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First detection and origin of multi-drug resistant *Klebsiella pneumoniae* ST15 harboring OXA-48 in South America

Cecilia Salazar[a], Verónica Antelo[a], Mariela Vieytes[b], Cristina Dávila[c], Fabio Grill[b], Antonio Galiana[b], Gregorio Iraola[a,d,e,*]

[a] *Microbial Genomics Laboratory, Institut Pasteur de Montevideo, Montevideo, Uruguay*
[b] *Hospital Maciel, Montevideo, Uruguay*
[c] *Casa de Galicia, Montevideo, Uruguay*
[d] *Center for Integrative Biology, Universidad Mayor, Santiago de Chile, Chile*
[e] *Wellcome Sanger Institute, Hinxton, United Kingdom*

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ABSTRACT

Objectives: The emergence and spread of carbapenem resistant clones is of major concern for global health. This study aimed to characterize the first detected *Klebsiella pneumoniae* ST15 harboring the epidemic carbapenemase OXA-48 in South America.
Methods: During a routine colonization screening with carbapenem-resistant bacteria, one *K. pneumoniae* strain (CGHM01) was isolated from the urine of a hospitalized patient suffering from a neurodegenerative disease in Uruguay. We used long-read whole-genome sequencing and a phylogenomic approach to characterize the emergence of *K. pneumoniae* CGHM01.
Results: *K. pneumoniae* CGHM01 is a multi-drug resistant strain carrying an IncL/M plasmid that encodes the carbapenemase gene $bla_{\text{OXA-48}}$ within the *Tn1999.2* transposon. Also, it carries an IncR plasmid harboring a class I integron with an array of antibiotic resistance genes including the extended-spectrum beta-lactamase $bla_{\text{CTX-M-15}}$. Two copies of $bla_{\text{CTX-M-15}}$ were also inserted in different positions of the chromosome. CGHM01 belongs to a ST15 sublineage that likely originated in continental Spain around 2012.
Conclusions: The asymptomatic carriage of this strain in the urinary tract warns of difficulties for detection and reporting of emerging carbapenem-resistant clones in new geographic areas where these are not endemic.

1. Introduction

Klebsiella pneumoniae is a major human pathogen that encloses a wide variety of high-risk clones distributed worldwide with differential incidence and pathogenic characteristics. Some specific clones contribute disproportionately to global disease burden and are referred to as 'global problem clones' [1]. These epidemic clones include ST15, which is among the most common third-generation cephalosporin-resistant clones reported in different countries. The ST15 clone has been mainly associated with the dissemination of the extended spectrum beta-lactamase (ESBL) CTX-M-15. Additionally, ST15 is frequently associated with the dissemination of carbapenem resistance. This represents an urgent problem for global health because carbapenems are among the last-line antibiotics to treat multidrug-resistant infections. Resistance to carbapenems is mostly mediated by carbapenem hydrolysis by enzymes known as carbapenemases. Among them, the oxacillinase-48 (OXA-48) is one of the most common and widely disseminated. This enzyme was first detected in Turkey in 2004 from *K. pneumoniae* and quickly became endemic [2]. By the end of that decade, OXA-48 had disseminated to Europe, North Africa and subsequently continued its epidemic expansion to the rest of the world [3].

Recently, we detected third-generation cephalosporin and carbapenem resistant *K. pneumoniae* ST15 harboring the epidemic beta-lactamases CTX-M-15 and OXA-48 for the first time in South America. Therefore, in this work we applied a phylogenomic approach to characterize this strain. Our results are relevant for pre-

* Corresponding author. Mailing address: Microbial Genomics Laboratory, Institut Pasteur Montevideo, Mataojo 2020, Montevideo 11400, Uruguay.
E-mail address: giraola@pasteur.edu.uy (G. Iraola).

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cisely understanding evolutionary and ecological patterns of *K. pneumoniae* global problem clones as they spread to new geographic areas, enabling us to improve the design of control strategies for multi-drug resistant bacteria.

2. Materials and Methods

2.1. Bacterial isolation and antibiotic resistance

Isolated colonies were obtained from urine catheter samples and identified at the species level using the VITEK 2 and VITEK MS system (bioMérieux, Marcy L'Etoile, France), confirming the presence of *Klebsiella pneumoniae*. Antimicrobial susceptibility was determined by VITEK 2, disk diffusion and E-test according to Clinical Laboratory and Standards Institute 2019 cutoffs. Detection of OXA-48 was performed with the Coris (BioConcept, Gembloux,Belgium) and NG-Test Carba 5 (NG Biotech, Guipry, France) immunochromatographic assays, growth in the selective agar medium for oxacillinase Carba Smart (bioMérieux, Marcy L'Etoile, France) and using the GeneXpert instrument at Hospital Maciel. Susceptibility to colistin was studied with the colistin broth disk elution test.

2.2. Whole-genome sequencing

Pair-end reads (2 × 150 bp) were generated with an Illumina HiSeq 4000 platform at the Wellcome Sanger Institute (United Kingdom) as described previously [4]. Long reads were generated on a MinION device (Oxford Nanopore, Oxford, UK) based on the Genomic DNA Ligation (SKQ-LSK109) protocol. The library was sequenced in a Flongle flow cell (FLO-FLG001) and base-called using the Guppy 3.2.9 software (high-accuracy mode). Hybrid assembly was performed with Unicycler [5] using default parameters and subsequently annotated with Prokka [6]. Sequence type was determined using MLSTar [7].

2.3. Phylogenomic analysis

We obtained 477 *Klebsiella pneumoniae* ST15 genomes from the PATRIC database (https://www.patricbrc.org, accessed 21 December 21 2019), representing isolates from 38 countries from 1980 to 2018 (Supplementary Table S1). Genomic characterization for traits of interest such as capsule type, AMR, and virulence genes was performed using Kleborate v0.3.0 (https://github.com/katholt/Kleborate). Phylogenetic analysis was performed calling core SNPs using Snippy (https://github.com/tseemann/snippy) and snp-sites [8] with the CGHM01 strain as reference genome. Recombinant regions were removed with Gubbins [9] using default parameters, and a phylogenetic tree was built with FastTree [10] using the GTR substitution model. A more restricted dataset including 78 European and Uruguayan ST15 strains was used to identify temporal signals using the root-to-tip regression approach (with 10,000 permutations) and a dated phylogeny as obtained with BactDating [11]. Visualizations were generated with the ggtree [12] package. Code to reproduce analyses is available at www.github.com/giraola/klebsCG15.

3. Results

3.1. Emergence of K. pneumoniae ST15 harboring OXA-48 in South America

In 2019, during a routine colonization screening, bacterial growth was observed in urine cultures from a bladder catheter of an 86-year-old female patient showing no symptoms of urinary infection. The patient was being treated with tamoxifen against a breast neoplasm, had a record of back pain requiring chronic pain treatment, was hospitalised in a tertiary care centre since 2017, and was diagnosed with Alzheimer's disease.

Genomic characterization of this strain, named CGHM01 (additional information in Supplementary Table S2), showed that it carried the siderophore yersiniabactin genotype 299-4LV (in a genomic island of type *ybt* 13; ICE*Kp2*), the capsular type KL112, and the O-locus type O1v1. The chromosome also encoded mutations in *parC* and *gyrA* genes that confer resistance to fluoroquinolones and three ESBLs coded by a gene similar to $bla_{\text{SHV-28}}$ gene and two copies of the $bla_{\text{CTX-M-15}}$ gene in different chromosomal positions next to ISEcp1 elements. The strain also carried a ~63 kb IncL/M plasmid (pCGHM01-63) that encoded the carbapenemase gene $bla_{\text{OXA-48}}$ within the *Tn1999.2* transposon, characterized by the insertion of an *IS1* element inside the *IS4* transposase placed upstream the carbapenemase gene (Supplementary Fig. S1, Supplementary Table S3). Also, it harbored a ~55 kb IncR plasmid (pCGHM01-55) encoding several antimicrobial resistance (AMR) genes in a 20 kb class I integron inserted next to a mercuric resistance operon. This array of antibiotic resistance genes included the fluoroquinolone-acetylating aminoglycoside 6'-N-acetyltransferase AAC(6')-Ib-cr, the aminoglycoside phosphotransferase APH(3'')-Ib and APH(6)-Id, the chloramphenicol O-acetyltransferase CatB4, the sulfonamide-resistant dihydropteroate synthase Sul2, and the ESBLs OXA-1, TEM-30, and CTX-M-15 (Supplementary Fig. S2, Supplementary Table S4).

3.2. Phylogenomic characterization of CGHM01

To understand the origin of CGHM01, we used a global collection of over 400 ST15 genomes that revealed the lack of genetic relatedness of this strain with other ST15 strains previously reported in the region. This is clear given that strains reported between 2014 and 2017 in the Caribbean Islands and Brazil are located outside the monophyletic clade conformed by strains with capsule type KL112, in which CGHM01 is placed (Fig. 1). More specifically, CGHM01 is embedded within a KL112 subclade conformed by European strains (Fig. 1, see dataset in Microreact [13]: https://microreact.org/project/3IJIY_QF0). These European strains were reported in Spain from 2013 to 2015. All strains in this clade were positive for $bla_{\text{OXA-48}}$ and carried the yersiniabactin sequence type (YbST) 299-4LV, the genomic island of type *ybt* 13; ICE*Kp2* and the O-locus type O1v1. Among KL112 genomes, this combination of genomic markers was only observed in this clade, indicating a potential Spanish origin of CGHM01.

To further investigate the epidemiological relatedness between CGHM01 and Spanish strains, we studied the evolutionary history of European KL112 strains performing a temporal analysis. First, we detected a significant temporal signal in a dataset of 78 dated European KL112 strains (including CGHM01). This is evidenced by a positive correlation between isolation date and root-to-tip distance ($r^2 = 0.56$, $r = 0.75$, $P = 2.7 \times 10^{-15}$) (Supplementary Fig. S3). Second, we found that CGHM01 falls within the 95% HPD (High Posterior Density) interval of the root-to-tip linear regression that supports the clock-like evolution of KL122 strains included in this dataset (Fig. 2A). According to this analysis, the amount of genetic change separating Spanish strains from CGHM01 is the expected, given the elapsed effective time (Fig. 2B). Together, this supports the Spanish origin of the CGHM01 detected for the first time in Uruguay in 2019. The time for the most recent common ancestor of this clade was traced back to 2010 (95% HPD: 2009-2011).

4. Discussion

K. pneumoniae ST15 is considered a global problem for being one of the most significant outbreak-causing clones. Accordingly,

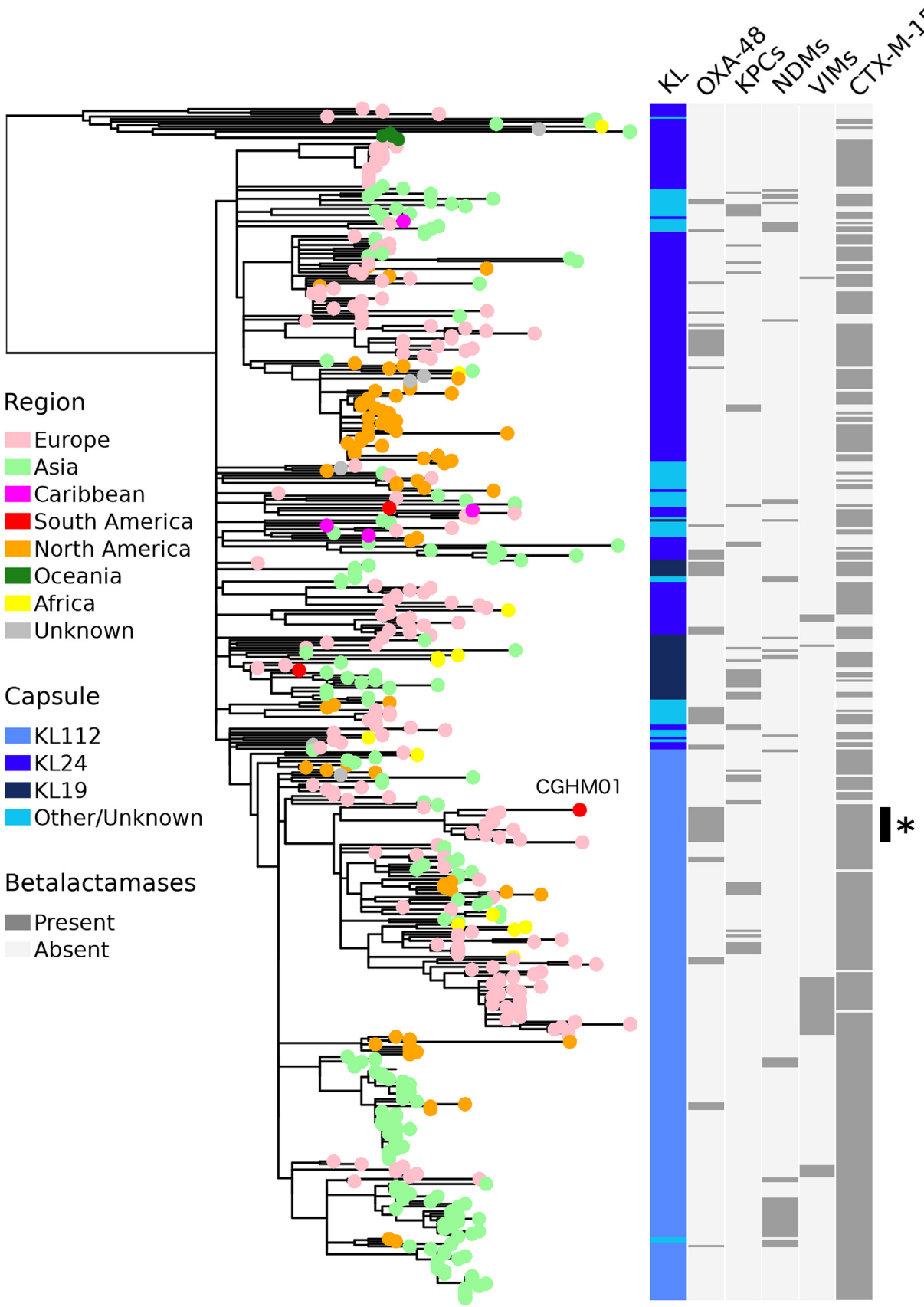

Fig. 1. Global phylogenetic analysis of *Klebsiella pneumoniae* CG15. Core genome phylogeny showing relevant metadata, such as: geographic region of origin (colored tip circles), capsule type (first vertical colored strip), and presence/absence of carbapenemases and ESBLs. The rightmost vertical black line with an asterisk highlights strains belonging to the European subclade including CGHM01.

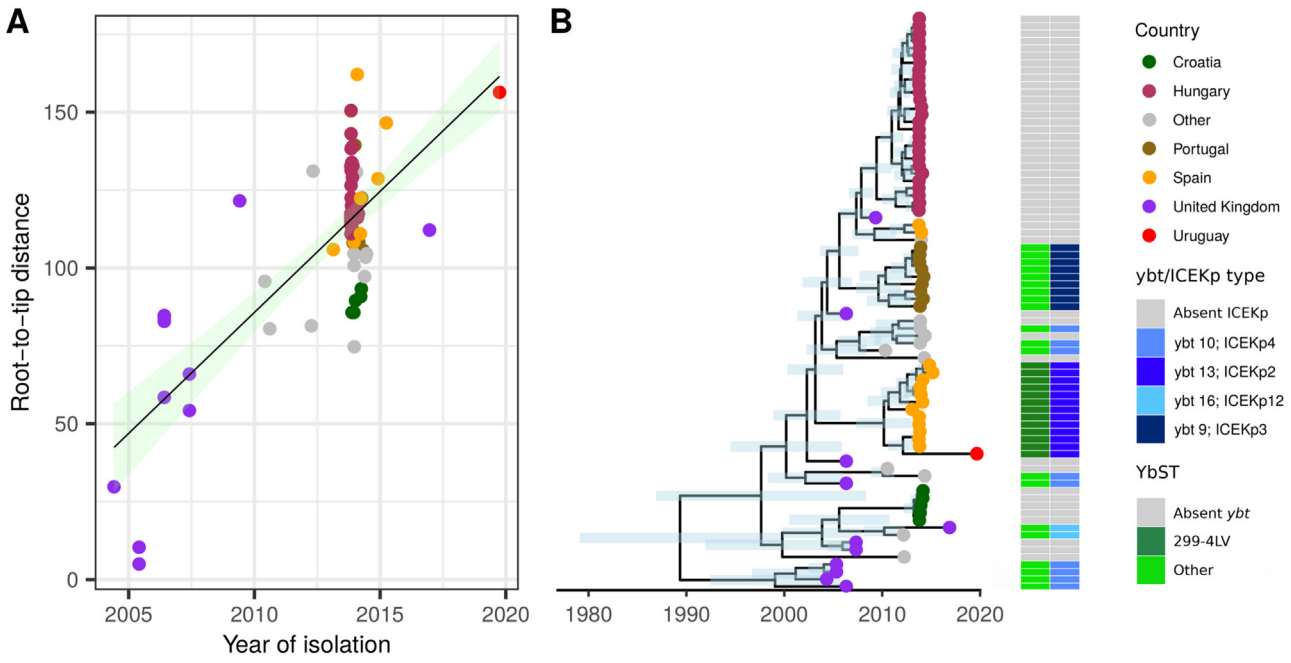

Fig. 2. Temporal analysis of KL112 strains. A) Root-to-tip distance regression analysis showing a positive and significant correlation (see Fig. S2) indicating strong temporal signal in the KL112 clade. B) Dated phylogeny of KL112 strains. Node bars represent confidence intervals for estimated dates. Tips are colored according to the country of origin of each strain. Vertical colored stripes show the presence/absence of ICE*Kp* and yersiniabactin characterization.

understanding its dissemination patterns and evolution are essential to improve prevention measures and treatment. In particular, ST15 is among the most common third-generation cephalosporin-resistant clones, mainly associated with the epidemic spread of the ESBL CTX-M-15. In South America, CTX-M-15-positive ST15 strains have been only reported recently in Brazil [14], but the precise genetic features of these strains are unknown due to the unavailability of high-quality genomes. Our long-read sequencing analysis of the CGHM01 strain found in Uruguay allowed us to precisely define the genomic context of the $bla_{\text{CTX-M-15}}$ gene. Specifically, we confirmed that an IncR plasmid is responsible for $bla_{\text{CTX-M-15}}$ dissemination. Additionally, we found chromosomal introgressions of $bla_{\text{CTX-M-15}}$, which is an uncommon feature in *K. pneumoniae*. Given that hyperproduction of ESBLs also leads to carbapenem resistance, the genomic expansion of $bla_{\text{CTX-M-15}}$ may represent a recent adaptation to stronger antibiotic pressures, reflecting the systemic increase in cephalosporin and carbapenem consumption over the past years, particularly in low- and middle-income countries [15].

K. pneumoniae strains commonly colonise human mucosal surfaces, including the urinary tract, constituting an important step in progression to infection. Indeed, asymptomatic colonisation with *K. pneumoniae* has been identified as a risk factor to develop active urinary infection in the follow-up year after colonisation is detected [16]. However, a different study reported that over 80% of patients showing urinary tract colonisation by carbapenem-resistant *K. pneumoniae* were asymptomatic, and adverse outcomes were not observed in a follow-up period of 3 months [17]. This highlights current difficulties in the clinical interpretation of urinary colonisation by *K. pneumoniae*. Importantly, this has a negative impact on the surveillance and reporting of carbapenem-resistant bacteria, since these strains can remain unnoticed in patients who can serve as asymptomatic carriers and spreaders.

The case presented here reflects the above-mentioned situation and probably is an example of many other yet unnoticed carbapenem-resistant clones that are circulating in South America. Indeed, despite being globally distributed and frequently associated with endemic infections, OXA-48 has been detected in South America only in a couple of *K. oxytoca* strains from Colombia [18] and *K. pneumoniae* ST-307 from Ecuador [19]. This probably reflects under-reporting of OXA-48, given that detection of these enzymes using phenotypic methods remains challenging for clinical laboratories and usually requires sequencing. Indeed, CGHM01 is the first OXA-48-producing strain isolated in South America whose complete genome is available. In fact, the analysis of this genome revealed that $bla_{\text{OXA-48}}$ was linked to the *Tn1999.2* transposon, a variant that enhances the expression of OXA-48 conferring increased resistance to carbapenems [20]. This can be informative to clinicians during antibiotic treatment selection or optimization. Additionally, the identification of similar strains previously reported in Spain demonstrates the importance of active genomic surveillance to trace the origin and spread of clinically relevant clones like *K. pneumoniae* ST15 as they acquire new antibiotic resistance mechanisms and colonise new geographic areas.

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Competing interests

The authors declare that there are no conflicts of interest.

Ethical approval

Not applicable.

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Data availability

Illumina and Oxford Nanopore reads were deposited at the European Nucleotide Archive (ENA) and Sequence Read Archive (SRA) under accession numbers ERR3797164 and PRJNA660033, respectively.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jgar.2022.08.005.

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