertion site of the integron harbouring the *bla*$_{VIM-1}$ MßL gene recently identified in *Pseudomonas aeruginosa* strain 62-2633 from Greece. The IRi of the *bla*$_{VIM-1}$ and *Tn*21 integrons are highlighted in bold. Note that insertion point is identical.

```
                    10        20        30
Sequen      TGCAGCGTCTTCTGAAAACGACAATGGAGGTGGTAGCCGAGGGTGTGGAA
            ::::::::::::::::::::::::::::::::::::::::::::::::::
EM_PRO GACGTTCAATGCAGCGTCTTCTGAAAACGACAATGGAGGTGGTAGCCGAGGGTGTGGAA
            10        20        30        40        50        60

      40        50        60        70        80        90
Sequen ACACCCGACTGCCTTGCGTGGTTGCGGCAGGCGGGTTGCGACACGGTGCAGGGTTCCTG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_PRO ACACCCGACTGCCTTGCGTGGTTGCGGCAGGCGGGTTGCGACACGGTGCAGGGTTCCTG
             70        80        90       100       110       120

     100       110       120       130       140       150
Sequen TTCGCCAGGCCGATGCCGGCGGCGCCTTCGTCGGCTTCGTCAACCATGGAGGAACACC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_PRO TTCGCCAGGCCGATGCCGGCGGCGCCTTCGTCGGCTTCGTCAACCATGGAGGAACACC
            130       140       150       160       170       180

     160       170       180       190       200       210
Sequen ACCATGAACGCCAATGAACCGAGCACCAGTTGCTTCGTGCTTCGCTGCTTGTA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_PRO ACCATGAACGCCAATGAACCGAGCACCAGTTGCTTCGTGCTTCGCTGCTTGTA
            190       200       210       220       230       240

EM_PRO GATGCCGCTTCACGCCGGAAGGGCCGAGTACGTGGAGCATTTCTGCGGCTGGAGTGC
            250       260       270       280       290       300
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Figure 2B. Results of a FASTA search of Genbank for sequences displaying highest identity to the insertion site of the integron harbouring the *bla*$_{VIM-2}$ MßL gene recently identified in *Pseudomonas aeruginosa* strain 301-5433 from France. The IRi of the *bla*$_{VIM-2}$ and *Tn*21 integrons are highlighted in bold. Note that insertion point is identical both to the resident integron of *Tn*21 and the *bla*$_{VIM-2}$ isolate from Greece.

```
Sequen      TGCAGCGTCTTCTGAAAACGACAATGGAGGTGGTAGCCGAGGGTGTGGAA
            ::::::::::::::::::::::::::::::::::::::::::::::::::
EM_PRO GACGTTCAATGCAGCGTCTTCTGAAAACGACAATGGAGGTGGTAGCCGAGGGTGTGGAA
            10        20        30        40        50        60

      40        50        60        70        80        90
Sequen ACACCCGACTGCCTTGCGTGGTTGCGGCAGGCGGGTTGCGACACGGTGCAGGGTTCCTG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_PRO ACACCCGACTGCCTTGCGTGGTTGCGGCAGGCGGGTTGCGACACGGTGCAGGGTTCCTG
             70        80        90       100       110       120

     100       110       120       130       140       150
Sequen TTCGCCAGGCCGATGCCGGCGGCGCCTTCGTCGGCTTCGTCAACCATGGAGGAACACC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_PRO TTCGCCAGGCCGATGCCGGCGGCGCCTTCGTCGGCTTCGTCAACCATGGAGGAACACC
            130       140       150       160       170       180

     160       170       180       190       200       210
Sequen ACCATGAACGCCAATGAACCGAGCACCAGTTGCTTCGTGCTTCGCTGCTTGTAA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_PRO ACCATGAACGCCAATGAACCGAGCACCAGTTGCTGCGTGTGCTGCAGGAAATCCCGCTC
            190       200       210       220       230       240

EM_PRO GATGCCGCTTCACGCCGGAAGGGCCGAGTACGTGGAGCATTTCTGCGGCTGGAGTGC
            250       260       270       280       290       300
```

Figure 2C. Results of a FASTA search of Genbank for sequences displaying highest identity to the insertion site of the integron harbouring the *bla*$_{VIM-1}$ MßL gene recently identified in *Pseudomonas aeruginosa* strain 62-5149 from Greece. The alignment shows perfect identity to the *Tn*5051 transposon responsible for the spread of *bla*$_{IMP-13}$ in Italy and *bla*$_{VIM-2}$ in Poland and also has an identical insertion site in the *tnp*R gene of *Tn*5051.

```
                   270       260       250       240
Sequen-                CAAGCAGGCAGCACGAAGCGGCGCCTTCGTCGACTCAA
                       :::::::::::::::::::::::::::::::::::::::
EM_PRO TCAATCAATTCGGAAGGCAACTCTATTCTGACGATTTAGCCCGGATGTCTGACCCAA
            620       630       640       650       660       670

     230       220       210       200       190       180
Sequen- GTTAGGGTATCCTCAATCTGACGGTAGCGAATCCGAGCGTTCGGATCGGCATCGGTTT
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_PRO GTTAGGGTATCCTCAATCTGACGGTAGCGAGTCGCAGGCGTTCGGATCGGCATCGGTTT
            680       690       700       710       720       730

     170       160       150       140       130       120
Sequen- CGTTCCCTGTCTTGGCAGCGCTCGAAGCGTTGATAACACTCCAACCGCAGAATGCT
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_PRO CGTTCCCTGTCTTGGCAGCGCTCGAAGCGTTGATAACACTCCAACCGCAGAATGCT
            740       750       760       770       780       790

     110       100        90        80        70        60
Sequen- CGACGTATTCCGCGCCTCCGGGGTGAAGCGGCATCGACGCGGGATTTCCTTCGAGCACA
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_PRO CGACGTATTCCGCGCCTCCGGGGTGAAGCGGCATCGACGCGGGATTTCCTTCGAGCACA
            800       810       820       830       840       850

      50        40        30        20        10
Sequen- CGCAGCAACTGGTGGTGGTCGAGTCTGTCGTTTCAGAAGACGGCTGCA
        ::::::::::::::::::::::::::::::::::::::::::::::::
EM_PRO CGCAGCAACTGGTGGTGGTCGAGTCTGTCGTTTCAGAAGACGGCTGCACTGAACGTCAG
            860       870       880       890       900       910
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Figure 3. Schematic of the genetic loci of *bla*$_{VIM}$ gene cassettes in isolates 62-2633 & 62-5149 from Greece and 301-5433 from France. Double vertical lines indicate the positions of the inverted repeats at the 5' ends of the transposons and integrons. Open boxes indicate the positions of the various genes with arrows indicating the direction of their transcription. Open ellipses represent the recombination target site *attI1* and filled circles indicate the 59 base elements of the MßL gene cassettes. The loci of isolates 62-2633 and 301-5433 indicate that the integrons harbouring the MßL genes *bla*$_{VIM-2}$ and *bla*$_{VIM-2}$ are inserted immediately before the start codon of the *tnp*M gene of *Tn*21 in an identical position to the resident integron of *Tn*21. The *tnp*M gene is therefore not truncated and should be fully functional. However, the *tnp*M gene of *Tn*5051 is truncated by the insertion of the *bla*$_{VIM-2}$ integron in isolate 62-5149 and is therefore non-functional.

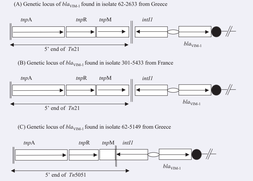

Figure 4. Alignment of insertion points of metallo-ß-lactamase containing integrons in the *tnp*M gene of transposon *Tn*5051.

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bla VIM-1 Greece  AGCACACGCAGCAACTGGTGGTGGTCGAGTCTGTCGTTTCAGAAGACGGCTGCAC
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||
bla IMP-13        AGCACACGCAGCAACTGGTGGTGGTCGAGTCTGTCGTTTCAGAAGACGGCTGCAC
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||
IN VIM-2 Poland   AGCACACGCAGCAACTGGTGGTGGTCGAGTCTGTCGTTTCAGAAGACGGCTGCAC
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||
IN 60 France      AGCACACGCAGCAACTG-TGGTGGTCGAGTCTGTCGTTTCAGAAGACGGCTGCAC
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CONCLUSIONS

- The highly mobile transposon *Tn*21 is associated with both the MßL-producing *A. baumannii* strain 62-2633 isolated from a Greek hospital and *P. aeruginosa* strain 301-5433 isolated in France.
- The transposon *Tn*5051 previously found to be responsible for the dissemination of *bla*$_{VIM-2}$ in Poland and *bla*$_{IMP-13}$ in Italy is found to harbour *bla*$_{VIM-1}$ in strain *P. aeruginosa* 62-5149 (Greece).
- The *Tn*21 transposons associated with *P. aeruginosa* 62-2633 and 301-5433 are likely to be more mobile than the MßL alleles mobilised by *Tn*5051. This is because the transposon modulator gene *tnp*M is truncated, whereas the *tnp*M gene associated with *Tn*21 is not. The *tnp*M gene encodes a protein that increases transposition frequency.

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