Global silent transmission of the class A ß-lactamase LAP-2 by plasmid transfer and clonal expansion of *Klebsiella pneumoniae* lineages

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BACKGROUND Narrow-spectrum antibiotics, such as narrow-spectrum β-lactams, have been pointed out as an efficient strategy to mitigate resistance and reach successful outcomes in treating enterobacterial infections. The narrow-spectrum class A β-lactamase LAP-2, conferring resistance to narrow-spectrum cephalosporins/penicillin derivatives, is spread among *Enterobacteriaceae* from Asia but is rarely reported in the Americas. Due to the lack of information concerning the bla_{LAP-2} genetic background involved with its dissemination, this study determined the bla_{LAP-2} genomic environment and contextualised the LAP-2-positive *Klebsiella pneumoniae* strains from Brazil in the current epidemiological scenario.

OBJECTIVES This study characterised LAP-2-positive *K. pneumoniae* strains, focusing on their genetic environment and epidemiology.

METHODS Whole-genome sequencing and *in silico* analyses were performed to identify the genetic context of bla_{LAP-2} in five clinical isolates from Brazil. Comparative genomics and phylogenomic analysis were conducted to investigate the transmission dynamics of bla_{LAP-2} globally.

FINDINGS The bla_{LAP-2} gene was embedded in a conserved genetic module (IS*3*-bla_{LAP-2}-*ftsI*), facilitating its dissemination among diverse *Enterobacteriaceae* species. The Brazilian strains harboured bla_{LAP-2} within a pXJ-K2 variant plasmid, a key vector in LAP-2 spread. Phylogenomic analysis revealed that, in Brazil, bla_{LAP-2} was carried by an ST11 *K. pneumoniae* lineage distinct from the Chinese lineage but globally disseminated.

MAIN CONCLUSIONS This study provides insights into the epidemiology and transmission dynamics of bla_{LAP-2}, revealing its silent spread via plasmid transfer and clonal expansion of ST11 lineages. The high transmission potential of bla_{LAP-2} may compromise the application of narrow-spectrum β-lactams as a viable treatment option for enterobacteria-causing infections.

Key words: bla_{LAP} - *qnrS1* - narrow-spectrum β-lactamase - extensively-drug resistance - genomic epidemiology - pandemic lineage

The antibiotic resistance crisis is one of the world's most urgent health challenges that has been attributed to the overuse and misuse of antimicrobial agents, contributing to the emergence of "superbugs". In this context, the use of narrow-spectrum antibiotics in clinical practice, such as narrow-spectrum cephalosporins and penicillin derivatives, has been pointed out as an efficient approach to optimise antibiotic use and mitigate the problem of resistance emergence to broad-spectrum antibiotics.[1] For example, the effectiveness of ampicillin (a narrow-spectrum penicillin derivative) in association with a natural constituent of green tea against multidrug-resistant (MDR) *Escherichia coli* strains has been demonstrated.[2] Similarly, another narrow-spectrum penicillin derivative (temocillin) has been proposed as an alternative to carbapenems for treating acute pyelonephritis and other infections due to extended-spectrum β-lactamase (ESBL)-producing *Enterobacteriaceae*.[3] Also, the cefoxitin, a narrow-spectrum cephalosporin, has been suggested as a definitive therapeutic option for bacteraemia caused by ESBL-producing *Klebsiella pneumoniae*, sparing carbapenems and reducing the selection of carbapenem-resistant strains.[4] This strategy would drastically reduce the ecological impact and the selection pressure on commensal flora, thereby containing the emergence of resistance.[5]

The bla_{LAP-1} and bla_{LAP-2} alleles, differing by one amino acid substitution, code for narrow-spectrum Class A β-lactamases, and were concomitantly identified in genetically unrelated *Enterobacter cloacae* strains from France, Vietnam, and China, respectively.[6,7,8] These alleles confer resistance to first-line cephalosporins and penicillin derivatives, and they are featured by their direct association with the *qnrS1* gene, a plasmid-mediated determinant conferring resistance to quinolones, which might account for resistance to both antimicrobial classes. The bla_{LAP-2} associated with *qnrS1* has been reported in several enterobacterial species across multiple countries.[9,10,11] However, despite bla_{LAP-2} widespread potential, this gene has never been reported in the Americas

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until recently, being identified only in one *E. coli* strain from Cuba and one *Leclercia adecarboxylata* strain from Brazil, both from animal sources.[12,13]

The intricate link of bla_{LAP-2} and *qnrS1* strongly suggests that these genes are in a specific genetic platform that is being transferred and disseminated by plasmids.[14,15,16] However, there is a lack of information concerning the intrinsic bla_{LAP-2} genomic context and the genetic elements involved with its dissemination. Until now, only one plasmid, pXJ-K2 (Accession number CP032243.1), recovered from a hypervirulent and MDR *K. pneumoniae* strain from China in 2018, was completely sequenced, revealing the direct genomic context of bla_{LAP-2}.[16] The lack of information on the occurrence and dissemination of certain antibiotic resistance genes, such as bla_{LAP-2}, sometimes leads to the erroneous conclusion that these genes are restricted to some niches or have a discrete distribution. For example, the mobilisable colistin resistance *mcr-1* gene, although reported in 2011, was silently widespread since the 2000s embedded in a highly conserved immediate genetic background that various plasmid types have harboured.[17] This scenario reveals the impact of this silent dissemination on public health, since the prevalence of these genes may be underestimated, as recently demonstrated for *mcr-9.1* gene.[18]

During a genomic study, we identified the bla_{LAP-2} in MDR and extensively drug-resistant (XDR) *K. pneumoniae* strains recovered from Roraima and Rio de Janeiro. This gene had never been reported in clinical cases in the Americas to date. Therefore, based on the presented gaps and the still open questions about LAP-2, and due to the rarity of reports of this gene in the continent, this study raised some relevant questions: Is the bla_{LAP-2} being spread in the context of a genetic module or in an independent way? Is the bla_{LAP-2} being spread by a specific plasmid? Is the bla_{LAP-2} being spread among *K. pneumoniae* by a specific lineage? To address these questions, we performed a large-scale genomic analysis to unravel the current epidemiological scenario of LAP-2 and to determine its genetic environment among bacterial species spatio-temporally distributed.

MATERIALS AND METHODS

Studied strains - during a global genomic study on MDR and XDR *K. pneumoniae* strains recovered from hospitals in Roraima and Rio de Janeiro, five genomes carrying the bla_{LAP-2} resistance gene were identified. The bla_{LAP-2}-positive strains were recovered in 2016 and 2017 from tracheal secretion (KP68.2RR, KP46.2RR and KP5133RR) and soft tissue (KP290RR) infections from inpatients placed in the General Hospital of Roraima, Boa Vista. One strain (KP205RJ) was isolated from a urinary tract infection (UTI) case in the São Francisco na Providência de Deus Hospital, Rio de Janeiro, 2022. These clinical strains belong to the Bacterial Culture Collection of the Laboratory of Molecular Genetics of Microrganisms, FIOCRUZ.

Antimicrobial susceptibility test - the resistance profile of the five bla_{LAP-2}-positive strains had been previously determined by the disc-diffusion method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines[19] for the following antibiotics: Ampicillin; Ampicillin+Sulbactam; Amoxicillin+Clavulanic acid; Ticarcillin+Clavulanic acid; Piperacillin+Tazobactam; Cefepime; Cefotaxime; Ceftazidime; Ceftriaxone; Cefoxitin; Cefuroxime; Cephalothin; Ciprofloxacin; Clarithromycin; Clindamycin; Chloramphenicol; Ertapenem; Meropenem; Imipinem; Doripenem; Gentamicin; Tobramycin; Netilmicin; Amikacin; Doxycycline; Tetracycline; Minocycline; Trimetropim+ Sulfamethazole; Aztreonam; Fosfomycin; Tigecycline. The minimum inhibitory concentration (MIC) of colistin was evaluated by broth microdilution and interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guideline.[20] The current criteria for resistance classification were applied.[21]

Whole-genome sequencing and in silico analysis - The genomes of the five strains from this study were obtained on the Illumina Hiseq 2500 using the Nextera paired-end library kit for library construction. SPAdes v3.15.2[22] was used for genome assembly and gene prediction and annotation were conducted with Prokka v1.14.6.[23] An *in silico* multilocus sequence typing (MLST) analysis was performed to determine the sequence type (ST) of the five *K. pneumoniae*. The resistome was obtained with the Comprehensive Antibiotic Resistance Database (CARD - https://card.mcmaster.ca/) using the Resistance Gene Identifier tool (RGI) criteria, in which only exact (Perfect) matches were considered (100% coverage and 100% amino acid identity). The genomes obtained in this study were deposited as follows: KP205RJ (Accession number: JBHGVP000000000), KP5133RR (Accession number: JBDXTF000000000), KP290RR (Accession number: JALIZR000000000), KP68.2RR (Accession number: JALIZT000000000), and KP46.2RR (Accession number: JALIZS000000000).

Determination of bla_{LAP-2} genetic context - Due to the lack of information on the bla_{LAP} genetic context involved with its dissemination, it would be interesting to determine whether this gene is being disseminated alone or as a stable module among organisms and geographic regions. For this purpose, all genomes of the species already reported in the literature as carrying bla_{LAP} alleles (either LAP-1 or LAP-2), such as *K. pneumoniae*, *K. aerogenes*, *E. coli*, *Salmonella enterica* and *Enterobacter* spp., were retrieved from GenBank. In the same way, all complete plasmid sequences were retrieved from the Plasmid Database (PLSDB). The bla_{LAP-1} and bla_{LAP-2} alleles present in the CARD database were used as references to screen these genomes and plasmids using the Abricate program, with a cutoff of 100% sequence coverage and 100% nucleotide identity. A total of 8,884 bla_{LAP}-positive genomes and 326 plasmids were retrieved. To investigate the direct genomic context where the bla_{LAP} gene is typically inserted, regions ranging from 5 kb upstream and 5 kb downstream from bla_{LAP} were analysed in all retrieved bla_{LAP}-positive sequences (8,884 genomes and 326 complete plasmids) by pairwise comparison using blastN. The sequences sharing the same bla_{LAP} direct genetic context were clustered and one

representative sequence of each cluster was chosen. The arrangements with more remarkable differences in gene synteny were visualised using the Clinker software.[24]

Comparison of bla_{LAP-2}-carrying plasmids - The presence of plasmid replicons in the genomes from Brazil obtained here was identified using PlasmidFinder v.2.0 (Available from: https://cge.cbs.dtu.dk/services/PlasmidFinder/). To trace the routes of bla_{LAP-2} transmission involved with its emergence in Brazil and to verify whether previously described plasmids carrying bla_{LAP-2} would be circulating in *K. pneumoniae* strains from Brazil, the five genomes from this study and all 326 complete bla_{LAP-2}-harbouring plasmids available in PLSDB database[25] were used in comparative analysis with the BLAST Ring Image Generator (BRIG).[26]

Phylogenomic analysis and genomic epidemiology of LAP-2-positive K. pneumoniae - Finally, to verify whether the bla_{LAP-2}-positive *K. pneumoniae* strains occurring in Brazil had already been reported in other countries, the Mash v2.2.2[27] software was used to estimate the distance between the five Brazilian genomes and the 6,734 bla_{LAP-2}-carrying *K. pneumoniae* genomes retrieved from GenBank. The genomes most closely related to those obtained in this study were selected [Supplementary data (Table)], and the core genome of this subset was estimated using Roary v3.13.0.[28] The sites with single nucleotide polymorphisms (SNPs) were extracted from the concatenated core genes using snp-sites v2.5.1.[29] The phylogenetic reconstruction was performed with IQTree v1.6.12,[30] using the best-fit substitution model (SYM+ASC+R4), to obtain a maximum likelihood tree with 1,000 ultrafast bootstrap replicates. The tree was drawn using the iTOL web platform (https://itol.embl.de).[31]

Ethics - Not required.

RESULTS AND DISCUSSION

The *in silico* MLST genomic analysis revealed that the five bla_{LAP-2}-positive strains from Brazil belonged to the pandemic high-risk ST11 clone spread worldwide.[32,33] These strains were resistant to a broad range of antibiotics, such as ampicillin, ampicillin/sulbactam, amoxicillin/clavulanic acid, ciprofloxacin, aztreonam, tigecycline, and all tested cephalosporins, accounting for an MDR (KP290RR, KP68.2RR, KP46.2RR) and an XDR (KP205RJ, KP5133RR) phenotype (Table). Fosfomycin was the only antibiotic to which all samples were sensitive. This resistance phenotype was compatible with the observed resistome, which was composed by antibiotic resistance genes (ARGs) associated with resistance to aminoglycosides (*aac(3)-IIa*, *aadA2*, *aph(3)-Ia*), fluoroquinolones (*aac(6')-Ib-cr*, *qnrS1*), chloramphenicol (*catB4*), trimethoprim (*dfrA12*), tetracyclines (*tet(A)*), sulphonamides (*sul1*), quaternary ammonium compounds (*qacEΔ1*), narrow- (bla_{LAP-2}, bla_{OXA-1}, bla_{TEM-1}), broad-spectrum β-lactams ($bla_{CTX-M-15}$, bla_{SHV-11}), and carbapenems (bla_{KPC-2}) (Table). It is worth mentioning the presence of three (bla_{LAP-2}, $bla_{CTX-M-15}$, bla_{SHV-11}) and four (bla_{LAP-2}, $bla_{CTX-M-15}$, $bla_{SHV-11,}$ bla_{KPC-2}) Ambler class A β-lactamase genes among these *K. pneumoniae* strains presenting multidrug and extensively drug resistance phenotypes. This contributes to an increase in the amounts of β-lactamases produced and, consequently, to higher MICs of β-lactams, accounting for a decrease in therapeutic options.

In spite of the spatio-temporal differences between *K. pneumoniae* strains from Roraima (2016-2017) and Rio de Janeiro (2022), the bla_{LAP-2} was embedded in the same genetic context in all of them, considering both gene synteny and nucleotide identity (Fig. 1). The bla_{LAP-2} was found in contigs of ~68 kb, and it was directly flanked by an IS*3* family transposase and the cell division-associated gene *ftsI,* coding for a PBP-3-like peptidoglycan transpeptidase. It was also found insertion sequences, transposases, several plasmid-borne genetic determinants such as conjugal transfer genes of the Type IV Secretion System, RelBE toxin-antitoxin coding genes, IncP type plasmid replication *repA*, and two additional ARGs, *tet(A)* and the *qnrS1* (Fig. 1). The presence of the entire conjugal transfer *tra* genes in the same contig where bla_{LAP-2}, *qnrS1*, and *tet(A)* were found demonstrates that these ARGs may be horizontally disseminated in a one-step fashion by conjugation to other enterobacterial genera. Moreover, blastN searches revealed that this ~68 kb LAP-2-containing module was also found in 21 publicly available *K. pneumoniae* genomes from Brazil recovered in Rio de Janeiro, São Paulo, and Amazonas states between 2014 and 2024, revealing that LAP-2 occurrence in Brazil was underestimated and that it has been carried in the same genetic environment for at least 10 years.

Interestingly, a recent study reported the occurrence of bla_{LAP-2} in a plasmid-related contig in a *L. adecarboxylata* strain recovered from a pigeon in Brazil.[13] When this contig was analysed against the ~68 kb bla_{LAP-2}-carrying modules of *K. pneumoniae* from Brazil, we observed that although they were different, the bla_{LAP-2} direct genomic context was shared among them (pRi-A4b-associated *orf* - recombinase - IS*Kpn19* - resolvase - *qnrS1* - IS*3 tnpA* - bla_{LAP-2} - *ftsI*) (Fig. 1, red dotted lines). Moreover, we verified that the bla_{LAP-2} found in *K. pneumoniae* strains from Brazil recovered between 2016 and 2022, and bla_{LAP-1} described in *E. cloacae* strains recovered from France and Vietnam between 2002 and 2005[7] shared a similar genetic environment (*qnrS1* - IS*3*-like *tnpA* - bla_{LAP} - *ftsI (pbp-3)*) (Fig. 1, blue dotted lines). This finding indicates that the direct bla_{LAP} genomic context has been conserved even among different species with different spatio-temporal distributions.

To check this, we then performed a comparative analysis of the 5 kb upstream and downstream regions surrounding bla_{LAP-2}, with the 8,884 available bla_{LAP-2}-carrying genomes and 326 complete plasmid sequences from several enterobacterial species. Twenty-five different bla_{LAP-2}-containing genetic modules were observed among the genomes analysed, demonstrating that a great proportion of genomes presented identical modules (Fig. 2). Moreover, the *ftsI* - bla_{LAP-2} - IS3 *tnpA* arrangement was observed in most modules, corroborating our hypothesis that bla_{LAP} is harboured

TABLE

General features of the bla_{LAP-2}-positive *Klebsiella pneumoniae* strains from Brazil

Strains	Sequence type	Isolation/year	Resistance classification	Antibiotic resistance profile	Resistome*
KP205RJ	11	Rio de Janeiro/2022	XDR	SAM, AMC, AMP, TIM/TIC, PIT, CPM, CTX, CFO,CAZ , CFL, CFZ, CRO, CRX , CIP, CLA, CLI, COL, ERT, IMP, MER, DOR, GEN, TE, DOX, MIN, SXT, ATM, C, TGC	*aac(3)-IIa, aac(6')-Ib-cr, aadA2, aph3-Ia,* bla_{KPC-2}, $bla_{CTX-M-15}$, *qnrS1*, bla_{LAP-2}, bla_{OXA-1}, bla_{SHV-11}, *catB4*, *sul1*, *tet(A), dfrA12*, *qacEΔ1*
KP5133RR	11	Roraima/2016	XDR	SAM, AMC, AMP, TIM/TIC, PIT, CPM, CTX, CFO,CAZ , CFL, CFZ, CRO, CRX , CIP, CLA, CLI, CLO,COL GEN, TOB, NET, TE, DOX, MIN, SXT, ATM, TGC	*aac(6')-Ib-cr, qnrS1,* $bla_{CTX-M-15}$, bla_{LAP-2}, bla_{OXA-1}, bla_{SHV-11}, *catB4, tet(A)*
KP46.2RR	11	Roraima/2016	MDR	SAM, AMC, AMP, TIM/TIC, PPT, CPM, CAZ, CRO, FOX, CXM, CFL, ETP, MER, IMI, GEN, TOB. TE, ATM, C, TGC, CIP	*aac(3)-IIa, aac(6')-Ib-cr, qnrS1,* bla_{KPC-2}, $bla_{CTX-M-15}$, bla_{LAP-2}, bla_{OXA-1}, bla_{SHV-11}, *catB4, tet(A)*
KP68.2RR	11	Roraima/2016	MDR	SAM, AMC, AMP, PPT, CPM, CAZ, CRO, FOX, CXM, CFL, GEN, TOB, SXT, ATM, C, TGC, CIP, CT	*aac(6')-Ib-cr, qnrS1,* $bla_{CTX-M-15}$, bla_{LAP-2}, bla_{OXA-1}, bla_{SHV-11}, *catB4*
KP290RR	11	Roraima/2017	MDR	SAM, AMC, AMP, TIM/TIC, PIT, CPM, CTX, CFO,CAZ , CFL, CFZ, CRO, CRX , CIP, CLA, CLI, CLO,COL, TOB, NET, TE, DOX, MIN, SXT, ATM, TGC	*aac(6')-Ib-cr, qnrS1,* $bla_{CTX-M-15}$, bla_{LAP-2}, bla_{OXA-1}, bla_{SHV-11}, *catB4*

SAM: Ampicillin+Sulbactam; AMC: Amoxicillin+Clavulanic acid; AMP: Ampicillin; TIM/TIC: Ticarcillin+Clavulanic acid; PIT: Piperacillin+Tazobactam; CPM: Cefepime; CTX: Cefotaxime; CAZ: Ceftazidime; CRO: Ceftriaxone; CFO: Cefoxitin; CRX: Cefuroxime; CFL: Cephalothin; CIP: Ciprofloxacin; CLA: Clarithromycin; CLI: Clindamycin; CLO: Chloramphenicol; COL: Colistin; ERT: Ertapenem; MER: Meropenem; IMP: Imipinem; DOR: Doripenem; GEN: Gentamicin; TOB: Tobramycin; NET: Netilmicin; AMI: Amikacin; DOX: Doxycycline; TE: Tetracycline; MIN: Minocycline; SXT: Trimetropim+ Sulfamethazole; ATM: Aztreonam; FOS: Fosfomycin; TGC: Tigecycline. *all antibiotic resistance genes presented 100% sequence coverage and 100% amino acid identity with the reference sequences compiled in CARD database.

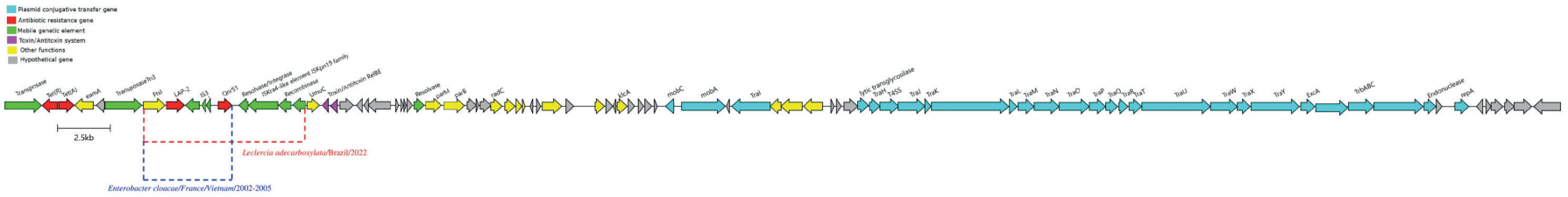

Fig. 1: schematic representation of the plasmid-derived contig (68.7 kb) carrying the bla_{LAP-2} characterised in the *Klebsiella pneumoniae* strains from this study and also found in 21 publicly available genomes from Brazil recovered between 2014 and 2024. Arrows represent gene orientation labelled with the gene name or product, and they are coloured according to the following functional categories: green, mobile genetic elements; red, antimicrobial resistance genes; blue, conjugal transfer genes; pink, toxin-antitoxin system; yellow, other functions such as repair, replication, permeases, and plasmid partition; grey, hypothetical genes. Red and blue dotted lines highlight the LAP-2-containing modules shared with *Leclercia adecarboxylata* (Brazil/2022)[13] and *Enterobacter cloacae* (France and Vietnam/2002-2005),[7] respectively.

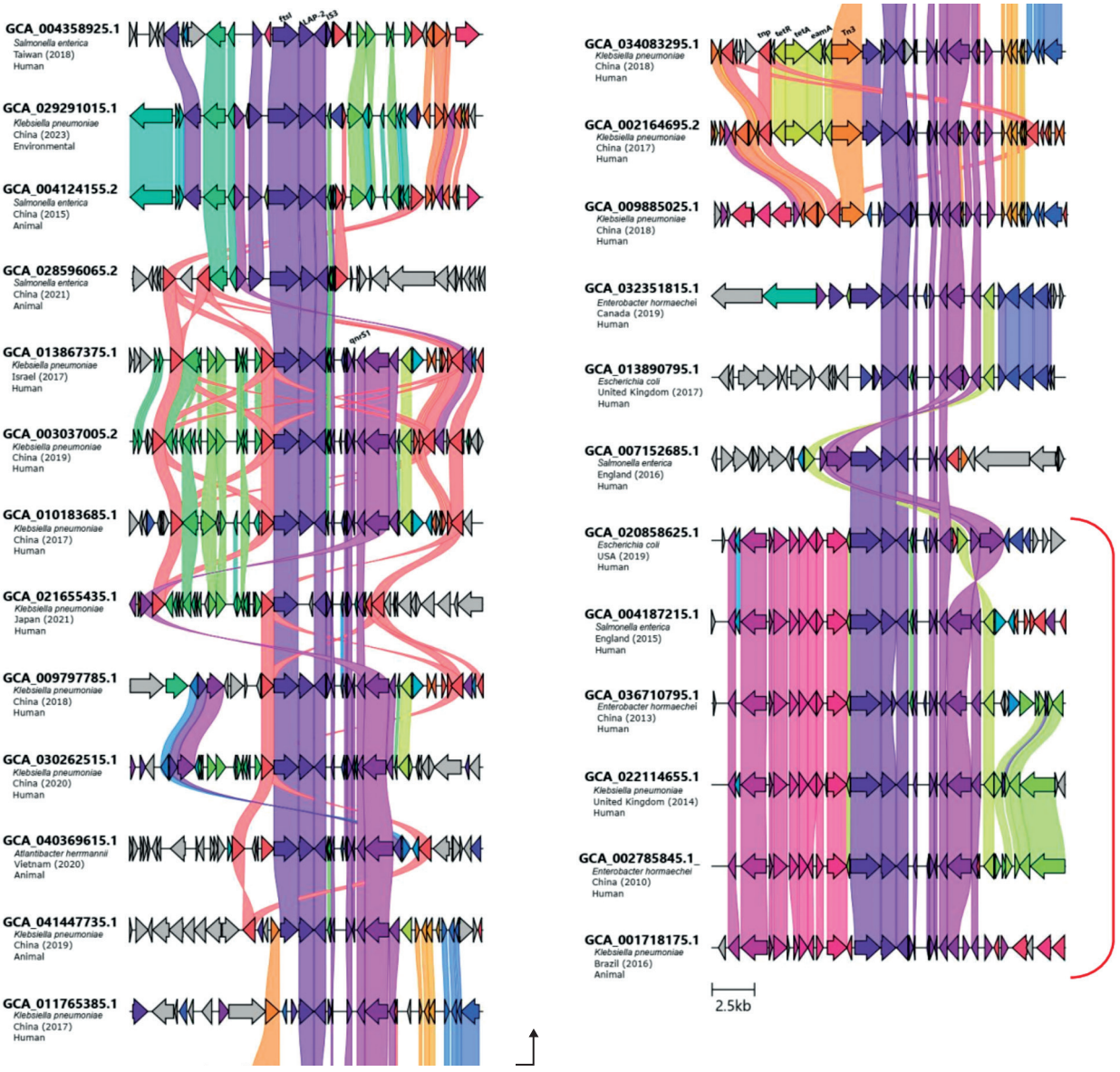

Fig. 2: genomic synteny comparisons of the bla_{LAP-2}-containing platforms present in the spatio-temporally distributed *Enterobacteriaceae* species. The alignment was created with Clinker using default settings. Each coding sequence is represented by an arrow, and shaded colour-coded vertical blocks show the conservation of gene synteny and the degree of sequence identity. Genes shared among the different platforms are shown in the same colour. The conserved LAP-2-containing core module (*ftsI* - bla_{LAP-2} - IS*3 tnpA*) is indicated as well as the *qnrS1* and *tet(A)* ARGs. Closely related platforms distributed among different species are highlighted with a red bracket.

and disseminated in a conserved core module. The direct association of bla_{LAP} and *qnrS1* was observed in 21/25 modules (Fig. 2), corroborating their strong link and co-transmission through space and time.

Interestingly, we observed that closely related bla_{LAP-2}-carrying modules were shared among different species with distinct spatio-temporal distributions (Fig. 2, red bracket). This finding indicates the significant role of horizontal gene transfer in the worldwide spread of LAP-2 among *Enterobacteriaceae* species. In conclusion, the aforementioned results altogether revealed that bla_{LAP} alleles (including bla_{LAP-1}) are found in a core genetic module, which has been disseminated throughout the years and among distinct enterobacterial genera.

Due to the relevance of horizontal gene transfer in the bla_{LAP-2} dissemination, it was verified whether there is a plasmid or a subset of plasmids involved with bla_{LAP-2} spread and its emergence in Brazil. The bla_{LAP-2}-positive genomes from this study were compared with all 326 complete bla_{LAP-2}-containing plasmids publicly available in PBSDB database. It was verified that the strains from Brazil carried an almost complete version (99.9% nucleotide identity and 88% - 92% sequence coverage) of the bla_{LAP-2}-containing plasmid pXJ-K2 (GenBank

accession number CP032243.1), recovered from a hypervirulent and MDR *K. pneumoniae* strain from China in 2018.[16] The slight differences observed among them corresponded to gain/loss of genetic elements, such as ISs and an integron (Fig. 3). Moreover, the vast majority of the bla_{LAP-2}-carrying plasmids corresponded to pXJ-K2 variants, which were almost exclusively found in *K. pneumoniae* genomes. These interesting findings revealed that bla_{LAP-2} is majorly disseminated among *K. pneumoniae* by variants of the persistent and widespread pXJ-K2 plasmid, including in Brazil.

Moreover, the genomic analysis revealed that, besides harbouring the pXJ-K2, the K205RJ strain also carried a second plasmid sharing 82% sequence coverage and 98.7% nucleotide identity with the LAP-2-carrying pK18An plasmid (GenBank accession number HF545435), present in a *K. pneumoniae* strain (K18An) recovered from a clinical case in Vietnam in 2004 (Fig. 4). Interestingly, different from pXJ-K2, which is spread among enterobacteria worldwide, the occurrence of pK18An is scarcer, since only a few significant hits (above 75% sequence coverage) were obtained with *Enterobacteriaceae* species recovered

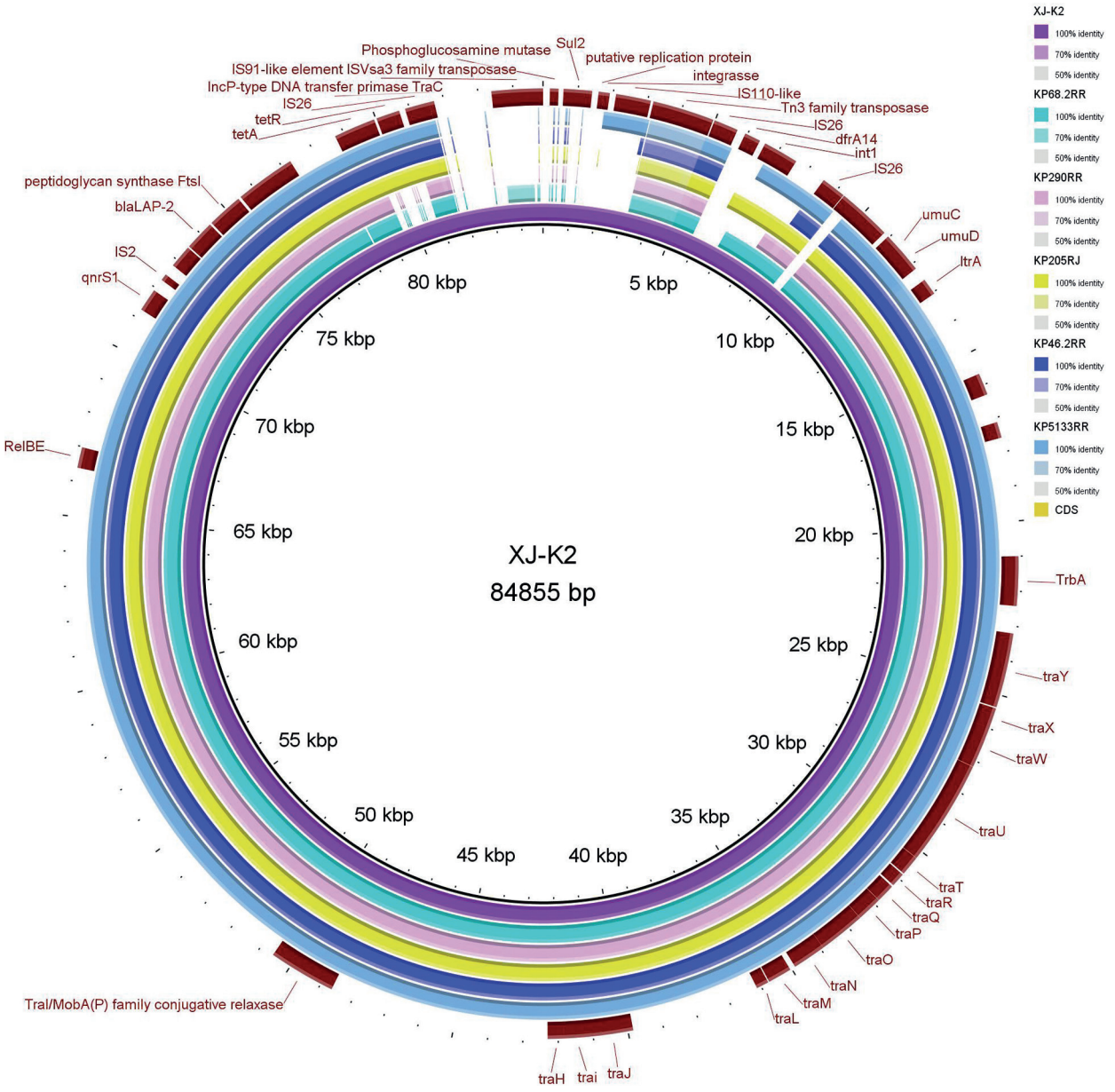

Fig. 3: BLAST Ring Image Generator (BRIG) comparative analysis of the bla_{LAP-2}-carrying pXJ-K2 plasmid and the Brazilian LAP-2-positive genomes from this study. The pXJ-K2, used as a reference, is shown in the innermost circle (purple). The plasmid architecture and composition of relevant genes, including bla_{LAP-2} and *qnrS1*, are highlighted in the outermost circle (burgundy). The plasmid-like sequences of the Brazilian genomes are plotted on each circle with different colour codes as shown in the legend. Major differences between pXJ-K2 and the plasmids of the strains from Brazil are associated with the presence/absence of mobile genetic elements.

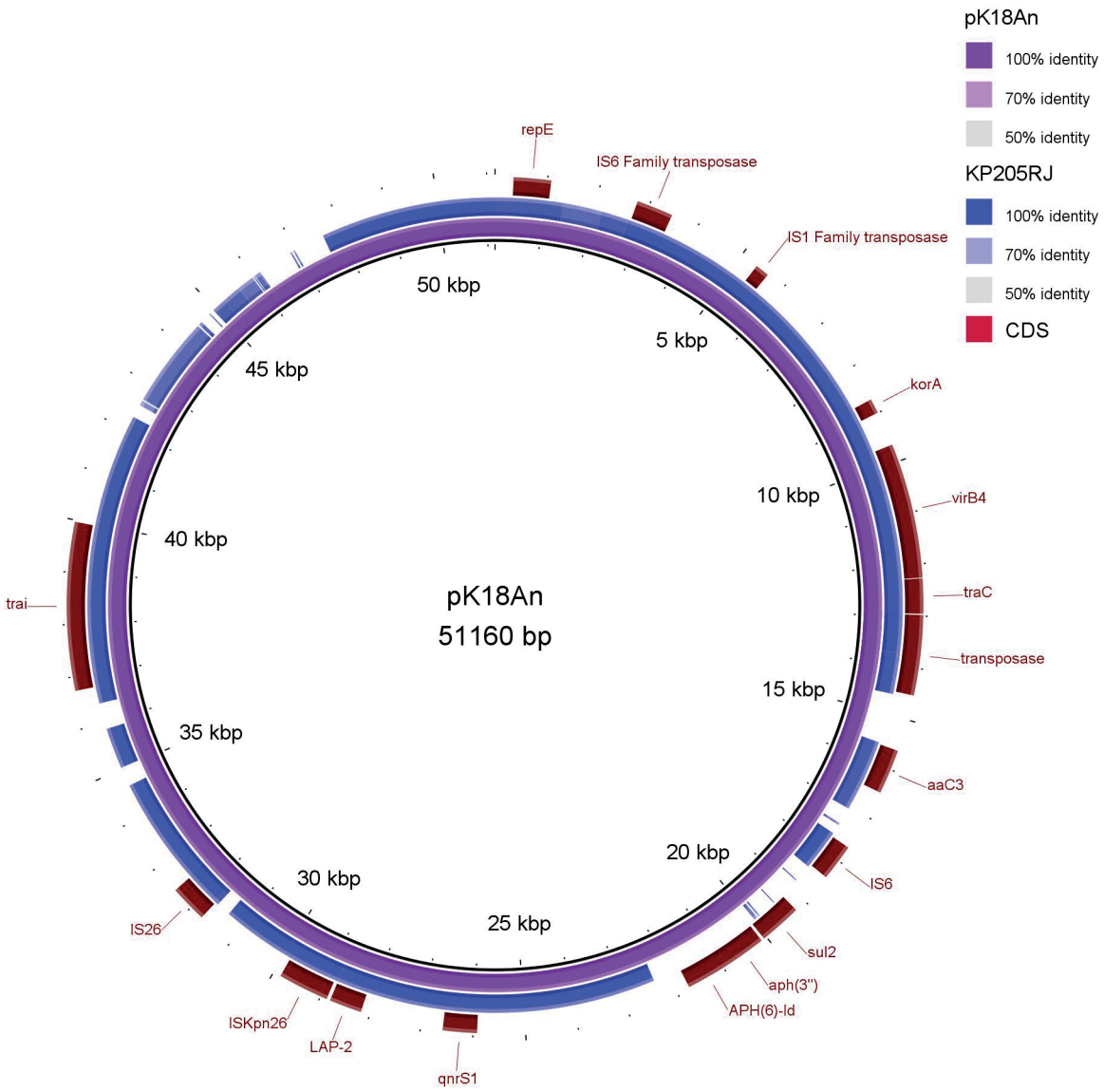

Fig. 4: BLAST Ring Image Generator (BRIG) comparative analysis of the bla_{LAP-2}-carrying pK18An plasmid and K205RJ genome. The pK18An, used as a reference, is shown in the innermost circle (purple). The plasmid architecture and composition of relevant genes, including bla_{LAP-2} and *qnrS1*, are highlighted in the outermost circle (burgundy). The K205RJ genome was plotted in the middle circle (blue). Major differences between pK18An and the plasmid harboured by K205RJ are associated with the presence/absence of mobile genetic elements.

from China (2018 - 2022), Japan (2014), Canada, USA. Interestingly, when analysing the main intrinsic differences between the globally disseminated pXJ-K2 plasmid and pKp18An, it was verified that the RelBE toxin-antitoxin (TA) system was present in the former but absent in the latter plasmid. This TA system has been associated with biofilm formation, virulence regulation,[34] and plasmid maintenance that prevents plasmid loss[35] and, therefore, its presence may contribute to the perpetuation and persistence of the pXJ-K2 plasmid.

The genetic relationship among the Brazilian strains and the closest LAP-2-positive *K. pneumoniae* circulating worldwide was evaluated to track the epidemiological routes involved with LAP-2 dissemination. The phylogenomic reconstruction, including only ST11 *K. pneumoniae* genomes, revealed three main clusters. Two of them included only genomes recently recovered in China (2023 and 2024), and the other, corresponding to the largest cluster, comprised genomes recovered worldwide (Brazil, USA, Japan, Norway, and Uganda) throughout 10 years (2014 to 2024) (Fig. 5). This data suggests that the transmission of bla_{LAP-2} in China is occurring independently relative to the rest of the world. Interestingly, additional analyses demonstrated that in all three ST11 lineages, the bla_{LAP-2} was in the context of pXJ-K2 variants, ratifying the spread potential of this plasmid and its important role in LAP-2 mobilisation. Therefore, these results demonstrated that bla_{LAP-2}, in the context of pXJ-K2 variants, has been dispersed by three ST11 lineages, two occurring in China and one globally disseminated worldwide, including in Brazil, where it has been disseminated and persisting since 2014.

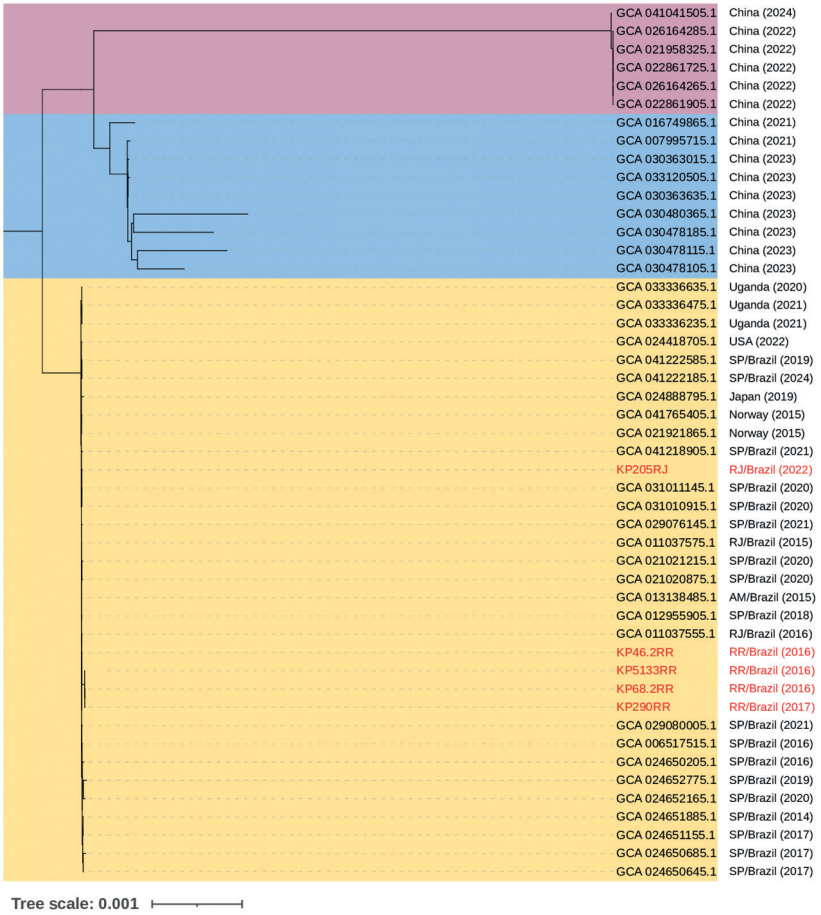

Fig. 5: single nucleotide polymorphism (SNP)-based phylogenomic tree assessing the genetic relationship among the genomes from Brazil obtained in this study and the most closely related LAP-2-positive *Klebsiella pneumoniae* publicly available genomes. These genomes were indexed with Mash software (all of them from ST11) and selected among 6,734 LAP-2-carrying *K. pneumoniae* genomes. The three clusters are delimited by different shaded colours. The genomes from Brazil obtained in this study are highlighted in red.

In conclusion, this study assessed the epidemiology and the transmission dynamics of $bla_{\text{LAP-2}}$. This gene is found in a core genetic module conserved among different species spatio-temporally distributed, and has been silently transmitted by both plasmid spread and clonal expansion, since it is majorly disseminated by variants of the persistent and widespread pXJ-K2 plasmid that is circulating among *K. pneumoniae* ST11 lineages distributed worldwide.

Although the *K. pneumoniae* species is intrinsically resistant to narrow-spectrum β-lactams due to the constitutive expression of bla_{SHV}, it may act as a reservoir of plasmids harbouring $bla_{\text{LAP-2}}$ and other ARGs that could be easily transferred in a one-step fashion to other enterobacteria. Therefore, in this scenario, $bla_{\text{LAP-2}}$ spread may compromise the use of narrow-spectrum β-lactams as an alternative stewardship strategy to treat MDR enterobacteria-causing infections.

ACKNOWLEDGEMENTS

To Dr Raquel Caldart and Caio Rodrigues from the Federal University of Roraima and the Federal Hospital of State Servants/RJ, respectively, for the isolation of strains analysed in this study.

AUTHORS' CONTRIBUTION

NMSB - formal analysis, investigation, methodology, software, writing - original draft; SMM - data curation, formal analysis, investigation, methodology, software; FSF - methodology; ACPV - funding acquisition, resources, validation; ELF - conceptualisation, data curation, supervision, validation, writing - original draft, writing - review and editing. The authors declare that there are no conflicts of interest.

DATA AVAILABILITY

The contents underlying the research text are included in the manuscript.

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OPEN PEER REVIEW

Memórias do IOC thanks the anonymous reviewers for their contribution to the peer review of this work.

FIRST REVIEW ROUND

REVIEWERS' COMMENTS

REVIEWER #1

The paper entitled "Global Silent transmission of the Class A B-lactamase LAP-2 by plasmid transfer and clonal expansion of Klebsiella pneumoniae lineages" describes for the first time the presence of blaLAP-2 in Brazil and provides a global context to the dissemination of the field. The paper brings important contributions to the field; however, there are several gaps in the methodological procedures that need to be clarified.

Major Review:

Abstract: Please italicize species names.

Introduction: Please further clarify and introduce better about the narrow-spectrum cephalosporins and penicillin derivatives. The authors used a single reference as a background source about this information, which is crucial to the paper. Also need to add on this first paragraph, references to sustain the discussion about the silent pandemic. The paper mentioned at the end of the first paragraph is related to the narrow-spectrum cephalosporins.

Methods/Results: More context is needed on the five Brazilian isolates. Are they of human origin? What type of infection? Where were they isolated as part of routine screening? The authors presented an antibiotic profile in the results (Table 1), but there is no description of the methodology for obtaining these results. What AST standards were used?

The authors mention that the 5 isolates belong to ST11, as well as mention the other publicly available similar genomes they found from ST11. How was ST estimated? Which program was used?

Does the author perform any quality checks on the 5 Brazilian genomes? How good was the assembly? How many contigs? N50? Do the authors assess completion and contamination of the 5 genomes?

The description of how the authors found other publicly available genomes is very poor. They mentioned CARD, but CARD was just a database. Did the authors download all publicly available genomes and screen through CARD? Did the authors use one of the tools inside CARD to find more genomes?

Please include the threshold of completeness and identity used to confirm the presence of LAP-2 on the genomes. Please also provide supplementary material showing completeness and identity for all AMR genes identified in the 5 Brazilian genomes.

Clinker requires as input either GFF or GBK files. Did the authors annotate all the genomes used in Clincker analysis? Did the authors retrieve the annotations from NCBI?

Are all blaLAP-2 genomes present on PLSDB the same genomes previously identified throughout CARD?

Please. Provide support data with information on all publicly available genomes used in this study, their accession numbers, their assembly level, and biosample metadata info. It is really unclear and poorly described about those genomes used in the paper, and understanding the dataset is a fundamental part to corroborate the dataset. The whole method is really short and summarized, hindering a lot of information that is crucial to the paper.

Images: Figure 1 is too small; the reviewer suggests putting this image in a landscape format. Also, this is not the appropriate way to show the comparisons among genomes. Why did the authors not create an image similar to Figure 2 to demonstrate this?

Did the authors perform Clinker analysis on the 8,884 genomes available to compare the region? How did the authors come up with twenty-five modules? It would really add to this paper if the author could further clarify the differences among those modules and what the predominant module is among the isolates.

Was the plasmid of the Brazilian strains assembled in a single contig?

Minor: The Results/Discussion section has more results. The authors do not properly discuss their results, bringing some literature context to that/

AUTHORS' RESPONSE TO THE REVIEWERS

Dear Editor,

We greatly appreciate the detailed comments and suggestions, which will undoubtedly enrich our manuscript. All suggestions were pertinent, and they were addressed and incorporated in the MS revised version, which are highlighted in the text. Find below the point-by-point response concerning reviewer recommendations:

Reviewer: 1

Reviewer comments: The paper entitled "Global Silent transmission of the Class A B-lactamase LAP-2 by plasmid transfer and clonal expansion of Klebsiella pneumoniae lineages" describes for the first time the presence of blaLAP-2 in Brazil and provides a global context to the dissemination of the field. The paper brings important contributions to the field; however, there are several gaps in the methodological procedures that need to be clarified.

Major Review:

Abstract: Please italicize species names.

Response: The corrections were made

Introduction: Please further clarify and introduce better about the narrow-spectrum cephalosporins and penicillin derivatives. The authors used a single reference as a background source about this information, which is crucial to the paper. Also need to add on this first paragraph, references to sustain the discussion about the silent pandemic. The paper mentioned at the end of the first paragraph is related to the narrow-spectrum cephalosporins.

Response: we included additional information on the state of the art concerning the importance of narrow-spectrum beta-lactams in the treatment of infections caused by MDR bacteria.

However, the term "Silent spread" was used in the title and in the text to call attention to the unnoticed dissemination of an antimicrobial resistance gene (blaLAP-2) that has the potential to become clinically significant and for which there was scarce epidemiological information. Even though, we included in the third paragraph of the introduction some references about the impact of silent dissemination of resistance genes.

Methods/Results: More context is needed on the five Brazilian isolates. Are they of human origin? What type of infection? Where were they isolated as part of routine screening? The authors presented an antibiotic profile in the results (Table 1), but there is no description of the methodology for obtaining these results. What AST standards were used?

Response: The reviewer is right, several methodological information concerning the studied strains was missing, and they are now provided in the material and methods section.

The authors mention that the 5 isolates belong to ST11, as well as mention the other publicly available similar genomes they found from ST11. How was ST estimated? Which program was used?

Response: In fact, this information was missing. The ST of the five Brazilian genomes and the publicly available genomes included in phylogenetic tree was estimated by in silico MLST. This information was included in the Material and Methods section.

Does the author perform any quality checks on the 5 Brazilian genomes? How good was the assembly? How many contigs? N50? Do the authors assess completion and contamination of the 5 genomes?

Response: The number of contigs and N50 of each assembly were, respectively: Kp290RR, 115 and 209 kb; Kp46.2RR, 170 and 167 kb; Kp68.2RR, 109 and 148 kb; Kp205RJ, 269 and 89 kb; Kp5133RR, 518 and 17 kb. The completeness and contamination were assayed using BUSCO with the enterobacteriaceae_odb12 dataset, where Kp5133RR presented 93.8% completeness and the other genomes ≥98.7% completeness.

The description of how the authors found other publicly available genomes is very poor. They mentioned CARD, but CARD was just a database. Did the authors download all publicly available genomes and screen through CARD? Did the authors use one of the tools inside CARD to find more genomes?

Response: we agree that this part should be presented in more detail. In fact, we downloaded from GenBank all genomes of the species that have already been reported to carry the blaLAP-1 or LAP-2 genes. We also downloaded all complete plasmid sequences available in PLSDB. Using the Abricate program, these genomes and plasmids were screened for the presence of blaLAP-1 or -2 using as queries the blaLAP-1 and blaLAP-2 reference genes present in the CARD database. This methodology is presented in more detail in the MS revised version.

Please include the threshold of completeness and identity used to confirm the presence of LAP-2 on the genomes. Please also provide supplementary material showing completeness and identity for all AMR genes identified in the 5 Brazilian genomes.

Response: All antibiotic resistance genes, including LAP-2, found in the five Brazilian genomes by CARD analysis presented 100% sequence coverage and 100% amino acid identity considering the CARD reference sequences. This information was included in the material and methods section and in Table 1 footnote. That is why we think providing a supplementary table with this information is unnecessary.

Clinker requires as input either GFF or GBK files. Did the authors annotate all the genomes used in Clincker analysis? Did the authors retrieve the annotations from NCBI?

Response: we only selected representative genomes for analysis in Clinker, and these genomes were annotated using Prokka.

Are all blaLAP-2 genomes present on PLSDB the same genomes previously identified throughout CARD?

Response: No, because only complete plasmid sequences are deposited in the PLSDB, and among the genomes there are fragmented sequences that cannot be directly assumed to be complete plasmids. This aspect in clearer in the revised version.

Please. Provide support data with information on all publicly available genomes used in this study, their accession numbers, their assembly level, and biosample metadata info. It is really unclear and poorly described about those genomes used in the paper, and understanding the dataset is a fundamental part to corroborate the dataset. The whole method is really short and summarized, hindering a lot of information that is crucial to the paper.

Response: Throughout this study, we analyzed hundreds of thousands of genomes. We think that providing the metadata of all these genomes would be useless. Instead we presented in the revised version a supplementary material containing the metadata of the genomes that gave support to our genomic and epidemiological analyses (Figure 5).

Images: Figure 1 is too small; the reviewer suggests putting this image in a landscape format. Also, this is not the appropriate way to show the comparisons among genomes. Why did the authors not create an image similar to Figure 2 to demonstrate this?

Response: The figure 1 format was modified as suggested.

The figure 1 was conceived with the unique purpose of showing the genetic context in which LAP-2 was found in the Brazilian genomes (the five described in the study and the other LAP-2-positive genomes retrieved from GenBank). We did not intend to compare genomes at any time. We just take advantage of this figure to call attention to the fact that the direct lap-2 genetic context (the surrounding regions) was conserved even in other species (Leclercia adecarboxylata and Enterobacter cloacae) with a distinct spatio-temporal distribution.

Did the authors perform Clinker analysis on the 8,884 genomes available to compare the region? How did the authors come up with twenty-five modules? It would really add to this paper if the author could further clarify the differences among those modules and what the predominant module is among the isolates.

Response; The region of the blaLAP genetic context was evaluated in the 8,884 genomes and 326 complete plasmids using blastN. Pairwise comparisons were made, and those genomes sharing synteny conservation in the LAP-2 surrounding regions (5kb upstream and 5 kb downstream of LAP-2) were grouped together. In this way, we obtained 25 main LAP-2 arrangements and one genome of each group was chosen as a representative genome to be visualized in Clinker. This methodology was detailed in the MS revised version.

Was the plasmid of the Brazilian strains assembled in a single contig?

Response: No! The full characterization in a single contig was not possible due to the genomic sequencing method used. But since the Brazilian genomes presented the blaLAP-2 inserted in a 68 kb region together with several plasmidial signatures, such as the IncP repA, the entire conjugal transfer tra genes, and toxin-antitoxin system, we suggested that the LAP-2 in Brazilian strains would be in the context of a plasmid. Moreover, when we performed a BlastN search of a LAP-2-carrying plasmid (pXJ-K2) against the five Brazilian genomes, we verified that a great portion of this plasmid (~90% of sequence coverage) with high nucleotide identity (99.9%) was present in the Brazilian strains, corroborating the possible plasmidial nature of LAP-2 in these strains.

Minor: The Results/Discussion section has more results. The authors do not properly discuss their results, bringing some literature context to that/

Response: we appreciate the reviewer's suggestion, but we disagree with this point. We think that our results are well discussed, given the limited information available in the literature concerning blaLAP-2 distribution, epidemiology, and evolution.

SECOND REVIEW ROUND

REVIEWERS' COMMENTS

REVIEWER #1

No comments.

DECISION: ACCEPT SUBMISSION | RECEIVED 14 MAY 2025 | ACCEPTED 13 OCTOBER 2025