

## Increased prevalence and clonal dissemination of multidrug-resistant *Pseudomonas aeruginosa* with the *bla*<sub>IMP-1</sub> gene cassette in Hiroshima

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**Objectives:** The aim of this study was to evaluate the dissemination of metallo- $\beta$ -lactamase (MBL)-encoding genes among multidrug-resistant (MDR) *Pseudomonas aeruginosa* isolates recovered from major hospitals in the Hiroshima region.

**Methods:** During July to December from 2004 to 2006, a surveillance of eight major hospitals in the Hiroshima region identified 387 non-duplicate isolates resistant to imipenem (MIC  $\geq$  16 mg/L). They were screened for resistance to amikacin (MIC  $\geq$  64 mg/L) and ciprofloxacin (MIC  $\geq$  4 mg/L) and MBL-encoding genes. The structure of the variable regions of the integrons was determined using PCR mapping. Clonality was assessed using PFGE and multilocus sequence typing (MLST).

**Results:** The frequency of MBL-positive isolates in MDR *P. aeruginosa* isolates significantly increased from 42.3% in 2004 to 81.4% in 2006. Most of the MBL-positive isolates produced IMP-1 followed by VIM-2. The *bla*<sub>IMP-1</sub> and *bla*<sub>VIM-2</sub> genes were present in class 1 integrons. Characterization of the variable regions of the integron showed the presence of six different gene cassette arrays in *bla*<sub>IMP-1</sub> cassettes and a single array in *bla*<sub>VIM-2</sub> cassettes. The IMP-1 producers belonged to two clonal lineages using PFGE and MLST analyses and the integron variations correlated well with the clonal complexes. Among them, strains positive for a newly identified In113-derived *bla*<sub>IMP-1</sub> gene cassette array were most widely distributed in Hiroshima.

**Conclusions:** This study shows a dramatic increase in MBL genes, primarily *bla*<sub>IMP-1</sub>, in MDR *P. aeruginosa* isolates in Hiroshima during these 3 years. In addition, MDR *P. aeruginosa* with the newly discovered In113-derived *bla*<sub>IMP-1</sub> gene cassette array appears to be clonally expanding.

Keywords: metallo- $\beta$ -lactamases, integrons, epidemiology

### Introduction

*Pseudomonas aeruginosa* continues to be a leading cause of nosocomial infections. Drug resistance is frequently found in

nosocomial isolates and often involves multiple antibiotic classes. Carbapenems are the most potent  $\beta$ -lactams against *P. aeruginosa*; however, intensive use of the carbapenems in the treatment of nosocomial *P. aeruginosa* infections has

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facilitated the emergence and development of carbapenem-resistant *P. aeruginosa*. Resistance to imipenem in *P. aeruginosa* is significantly associated with reduced uptake of the agent; this results from the loss or reduced expression of the OprD porin.<sup>1</sup> Resistance to meropenem may also arise via AmpC hyperproduction and/or overexpression of the intrinsic efflux systems.<sup>2</sup>

Acquisition of genes encoding Ambler's class B metallo- $\beta$ -lactamases (MBLs) confers resistance to  $\beta$ -lactams including penicillins, cepheims and carbapenems and contributes to high-level carbapenem resistance.<sup>3</sup> Carbapenem-resistant *P. aeruginosa* producing MBLs were first discovered in Japan<sup>4,5</sup> and since then they have been reported in many countries all over the world.<sup>6</sup> Four types of MBLs, IMP, VIM, SPM-1 and GIM-1, have been reported in *P. aeruginosa*.<sup>4,7–9</sup> Among them, IMP and VIM are the most common types of MBL with worldwide distribution.<sup>10,11</sup> The MBL genes are frequently carried on mobile gene cassettes together with other classes of drug and/or antiseptic resistance determinants that are inserted into the chromosome- or plasmid-borne class 1 or 3 integrons.<sup>11–13</sup> Integrons are often found on the plasmid or as a part of the transposons or mobile genetic elements called ISCR elements that are able to mobilize chromosomal elements resulting in an increase in the number of resistant Gram-negative bacilli.<sup>14,15</sup> Therefore, MBL-producing *P. aeruginosa* often behave as multidrug-resistant (MDR) strains. Here, we report a survey for the dissemination of MBL genes in MDR *P. aeruginosa* and a molecular epidemiology study of the *bla*<sub>IMP-1</sub>-positive *P. aeruginosa* strains during a 3 year period from 2004 to 2006 in Hiroshima, Japan. Our data clearly indicate the dramatic change in the genetic make-up of resistance in MDR *P. aeruginosa* during a 3 year period and the dissemination of a novel MDR *P. aeruginosa* clone with an *In13*-derived gene cassette array in Hiroshima.

## Materials and methods

### *Collection of P. aeruginosa* isolates and antimicrobial susceptibility testing

Non-duplicate *P. aeruginosa* isolates were obtained from patients from eight general hospitals in Hiroshima during July to December from 2004 to 2006. Identification of *P. aeruginosa* was performed by the submitting laboratories. They were cultured on nalidixic acid/cetrimide (NAC) agar (Eiken, Tokyo, Japan) and verified using PCR amplification with primers specific for *P. aeruginosa* 16S rRNA [Table S1, available as Supplementary data at *JAC* Online (<http://jac.oxfordjournals.org/>)].<sup>16</sup> Imipenem-resistant *P. aeruginosa* were selected when an MIC of  $\geq 16$  mg/L occurred. MICs were determined using the microdilution broth method of the CLSI.<sup>17</sup> The antibiotics tested were ciprofloxacin (Meiji Seika Kaisha, Ltd, Tokyo), imipenem (Banyu Pharmaceutical Co., Ltd, Tokyo) and amikacin (Banyu Pharmaceutical Co., Ltd, Tokyo).

Criteria for MDR *P. aeruginosa* were in accordance with the Law Concerning the Prevention of Infections and Medical Care for Patients with Infections from the Japanese Ministry of Health, Labor, and Welfare where the criteria are resistance to imipenem (MIC  $\geq 16$  mg/L), amikacin (MIC  $\geq 64$  mg/L) and ciprofloxacin (MIC  $\geq 4$  mg/L). The criterion for amikacin resistance (MIC  $\geq 64$  mg/L) was different from that of the CLSI guidelines (MIC  $\geq 32$  mg/L).<sup>17</sup>

### *MBL screening test with an inhibitor using a double-disc synergy test with two Kirby–Bauer discs*

Screening for the production of MBL was performed using sodium mercaptoacetic acid (SMA) as described previously.<sup>8</sup>

### *PCR detection and characterization of the variable regions of the bla<sub>IMP-1</sub>- or bla<sub>VIM-2</sub>-containing integrons and quinolone resistance-determining regions (QRDRs)*

The primers used are shown in Table S1. PCR amplification was performed using Takara EX *Taq* DNA polymerase (Takara, Tokyo, Japan) with 25 cycles of: denaturing at 98°C for 10 s; annealing at 50°C for 30 s and polymerization at 72°C for 1 min. The structure of the variable regions of the *bla*<sub>IMP-1</sub>- or *bla*<sub>VIM-2</sub>-containing integrons was determined using a PCR mapping approach with primers designed from the conserved integron sequences flanking the cassette array as shown in Table S1.<sup>18</sup> To investigate mutations in QRDRs, the *gyrA* and *parC* QRDRs were determined using PCR with primers described previously.<sup>19</sup> For sequencing of the PCR products, the amplicons were directly sequenced using both DNA strands using an ABI PRISM 3100 Genetic Analyzer (AB Applied Biosystems, Foster City, CA, USA) using an ET terminator reaction (Amersham-Pharmacia Biotech, Piscataway, NJ, USA). Comparison of experimentally determined nucleotide sequences to sequence databases was performed using BLAST (<http://www.ncbi.nlm.nih.gov/blast>).

### *PFGE and Southern blot analyses*

PFGE was performed using the method described previously.<sup>20</sup> To identify the location of the MBL genes on the genome, Southern blot hybridization using the *bla*<sub>IMP-1</sub> probe or *bla*<sub>VIM-2</sub> probe was performed on the PFGE-separated DNA after transfer to a Hybond N<sup>+</sup> membrane using an ECL direct nucleic acid labelling and detection kit (Amersham-Pharmacia).

### *Multilocus sequence typing (MLST)*

MLST was performed using the protocol published by Curran *et al.*<sup>21</sup> PCRs and DNA sequencing for the housekeeping genes *acsA*, *aroE*, *guaA*, *mutL*, *nuoD*, *ppsA* and *trpE* were performed using the method described previously.<sup>21</sup> The nucleotide sequences were determined using both strands and compared with existing sequences in the MLST database ([www.pubmlst.org](http://www.pubmlst.org)) for assignment of allelic numbers and sequence types (STs). Isolates having five or more identical alleles were considered a part of the same clonal complex.

### *Conjugation*

The filter mating method was used to determine the transferability of the carbapenem resistance determinant using the rifampicin-resistant mutant *P. aeruginosa* PAO1Rp as a recipient.<sup>22</sup> Mueller–Hinton agar containing 100 mg/L rifampicin and 2 mg/L imipenem was used to select for conjugation.

## Results

### *Screening for drug-resistant strains*

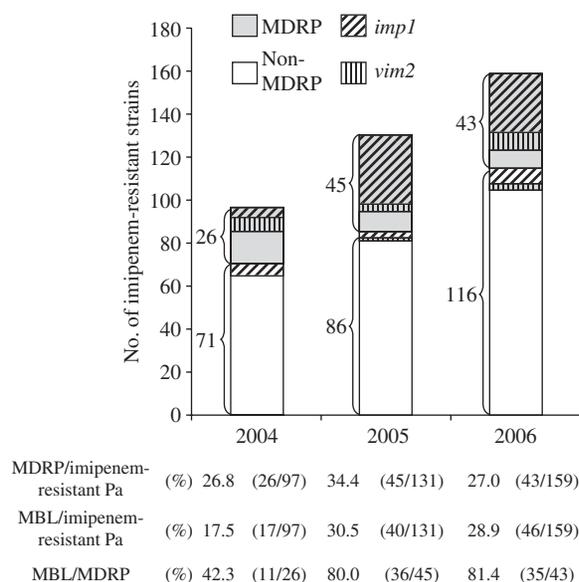
A total of 3004 isolates of *P. aeruginosa* were processed at eight hospitals (a–h) in Hiroshima during July to December

2004–2006. Of these 3004 isolates, 387 single patient isolates had an MIC of imipenem  $\geq 16$  mg/L. Drug-resistant *P. aeruginosa* were isolated more frequently from either urine or sputum samples. Most of the patients were hospitalized except for 19 outpatients. Among the 387 strains, 114 strains (29.5%, 3.8% of the total) were MDR *P. aeruginosa*. Annual numbers of the imipenem-resistant strains continuously increased during the 3 years of the study, from 97 in 2004 to 159 in 2006, but the ratio of MDR *P. aeruginosa* among imipenem-resistant strains did not change significantly (Figure 1).

#### Prevalence of MBL-positive strains

Phenotypic testing using the inhibitor SMA showed that 103 of the 387 *P. aeruginosa* isolates (26.6%) were MBL positive. Eighty-two of the MBL-positive strains (79.6%) were MDR *P. aeruginosa*. We further analysed the 387 strains for the MBL genes using PCR and subsequent direct sequencing with universal primer sets to identify the three types of MBL genes: *bla*<sub>IMP-1</sub>, *bla*<sub>VIM-2</sub> and *bla*<sub>SPM</sub>. Eighty strains (20.7%) were positive for the *bla*<sub>IMP-1</sub> allele and 23 strains (5.9%) were positive for the *bla*<sub>VIM-2</sub> allele. No *bla*<sub>SPM</sub> alleles were detected in the 387 strains. During the 3 years, the rate of isolation of the MBL-positive strains among imipenem-resistant strains increased slightly from 17.5% in 2004 to 28.9% in 2006 (Figure 1). The rate of isolation of MBL-positive strains among MDR *P. aeruginosa* increased dramatically during the 3 years and almost doubled from 42.3% (11/26) in 2004 to 81.4% (35/43) in 2006.

We determined whether the 387 strains possessed homologues of the *bla*<sub>IMP</sub> or *bla*<sub>VIM</sub> gene. Genomic Southern hybridization was performed under mildly stringent conditions using either a *bla*<sub>IMP-1</sub> or a *bla*<sub>VIM-2</sub> probe. The strains with positive PCR results showed hybridized bands, and no other bands were detected in the other strains.



**Figure 1.** Annual number of isolates of imipenem-resistant *P. aeruginosa*. Annual number of MDR *P. aeruginosa* (MDRP) or MBL-producing strains in imipenem-resistant strains and annual number of MBL-producing strains in MDR *P. aeruginosa* strains are shown. Pa, *P. aeruginosa*.

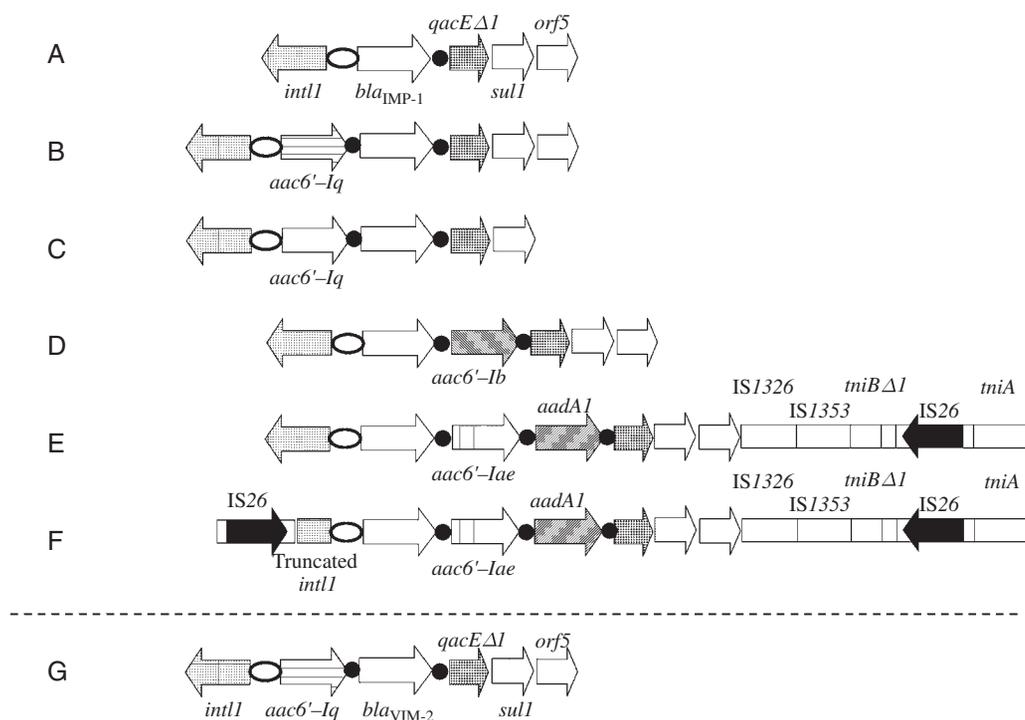
#### Integron structures

To investigate the genetic content in the *bla*<sub>IMP-1</sub> and *bla*<sub>VIM-2</sub> integrons, we performed PCR mapping and selected seven isolates representing all of the integron polymorphs identified. The structures of the gene cassette arrays are shown according to the sequence of the entire variable regions in Figure 2. The inserted gene cassettes in the arrays show one to three gene cassettes, and the genes with known functions, excluding *bla*<sub>IMP-1</sub> and *bla*<sub>VIM-2</sub>, were exclusively aminoglycoside acetyltransferases and aminoglycoside adenytransferase. In the integron polymorph A, the *bla*<sub>IMP-1</sub> cassette was accompanied by the quaternary ammonium compound resistance gene (*qacEΔ1*), the *sulI* gene and *orf5* (encoding the so-called 3'-conserved region). The integron polymorphs A and B differed from each other only by the presence of an aminoglycoside acetyltransferase gene (*aac6'-Iq*) between *bla*<sub>IMP-1</sub> and *intI1* in polymorph B. The integron polymorph C contained the same genes as the integron polymorph B, except that *orf5* is missing in the 3'-conserved region. The integron polymorph D possessed *bla*<sub>IMP-1</sub> and aminoglycoside acetyltransferase gene (*aac6'-Ib*) cassettes between the 5'-conserved region and the 3'-conserved region. The integron polymorph E had three gene cassettes, *bla*<sub>IMP-1</sub> followed by the aminoglycoside acetyltransferase gene (*aac6'-Iae*) and the aminoglycoside adenytransferase gene (*aadA1*) in the class 1 integron (*intI1-qacEΔ1-sulI-orf5*). Downstream of the 3'-conserved region, two insertion sequences, IS1326 and IS1353, were located in tandem followed by a *tni* module of Tn21 with insertion of IS26 into the *tniA*-coding region. The integron polymorph F shared identical gene cassettes in the 3'-conserved region and further downstream sequences with the integron polymorph E; however, the *intI1* in the 5'-conserved region is truncated due to an IS26 insertion. Among the six variants in the *bla*<sub>IMP-1</sub> integron, integron polymorph F is unusual. In Hiroshima, MDR *P. aeruginosa* with integron polymorph F were not isolated in 2004 but is the most frequent isolate occurring recently and accounted for 62.8% in 2005 and 55.9% in 2006 of the *bla*<sub>IMP-1</sub>-positive isolates. Polymorph F was isolated from five (a, b, c, f and g) of the eight hospitals during the 2 years. The *bla*<sub>VIM-2</sub> integron in polymorph G possessed the aminoglycoside acetyltransferase gene (*aac6'-Iq*) accompanied by a 3'-conserved region (Figure 2). Most of the polymorph G strains, 16 out of 22, were from hospital e suggesting nosocomial spread of a single clone.

#### Cluster analysis using PFGE patterns, MLST and molecular epidemiology

We focused on isolates carrying *bla*<sub>IMP-1</sub> and further analysed the genomic background of the strains. Data from PFGE using cluster analysis, MLST and genetic and phenotypic characterizations of the 80 *bla*<sub>IMP-1</sub>-positive strains are summarized in Figure S1 [available as Supplementary data at JAC Online (<http://jac.oxfordjournals.org/>)]. Cluster analysis of the PFGE restriction patterns identified at least three large clusters, I, II, III and one singleton. Clusters I and II were split further into several subtypes. MLST showed that all the isolates belong to one of two STs, ST357 and ST235. ST357 contained all of the strains belonging to cluster I, and ST235 contained the rest of the strains belonging to clusters II, III and the singleton. We performed Southern hybridization with the PFGE gels using the

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**Figure 2.** Schematic representation of the genetic content in the *bla*<sub>IMP-1</sub> and *bla*<sub>VIM-2</sub> integrons. ORFs are shown as arrows indicating the orientation of the coding sequence and with the gene name. Open ellipses, *attI1* site; black circles, 59 bp elements/*attC*; *intI1*, integrase; *aac*, aminoglycoside acetyltransferase; *aad*, aminoglycoside adenyltransferase; *qacEΔ1*, QacEΔ1 multidrug exporter; *sul1*, Sul1 dihydropteroate synthase; *orf5*, hypothetical protein; and IS, insertion sequence.

*bla*<sub>IMP-1</sub> probe, and the hybridized bands were visualized using different colours according to the polymorph types (Figure S1). The *bla*<sub>IMP-1</sub> genes are located on variable sizes of DNA fragments from <100 to ~550 kb. There was a strong correlation between polymorph types and STs: strains positive for polymorphs A, B and C belonged to ST357 and those for polymorphs D, E and F belonged to ST235.

The *bla*<sub>IMP-1</sub> genes of most strains are in cluster I and, except for those in cluster IA and IE, are located on DNA fragments of <100 kb. Strains positive for polymorphs D and E showed similar DNA profiles, and they were localized in the subcluster IIB and a part of subcluster IIA, respectively. The *bla*<sub>IMP-1</sub> genes of polymorphs D and E are located on DNA fragments of ~120 kb and ~240 kb, respectively. DNA profiles of most strains with polymorph F from hospital g isolated in 2005 were very similar, forming a cluster with a Dice coefficient of ≥90% (part of IIC). Further, the *bla*<sub>IMP-1</sub> gene probe hybridized to DNA fragments with very similar sizes of ~300 kb. Conversely, DNA profiles of the rest of the strains within polymorph F were highly heterogeneous, as were the DNA fragments hybridizing with the *bla*<sub>IMP-1</sub> gene probe. For some of the strains with polymorph A and those with polymorph F, the *bla*<sub>IMP-1</sub> probe hybridized with two DNA bands suggesting a duplication of the *bla*<sub>IMP-1</sub> gene.

### Resistance to fluoroquinolones

Alterations in *gyrA* and *parC* caused by mutations in QRDRs are suggested to play a major role in fluoroquinolone resistance in clinical isolates of *P. aeruginosa*.<sup>19,23</sup> Fragments including the putative QRDRs were amplified using PCR and sequenced (isolates marked by asterisks in Figure S1). They all had the same

pattern of amino acid replacement (*GyrA*, 83Thr→Ile and 87Asp→Asn; and *ParC*, 87Ser→Leu) in the QRDRs.

### Discussion

Here we show that the genetics of resistance in MDR *P. aeruginosa* have dramatically changed during a 3 year study in Hiroshima. The ratio of MDR *P. aeruginosa* in drug-resistant *P. aeruginosa* (imipenem resistance) did not change significantly, but the ratio of MBL-positive strains increased significantly for MDR *P. aeruginosa*. In 2004, most of the MDR *P. aeruginosa* did not carry MBL genes, and the resistance to imipenem might be attributed to the reduced expression of the OprD porin, combined with derepression of the chromosomal *ampC* β-lactamase gene and/or overexpression of the efflux pump systems.<sup>20</sup> However, in 2006, ~80% of the MDR *P. aeruginosa* were carriers of MBL genes that conferred the extrinsic drug resistance to imipenem (Figure 1). Many of them had both aminoglycoside-inactivating genes, showing resistance to imipenem and amikacin (Figure 2). This suggests that the acquisition of the MBL gene-containing integron facilitates the swift propagation of the MDR determinants in *P. aeruginosa* causing the outbreaks.

Earlier studies showed that *bla*<sub>IMP-1</sub> was found on conjugative plasmids.<sup>11</sup> Repeated attempts to detect *bla*<sub>IMP-1</sub> on plasmids using a conjugation experiment were unsuccessful. Rather, fragments of genomic DNA separated by PFGE hybridized with a gene probe for *bla*<sub>IMP-1</sub>, suggesting a chromosomal location. However, we cannot exclude the possible carriage of the *bla*<sub>IMP-1</sub> determinant by a large plasmid that may be strongly associated with the chromosome.

The spread of *bla*<sub>IMP-1</sub>-positive isolates observed during the 3 years was caused by members of two clonal lineages using MLST analysis, suggesting that clonal expansion played a predominant role in the dissemination of these isolates. However, diversity was observed among the isolates. In the clonal lineage of ST357, acquisition of *aac6'-Iq* and subsequent loss of *orf5* in the 3'-conserved region possibly created two different integron structures, B and C, from A (Figure 2). *aac6'-Iq* was reported in 1998 from *Klebsiella pneumoniae* and was located in tandem in the same direction as the *bla*<sub>IMP-1</sub> gene.<sup>24</sup> This is the first report that *P. aeruginosa* harbours the *aac6'-Iq* gene. In the clonal lineage ST235, two different integron structures, D and E, were present. The array of integron polymorph E is identical to In113 that was recently discovered in MDR *P. aeruginosa* strain IMCJ2.S1 that occurred in an outbreak in a neurosurgery ward of a hospital located in the Tohoku area of Japan.<sup>25</sup> Since Hiroshima and the Tohoku area are 800 km apart, this suggests that the gene cassette is possibly widespread in Japan. In 2002, 594 MBL-producing *P. aeruginosa* isolated from 60 hospitals throughout Japan were evaluated.<sup>26</sup> That report suggested a clonal spread of non-impipenem-susceptible *P. aeruginosa* carrying the *bla*<sub>IMP-1</sub> integron with *aacA4* (*aac6'-Ib*) accompanied by a 3'-conserved region, which corresponds to polymorph type D in this study. These data suggest relatively recent dissemination of the strains carrying the *bla*<sub>IMP-1</sub> gene cassette array described in this study. The presence of two different clonal lineages carrying the *bla*<sub>IMP-1</sub> gene in different integron structures clearly points to a different phylogeny of these isolates and to different events in the acquisition of the *bla*<sub>IMP-1</sub> gene in these strains.

In Hiroshima, it appears that *bla*<sub>IMP-1</sub>-positive strains carrying polymorph F began to disseminate in 2005 and by 2006 they were found in at least five different hospitals out of the eight, contributing to ~60% of all *bla*<sub>IMP-1</sub>-positive isolates. The reason why the *bla*<sub>IMP-1</sub>-positive strains carrying polymorph F showed a high dissemination potential remains unexplained. Interestingly, *bla*<sub>IMP-1</sub>-positive strains carrying polymorph F isolated in 2005 in hospital g were genotypically very similar and the *bla*<sub>IMP-1</sub> probe hybridized to an almost identical 300 kb DNA fragment (Figure S1). This clearly suggests the nosocomial spread of a particular clone in the hospital. In contrast, the *bla*<sub>IMP-1</sub>-positive strains carrying polymorph F in 2006 from various other hospitals were genotypically diverse and the DNA band size hybridizing with the *bla*<sub>IMP-1</sub> probe was highly heterogeneous. Whether or not this is due to genomic rearrangement of the preceding clone or multiple independent events of acquisition of *bla*<sub>IMP-1</sub>-containing integron polymorph F by different sublineages remains to be determined. It is interesting to note that truncation of *int11* by IS26 resulted in the disruption of the integron structure and the formation of a composite transposon containing two IS26 elements bracketing resistance genes. This may facilitate IS26-mediated mobilization of *bla*<sub>IMP-1</sub> among the *bla*<sub>IMP-1</sub>-positive strains carrying polymorph F. Detailed characterization of the overall structure of the mobile element in strains carrying *bla*<sub>IMP-1</sub>-containing integron polymorph F may be necessary to understand this phenomenon.

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## Transparency declarations

None to declare.

## Supplementary data

Table S1 and Figure S1 are available as Supplementary data at JAC Online (<http://jac.oxfordjournals.org/>).

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