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Novel Phenotypic Screening Method Accurately Detects Emerging *P. aeruginosa* Metallo-ß-Lactamases (mßl) Isolates in Italy: Report from the SENTRY Antimicrobial Surveillance Program

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ABSTRACT

Background: mßl as a resistance (R) mechanism in multi-drug resistant (MDR) *P. aeruginosa* (PSA) has been documented in Asia, Canada and Europe. However, detection requires molecular studies, often needing extended time frames. Recently an Etest (AB BIODISK) strip was developed for metallo-ß-Lactamase (mßl) diagnosis, a method routinely applied to selected isolates by the SENTRY Program (2000-2002).
Methods: Strains of MDR-PSA were selected by Senda criteria (R to ceftazidime, imipenem and meropenem [MER]) and tested with the Etest mßl strip; imipenem MIC decreases ≥ 8-fold = positive (+) test. 27 candidates from SENTRY Italian sites (Catania, Genoa, Rome) were screened, 21 were mßl (+). These strains were studied by riboprinting, PFGE, PCR primer sets for *imp*, *vim*, and *spm*; enzyme hydrolysis assays and sequencing. MICs were tested by NCCLS methods.
Results: mßl (+) MDR-PSA were detected in all 3 Italian sites, first noted in 2000. Among 16 mßl (+) strains at site A, all were clonally related (ribogroup 1034.2/PFGE pattern A). At site B, 2 of 4 mßl (+) strains were similar and the same as site A. Site C mßl (+) isolates were unrelated (115.5, 219.7), but clones of mßl (-) MDR-PSA were noted. mßl genotypic characterization showed: site A (16 VIM-1), site B (VIM-1), site C (VIM-like). All strains readily hydrolyze imipenem and were sensitive to EDTA inhibition indicating that site C has a novel mßl. Most mßl (+) strains were isolated from patients with pneumonias (45%) > SSTI (27%) > bacteremias (23%); only polymyxins remained active.
Conclusions: The Etest mßl strip was a sensitive and specific test, detecting clonal or novel mßl (VIM-1, VIM-like) strains at 3 hospitals in Italy. These emerging MDR-PSA with associated mßls have reached alarming proportions indicating need for local interventions and development of novel agents.

INTRODUCTION

Metallo-ß-Lactamase (mßl) determinants have been acquired by *P. aeruginosa* and other non-enteric Gram-negative bacilli. These enzymes effectively hydrolyze carbapenems, extended-spectrum cephalosporins and penicillins, and the mßls are not inhibited by clinically-available inhibitors such as clavulanic acid or penicillanic acid sulfones.

The number of mßl enzymes has rapidly expanded to include IMP, VIM, and SPM and their occurrence in clinical isolates appears to be expanding in some geographic regions like Korea, parts of Western Europe and possibly Japan where the IMP-1 was first widely studied. The epidemiology studies, however, require simple and accurate phenotype screens to direct more indepth epidemiologic interventions and molecular characterization. This report summarizes the application of a novel, quantitative mßl screen using the stable gradient technology of the Etest (AB BIODISK, Solna, Sweden). The developmental details of this method have been described by Walsh et al. (JCM 40:2755-2759, 2002). This method was utilized experimentally by the SENTRY Antimicrobial Surveillance Program beginning in late 2000 to detect mßl-producing *P. aeruginosa* and Acinetobacter spp. among the subset of strains that were multi-drug-resistant (resistant to ≥ 4 agents), and additionally met phenotypic criteria used in earlier studies in Japan. The experience with the Etest mßl screen utilized in the testing of strains from Italy is used to document the accuracy of the method.

MATERIALS AND METHODS

Beginning in 2000, the SENTRY Antimicrobial Surveillance Program initiated a protocol to evaluate MDR *P. aeruginosa* including strains suspected of possessing metallo-ß-Lactamases (Figure 1). Strains with MICs of > 8 µg/ml for imipenem and meropenem and a ceftazidime MIC of > 16 µg/ml ("Senda criteria") were tested for metallo-ß-Lactamase expression.

Detection of Metallo-ß-Lactamases. Isolates were plated onto nutrient agar containing 10 µg/ml imipenem and incubated overnight. The following day, several colonies were suspended into sterile deionized water and plated onto Mueller-Hinton agar such that the final inoculum was equivalent to a density of a 1 McFarland standard. An Etest strip (AB BIODISK) was applied that incorporates imipenem with and without EDTA (an inhibitor of metallo-ß-Lactamases) formulated for the specific detection of metallo-ß-Lactamases. If the presence of EDTA reduced the imipenem MIC by ≥ eight-fold, then the organism was categorized as metallo-ß-Lactamase screen-positive.

Confirmatory Test for the Presence of Metallo-ß-Lactamases. Colonies from the plate containing 10 µg/ml imipenem were used to inoculate 10 ml of nutrient broth and grown overnight at 37ºC. The following day, the cells were harvested at 13,000 g by centrifugation, resuspended in 1.5 ml of assay buffer (30 mM cacodylate buffer, pH 7.0, 100 µg/l $ZnCl_2$) and sonicated (Sonics Bira Cell, Basingstoke, UK). The sonicated cells were centrifuged at 13,000 g for 30 minutes at 4ºC. The crude cell extract was examined spectroscopically for its ability to hydrolyze imipenem at 298 nm. The enzyme activity (with and without EDTA) was expressed as nmol substrate hydrolyzed/ml/min/µg protein.

Ribotyping. All the metallo-ß-Lactamase positive phenotypes were processed on the automated Riboprinter® Microbial Characterization System (Qualicon, Wilmington, DE) using PvuII.

Pulsed Field Gel Electrophoresis (PFGE). The *P. aeruginosa* isolates showing same riboprint patterns were subjected to PFGE. Chromosomal DNA digested with SpeI was electrophoresed on 1% agarose (switch times 5 - 90 sec, 24 hr) with a CHEF-DRII instrument (BioRad, Hercules, CA). Ethidium Bromide stained band patterns were compared visually. Strains showing ≤ 3 bands difference were considered to be clonally related.

DNA Sequencing and Sequence Analysis. Sequencing of PCR amplicons was undertaken on both strands by the dideoxy-chain termination method with a Perkin Elmer Biosystems 377 DNA sequencer. Sequence analysis was performed using the Lasergene DNASTAR software package. Alignments and phylogenetic analysis was obtained using Clustal W and PAM 250 matrix.

Oligonucleotide Primers for Sequencing/PCR.

VIM forward	TTATGGAGCAGCAAGCAGTG
VIM reverse	CGAATGCGCAGGACCAGG
IMP forward	ATGAGCAAGTTATCCTTATTC
IMP reverse	GCTGCAACGACTTGTTAG
SPM forward	ATACAGAGCCGAAGGTCGAC
SPM reverse	AACGGCGAAGAGACAATGAC

PCR Conditions. For amplification using IMP, VIM, and SPM primers, PCR was performed using AB-gene Expand Hi-fidelity master mix containing a mix of PFU/non proof reading TAQ polymerases and dNTPs. Primers were used at 10 pmolar concentrations and 1 ml of bacterial culture at density OD 1 at 600nm was used as template. Cycling parameters were: 95ºC for 5 minutes followed by 30 cycles of 95ºC for 1 minute, annealing at 45ºC for 1 minute and extension 68ºC for 1 minute and ending with a 5 minute incubation at 68ºC. PCR products were visualized by electrophoresis on 0.8% agarose gels in Tris Boric Acid/EDTA buffer (pH 7.0) and stained with 1% ethidium bromide as previously described.

RESULTS

- The distribution of the mßl-positive isolates was from several infection types: bacteremia (five episodes; 22.7%), pneumonia (10 episodes; 45.5%), wound infections (six episodes; 27.3%) and one UTI (4.5%).
- The novel Etest mßl screen accurately detected numerous mßl-producing *P. aeruginosa* strains within the SENTRY Program (Table 1). When applied to monitored centers (3) in Italy, 22 mßl strains were detected ranging from 3.3 - 4.1% of *P. aeruginosa* isolates in sites 085 and 086 to 14.8% of isolates in site 075 (average imipenem MIC at ≥ 192 µg/ml and with EDTA at 3 - 16 µg/ml).
- Nearly all Etest mßl screen-positive strains were proven to be metallo-enzymes by hydrolysis and inhibition assays, screening PCR and gene sequencing (VIM-1), 100.0% sensitivity and high specificity (81.5%) in this hospital grouping. One novel mßl was observed in site 086 (characterization in progress).
- Epidemiologically related clusters of mßl-positive and -negative, multi-drug-resistant *P. aeruginosa* isolates were documented including 16 strains in site 075 (ribotype 1034.2/PFGE A, A1, A2). A cluster in site 085 shared the same ribotype with site 075 (1034.2), but was unique by PFGE (Figure 2). All other mßl-positive strains were unique. Three non-mßl epidemic clusters were also noted (Table 2).
- The VIM-1 enzyme was detected in all mßl-positive strains except those from site 086.

Table 1. Screening results and confirmatory tests for metallo-ß-Lactamase-producing strains in Italy (three medical centers in the SENTRY Program, 2000-2001).

Medical center/ organism no.	Imipenem MIC (µg/ml) Alone	+EDTA	Hydrolytic activity Imipenem[a]	+EDTA (%)[b]	PCR screen result *vim*	*imp*	*spm*[c]
075							
3755A	>256	3	315	97	+	-	-
3608C	192	6	410	96	+	-	-
3626C	>256	4	400	96	+	-	-
3632C	>256	4	692	94	+	-	-
3634C	>256	4	496	98	+	-	-
3636C	>256	4	668	95	+	-	-
3647C	>256	4	444	98	+	-	-
3660C	192	6	702	95	+	-	-
3677C	>256	6	400	99	+	-	-
3679C	>256	4	342	96	+	-	-
2451D	>256	6	564	98	+	-	-
2457D	>256	3	324	97	+	-	-
2458D	>256	4	595	99	+	-	-
2459D	>256	3	506	98	+	-	-
2479D	>256	4	428	99	+	-	-
2489D	>256	4	396	94	+	-	-
085							
4744A	>256	3	548	98	+	-	-
14297A	>256	12	668	99	+	-	-
2966C	>256	4	514	98	+	-	-
2394E	>256	4	955	99	+	-	-
086							
10117A	192	8	826	96	-	-	-
14571A	128	16	925	90	-	-	-

a. nmol substrate/ml/min/µg protein.
b. Represents % inhibition in the presence of EDTA.
c. Recently described enzyme from Sao Paulo (SP), Brazil.

Table 2. Epidemiologic investigations of documented mßl-positive strains and multi-drug-resistant *P. aeruginosa* at three medical centers in Italy (SENTRY Program, 2000-2001).

Medical center/ Organism no.	Site of infection	mßl-screen	Pattern for: Ribotype	PFGE	mßl by sequencing
075					
3755[a]	Blood	+	1034.2	A	VIM-1
3608[a]	Lung	+	1034.2	A1	VIM-1
3626[a]	Lung	+	1034.2	A1	VIM-1
3632[a]	Lung	+	1034.2	A1	VIM-1
3634[a]	Lung	+	1034.2	A1	VIM-1
3636[a]	Lung	+	1034.2	A1	VIM-1
3647[a]	Lung	+	1034.2	A1	VIM-1
3660[a]	Lung	+	1034.2	A1	VIM-1
3677[a]	Lung	+	1034.2	A1	VIM-1
3679[a]	Lung	+	1034.2	A2	VIM-1
2451[a]	Wound	+	1034.2	A1	VIM-1
2457[a]	Wound	+	1034.2	A	VIM-1
2458[a]	Wound	+	1034.2	A	VIM-1
2459[a]	Wound	+	1034.2	A1	VIM-1
2479[a]	Wound	+	1034.2	A1	VIM-1
2489[a]	Wound	+	1034.2	A	VIM-1
3610[a]	Lung	-	44.1	B[b]	NT[e]
3620	Lung	-	44.2	C[c]	NT[e]
3628	Lung	-	1418.5	NT	NT[e]
3668	Lung	-	44.1	B[b]	NT[e]
3672	Lung	-	44.2	C[c]	NT[e]
6692	Blood	NT	218.7	D[d]	NT[e]
6700	Blood	NT	218.7	D[d]	NT[e]
085					
4744	Blood	+	1034.2	A	VIM-1
14297	Blood	+	60.2	NT	VIM-1
2966	Lung	+	1034.2	A	VIM-1
2394	Urine	+	45.6	NT	VIM-1
086					
10117	Blood	+	115.5	NT	**[f]
14571	Blood	+	1033.3	NT	**[f]

a. Epidemic cluster of 16 *P. aeruginosa* strains from three infection sites spaning two years. All contained a VIM-1.
b. Epidemic cluster of mßl-negative strains (3616 and 3668)
c. Epidemic cluster of mßl-negative strains (3620 and 3672).
d. Epidemic cluster of mßl-negative strains (6692 and 6700).
e. NT = not tested, did not meet phenotype criteria.
f. Novel enzyme of the mßl type, characterization in progress.

Figure 1: Flow diagram of the criteria used and the phenotypic and genotypic confirmation of resistance mechanisms and epidemiology of multi-drug resistant *P. aeruginosa*.

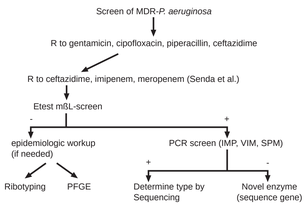
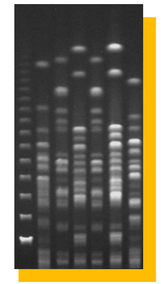


Figure 2: PFGE profiles of carbapenem resistant *P. aeruginosa* showing complimentary results to those of ribotyping within medical center. Lane 1, 48.5 kb λ ladder; lane 2, 3660C (ribotype 1034.2); lane 3, 3616C (44.1); lane 4, 3620C (44.2); lane 5, 3668C (44.1); lane 6, 3672C (44.2); lane 7, 2996C (1034.2). Isolates with ribotypes 44.1 and 44.2 were from the same site (75) and showed identical PFGE patterns. Isolates 3660C (site 75) and 2966C (site 85) shared the same ribotype (1034.2), but quite different PFGE patterns, ruling out the possibility of inter-hospital clonal spread.



CONCLUSIONS

- A simple and accurate phenotypic test (Etest mßl strip) can effectively detect epidemic clusters of mßl-producing *P. aeruginosa* from among other multi-drug-resistant endemic strains.
- Dissemination of mßl-producing *P. aeruginosa* has emerged in Italian medical centers monitored by the SENTRY Program, documented by sequencing to be VIM-1, but a novel type also appeared in Rome.
- Wider use of this screen seems prudent in geographic regions or individual hospitals where metallo-enzymes have been previously described or where multi-drug-resistant non-fermentative Gram-negative bacilli have become endemic.

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