P1724

*Tn*501-Like Transposons Carrying Novel *bla*$_{VIM-4}$ Metallo-ß-Lactamase (MßL) Gene Cassettes in Poland and *bla*$_{GIM-1}$ in Germany: Report from the SENTRY Antimicrobial Surveillance Programme

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AMENDED ABSTRACT

Objective: Analysis of the genetic context of MßL gene cassette containing integrons in carbapenem-resistant *Pseudomonas aeruginosa* strains isolated from Poland and Germany.
Methods: Carbapenem-resistant strains were analyzed by ribotyping and pulsed-field gel electrophoresis. The MßL containing integrons from these strains were amplified using primers designed to Class 1 integron specific 5' and 3' conserved sequences (CS). Upstream sequences were amplified by PCR by a novel degenerate primer approach using primers anchored to the 5' CS sequences and degenerate primers designed to randomly hybridize to upstream sequences. Sequencing was performed on both strands by the dideoxy-chain termination method.
Results: All eleven Polish isolates contained an identical Class 1 integron with a novel VIM-4 cassette which included a 5' direct repeat of 169bp of the 3' portion of the *bla*$_{VIM-4}$ gene. The eleven strains represented four different PFGE types. In all of these isolates the Class 1 integron was inserted into the *tnp*A gene of a *Tn*501 type transposon, the *tnp*A gene having 98% identity to the *tnp*A gene of *Tn*501. The German isolates were all of an identical ribotype and contained a Class 1 integron harbouring the GIM-1 MßL. Interestingly, this Class 1 integron was also inserted into the *tnp*A gene of a *Tn*501-like transposon (93% identity) but at a different site.
Conclusions: The Polish integrons harbour an unusual *bla*$_{VIM-4}$ gene cassette that has a 3' duplication which can be explained by a mechanism involving deletion of a segment of an ancestral tandem repeat of *bla*$_{VIM-4}$ via slipped strand replication, mediated by a combination of polymerase and integrase. Interestingly both the Polish and German MßL producing isolates contain Class 1 integrons that are harboured by *Tn*501-like transposons. In all cases the integrons are inserted into the *tnp*A gene of the transposon. This is the first time that MßL gene cassettes have been associated with *Tn*501-like transposons and this observation adds another level of mobility to these gene cassettes.

INTRODUCTION

Pseudomonas aeruginosa is characterized by inherent resistances to a wide variety of antimicrobials. Its resistance to anti-pseudomonal ß-lactams, advanced generation cephalosporins, monobactams and carbapenems is also an increasing clinical problem. Mechanisms of low-level resistance to carbapenems (MIC, 8-32 mg/L) in *P. aeruginosa* are associated with reduced uptake as a result of loss of the OprD porin combined with de-repression of the chromosomal *amp*C ß-lactamase gene or by over-expression of an efflux pump system. High-level resistance to carbapenems (MIC, >32 mg/L) is still uncommon in *P. aeruginosa,* but can be caused by the presence of class B ß-lactamases, the metallo-ß-lactamases (MßL).

Since their initial discovery in *P. aeruginosa* strains from Japan in the early 1990s, MßL genes have been found in many parts of the world and almost exclusively as gene cassettes in Class 1 integrons. Four families of acquired MßLs have been reported, including IMP, VIM, SPM, and the very recently identified GIM. However, little information is available concerning the genetic context of these integrons, or the mobile genetic elements that are responsible for their dissemination. Recently we have characterized a novel *bla*$_{VIM-4}$ MßL gene cassette from clinical strains isolated from hospitalized children in Poland. This novel gene cassette was harboured on a Class 1 integron and found in 11 strains of *P. aeruginosa* with various PFGE patterns. We have also very recently characterized the newly identified GIM-1 MßL gene cassette and its genetic environment (see poster P1722).

This study used a random primer PCR approach to identify DNA loci surrounding the insertion site of the integrons carrying the *bla*$_{VIM-4}$ and *bla*$_{GIM-1}$ gene cassettes.

METHODS

Bacterial strains. *P.aeruginosa* strains: 474/98, 101/99, 419/99, 161/00, 226/00, 301/00, 404/00, 170/01, 342/01, 486/01 and 597/01 were clinical isolates collected in 1998-2001 from children hospitalized in Warsaw, Poland and strains 73-12198, 73-15480, 73-15553, 73-15574 and 73-5671 were clinical isolates from the University Hospital Dusseldorf, Germany isolated in 2002. All isolates were characterized as MßL-producers by using the Etest MßL strips and typed by pulsed-field gel electrophoresis (PFGE).

PCR screening for integrons and *bla*$_{VIM}$/*bla*$_{IMP}$ MßL genes. Primers used for amplification of *bla*$_{VIM}$/*bla*$_{IMP}$ genes and Class 1 integron were VimF/R, and QACR and VAF, respectively and they 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.

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). Primers used to sequence the variable region of the Class1 integron are listed in Table 2. 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 404/00 and 73-5671 containing *bla*$_{VIM-4}$ and *bla*$_{GIM-1}$ respectively were amplified by a random primer PCR approach (Figure 1). This consisted of two nested primers designed within the Class 1 integron sequence approximately 200bp and 100bp downstream from the IRi inverted repeat of the Class 1 integron. The primer at position 200bp 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.

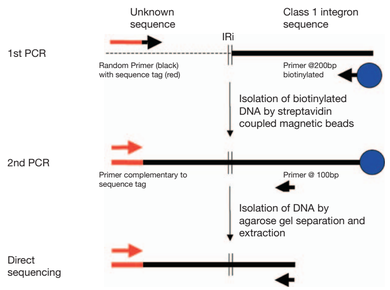
Step 1 included 4 PCR reactions with the biotinylated primer at position 200bp and one of each of the random primers. The products of the PCR reaction were then incubated with Dynal streptavidin linked beads, washed and then denatured with 0.1M NaOH. The beads were then separated using a magnetic rack and the supernatant neutralised with 0.2M HCL and diluted prior to use as a template in the second step PCR.

Step 2 PCR 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 100bp. Individual products from Step 2 PCR were isolated from gels after electrophoresis and sequenced with the primer at position 100bp.

RESULTS

- Four PFGE patterns were identified among the 11 *P. aeruginosa* isolates from Poland (Figure 3). All the isolates from Germany had an identical PFGE pattern.
- All 11 Polish isolates contain an identical Class 1 integron with a novel *bla*$_{VIM-4}$ cassette (Figure 2).
- Both *bla*$_{VIM-4}$ and *bla*$_{GIM-1}$ containing integrons are inserted into the *tnp*A gene of a transposon *Tn*501-like element (Figures 4 and 5).
- The insertion sites of the Polish *bla*$_{VIM-4}$ and the *bla*$_{GIM-1}$ containing integrons are at positions 1553bp and 31bp of the *tnp*A gene respectively (Figure 5).

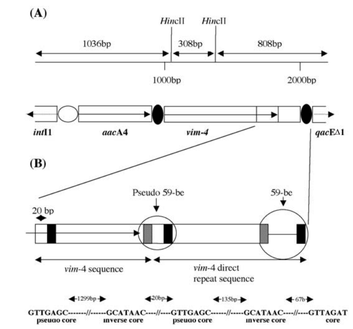
Figure 1: Strategy for amplification of unknown DNA flanking the insertion site of Class 1 integrons-see methods.



METHODS (Continued)

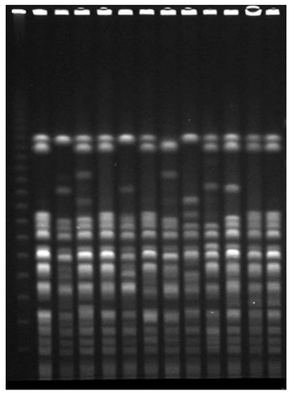
RESULTS

Figure 2: Schematic of the *bla*$_{VIM-4}$ containing integron amplified with the primers VAF and QACR.



Legend to Figure 2
(A) The amplicon is 2148bp long and contains partial sequence of the class 1 conserved sequences *int*I1 and *qac*EΔ1 at the 5' and 3' ends of the integron respectively, together with the two gene cassettes found in this integron. The position of *Hin*cII restriction enzyme sites and the expected sizes of fragments that would be generated by a *Hin*cII digest of this amplicon are shown above. Open boxes represent the positions of the various genes and the arrows show the direction of their transcription. The *att*I1 site of this integron is represented by an open ellipse and the 59-be of the gene cassettes denoted by filled ellipses. The position of the 169 bp direct repeat of the 3' end of *bla*$_{VIM-4}$ is shown by an empty box at the 3' end of the *bla*$_{VIM-4}$ gene.
(B) Represents an enlarged diagram of the *bla*$_{VIM-4}$ gene and its putative 59 base elements. Filled rectangles represent the pseudo-core sequence found within the *bla*$_{VIM-4}$ structural gene, the pseudo-core sequence of the 3' *bla*$_{VIM-4}$ repeat and the conserved core site of the 59-be. Checked rectangles represent inverse core sequences and the circled regions represent possible 59base elements. The sequences of the pseudo-core sites, the core site and the inverse core sites are given below.

Figure 3: PFGE profiles of *P. aeruginosa* isolates producing metallo-ß-lactamases obtained after *Spe*I digestion.



Legend to Figure 3.
PFGE profiles of DNA from *P. aeruginosa* isolates producing metallo-ß-lactamases following *Spe*I digestion. Lane 1 is the Llambda ladder PFG molecular marker, Lanes 2- 13 left to right are *Pseudomonas aeruginosa* strains 474/98, PFGE type A, 419/99 PFGE type B, 101/99 PFGE type C, 161/00 PFGE type A, 226/00 PFGE type B, 301/00 PFGE type A, 404/00 PFGE type D, 170/01 PFGE type B1, 342/01 PFGE type A1, 486/01 PFGE type A2, 597/01 PFGE type A and 163/02 PFGE type A.

Figure 4A: Results of a FASTA search of Genbank for sequences displaying highest identity to the insertion site of the integron harbouring the *bla*$_{VIM-4}$ MßL gene recently identified in *Pseudomonas aeruginosa* isolates recovered from hospitalised children in Poland.

EM_PRO: ISTN501 Z00027.1 Transposon Tn501 from Pseudomo (8355 nt)
initn: 1047 init1: 1047 opt: 1865 Z-score: 1455.6 bits: 282.6 E(): 2.7e-74
banded Smith-Waterman score: 1865; 96.962% identity (98.205% ungapped) in 395 nt overlap (12-406:6901-7294)

```
Sequen                                  10        20        30        40
                                        TCTGAAAACGACAGGAAGAGCGCTTGCAGCTGCTGGACGAG
                                        : ::::::::::::::::::::::::::::::::::::::
EM_PRO CTGGCGATCAACCCGAACAGCGACCAGTATCTGGAAGAGCGCTTGCAGCTGCTGGACGAG
          6880      6890      6900      6910      6920      6930

          50        60        70        80        90       100
Sequen CAGTTGGCCACTGTCGCCCGCCTGGCCAAGGACAACGAGCTGCCCGATGCCATCCTCACC
       :::: ::::::::::::::: :::::::::::::::::::::::::::::::::::::
EM_PRO CAGTTGGCCACTGTCGCCCGCCTGGCCAAGGACAACGAGCTGCCCGATGCCATCCTCACC
          6940      6950      6960      6970      6980      6990

         110       120       130       140       150       160
Sequen GAGTCCGGGTTGAAGATCACCCCGCTAGAATGCGGCGGTGCCGGATCGGGCGCAGGCGCTG
       :::::::::::::::::::::::::::::: :::::::::::::::::::::::::::::
EM_PRO GAGTCCGGGTTGAAGATCACCCCGCTGGATGCGGCGGTGCCGGATCGGGCGCAGGCGCTG
          7000      7010      7020      7030      7040      7050

         170       180       190       200       210       220
Sequen ATCGACCAGACCAGTCAGTTACTGCCGCCGCATCAAGATCACCGAACTGCTGATGGACCTG
       :::::::::::::::::::::::::::::::::::::::::::::::::::::::::: ::
EM_PRO ATCGACCAGACCAGTCAGTTACTGCCGCGCATCAAGATCACCGAACTGCTGATGGACGTG
          7060      7070      7080      7090      7100      7110

         230       240       250       260       270       280
Sequen GACGAACTGGACGGGCTTCAGCCGCCACTTCACCCACCTGAAGGACGGGGCCGAGGCCAA
       ::: ::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_PRO GACG-ACTGGACGGGCTTCAGCCGCCACTTCACCCACCTGAAGGACGGGGCCGAGGCCAA
            7120      7130      7140      7150      7160

         290       300       310       320       330       340
Sequen AGACCGGACGTTGCTGCTGTCCGCGATTCTCGGTGATGCAATCAACCTCGGGCTGACCAA
       :::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_PRO AGACCGGACGTTGCTGCTGTCCGCGATCCTCGGTGATGCGATCAACCTCGGGCTGACCAA
    7170      7180      7190      7200      7210      7220

         350       360       370       380       390       400
Sequen NATGGCCGAGTCNAGCCCCGGACTGACCTACNCCAAGCTGTCCTGGCTGCAANCCTGGCA
       ::::::::: :::::::: :::::::::: :::::::::: :::::::::::::::: : :::
EM_PRO GATGGCCGAGTCGAGCCCCGGTCTGACCTACGCCAAGCTGTCCTGGCTGCAAGCCTGGCA
    7230      7240      7250      7260      7270      7280

Sequen CATCC
       :::::
EM_PRO CATCCGCGACGAGACCTATTCGGCGGCCCTGGCCGAGCTGGTCAACCACCAGTACCAGCA
    7290      7300      7310      7320      7330      7340
```

Figure 4B: Results of a FASTA search for sequences displaying highest identity to regions upstream of the insertion site of the integron harbouring the *bla*$_{GIM-1}$ MßL gene. Alignments display highest identity to a *Tn*501-type transposon (*Tn*4653 93% identity) found in a plasmid harboured by *Pseudomonas putida* and transposon *Tn*501 from *Pseudomonas aeruginosa* (91% identity).

>>EM_PRO: PPU344068 AJ344068.1 Pseudomonas putida plasmid (116580 nt)
initn: 668 init1: 590 opt: 609 Z-score: 485.1 bits: 103.9 E(): 7e-21
banded Smith-Waterman score: 609; 93.478% identity (93.478% ungapped) in 138 nt overlap (23-160:89687-89824)

```
Sequen                  10        20        30        40        50
                        TGCAGCCGTCTTCTGAAAACGACAGCTACGGAGCGCGACACCTTGCTTGCGT
                        : :: :::::::: :::::: ::::::::::
EM_PRO ACTGATACATGCGCGTCGCTTGATCCTCTCGGCCACGGAGCGGGACACCCTGCTTGCGT
       89660     89670     89680     89690     89700     89710

                60        70        80        90       100       110
Sequen TGCCGGAAAGCCAGGATGACCTGATCCGCTACTACACCTTCAACGACTCCGACCTGTCGC
       :::: ::::::::::::::: :::::::::::::::::::::::::::::::::::::::
EM_PRO TGCCAGAAAGCCAGGATGACATGATCCGCTACTACACCTTCAACGACTCCGACCTGTCGC
       89720     89730     89740     89750     89760     89770

         120       130       140       150       160
Sequen TGATCCGCCAGCGGCGCGGCGACGCCAACCGCCTGGGCTTCGTGCTGCCTGCTTGTA
       :::::::::::::::::::::::::::::::::: ::::::: : :::
EM_PRO TGATCCGCCAGCGGCGCGGCGACGCCAACCGCCTAGGCTTCGCGGTGGCAGCTCAGCCTGC
       89780     89790     89800     89810     89820     89830

EM_PRO TGCGCTACCCCGGCTATGCGTTGGGCACCGACAGCGAGCCGCCCGAGCCGGTCATCCTGT
       89840     89850     89860     89870     89880     89890
```

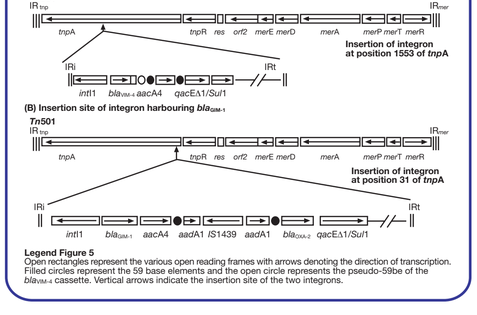
>>EM_PRO: ISTN501 Z00027.1 Transposon Tn501 from Pseudomo (8355 nt)
initn: 572 init1: 572 opt: 582 Z-score: 469.7 bits: 98.9 E(): 2.2e-19
banded Smith-Waterman score: 582; 91.304% identity (91.304% ungapped) in 138 nt overlap (23-160:5378-5515)

```
Sequen                  10        20        30        40        50
                        TGCAGCCGTCTTCTGAAAACGACAGCTACGGAGCGCGACACCTTGCTTGCGT
                        : :: :::::::: : :::: :::: ::::
EM_PRO ACTGACACATGCCGCGTCGCTTGATCCTCTCGGCCACGGAGCGGGACACCCTGCTCGCGT
       5350      5360      5370      5380      5390      5400

                60        70        80        90       100       110
Sequen TGCCGGAAAGCCAGGATGACCTGATCCGCTACTACACCTTCAACGACTCCGACCTGTCGC
       :::: ::::::::::::::: :::::::::::::::::::::::::::::::::::::::
EM_PRO TGCCAGAAAGCCAGGATGACCTGATCCGCTACTACACCTTCAACGACTCCGACCTGTCGC
       5410      5420      5430      5440      5450      5460

         120       130       140       150       160
Sequen TGATCCGCCAGCGGCGCGGCGACGCCAACCGCCTGGGCTTCGTGCTGCCTGCTTGTA
       :::::::::::::::::::::::::::::::::: ::::::: : :::
EM_PRO TGATCCGTCAGCGGCGCGGCGACGCCAACCGCCTCGGCTTCGCCGTGACAACTCGCCTGC
       5470      5480      5490      5500      5510      5520

EM_PRO TGCGCTACCCCGGCTATGCGTTGGGCACTGACAGCGAGCTGCCCGAACCGGTCATCCTGT
       5530      5540      5550      5560      5570      5580
```

Legend Figure 4A- The alignment displays highest identity (98%) to the *tnp*A gene of transposon *Tn*501 and is inserted at position 1553 of the *tnp*A gene. The inverted repeat IRi of the integron carrying *bla*$_{VIM-4}$ is underlined and highlighted in bold in the alignment.

Legend Figure 4B- The inverted repeat IRi of the integron carrying *bla*$_{GIM-1}$ is underlined and highlighted in bold in the alignment.

Figure 5: Insertion points of the *bla*$_{VIM-4}$ and *bla*$_{GIM-1}$ integrons into the *Tn*501-type transposon are depicted in A and B respectively.



Legend Figure 5
Open rectangles represent the various open reading frames with arrows denoting the direction of transcription. Filled circles represent the 59 base elements and the open circle represents the pseudo-59be of the *bla*$_{VIM-4}$ cassette. Vertical arrows indicate the insertion site of the two integrons.

CONCLUSIONS

- The finding of distinct PFGE patterns associated with the Polish isolates indicate that the *bla*$_{VIM-4}$ MßL containing integron is mobile or at least was mobile in the past (Figures 2 and 3).
- The insertion of the integrons into the coding sequence of the *tnp*A gene is likely to render this gene non functional and the transposon itself non self-mobile. However, the insertion of these integrons within the *Tn*501-like transposons is likely to confer an additional level of genetic movement by virtue of inserting between *Tn*501 inverted repeats if the transposition function is supplied in *trans*.

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