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Molecular identification of multidrug resistant *Enterobacter hormaechei* in Venezuela.

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Key words: *Enterobacter hormaechei*; 16S rRNA; KPC; VIM.

Abstract. Besides the importance of *Enterobacter cloacae* species complex as a nosocomial pathogen, little is known about the frequency of each species/genotype. Here, we describe a strain of *E. hormaechei subsp. hormaechei* isolated from a bronchial secretion of a patient, in the Intensive Care Unit at the General Hospital of Cumaná, Venezuela, who died due to complications of his infection. The molecular identification was done by sequencing the 16S rRNA gene and comparing it to sequences from the GenBank. This strain showed resistance to multiple families of antibiotics (MDR), and the genes blaKPC and blaVIM were detected by PCR. This is the first time *E. hormaechei* has been identified in Venezuela.

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Identificación molecular de *Enterobacter hormaechei* multidrogoresistente en Venezuela.

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Palabras clave: *Enterobacter hormaechei*; ARNr 16S; KPC; VIM.

Resumen. A pesar de la importancia de las especies del complejo *Enterobacter cloacae* como patógeno nosocomial, poco se conoce sobre la frecuencia de cada especie/genotipo. Aquí se describe una cepa de *E. hormaechei subsp. hormaechei* aislada de una secreción bronquial de un paciente internado en la Unidad de Cuidados Intensivos del Hospital General de Cumaná, Venezuela, quien murió producto de complicaciones de su infección. La identificación molecular fue hecha por secuenciación del gen ARNr 16S y porcomparación con las secuencias del GenBank. Esta cepa mostró resistencia a múltiples familias de antibióticos (MDR) y se detectaron los genes blaKPCyblaVIMpor PCR. Este es el primer reporte de *E. hormaechei* en Venezuela.

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INTRODUCTION

The species of the *Enterobacter* cloacae complex are considered as emerging pathogens, show genomic heterogeneity and comprise six species: *E. cloacae, E. hormaechei, E. asburiae, E. kobei, E. ludwigii and E. nimipressuralis*, out of which, the first two are the most frequently isolated from clinical samples (1). Phenotypic identification of all species belonging to this taxon is usually difficult and not always reliable; therefore, molecular methods are often used, and despite the importance of the species complex as a nosocomial pathogen, little is known in the contribution of each species/genotype as an infectious agent (2).

E. hormaechei consists of three different subspecies: *E. hormaechei* subsp. *oharae*, *E. hormaechei* subsp. *hormaechei* and E. *hormaechei* subsp. *steigerwaltii*, and their differentiation could be based on their particular properties and biochemical tests (3). Most studies on antimicrobial susceptibility are focused on *E. cloacae, E. hormaechei* and *E. asburiae*; these studies reported small variations between the species (2). The most important resistance mechanism against β-lactam drugs is the production of carbapenemases in *E. hormaechei* (4).

Clinical history

A strain of *Enterobacter sp.* was isolated from a bronchial secretion of a 52 years-old male patient at the Intensive Care Unit of the General Hospital of Cumaná, Venezuela. This patient was admitted to the Surgery Unit Specialized in Soft Tissue after an exploratory laparotomy to perform an appendectomy. After complications due to an infection, he was transferred to the Intensive Care Unit, and died after28 days of hospitalization. The autopsy showed as cause of death, abdominal sepsis, necrohemorrhagic pancreatitis, lower respiratory tract infection and urinary infection. No species identification was possible by biochemical tests at the time of the diagnosis.

Laboratory results

Biochemical tests showed that the strain grew on media supplemented with sucrose, lactose, sorbitol, maltose, mannitol and malonate, but did not grew on media supplemented with inositol. It did not produce indole, H2S, nor showed the presence of urease, ornithine or lysine decarboxylase. It did show the presence of citrate and arginine decarboxylase (although it showed a weak reaction), and showed motility in SIM-agar at 37°C. The biochemical tests agreed with the identification of *E. hormaechei*, but it had an aninfrequent result for urease, as 83% of the strains have been reported to show positive results (3).The *Enterobacter sp.* strain showed resistance to the following: ampicillin (AMP), amoxicillin-clavulanate (AMC), cefalotin (CF), piperacillin (PIP), piperacillin-tazobactam (TZP), cefuroxime (CXM), cefoxitin (FOX), cefotaxime (CTX), ceftriaxone (CRO), aztreonam (ATM), ceftazidime (CAZ), cefepime (FEP), imipenem (IPM), meropenem (MEM), ertapenem (ETP), trimethoprim-sulphamethoxazole (SXT), amikacin (AK), tobramycin (NN), netilmicin (NET), ampicillin-sulbactam (SAM), ciprofloxacin (CIP), chloramphenicol (C). But the strain was sensitive to gentamicin (GM), tigecycline (TGC) and colistin (CL) (5). The combined test with IPM, MEM and EDTA/SMA discs (0.5 M/300 µg/mL), as well as with IPM, MEM and phenylboronic acid (300 µg), were positive, which implied the presence of both metallo-beta-lactamase (MBL) and serine-carbapenemase (KPC) enzymes (6,7).

Total DNA was extracted from the strain isolated after incubation in LB broth for 20 hours at 37 ºC, using the Wizard Genomic DNA kit (Promega).The polymerase chain reaction (PCR) of the blaVIM, and blaKPC genes was carried out using previously published primers (8), following the established protocols (9). The strain showed the amplification of the genes blaVIM and blaKPC (Fig. 1). Since the presence of MBL- and KPC-producing *Enterobacter* strains has high clinical and epidemiological importance, we investigated in further detail the identification of this strain. For this, the 16S ribosomal RNA gene (rRNA) was amplified using the primers fD2: 5´-AGAGTTTGATCATGGCTCAG-3´ and rPl: 5´-ACGGTTACCTTGTTACGACTT-3´ (10). The amplification was done as follows: an initial denaturation at 94ºC (5 min) was followed by 30 cycles of denaturation at 94ºC (2 min), annealing at 42°C (30 s), extension at 72°C (4 min), and a final extension at 72ºC (10 min). The amplified products were visualized electrophoretically on 2% agarose gel containing GelRed Nucleic Acid Stain (Biotium) and run in a 1X TBE buffer. The amplified fragment was sequenced directly, using both primers, in an automated sequencer ABI 3130X2, by the BigDye terminator method (Life Technologies), at the Centro de Secuenciación y Procesamiento de Ácidos Nucléicos (CeSAAN) of the Instituto Venezolano de Investigaciones Científicas (IVIC), Caracas, Venezuela. The sequences obtained were aligned with theClustalX program included in Mega 6.0 (11), and the consensus sequence was used to search homologous sequences at the GenBank. A phylogenetic analysis was carried out, to infer the relationships of the identified *Enterobacter* sp. strain with the published sequences with highest homologies, using the UPGMA method in MEGA6.0 (11), calculating the percentage of replicate trees, in which the associated taxa clustered together in the bootstrap test using 1000 replicates. The evolutionary distances were computed using the Maximum Composite Likelihood method and the number of base substitutions per site was used as the units of the tree. All positions containing gaps and missing data were eliminated. According to this phylogenetic analysis, the strain of *Enterobacter* sp. can be classified as *E. hormaechei* subsp. hormaechei (Fig. 2).

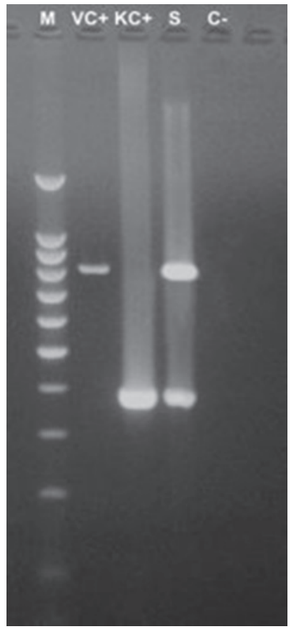

Fig.1. Detection of VIM type metallo-beta-lactamases and KPC type serin-carbapenemase by PCR in the strain of *E. hormaechei* subsp. *hormaechei.* Amplification of both fragments of 390 bp and 798 bp, typical of blaVIM and blaKPC can be seen in the studied isolate. M: molecular weight marker of 100 bp, VC+: positive control for blaVIM gene (*E. cloacae* amplified only with primers for blaVIM) (11), KC+: positive control for blaKPC gene (*E. cloacae* amplified only with primers for blaKPC) (11), S: studied isolate of E. *hormaechei* subsp. *hormaechei*, C-: negative control (water).

Ethical approval

The treatment of the patients, analysis of the isolates and information generated was conducted according to the bioethical and biosafety guidelines as stated by the Bioethics and Biosafety Commission of the IIBCAUDO (CoBios). We obtained a written consent from the patient's relatives for publication of this case.

DISCUSSION

The presence of MDR *E. hormaechei* subsp. *hormaechei* in the Cumaná hospital, carrying blaVIM and blaKPC genes, is of great relevance, both clinically and epidemiologically. The gene blaVIMhas been reported before in this hospital in three strains of *Enterobacter* and blaKPC in one strain of *E. cloacae* (9,12). They reported an unclassified strain of *Enterobacter* which resulted to have asimilar pattern of resistance. Metallo-β-lactamases (MBLs) have emerged among several Gram-negative pathogens, namely, species of the Enterobacteriaceae, as well as *Pseudomonas aeruginosa* and *Acinetobacter baumannii* (13). VIM types are the most frequently acquired MBLs and in some regions are widespread (14).

MBL- and KPC-producing E. cloacae are unusual causes of severe infections in compromised hosts, and empiric therapy with carbapenems may be inappropriate; therefore, colistin, although nephrotoxic, alone or in combination with other antibiotics (cephalosporins, quinolones, aminoglycosides or carbapenems), may represent the only effective therapeutic option against MDR *Enterobacter spp* (2). Until recently, these genes have been reported in Venezuela, but only in strains of *Klebsiella pneumoniae* (both genes) and *Pseudomonas aeruginosa* (blaVIM only) (15-17).

In 2001 alone, species of *E. cloacae* complex caused 7% of nosocomial infections in intensive care units in the USA (18). Therefore, the *E. cloacae* complex can be considered as an emerging pathogen, of which *E. hormaechei* is the most commonly isolated nosocomial pathogen (1). *E. hormaechei* has been shown to be of clinical significance by the report of several outbreaks of sepsis in neonatal intensive care

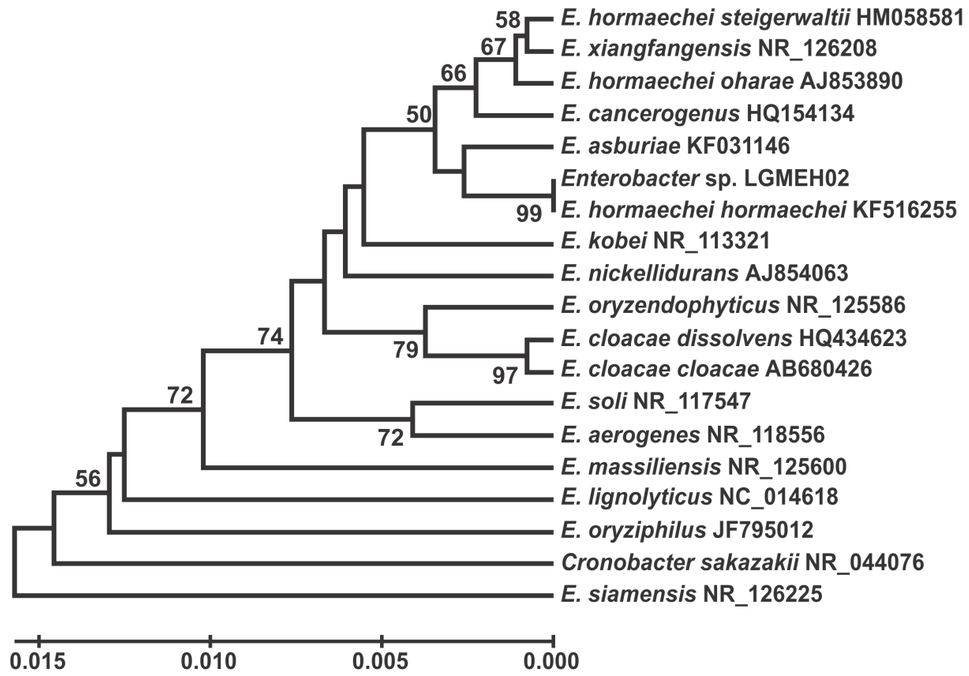

Fig.2. The phylogenetic relationships of the studied isolate of *E. hormaechei* subsp. hormaechei with the sequences of *E. cloacae* complex published in the GenBank, inferred using the UPGMA method. The bootstrap test values are shown next to the branches only when they are above 50%. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

units in Brazil and the USA (19,20). In addition, sepsis and meningitis have been more recently associated with *Enterobacter spp* (21).

When sequencing rRNA genes from strains of *Cronobacter (Enterobacter) sakazakii* causing nosocomial infections, 10 strains of *E. hormaechei* were identified from different origins (21), which points out an important misidentification of this species. In surveillance studies, *Enterobacter* species are often not further classified beyond the genus level probably because identification is difficult (1). Their clinical significance, especially over the last 15 years, have been reported in many publications, demonstrating their remarkable ability to upregulate or acquire resistance determinants, and making them some of the most worrisome microorganisms of the current antibiotic era (2).

The biochemical tests agreed with the identification of *E. hormaechei* subsp. *hormaechei*, although in the test for sorbitol, only 17% of the strains have been reported showing positive results (3). The identification of the species within the *E. cloacae* complex in clinical specimens is

important because it gives more information on their clinical relevance that otherwise could remain underestimated (2). Most clinical laboratories in developed countries routinely identify *Enterobacter spp.* by phenotypic methods employing commercially available kits or semi-automated systems that are limited to *E. cloacae* and *E. asburiae*, while for further identification and discrimination of the other species in this genus, biochemical tests or molecular methods such as 16S rRNA, rpoB and hsp60 gene sequencing should be used.

This study demonstrates the usefulness of molecular techniques for a more accurate identification of the *Enterobacter* species implicated in the clinical case of patients, as well as for the detection of resistance genes carried by this strain of *E. hormaechei subsp. hormaechei*, which is critical for more precise control measures of nosocomial infections caused by the species.

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REFERENCES

1. **Paauw A, Caspers MP, Schuren FH, Leverstein-van Hall MA, Delétoile A, Montijn RC, Verhoef J, Fluit AC.** Genomic diversity within the *Enterobacter cloacae* complex. PLoS One 2008;3(8):e3018.
2. **Mezzatesta ML, Gona F, Stefani S.** *Enterobacter cloacae* complex: clinical impact and emerging antibiotic resistance. Future Microbiol 2012;7(7): 887–902.
3. **Hoffmann H, Stindl S, Ludwig W, Stumpf A, Mehlen A, Monget D, Pierard D, Ziesing S, Heesemann J, Roggenkamp A, Schleifer KH.** *Enterobacter hormaechei subsp. oharae subsp. nov., E. hormaechei subsp. hormaechei comb. nov., and E. hormaechei subsp. steigerwaltii subsp. nov.*, three new subspecies of clinical importance J Clin Microbiol 2005;43:3297-3303.
4. **Pereira PS, Borghi M, Albano RM, Lopes JC, Silveira MC, Marques EA, Oliveira JC, Asensi MD, Carvalho-Assef AP.** Coproduction of NDM-1 and KPC-2 in *Enterobacter hormaechei* from Brazil. Microb Drug Resist 2015;21(2):234-236.
5. **Clinical and Laboratory Standards Institute. Document M100-S25.** Performance standards for antimicrobial susceptibility testing; twenty-fifth informational supplement. Wayne, PA: CLSI. 2015. p. 44-61.
6. **Lee K, Lim Y, Yong D, Yum J, Chong Y.** Evaluation of the Hodge test and the imipenem-EDTA double-disk synergy test for differentiating metallo-ß-lactamase-producing isolates of *Pseudomonas spp.* and *Acinetobacter spp.* J Clin Microbiol 2003;41:4623-4629.
7. **Pasteran F, Mendez T, Guerreiro L, Rapoport M, Corso A.** Sensitive screening for suspected class A carbapenemase production in species of Enterobacteriaceae. J Clin Microbiol 2009;47:1631-1639.
8. **Poirel L, Walsh TR, Cuvillier V, Nordmann P.** Multiplex PCR for detection of acquired carbapenemase genes. Diagn Microbiol Infect Dis 2011;70(1):119-123.
9. **Martínez D, Rodulfo HE, Rodríguez L, Caña LE, Medina B, Guzman M, Carreño N, Marcano D, De Donato M.** First report of metallo-β-lactamases producing *Enterobacter* spp. strains from Venezuela. Rev Inst Med Trop Sao Paulo 2014;56(1):67-69.
10. **Weisburg WG, Barns SM, Pelletier DA, Lane DJ.** 16S ribosomal DNA amplification for phylogenetic study. J Bacteriol 1991;173(2):697-703.
11. **Tamura K, Stecher G, Peterson D, Filipski A, Kumar S.** MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol Biol Evol 2013;30(12):2725-2729.
12. **Martínez D, Marcano D, Rodulfo HE, Salgado N, Cuaical N, Rodríguez L, Caña LE, Medina B, Guzman M, De Donato M.** KPC and VIM producing *Enterobacter* cloacae strain from a hospital in northeastern Venezuela.Invest Clin 2015;56(2):182-187.
13. **Walsh TR, Toleman MA, Poirel L, Nordmann P.** Metallo-beta-lactamases: the quiet before the storm? Clin Microbiol Rev 2005;18(2):306-325.
14. **Deshpande LM, Jones RN, Fritsche TR, Sader HS.** Occurrence and characterization of carbapenemase-producing Enterobacteriaceae: report from the SENTRY Antimicrobial Surveillance Program (2000-2004). Microb Drug Resist 2006;12(4):223-230.
15. **Guevara A, de Waard J, Araque M.** Detección del gen blaVIM-2 en cepas de Pseudomonas aeruginosa productoras de metalo β-lactamasa aisladas en una unidad de cuidados intensivos en Ciudad Bolívar, Venezuela. Rev Chil Infect 2009;26:336-341.
16. **Marcano D, Pasterán F, Rapoport M, Faccone D, Ugarte C, Salgado N, Payares D, Spadola E, López Y, Maggi G, Galas M, Sánchez D.** First isolation of a VIM-producing Klebsiella pneumoniae from a seven-year-old child in Venezuela. J Infect Dev Ctries 2008;2:241-244.
17. **Marcano, D.; De Jesús, A.; Hernández, L. y Torres, L.** Frequency of enzymes associated with reduced sensitivity to β-lactam antibiotics in enterobacteria isolates, Caracas, Venezuela. Rev Panam Salud Pública 2011; 30(6): 529-534.
18. **Streit JM1, Jones RN, Sader HS, Fritsche TR.** Assessment of pathogen occurrences and resistance profiles among infected patients in the intensive care unit: report from the SENTRY Antimicrobial Surveillance Program (North America, 2001). Int J Antimicrob Agents 2004;24(2):111-118.
19. **Campos LC, Lobianco LF, Seki LM, Santos RM, Asensi MD.** Outbreak of *Enterobacter* hormaechei septicaemia in newborns caused by contaminated parenteral nutrition in Brazil. J Hosp Infect 2007;66(1):95-97.
20. **Wenger PN, Tokars JI, Brennan P, Samel C, Bland L, Miller M, Carson L, Arduino M, Edelstein P, Aguero S, Riddle C, O'Hara C, Jarvis W.** An outbreak of *Enterobacter* hormaechei infection and colonization in an intensive care nursery. Clin Infect Dis 1997;24(6):1243-1244.
21. **Townsend SM, Hurrell E, Caubilla-Barron J, Loc-Carrillo C, Forsythe SJ.** Characterization of an extended-spectrum beta-lactamase *Enterobacter* hormaechei nosocomial outbreak, and other *Enterobacter* hormaechei misidentified as Cronobacter (*Enterobacter*) sakazakii. Microbiology 2008; 154(Pt 12):3659-3667.