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First Survey of Metallo-β-Lactamases in Clinical Isolates of *Pseudomonas aeruginosa* in a German University Hospital[▽]

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A total of 489 clinical isolates of *Pseudomonas aeruginosa* was investigated for metallo-β-lactamase (MBL) production. Molecular analysis detected a bla_{VIM-1} gene in the chromosome of one isolate and a bla_{VIM-2} gene carried on the plasmid in seven isolates. Moreover, we showed that an initial screening by combined susceptibility testing of imipenem and ceftazidime followed by a confirmatory EDTA combination disk test represents a valid alternative to the molecular investigation of MBL genes, making MBL detection possible in routine diagnostic laboratories.

Metallo-β-lactamase (MBL)-producing Gram-negative bacteria are an increasing public health problem worldwide because of their resistance to all β-lactams except aztreonam (3). MBL genes are typically part of an integron and are either carried on transferable plasmids or are part of the bacterial chromosome (28). The most common transferable MBL families include the VIM-, IMP-, GIM-, SPM-, and SIM-type enzymes which have been detected primarily in *Pseudomonas aeruginosa* but were also found in other Gram-negative bacteria, including nonfermenters and members of the family *Enterobacteriaceae* (22). Recently, two new subgroups of MBLs, designated NDM-1 and DIM-1, were identified in a clinical isolate of *Klebsiella pneumoniae* in India and in a clinical isolate of *Pseudomonas stutzeri* in the Netherlands, respectively (21, 31).

In most studies, reduced susceptibility to imipenem has been adopted as the sole criterion for further phenotypic or molecular investigations in order to detect MBLs (11, 19, 20, 23). However, this criterion seems to be suboptimal, as it does not allow exclusion of isolates characterized by the loss of the OprD porin, the most common mechanism of resistance to imipenem in *P. aeruginosa* (14, 22). Moreover, several phenotypic tests for detecting MBLs, such as the MBL Etest (20, 25), EDTA combination disk test (20, 25, 30), EDTA disk synergy test (10), and imipenem lysate MBL assay (27), have been developed and evaluated. However, the performance of each of these tests seems to be strongly affected by the local rate of MBL-producing isolates.

In Germany, VIM-, and GIM-type enzymes in *P. aeruginosa* isolates have already been detected (1, 8, 26). Recently, Elias et al. described a nosocomial outbreak caused by a bla_{VIM-2}-positive *P. aeruginosa* in patients of the Department of Urology of the university hospital of the University of Würzburg, Würzburg, Germany, between November and December 2007 (4). However, to the best of our knowledge, no systematic surveys of the occurrence of MBLs in clinical isolates of *P. aeruginosa* have been conducted in Germany so far.

Accordingly, this study was designed with the following aims: (i) to develop improved screening criteria for the detection of MBL-producing *P. aeruginosa*; (ii) to determine the proportion of MBL-producing isolates in clinical isolates of *P. aeruginosa* in the university hospital of the University of Würzburg in Germany; (iii) to assess the relatedness of MBL-producing isolates; (iv) to determine the locations of the MBL genes detected; and (v) to evaluate two phenotypic tests as confirmatory tests for the detection of MBL production.

Since no standard imipenem MIC breakpoints for MBL producers are available, we first analyzed the MICs of imipenem in 10 well-characterized *P. aeruginosa* control strains shown previously to produce IMP-, VIM-, GIM-, SIM-, and SPM-type enzymes (Table 1). Identification to the species level was confirmed using Vitek 2 GN cards (bioMérieux, Nürtingen, Germany). Antimicrobial susceptibility testing was carried out with Vitek 2 AST-N021 and AST-N110 cards (bioMérieux). MICs were interpreted as recommended by the CLSI (2). All MBL-producing positive-control strains were intermediate sensitive or resistant to imipenem (MIC $\geq$ 8 μg/ml). Subsequently, we investigated 26 consecutive nonreplicate clinical isolates of *P. aeruginosa* with an imipenem MIC of $\geq$8 μg/ml for the presence of MBL genes by multiplex PCR as described by Ellington et al. (5). All clinical isolates were collected in our laboratory throughout 2007 before the outbreak at the university hospital of the University of Würzburg, Würzburg, Germany (4), and all isolates were shown by multiplex PCR to be negative for MBL genes. Since the loss of the OprD porin, the most common mechanism of resistance to imipenem in *P. aeruginosa*, does not confer ceftazidime resistance (15), we analyzed the ceftazidime MICs in the MBL-producing positive-control strains and in the 26 MBL-negative isolates. While all 10 MBL-producing positive-control strains were resistant to ceftazidime (MIC $\geq$ 32 μg/ml), only 6 of the 26 MBL-negative isolates were resistant to ceftazidime ($P <$

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TABLE 1. Comparison of imipenem and ceftazidime MICs in well-characterized MBL-producing positive-control strains and in MBL-negative clinical isolates[a]

Strain or isolate[b]	MBL enzyme[c]	Geographic origin	Reference	MIC (μg/ml)	
				Imipenem	Ceftazidime[d]
Control strains					
PA431	IMP-1	Turkey	17	8	≥64
PA552	IMP-1	UK	5	≥16	≥64
PA386	IMP-13	Italy	18	≥16	≥64
PA550	VIM-1	UK	5	≥16	≥64
PA373	VIM-2	Italy	9	≥16	≥64
PA399	VIM-2	Germany	4	≥16	≥64
PA430	VIM-4	Hungary	13	≥16	≥64
PA554	GIM-1	Germany	1	≥16	≥64
AB551	SIM-1	Korea	12	≥16	≥64
PA553	SPM-1	Brazil	6	≥16	≥64
Clinical isolates					
PA339	−	Germany	This study	≥16	8
PA340	−	Germany	This study	8	4
PA341	−	Germany	This study	≥16	≥64
PA342	−	Germany	This study	≥16	4
PA346	−	Germany	This study	≥16	4
PA349	−	Germany	This study	≥16	8
PA350	−	Germany	This study	≥16	4
PA352	−	Germany	This study	≥16	4
PA355	−	Germany	This study	≥16	4
PA356	−	Germany	This study	≥16	4
PA360	−	Germany	This study	8	16
PA361	−	Germany	This study	≥16	≥64
PA362	−	Germany	This study	8	32
PA364	−	Germany	This study	8	4
PA368	−	Germany	This study	≥16	4
PA370	−	Germany	This study	≥16	8
PA376	−	Germany	This study	≥16	16
PA377	−	Germany	This study	≥16	8
PA380	−	Germany	This study	8	4
PA381	−	Germany	This study	≥16	4
PA382	−	Germany	This study	≥16	4
PA395	−	Germany	This study	≥16	≥64
PA734/CF	−	Germany	This study	≥16	4
PA736/CF	−	Germany	This study	8	≥64
PA758/CF	−	Germany	This study	≥16	4
PA774/CF	−	Germany	This study	≥16	≥64

[a] Ten well-characterized MBL-positive strains (9 *P. aeruginosa* strains and one *Acinetobacter baumanii* strain) (control strains) and 26 MBL-negative *P. aeruginosa* isolates with reduced susceptibility to imipenem (clinical isolates) are compared.

[b] *P. aeruginosa* strains and isolates are indicated by PA at the beginning of the strain or isolate designation, and *P. aeruginosa* isolates from patients with cystic fibrosis are indicated by CF after a slash at the end of the isolate designation. One *Acinetobacter baumanii* (AB) control strain is shown.

[c] The type of MBL enzyme is given for the control strains that produce MBL. The clinical isolates were MBL negative (−).

[d] All 10 MBL-producing positive-control strains were resistant to ceftazidime (MIC ≥ 32 mg/ml), but only 6 of the 26 MBL-negative clinical isolates were resistant to ceftazidime ($P < 0.01$ by Fisher's exact test).

0.01) (Table 1). Consequently, we adopted MIC breakpoints for the initial screening of MBL producers of ≥8 μg/ml for imipenem and ≥32 μg/ml for ceftazidime.

From June 2008 until May 2009, a total of 489 consecutive nonreplicate isolates of *P. aeruginosa* from diverse clinical specimens were screened for MBL production. Sixty-eight of these isolates (13.9%) showed reduced susceptibility to imipenem (MIC ≥ 8 μg/ml). Adding resistance to ceftazidime (MIC ≥ 32 μg/ml) as an additional screening criterion for MBL production reduced the number of isolates to be consecutively tested by multiplex PCR (5) to 15. Following PCR, sequencing of the purified amplicons (QIAquick PCR purification kit; Qiagen, Hilden, Germany) was performed with an ABI 3130 genetic analyzer (Applied Biosystems, Foster City, CA). Molecular analysis revealed a $bla_{\text{VIM-1}}$ gene in one isolate and a $bla_{\text{VIM-2}}$ gene in seven additional isolates. These results were confirmed by amplification and sequencing of the entire VIM gene of isolates PA500 ($bla_{\text{VIM-1}}$) and PA399 ($bla_{\text{VIM-2}}$) by using the primer pairs VIM-F (5′-GTTATGCCGCACTC ACCCCCA-3′)/VIM-R (5′-TGCAACTTCATGTTATGCCG-3′) (29) and VIM2004A/VIM2004B (20). Furthermore, we could demonstrate the association of the MBL genes with a class 1 integron by PCR using the integron-specific primers 5-CS and 3-CS in combination with the MBL-specific primers VIM2004A and VIM2004B (20). The clinical origins and antimicrobial susceptibilities of the MBL-positive isolates are shown in Table 2.

On the basis of these results, the proportion of isolates producing MBL was 1.6% with regard to all *P. aeruginosa* isolates investigated and 11.7% with regard to the isolates with

TABLE 2. Epidemiological data and resistance phenotypes of all *P. aeruginosa* isolates with reduced susceptibility to imipenem and resistance to ceftazidime

Isolate	Specimen	Date of recovery	Ward[a]	MBL enzyme[b]	MIC (μg/ml)[c]											
					PIP	TZP	CAZ	FEP	ATM	IMP	MEM	COL	AMK	GEN	TOB	CIP
MBL-producing isolates																
PA399	Urine	November 2007	Urology	VIM-2	≥128	≥128	≥64	≥64	16	≥16	≥16	1	8	≥16	≥16	≥4
PA462	Ascitic fluid	June 2008	General surgery	VIM-2	≥128	≥128	≥64	≥64	16	≥16	≥16	1	4	≥16	≥16	≥4
PA465	Drainage	July 2008	General surgery	VIM-2	≥128	64	≥64	≥64	16	≥16	≥16	2	8	≥16	≤1	≥4
PA469	Wound	August 2008	General surgery	VIM-2	≥128	≥128	≥64	≥64	16	≥16	≥16	1	8	≥16	≥16	≥4
PA475	Wound	September 2008	General surgery	VIM-2	≥128	≥128	≥64	≥64	16	≥16	≥16	1	4	≥16	≥16	≥4
PA477	Drainage	September 2008	General surgery	VIM-2	≥128	≥128	≥64	≥64	16	≥16	≥16	1	8	≥16	≥16	≥4
PA481	Urine	October 2008	Urology	VIM-2	≥128	≥128	≥64	≥64	16	≥16	≥16	1	8	≥16	≥16	≥4
PA500	Tracheal secretion	February 2009	Surgical ICU	VIM-1	≥128	≥128	≥64	≥64	4	≥16	≥16	1	≤2	≥16	8	≥4
PA510	Tracheal secretion	May 2009	Medical ICU	VIM-2	≥128	≥128	≥64	≥64	≥64	≥16	≥16	1	4	≥16	≥16	≥4
MBL-negative isolates																
PA459	Tracheal secretion	June 2008	Surgical ICU	−	≥128	≥128	32	8	16	≥16	8	1	2	1	1	0.25
PA460	Wound	June 2008	General surgery	−	≥128	≥128	32	16	16	≥16	8	1	4	≥16	≥16	≥4
PA479	Urine	October 2008	General surgery	−	≥128	≥128	≥64	≥64	32	8	≥16	1	8	≥16	≥16	≥4
PA483	Wound	October 2008	Surgical ICU	−	≥128	≥128	≥64	32	64	≥16	≥16	1	2	1	1	0.25
PA494	Urine	December 2008	Neurosurgery	−	≥128	≥128	32	8	16	≥16	8	1	8	8	1	≥4
PA507	Drainage	April 2009	Surgical ICU	−	≥128	≥128	32	16	16	≥16	4	1	16	≥16	1	0.25
PA509	Wound	May 2009	Internal medicine	−	≥128	≥128	32	16	8	≥16	≥16	2	64	≥16	≥16	≥4

[a] ICU, intensive care unit.
[b] The type of MBL enzyme is given for the isolates that produce MBL. Some isolates did not produce MBL (−).
[c] Abbreviations for antimicrobial agents: PIP, piperacillin; TZP, piperacillin-tazobactam; CAZ, ceftazidime; FEP, cefepime; ATM, aztreonam; IMP, imipenem; MEM, meropenem; COL, colistin; AMK, amikacin; GEN, gentamicin; TOB, tobramycin; CIP, ciprofloxacin.

reduced susceptibility to imipenem. These data are in accordance with the MBL-producing isolate proportions recently reported for, e.g., Italy and Korea. However, we observed only the presence of the VIM-type MBLs, not the IMP-type MBLs, which are also commonly detected worldwide (11, 23).

All *P. aeruginosa* isolates with reduced susceptibility to imipenem and resistance to ceftazidime were analyzed for genomic relatedness by a randomly amplified polymorphic DNA (RAPD) typing technique using primers 208 and 272 (16). From the banding pattern, a dendrogram using the Ward clustering algorithm was generated based on the Dice coefficients with 0.5% optimization and 0.5% position tolerance for band matching and comparison using the GelComparII software program (Applied Maths, Sint-Martens-Latem, Belgium). RAPD typing with primer 208 revealed that all bla_{VIM-2}-positive isolates clustered together (Fig. 1). The cluster also included the first isolate of the outbreak at the university hospital of the University of Würzburg (PA399) (4). These results were con-

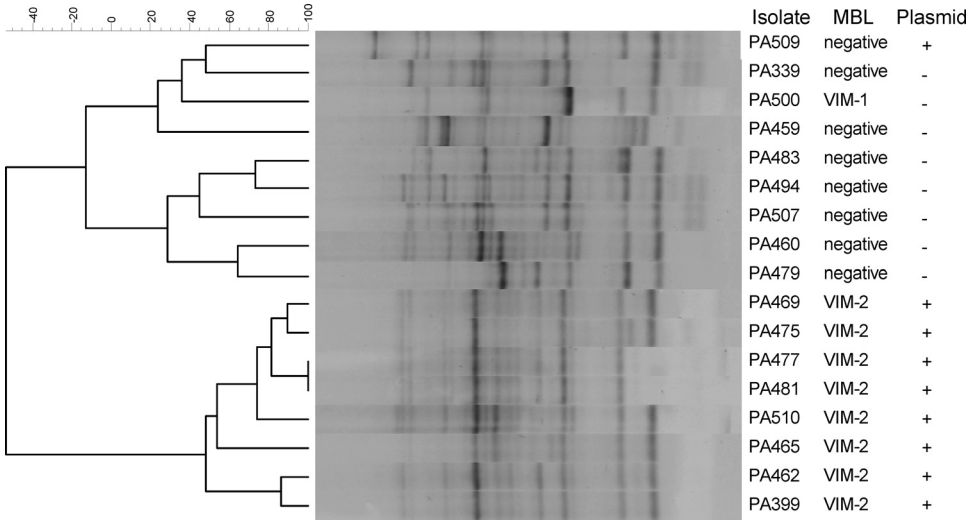

FIG. 1. Genotypic comparison of imipenem-nonsusceptible and ceftazidime-resistant isolates based on the RAPD profile using primer 208. The isolate designation, presence and type of MBL, and presence (+) or absence (−) of a plasmid in the *P. aeruginosa* isolate is shown to the right of the RAPD profile. The clusters are shown to the left of the RAPD profile.

firmed by RAPD typing with primer 272 (data not shown) and suggest a common clonal origin of all bla_{VIM-2}-positive isolates.

Plasmid extraction was attempted from all imipenem-nonsusceptible and ceftazidime-resistant isolates by the alkaline lysis method (24). Extracted plasmid DNA was subsequently subjected to 0.7% agarose gel electrophoresis, followed by ethidium bromide staining, which revealed the presence of a plasmid in all bla_{VIM-2}-positive isolates but not in isolate PA500, which therefore carried the bla_{VIM-1} gene on its chromosome. To further characterize the genetic location of the bla_{VIM-2} genes by the Southern blot technique, total DNA from all imipenem-nonsusceptible and ceftazidime-resistant isolates was subjected to pulsed-field gel electrophoresis (PFGE) using a modified version of the protocol obtained from the home page of the Health Protection Agency of the United Kingdom (http://www.hpa.org.uk/) and hybridized using digoxigenin (DIG)-labeled probes for bla_{VIM-2} and 16S rRNA gene. The probes were generated using the DIG-DNA labeling kit (Roche Diagnostics, Mannheim, Germany) with the purified PCR product amplified with the primer pair VIM2004A and VIM2004B (20) for bla_{VIM-2} and primer pair pc3 and bak for the 16S rRNA gene (7). Hybridization with the bla_{VIM-2}-specific gene probe revealed a band about 160 kb in size in all bla_{VIM-2}-positive isolates, corresponding to a plasmid carrying bla_{VIM-2}. The presence of the plasmid was further confirmed by large-scale preparation of plasmid DNA from isolates PA399 and PA462 using CsCl density gradient ultracentrifugation (24), followed by PFGE of the purified plasmids for size determination.

Furthermore, we successfully performed the conjugative transfer of the bla_{VIM-2} gene from isolate PA462 (MBL positive), which showed meropenem and amikacin MICs of $\geq$16 and 4 μg/ml, respectively, to isolate PA507 (MBL negative), which showed meropenem and amikacin MICs of 4 and 16 μg/ml, respectively. In detail, bacterial suspensions of both isolates in brain heart infusion (BHI) were adjusted to a McFarland standard of 0.5, and 3 ml of each suspension was mixed together and incubated at 37°C for 2 h without shaking. Transconjugant selection was performed on Mueller-Hinton (MH) agar plates containing 6 μg/ml each of meropenem and amikacin. The transconjugant was positive for bla_{VIM-2} as revealed by PCR with primers VIM2004A and VIM2004B. Moreover, RAPD typing using primers 208 and 272 and colony morphology on Mueller-Hinton agar revealed that the transconjugant and the MBL-negative isolate PA507 were geno- and phenotypically closer to each other than to isolate PA462, which clearly demonstrated that the plasmid carrying bla_{VIM-2} was transferred to the MBL-negative isolate PA507. The molecular analyses thus demonstrate the spread of a clone of *P. aeruginosa* harboring bla_{VIM-2} as part of a class 1 integron on a large conjugative plasmid in the geographically and temporarily restricted setting of a German university hospital.

In addition, all isolates with reduced susceptibility to imipenem and resistance to ceftazidime were also subjected to a phenotypic analysis by MBL Etest (AB Biodisk, Solna, Sweden) and EDTA combination disk test. The EDTA combination disk test was performed as previously described (20) using antibiotic disks (Oxoid, Wesel, Germany) containing 10 μg imipenem alone and in combination with 930 μg EDTA. The MBL Etest correctly identified all MBL-positive isolates but falsely identified six of the seven MBL-negative *P. aeruginosa* isolates as MBL positive. In contrast, the EDTA disk test was able to discriminate between all MBL-positive and MBL-negative isolates by using a breakpoint of $\geq$14 mm. Of note, the same test interpreted with a breakpoint of $\geq$7 mm as suggested by Pitout et al. (20) falsely identified all MBL-negative isolates as MBL positive. These data therefore suggest that the optimal breakpoint may depend on the strain collection studied. An initial screening by combined susceptibility testing of imipenem and ceftazidime, followed by a confirmatory EDTA combination disk test, thus represents a valid and less expensive alternative to the molecular investigation of MBL genes. This aspect is particularly important, as it makes MBL detection possible not only in reference laboratories but also in routine diagnostic microbiology laboratories.

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