FIRST REPORT OF METALLO-β-LACTAMASES PRODUCING Enterobacter spp. STRAINS FROM VENEZUELA

Dianny MARTÍNEZ(1,2), Hectorina E. RODULFO(1), Lucy RODRÍGUEZ(2), Luisa E. CAÑA(2), Belkis MEDINA(2), Militza GUZMAN(3), Numirin CARREÑO(1), Daniel MARCANO(4) & Marcos DE DONATO(1)

SUMMARY

Clinical strains of Enterobacter were isolated from Cumana’s Central Hospital in Venezuela, and classified as E. cloacae (21), E. aerogenes (7), E. intermedium (1), E. sakazakii (1) and three unclassified. The strains showed high levels of resistance, especially to SXT (58.1%), CRO (48.8%), CAZ (46.6%), PIP (46.4%), CIP (45.2%) and ATM (43.3%). This is the first report for South America of bla\textit{VIM} in two E. cloacae and one Enterobacter sp., which also showed multiple mechanisms of resistance. Both E. cloacae showed bla\textit{TEM-1}, but only one showed bla\textit{CTX-M-15} gene, while no bla\textit{SHV} was detected.

KEYWORDS: Carbapenemase; Metallobeta-lactamase; VIM; Enterobacter; Carbapenems.

INTRODUCTION

Species of the genus Enterobacter have been reported as an important source of intrahospital infections, especially those showing resistance to beta-lactams by the production of enzymes like extended spectrum beta-lactamases (ESBL) such as TEM, SHV, CTX, VEB, and carbapenemases such as VIM, KPC and GES. This represents an important therapeutic challenge because of the few remaining treatment options, which gives rise to morbimortality and hospital expenses.

There are reports of Enterobacter strains producing metallo-β-lactamase (MBL) in different parts of the world, such as E. cloacae in Japan, Taiwan, Korea and Italy, as well as E. aerogenes in Japan and France. However, no MBL-producing strains of Enterobacter have been reported anywhere in the Americas, except in Mexico and Argentina.

METHODS

During August 2010 and March 2011, clinical strains of Enterobacter were isolated in the University Hospital Antonio Patricio de Alcala in Cumana, Venezuela. The use of the strains was approved by the patients or their relatives, according to the recommendations of the Bioethics and Biosecurity Committee of IIBCA, Universidad de Oriente, Cumana, Venezuela.

Isolated strains were inoculated in BHI broth, incubated for 12 hours at 37 °C and later in MacConkey agar for 24 hours, in order to evaluate the morphological characteristics of the colonies to verify purity. For the classification of Enterobacter species tests for the fermentation of glucose and lactose in Kliger medium, use of citrate, arginine and malonate, use of MIO, LIA, and methyl red and Voges-Proskauer medium according to standard biochemical tests established for Enterobacteriaceae.

Antimicrobial susceptibility was assessed by Kirby-Bauer disk diffusion susceptibility test following the recommendations of the Clinical and Laboratory Standards Institute (CLSI). For tigecycline (TGC), we used the cutoff of the attached insert (Pfizer, INC).

Screening of extended spectrum beta-lactamases (ESBL), were carried out using the synergy effect between the antimicrobial disks CAZ, FEP, CRO, ATM and CTX surrounding AMC as well as the confirmatory test for ESBL was carried out using the combined disc test. Additionally, in order to detect the presence of ESBL enzymes we used the modification suggested by SONG et al. (2007), in order to avoid the masking effect that a derepressed AmpC gene could produce. For this, disks containing CAZ (30 μg) with and without clavulanic acid (10 μg) were added 3-aminophenyl boronic acid with a final amount of 400 μg.

The phenotypic detection of MBLs was carried out using IPM and MER disks on each side of a disk with ethylendiaminotetraacetic acid-sodium mercaptoacetate (EDTA-SMA, 0.5 μmoles-3 μg) and the combined disc test (IMP, IMP-EDTA and MER, MER-EDTA).

DNA extraction was carried out using the Wizard Genomic DNA kit (Promega) from the strains isolated after incubation in LB broth for 20 hours at 37 °C. The \textit{bla\textit{VIM}} gene was detected by PCR according
to MENDEZ et al.10. Additionally, we detected the type 1 and 2 bla<sub>VM</sub> according to FIETT et al.7. The genes bla<sub>CTX-M</sub> (EDELSTEIN et al. unpublished results, http://www.antibiotic.ru/en/pdfs/006-51.pdf), bla<sub>TEM</sub> and bla<sub>SHV</sub>12 were also detected. Finally, in order to determine the clonality of the three Enterobacter strains showing a phenotype consistent with MBL by the IMP/MER/EDTA synergy test, we used ERIC-PCR13.

RESULTS

The 33 strains of Enterobacter isolated were classified as E. cloacae (21), E. aerogenes (7), E. intermedium (1), E. sakazakii (1) and three were not possible to classify.

Ten of the strains were from infections acquired outside the hospital and most of the intrahospitalary strains were isolated in ICU (5), internal medicine areas A and B (4), soft surgery hall (4), pediatric area (3) and neonatology (3).

Antimicrobial susceptibility tests show high levels of resistance in most of the strains, with resistance to SXT (58.1%), CRO (48.8%), CAZ (46.6%), PIP (46.4%), CIP (45.2%) and ATM (43.3%) being the highest (Fig. 1). However, all the strains were sensitive to TGC. Results of the synergy test between CAZ/FEP/CTX/ATM with AMC in 16 of the strains were compatible with ESBL enzymes. We found that these strains showed the typical phenotypic effect for ESBL enzymes when using the combined disc test as a confirmatory. Additionally, three of the Enterobacter strains (two E. cloacae and one Enterobacter sp.) were resistant to carbapenems showing also synergy between IPM/MER and EDTA, typical of MBL enzymes (Table 1). ERIC-PCR patterns show no similarities among these strains. They showed resistance to multiple families of antibiotics (MDRs) and two of them also showed presence of ESBL by the synergy assay. These strains amplified for bla<sub>VM</sub> fragments (801 bp). Furthermore, both strains of E. cloacae amplified the typical fragment of the bla<sub>TEM</sub> gene (1080 bp), but only one of them amplified the fragment of the bla<sub>CTX-M</sub> gene (543 bp), while no bla<sub>SHV</sub> gene was detected.

Sequencing of the fragment of the bla<sub>VM</sub> gene, amplified using primers for type 2, produced sequences 100% homologous to bla<sub>VM-2</sub> reported in the GenBank in P. aeruginosa and other bacteria. Also, the sequences of the bla<sub>TEM</sub> gene were 100% homologous to type 1 reported for many Enterobacteria. In addition, the fragment of the bla<sub>CTX-M</sub> gene sequenced showed 100% homology with CTX-M-15 found in E. coli and other Enterobacteria.

DISCUSSION

According to our phenotypic tests, BLEA and ESBL-type of enzymes was very prevalent. On the other hand, we are not aware of previous reports of the presence of VIM-producing Enterobacter strain in any South American country. In Mexico, strains of E. cloacae

Table 1

Resistance pattern and epidemiological data of the three strains of Enterobacter showing bla<sub>VM</sub> type 2 MBLs

| Species          | Isolation date | Hospital area  | Type of sample      | Resistance pattern                      | Synergy tests | Detected genes   |
|------------------|----------------|----------------|---------------------|-----------------------------------------|---------------|------------------|
| E. cloacae       | August 2010   | Soft Surgery   | catheter            | CAZ, FEP, IPM, MER, TZP, PIP, CRO, ATM, SXT, AK, GM | ESBL, MBL     | bla<sub>VM-2'</sub>, bla<sub>TEM-1'</sub>, bla<sub>CTX-M-15</sub> |
| Enterobacter sp. | March 2011    | ICU            | bronchial secretion | CAZ, FEP, IPM, MER, TZP, PIP, CRO, ATM, SXT, CIP | AmpC, MBL     | bla<sub>VM-2</sub> |
| E. cloacae       | April 2011    | Internal Medicine B | urine               | CAZ, FEP, IPM, MER, TZP, PIP, CRO, ATM, SXT, CIP | ESBL, MBL     | bla<sub>VM-2'</sub>, bla<sub>TEM-1</sub> |

ESBL: extended-spectrum betalactamase, MBL: metallo-β-lactamase, AmpC: de-repression of the chromosomal AmpC gene. ICU: intensive care unit. Acronyms of antibiotic as shown in the legend of Fig. 1.
have been shown to produce MBL. These strains produced bla VIM-2 MBPs, the same type we found in this study. In Argentina, one strain of E. cloacae was reported containing bla SHV gene, along with bla TEM and genes that confer resistance to aminoglycosides and quinolones. This type of gene has been reported in Venezuela but only in strains of Pseudomonas aeruginosa and Klebsiella pneumoniae. VIM-producing P. aeruginosa strains were previously found in Cumana hospital (ENSONY TOVAR & MARCOS DE DONATO, unpublished results). It seems very likely that the gene found in P. aeruginosa could have been transferred through mobile elements such as plasmid and/or integrons between these two species which are sharing the same environment, as previously reported, making possible the spread of this gene to many other bacteria species causing infection in this hospital environment.

The presence of multiple mechanisms of resistance in the bacteria isolated in the Cumana hospital causing intrahospital infections, especially in species of Enterobacter, which have natural resistance to several antibacterial drugs, suggests that more efficient preventive measures must be put in place in this hospital to avoid the survival and transmission of these strains. However, all the strains were susceptible to ticarcillin, making it a suitable treatment for infections caused by MBL-producing enzymes in this hospital. This result agrees with numerous reports describing the use of ticarcillin to treat infections caused by multidrug resistant bacteria, including those producing carbapenemases.

**AUTHOR CONTRIBUTIONS**

DM, LR and LC isolated the strains. HER, DM and MDD carried out the molecular analysis, BM, MG and NC helped in the bacteriological analysis. DM, HER and MDD wrote the manuscript and everyone reviewed the manuscript.

**RESUMEN**

Primero reporte de cepas de Enterobacter spp productoras de metalobeta-lactamasas de Venezuela

Cepas clínicas de Enterobacter fueron aisladas del Hospital Central de Cumaná en Venezuela, y se clasificaron como E. cloacae (21), E. aerogenes (7), E. intermedium (1), E. sakazakii (1) y 3 sin clasificar. Las cepas mostraron altos niveles de resistencia, especialmente a SXT (58.1%), CRO (48.8%), CAZ (46.6%), PIP (46.4%), CIP (45.2%) y ATM (43.3%). Este es el primer reporte de América del Sur de bla VIM-2 en dos cepas de E. cloacae y una de Enterobacter sp., las cuales también mostraron múltiples mecanismos de resistencia. Ambas especies de E. cloacae mostraron genes bla VIM-1 pero solo una mostró el gen bla CTX-M-15 mientras que bla SHV no fue detectado.

**REFERENCES**

1. Betriu C, Rodríguez-Avial I, Gómez M, Culebras E, López F, Alvarez J, et al. Antimicrobial activity of tigecycline against clinical isolates from Spanish medical centers. Second multicenter study. Diagn Microbiol Infect Dis. 2006;56:437-44.

2. Falcone M, Mezzatesta ML, Perilli M, Forcella C, Giordano A, Calso V, et al. Infections with VIM-1 metallo-beta-lactamase-producing Enterobacter cloacae and their correlation with clinical outcome. J Clin Microbiol. 2009;47:3514-9.

3. Fiet J, Baraniak A, Mróžka A, Fleischer M, Drulis-Kawa Z, Naumiuk L, et al. Molecular epidemiology of acquired-metallo-beta-lactamase-producing bacteria in Poland. Antimicrob Agents Chemother. 2006;50:880-6.

4. Gómez S, Rapoport M, Togneri A, Viegas-Caetano J, Faccone D, Corso A, et al. Emergence of metallo-beta-lactamasas in Enterobacteriaceae from Argentina. Diagn Microbiol Infect Dis. 2011;69:94-7.

5. Guevara A, de Waard J, Araque M. Deteción del gen bla VIM-2 en cepas de Pseudomonas aeruginosa productoras de metalo-beta-lactamasa aisladas en una unidad de cuidados intensivos en Ciudad Bolívar, Venezuela. Rev Clin Infect. 2009;26:336-41.

6. Lee K, Lim Y, Yong D, Yun J, Chong Y. Evaluation of the Hodge test and the imipem-EDTA double-disc synergy test for differentiating metallo-beta-lactamase-producing isolates of Pseudomonas spp. and Acinetobacter spp. J Clin Microbiol. 2003;41:4623-9.

7. Lezama L., González-Escalante E, Tamazir LH. Comparación de cuatro métodos fenotípicos para la detección de beta-lactamasas de espectro extendido. Rev Peru Med Exp Salud Publica. 2010;27:345-51.

8. MacFaddin JE. Biochemical tests for identification of medical bacteria. 3rd ed. Lippincott Williams & Wilkins; 2000.

9. Marcano D, Pasterán F, Rapoport M, Faccone D, Ugarte C, Salgado N, et al. First isolation of a VIM-producing Klebsiella pneumoniae from a seven-year-old child in Venezuela. J Infect Dev Ctries. 2008;2:241-4.

10. Mendes RE, Kiyota KA, Monteiro I, Castanheira M, Andrade SS, Gales AC, et al. Rapid detection and identification of metallo-beta-lactamase-encoding genes by multiplex real-time PCR assay and melt curve analysis. J Clin Microbiol. 2007;45:544-7.

11. Morfin-Otero R, Rodriguez-Noriega E, Deshpande LM, Sader HS, Castanheira M. Dissemination of a bla VIM-2-carrying integron among Enterobacteriaceae species in Mexico: report from the SENTRY Antimicrobial Surveillance Program. Microb Drug Resist. 2009;15:33-5.

12. Quinteros M, Radice M, Gardella N, Rodriguez MM, Costa N, Koebenfeld D, et al. Extended-spectrum beta-lactamasas in Enterobacteriaceae in Buenos Aires, Argentina, public hospitals. Antimicrob Agents Chemother. 2003;47:2864-7.

13. Reboli AC, Houston ED, Monteforte JS, Wood CA, Hamill RJ. Discrimination of epidemic and sporadic isolates of Acinetobacter baumannii by repetitive element PCR-mediated DNA fingerprinting. J Clin Microbiol. 1994;32:2635-40.

14. Sader HS, Castanheira M, Mendes RE, Toleman M, Walsh TR, Jones RN. Dissemination and diversity of metallo-beta-lactamases in Latin America: report from the SENTRY Antimicrobial Surveillance Program. Int J Antimicrob Agents. 2005;25:57-61.

15. Song W, Bae I, Lee YN, Lee CH, Lee SH, Jeong SH. Detection of extended-spectrum beta-lactamases by using boronic acid as AmpC beta-lactamase inhibitor in clinical isolates of Klebsiella spp. and Escherichia coli. J Clin Microbiol. 2007;45:1180-4.

16. Winn WC, Allen S, Janda W, Koneman E, Procop G, Schreckenberger P, et al. Koneman’s color atlas and textbook of diagnostic microbiology. 6th ed. Bloomingtn: Lippincott Williams & Wilkins; 2005.