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New sequence type of an *Enterobacter cloacae* complex strain with the potential to become a high-risk clone

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ABSTRACT

Objectives: *Enterobacter cloacae* complex (ECC) has awakened interest recently because of its increasing resistance to carbapenems codified by several genes all over the globe. Even though there are some sequence types (STs) which represent high-risk clones, there is substantial clonal diversity in the ECC. This work aimed to perform whole-genome sequencing (WGS), genomic analysis, and phylogenetic studies of a *Klebsiella pneumoniae* carbapenemase (KPC) -producing multidrug-resistant (MDR) ECC isolate from Argentina.

Methods: We analysed the genome of an MDR KPC-producing ECC strain isolated from a urine sample from a patient in a hospital in Argentina. The WGS was done by Illumina MiSeq-I (Illumina, San Diego, CA). The genome was assembled with SPAdes 3.9.0, and annotated with PROKKA, RAST, and Blast. Plasmids were identified with PlasmidFinder. Antibiotic resistance genes were detected using RESfinder, CARD, and Blastn. STs were identified with pubMLST.

Results: The strain was identified as *Enterobacter hormaechei*, an important emerging human pathogen. No ST could be assigned; six of seven alleles of multilocus sequence typing (MLST) were the same as for *E. hormaechei* ST66, which is a high-risk clone. We found multiple acquired antibiotic resistance genes, including *bla*$_{KPC-2}$ in an IncM1 plasmid, and a secretion system VI, which can favour the prevalence of ECC strains while competing with other bacteria.

Conclusion: Because of its MLST profile being so close to that of *E. hormaechei* ST66, the acquisition of multiple resistance genes, and the presence of the secretion systems, the potential of this strain for becoming a new high-risk clone cannot be discarded.

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1. Introduction

Carbapenem-resistant *Enterobacteriaceae* (CRE) bacteria belong to the highest priority group for the development of new antibiotics. Genome analysis and molecular epidemiology are fundamental tools for developing strategies to identify and prevent the spreading of antibiotic resistance mechanisms. Members of the *Enterobacter cloacae* complex (ECC) gained more attention in the last 10 years because of the rise in carbapenem-resistant strains. Although there are some sequence types (STs) that are more frequently found and represent high-risk clones, there is considerable clonal diversity in the ECC [1]. Previous multinational surveillance studies employing MLST found significant clonal diversity with evidence for several potential high-risk clones [2]. Some of the most frequent STs are *E. hormaechei* ST66, 78, and 171, all of which have been reported to carry *bla*$_{KPC-2}$ [3]. In Argentina, there is not yet evidence for a predominant ST in the ECC [4]. The aim of this work was to perform whole-genome sequencing (WGS), genomic, and phylogenetic analysis of a multidrug-resistant ECC strain (HA16Eho). ECC HA16Eho was isolated from a hospitalized patient with a urinary tract infection in Buenos Aires, Argentina in February 2019. An antibi-

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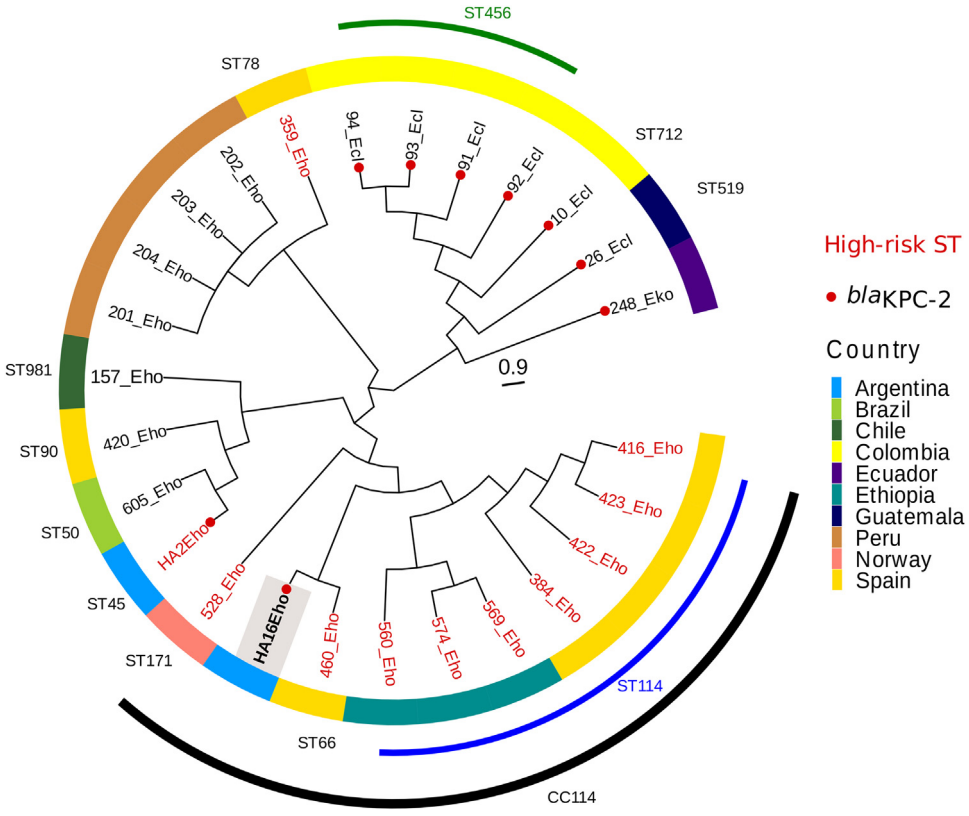

Fig. 1. Maximum likelihood phylogenetic tree based on the single nucleotide polymorphisms (SNPs) of the core genome of *Enterobacter cloacae* complex (ECC) strains from Latin America and ECC high-risk clones phylogenetically close to *E. hormaechei* HA16Eho. The phylogenetic tree was created with the Jukes-Cantor model and 100 bootstraps using the packages ggtree and ggtreeExtra in R. The labels at the tips of the tree show isolate ID on pubMLST and abbreviations for the species (Eho: *E. hormaechei*, Ecl: *E. cloacae*, and Eko: *E. kobeii*), with the exceptions of *E. hormaechei* HA16Eho, described in this study, and *E. hormaechei* HA2Eho, previously isolated in our institution. Labels in red indicate high-risk ECC ST, and a red dot at the tip point means that the isolate carried the bla_{KPC-2} gene. Selection criteria for the isolates from pubMLST were all ECC isolates from Latin America whose whole-genome sequencing (WGS) data were available at pubMLST, and all isolates which belonged either to *E. hormaechei* ST66 or ST114 (because these STs had the most similar MLST profiles to our strain) or *E. hormaechei* ST78 and ST171 (because these STs are common high-risk clones). *Enterobacter hormaechei* ST90 (420_Eho) was included because this ST was previously found in Argentina [4]. Because the WGS data of Argentinian isolates were not available, we chose another *E. hormaechei* ST90 isolate from the database. CC, clonal complex; ST, sequence type.

otic susceptibility profile was achieved with the BD Phoenix system (Sparks, MD, USA) following Clinical and Laboratory Standards Institute (CLSI) guidelines. The strain showed susceptibility to amikacin, gentamycin, and fosfomycin, and was resistant to cefotaxime, ceftazidime, cefepime, meropenem, imipenem, ertapenem, piperacillin-tazobactam, ciprofloxacin, norfloxacin, trimethoprim-sulfamethoxazole, and nitrofurantoin. Whole-genome sequencing of ECC HA16Eho was performed using Illumina MiSeq-I (Illumina, San Diego, CA). Assembly and annotation were done using FastQC v0.11.9 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/), Trimmomatic v0.39 (http://www.usadellab.org/cms/?page=trimmomatic), SPAdes v3.15.3 (https://cab.spbu.ru/software/spades/), QUAST v5.0.2 (http://quast.sourceforge.net/), and Prokka v1.14.5 (https://github.com/tseemann/prokka). Identification to the species level was achieved at https://pubmlst.org/species-id. HA16Eho was identified as *Enterobacter hormaechei*, which is an important emerging human pathogen [1]. Antibiotic resistance genes were searched using Resfinder (https://cge.cbs.dtu.dk/services/ResFinder/) and CARD (https://card.mcmaster.ca/). Beta-lactamase alleles were confirmed with the BLDB Database (http://www.bldb.eu/). Apart from carrying the gene bla_{ACT-45}, which is naturally harboured by this species, *E. hormaechei* HA16Eho carried the acquired antibiotic resistance genes bla_{KPC-2}, *aac(6')-Ib-cr5*, bla_{OXA-1}, *fosA2, catA, qnrB1*, and *dfrA14*. The bla_{KPC-2} gene was found surrounded by Tn*3*-*tnpA*-IS*Apu1*-IS*Apu2*-IS*Kpn27*-bla_{KPC-2}-IS*Kpn6*-Tn*3*-ΔTn*10* (18176 bp length). This DNA sequence was embedded in an IncM1 plasmid, named pDCCK2-KPC (JAMQJW010000022.1), and is similar to that of pDCCK1-KPC, which was previously described by our institution (JAMPTY010000024.1). In comparison with pDCCK1-KPC (77218 bp), pDCCK2-KPC (67,953 bp) lacked the genes *trbA-C, trbN, mcmM, pemI, pemK*, and Tn*3* from nt 11421 to nt 20610 in pDCCK1-KPC. The *aac(6')-Ib-cr5* and bla_{OXA-1} gene cassettes (GCs) were part of a CALIN (i.e. had their *attC* sites, but lacked the integron integrase gene); therefore, the Pc and Pc2 promoters from where GCs are usually transcribed were missing. This could have been the reason why *E. hormaechei* HA16Eho remained susceptible to amikacin. While *fosA2* was located in the chromosome, *catA* and *qnrB1* were likely to be on plasmids (although this cannot be assured, as these genes were found in short contigs). This assumption is based on the results from searches at the NCBI and CARD databases. The *dfrA14* GC was in the variable region of a class 1 integron lacking its 3' conserved sequence. Apart from the IncM1 plasmid carrying bla_{KPC-2}, PlasmidFinder identified three additional plasmids belonging to the incompatibility groups Col(pHAD28), IncFIB, and IncFII. A phylogenetic tree of all ECC isolates from Latin America whose WGS data were available at pubMLST was built using roary, snap-gene, and R (Fig. 1). *E. hormaechei* HA16Eho could not be classified as any known sequence type (ST) by pubMLST, but the ST with the most similar MLST profile was ST66. *E. hormaechei* HA16Eho shared six of seven alleles with ST66: *dnaA*=52, *fusA*=21, *gyrB*=20, *leuS*=44, *rplB*=4, and *rpoB* = 6, evidencing that *E. hormaechei* HA16Eho belonged to clonal complex (CC) 114. The seventh gene of the MLST profiling, *pyrG*, did not match any known allele. Apart from *E. hormaechei*

ST66, high-risk clone *E. hormaechei* ST114 was the closest to *E. hormaechei* HA16Eho [3]. High-risk clone *E. hormaechei* ST45 was previously found in our institution (JAMQJX000000000.1), while *E. hormaechei* ST90 was present in the study by De Belder (2017) in Argentinean isolates.

When tested with PathogenFinder (https://cge.food.dtu.dk/services/PathogenFinder/), we found that *E. hormaechei* HA16Eho possessed secretion systems I, II, and VI. Secretion system I was represented by the genes *lapE-lapB-lapC* in one contig and *lapA* in another contig. The 12 core genes of secretion system II were found in one single contig. *E. hormaechei* HA16Eho had three clusters of secretion system VI (SST6) genes distributed in four contigs. The largest sequence within a contig showed the following order: *tssJ-tssK-tssL-tssM-tagF-tssA-tssB-tssC-hcp1-tagH-impE-tssE-tssF-tssG-tssH*. T6SS is important for bacterial competition as well as virulence in many Gram-negative bacteria [5].

Because of the closeness of its MLST to that of *E. hormaechei* ST66, its ability to acquire multiple antibiotic resistance genes, and its secretion systems, the potential for *E. hormaechei* HA16Eho to become a new high-risk clone cannot be dismissed.

Nucleotide sequence accession no: This Whole Genome Shotgun project has been deposited at GenBank under the accession number JAMQJW000000000.1.

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Competing interests

None declared

Ethical approval

Patient consent was waived because of the study's retrospective nature and that no identifiable patient information was collected or presented. Patient confidentiality was maintained throughout the study and no additional risks were presented. Participation in the study did not interfere with patient management.

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