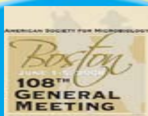



C-281

# Prevalence of metallo-β-lactamases in *Acinetobacter baumannii* clinical isolates: a report from the BORIS project


**J. Block**[1], J. Lavrrar[1], V. Labombardi[2], G. Procop[3], S. Novak-Weekley[4], P. Schreckenberger[5]

[1]bioMérieux, Inc., St. Louis, MO. [2]St. Vincent's Hospital, New York, NY, [3]University of Miami, Miami, FL. [4]SCPMG Regional Reference Laboratories, North Hollywood, CA. [5]Loyola University, Chicago, IL.

Contact info:
Jeanette Block
bioMérieux, Inc.
595 Anglum Dr.
Hazelwood, MO 63042
jeanette.block
@na.biomerieux.com



## ABSTRACT

**Background:** Acquired metallo-β -lactamases (MBLs) are carbapenemases requiring $Zn^{2+}$ for activity. With the exception of aztreonam, MBLs can hydrolyze β-lactams. Therefore, bacterial isolates producing MBLs can be highly resistant to treatment. Levels of MBL-producing *Acinetobacter baumannii* (AB) are increasing in nosocomial infections worldwide, though very few have been reported in the US. The BORIS project (bioMérieux Organism Resistance Surveillance network) was established to collect and study emerging infectious microorganisms from clinical laboratories throughout the US. The purpose of this study is to determine the prevalence of MBLs in clinical isolates of multi-drug resistant AB.

**Methods:** A total of 354 AB isolates were selected from four clinical laboratories representing the Southeast (SE), Southwest (SW), Northeast (NE) and Midwest (MW1) regions of the US. All AB isolates chosen were resistant to ≥3 antibiotic classes. Identity was verified by PCR assay for the *oxa-51* gene. Susceptibility was confirmed using CLSI broth microdilution assays. MBL production was determined using disk diffusion tests, and real-time PCR was used to detect and classify possible MBL genes. Automated rep-PCR was performed to show how isolates are related.

**Results:** AB isolates showed marked resistance to antibiotics tested in broth microdilution assays. Resistance to carbapenems was high overall, with 56% resistant to imipenem and 83% resistant to meropenem. Phenotypic MBL production averaged 16%, with the highest levels in the SW (39%) and the lowest in the SE (1%). Several of these isolates did possess MBL genes, as shown by real-time PCR. However, in a number of "MBL-phenotype" isolates, MBL genes were not detected. The discrepancy between phenotypic and genotypic MBL detection data will be examined. Finally, strain typing shows a high degree of heterogeneity, suggesting that isolates did not result from a single outbreak.

**Conclusion:** Though not widespread, MBL-producing AB are emerging in the US, and further studies of these MBL producing AB are necessary for proper treatment and prevention of spread.


## INTRODUCTION

***Acinetobacter baumannii*: an opportunistic pathogen**

- *Acinetobacter baumannii* (AB) is a common cause of nosocomial infections.
- Multi-drug resistant AB are becoming more prevalent, and levels of carbapenem-resistant AB are increasing dramatically.
- AB have several built-in antibiotic resistance mechanisms. For example, chromosomal AmpC and OXA-51 are commonly found in AB isolates.

**Metallo-β-lactamases (MBLs)**

- MBLs are Class B carbapenemases requiring $Zn^{2+}$ for activity; therefore, these enzymes can be inactivated by EDTA and mercaptopropionic acid (MPA).
- There are five classes of MBLs: VIM-like, IMP-like, SIM-1, SPM-1, and GIM-1.
  - VIM-like and IMP-like are the most common and have many variants.
  - SIM-1 (Korea), SPM-1 (Brazil), and GIM-1 (Germany) have only been found abroad (1).
- MBLs are rare in North America (1).
  - IMP-like—found in Mexico and New Mexico.
  - VIM-like—found in Canada and Texas.

**Purpose of this study**

- The **b**io**M**érieux **O**rganism **R**es**i**stance **S**urveillance network (BORIS) was established to collect currently significant microorganisms from US clinical laboratories in order to study their antibiotic resistance patterns and possible resistance mechanisms utilized.
- AB isolates were collected and tested for the presence of MBLs so as to establish the incidence of MBLs in the United States.

## METHODS

**AB isolate submission.** A total of 354 AB isolates, resistant to ≥3 classes of antibiotics, were sent to BORIS from four clinical laboratories representing the midwest (MW1), northeast (NE), southeast (SE), and southwest (SW) regions of the US. Antimicrobial resistance was determined at each site using VITEK® and VITEK2® susceptibility cards.

**Broth microdilution assays.** BMD microplates were made onsite at bioMérieux, using Mueller Hinton broth and varying concentrations of antibiotics, following the procedure outlined in CLSI publication M7-A7 (2). Interpretive standards for tigecycline were based on FDA breakpoints for *Enterobacteriaceae*. All other interpretations (S=Susceptible, I=Intermediate, R=Resistant) were assigned using CLSI breakpoints (3). For quality control, *E. coli* ATCC 25922, *E. coli* ATCC 35218, and *P. aeruginosa* ATCC 27853 were also assayed with each run.

**Multiplex PCR for OXA genes.** Multiplex PCR for detection of OXA-51, OXA-23, and Int1 was performed as previously described (4). Briefly, genomic DNA was isolated using the UltraClean Microbial DNA Isolation Kit (Mo Bio Laboratories, Inc., Carlsbad, CA). DNA was used in 25 μl reactions at a final concentration of 25ng/μl. Reactions also contained 200 μM each dNTP, 1.5 U Taq polymerase, 1X Taq Buffer, and 0.25 pmol each of primers OXA-51-like-F, OXA-51-like-R, OXA-23-like-F, OXA-23-like-R, Int1F, and Int1R. Cycling reactions were carried out as follows: 94°C 3 min, followed by 35 cycles of 94°C 45s, 57°C 45s, 72°C 1 min, followed by 5 min extension at 72°C. Reaction products were examined on Egels (Invitrogen Corp., Carlsbad, CA).

## METHODS

**MBL phenotypic tests.** Phenotypic tests for MBL production were performed as previously described (5). A 1:1 dilution of 100X Tris-EDTA (Sigma, St. Louis, MO) was prepared in $dH_2O$ and 20 μl was then added to blank disks. Disks were dried overnight. A 0.5 MacFarland suspension of test organism was inoculated on Mueller-Hinton plates. A 10 μg IPM disk (BD, Sparks, MD) was added to the center of the lawn. Tris-EDTA disks were rehydrated with 20 μl of a 1:320 dilution of mercaptopropionic acid (MPA, Sigma, St. Louis, MO) in $dH_20$, and the disk was placed exactly 10 mm from the IPM disk. Plates were incubated overnight at 35°C, and results were read the following day. A positive result was indicated as a shift in the zone of inhibition toward the MPA/EDTA disk.

**Real-time PCR for MBL genes.** Genomic DNA was prepared from each isolate using the UltraClean Microbial DNA Isolation Kit following manufacturer's instructions. Resulting DNA was used in three separate reactions for MBL detection, adapting a previously published procedure using multiplex real-time PCR for MBL detection (6). All reactions were run on the Roche LightCycler 2.0, and results were analyzed using the LightCycler 4.0 program. Protocols for the reactions are as follows: (1) VIM-like/IMP-like detection: 0.5 ng DNA was added to 20 μl reactions containing 5 mM $MgCl_2$, 0.1 μM primers VIMgen-F1 and VIMgen-R1, 1 μM primers IMPgen-F1 and IMPgen-R1, and 2 μl of 1X LightCycler FastStart DNA Master SYBR Green I. Samples were pre-incubated at 95 °C for 10 minutes, followed by 28 cycles of 95° C 15s, 56 °C 5s, and 72 °C 30s. (2) SIM/GIM/SPM detection: 0.5 ng DNA was added to 20 μl reactions containing 6 mM $MgCl_2$, 2 μl of 1X LightCycler FastStart DNA Master SYBR Green I, and 0.1 μM each of primers SIM-F1, SIM-R1, SPM-F1, SPM-R1, GIM-F1, and GIM-R1. Cycling conditions were as follows: 95°C for 10 min, followed by 30 cycles of 95°C 15s, 60°C 5s, and 72°C 25s. (3) 16S control for DNA quality: 0.5 ng DNA was added to 20 μl reactions containing 4 mM $MgCl_2$, 2 μl of 1X LightCycler FastStart DNA Master SYBR Green I, and 0.1 μM each of primers 16SF and 16SR. Cycling conditions were as follows: 95°C for 10 min, followed by 25 cycles of 95°C 15s, 54°C 5s, and 72°C 40s. DNA from strains *P. aeruginosa* 111615 (IMP-2), *E. coli* 111564 (VIM-1), *A. baumannii* 03-9-T/04 (SIM-1), and *P. aeruginosa* 111612 (SPM-1) was used as positive controls in the appropriate reactions. *E. coli* 25922 DNA was used as negative control.

**Strain typing.** Genomic DNA was prepared using the UltraClean Microbial DNA Isolation Kit following the manufacturer's protocol. Strain typing was performed using the DiversiLab™ Acinetobacter kit for automated rep-PCR (Bacterial BarCodes, Inc., Athens, GA). PCR reactions were run according the kit protocol, and 1 μl of resulting product was analyzed using DiversiLab™ LabChip kits (Bacterical BarCodes), following manufacturer's instructions. Chips were run on an Agilent Bioanalyzer, and results were analyzed using DiversiLab™ software version 3.3. Isolates were classified as indistinguishable (no band difference), similar (1-2 band differences), or different (3+ band differences).

**TABLE 1. Primers used in this study**

| PRIMER | PURPOSE | SEQUENCE |
|---|---|---|
| IMPgen-F1 | IMP-like detection | 5´-GAA TAG RRT GGC TTA AYT CTC-3´ |
| IMPgen-R1 | IMP-like detection | 5´-CCA AAC YAC TAS GTT ATC-3´ |
| VIMgen-F1 | VIM-like detection | 5´-GTT TGG TCG CAT ATC GCA AC-3´ |
| VIMgen-R1 | VIM-like detection | 5´-AAT GCG CAG CAC CAG GAT AG-3´ |
| GIM-F1 | GIM-1 detection | 5´-TCA ATT AGC TCT TGG GCT GAC-3´ |
| GIM-R1 | GIM-1 detection | 5´-CGG AAC GAC CAT TTG AAT GG-3´ |
| SIM-F1 | SIM-1 detection | 5´-GTA CAA GGG ATT CGG CAT CG-3´ |
| SIM-R1 | SIM-1 detection | 5´-TGG CCT GTT CCC ATG TGA G-3´ |
| SPM-F1 | SPM-1 detection | 5´-CTA AAT CGA GAG CCC TGC TTG-3´ |
| SPM-R1 | SPM-1 detection | 5´-CCT TTT CCG CGA CCT TGA TC-3´ |
| 16SF | Quality control | 5'-AGA GTT TGA TCC TGG CTC AG-3' |
| 16SR | Quality control | 5'-GCC AGC AGC CGC GGT AAT-3' |

## RESULTS

- AB isolates collected in this study are highly resistant to many antibiotics:
  - Carbapenem resistance: 56% R to IPM; 83% R to MER.
  - ≥87% resistant to all β-lactams and fluoroquinolones tested.
  - Moderate resistance to GM (55% overall).
  - Only susceptible to PB and TGC.
- Inhibition of growth by EDTA/MPA (a phenotypic test for MBL production):
  - 17% AB show reduced growth near EDTA-MPA disk.
  - Highest levels in SW (39%); lowest in SE (1%).
- Real-time PCR shows that only 2 isolates possess MBL genes (IMP-like genes); neither isolate was shown as positive in phenotypic tests.
  - IPM resistance in both isolates was very high; therefore, no zone of inhibition seen in phenotypic test.
- Strain typing shows that AB isolates are quite diverse; MBL-producers (NE31 and NE101) are not related, though were isolated only two months apart.

**TABLE 2. *Acinetobacter baumannii* broth microdilution antibiogram**

| Site | n | GM %S | GM %I | GM %R | IPM %S | IPM %I | IPM %R | MEM %S | MEM %I | MEM %R | TZP %S | TZP %I | TZP %R | CRO %S | CRO %I | CRO %R |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| NE | 147 | 54 | 19 | 27 | 27 | 46 | 27 | 10 | 7 | 83 | 5 | 18 | 77 | 1 | 5 | 93 |
| SW | 86 | 12 | 12 | 76 | 51 | 26 | 23 | 38 | 9 | 53 | 4 | 8 | 88 | 0 | 2 | 98 |
| SE | 95 | 26 | 4 | 69 | 1 | 2 | 97 | 1 | 0 | 99 | 1 | 1 | 98 | 0 | 3 | 97 |
| MW1 | 26 | 0 | 8 | 92 | 8 | 15 | 77 | 4 | 0 | 96 | 0 | 0 | 100 | 0 | 8 | 92 |
| TOTAL | 354 | 32 | 12 | 55 | 24 | 28 | 56 | 14 | 5 | 83 | 3 | 10 | 87 | 1 | 4 | 95 |
| MIC breakpoints | | ≤ 4 | 8 | ≥ 16 | ≤ 4 | 8 | ≥ 16 | ≤ 4 | 8 | ≥ 16 | ≤ 16 | 32-64 | ≥ 128 | ≤ 8 | 16-32 | ≥ 64 |

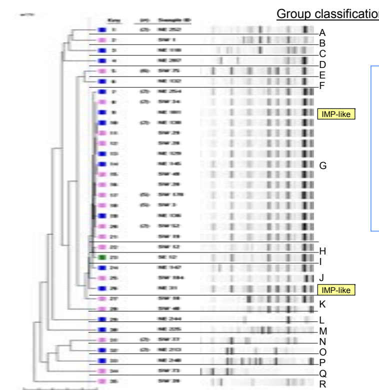
| Site | n | CAZ %S | CAZ %I | CAZ %R | FEP %S | FEP %I | FEP %R | CIP %S | CIP %I | CIP %R | PB %S | PB %I | PB %R |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| NE | 147 | 7 | 1 | 92 | 8 | 5 | 87 | 5 | 0 | 95 | 100 | 0 | 0 |
| SW | 86 | 2 | 0 | 98 | 10 | 14 | 76 | 0 | 1 | 99 | 56 | 17 | 27 |
| SE | 95 | 2 | 4 | 94 | 1 | 0 | 99 | 1 | 0 | 99 | 97 | 0 | 3 |
| MW1 | 26 | 4 | 0 | 96 | 4 | 8 | 88 | 0 | 0 | 100 | 96 | 0 | 4 |
| TOTAL | 254 | 4 | 2 | 94 | 6 | 6 | 88 | 3 | 0 | 97 | 99 | 0 | 1 |
| MIC breakpoints | | ≤ 8 | 16 | ≥ 32 | ≤ 8 | 16 | ≥ 32 | ≤ 1 | 2 | ≥ 4 | ≤ 2 | | ≥ 4 |

GM=Gentamicin; IPM=Imipenem; MEM=Meropenem; TZP=Piperacillin/Tazobactam; CRO=Ceftriaxone; CAZ=Ceftazidime; FEP=Cefepime; CIP=Ciprofloxacin; PB=Polymyxin B

**TABLE 3. Phenotypic testing for metallo-β-lactamase production**

| PHENOTYPIC RESULT | NE # in region | NE % in region | SW # in region | SW % in region | SE # in region | SE % in region | MW1 # in region | MW1 % in region | TOTAL Total # | TOTAL % of Total |
|---|---|---|---|---|---|---|---|---|---|---|
| MBL positive | 21 | 14.3% | 34 | 39.4% | 1 | 1.1% | 0 | 0% | 56 | 15.8% |
| MBL negative | 126 | 85.7% | 52 | 60.6% | 94 | 99.0% | 26 | 100% | 298 | 84.2% |
| TOTAL | 147 | 100% | 86 | 100% | 95 | 100% | 26 | 100% | 354 | 100% |

**FIGURE 1. Representative dendrogram of MBL phenotypic and genotypic positive AB isolates using automated rep-PCR**




- Isolates that tested positive for MBL production in phenotypic tests,as well as isolates genotypically positive for IMP-like genes, were typed.
- This dendrogram is representative of 58 "MBL-positive" isolates.
- These isolates were classified into 18 distinct groups.

**TABLE 4. MBL detection using real-time PCR**

| | MBL Positive # in region | MBL Positive % in region | MBL Negative # in region | MBL Negative % in region | False positives | False negatives |
|---|---|---|---|---|---|---|
| NE | 2 | 1.4 | 145 | 98.6 | 21 | 2 |
| SW | 0 | 0 | 86 | 100 | 34 | 0 |
| SE | 0 | 0 | 95 | 100 | 1 | 0 |
| MW1 | 0 | 0 | 26 | 100 | 0 | 0 |
| TOTAL | 2 | 0.60% | 352 | 99.40% | 56 | 2 |

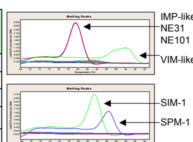
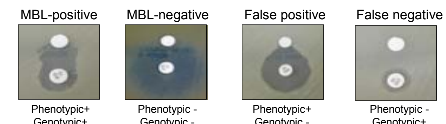



**FIGURE 2. Phenotypic classifications based on genotypic confirmation**




## CONCLUSIONS

- MBLs are not common in AB isolates in US.
- Though AB isolates collected in this study are resistant to carbapenems, only 0.6% have MBL genes for resistance.
- Inconsistency between phenotypic and genotypic testing for MBL presence can be explained:
  - FALSE NEGATIVES: Isolates negative in the phenotypic test but positive in the genotypic test were highly resistant to IPM. Therefore no zone of inhibition was produced in the phenotypic test and the results were incorrectly scored as "negative".
  - FALSE POSITIVES: Isolates positive in the phenotypic test but negative in the genotypic test showed only a minor shift in the zone of inhibition toward the MPA/EDTA disk.
    - "Shift" not as dramatic as that seen for positive control.
    - MPA/EDTA could be slightly inhibiting another enzyme needed for growth.
    - Possibly an unidentified MBL.
- Genotypic testing is highly recommended for detection of MBL production.
- Genotypic testing should be performed on isolates showing high resistance to IPM in phenotypic tests (i.e. no zone of inhibition produced).

## REFERENCES


1. Queenan, A.M. and K. Bush. 2007. Carbapenemases: the versatile β-lactamases. Clin Microbiol Rev. 20: 440-58.
2. Clinical and Laboratory Standards Institute. 2007. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; Approved standard – 6th ed. CLSI document M7-A7.
3. Clinical and Laboratory Standards Institute. 2008. Performance Standards for antimicrobial susceptibility testing; 18th information supplement. CLSI document M100-S18.
4. Turton, J.F., N. Woodford, J. Glover, S. Yarde, M.E. Kaufmann, and T.L. Pitt. 2006. Identification of *Acinetobacter baumannii* by detection of the blaOXA51-like carbapenemase gene intrinsic to this species. J. Clin. Microbiol. 44: 2974-2976.
5. Hong, S.G., S.Y. Kim, E.S. Moland, and K.S. Thompson. 2006. Tris-EDTA (TE) disk test for detection of metallo-β-lactamases (MBLs) in clinical isolates. 46th Intersci. Conf. Antimicrob. Agents Chemother, poster #D-445.
6. Mendes RE, Kiyota KA, Monteiro J, Castanheira M, Andrade SS, Gales AC, Pignatari AC, Tufik S. 2007. Rapid detection and identification of metallo-beta-lactamase-encoding genes by multiplex real-time PCR assay and melt curve analysis. J. Clin. Microbiol. 45: 544-7.