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Identification of pandemic ST147, ESBL-type β-lactamases, carbapenemases, and virulence factors in *Klebsiella pneumoniae* isolated from southern Peru

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Multidrug-resistant *Klebsiella pneumoniae* (MDR *K. pneumoniae*) is a significant pathogen associated with nosocomial infections, often leading to high morbidity and mortality. This resistance is largely due to the efficient horizontal transfer of mobile genetic elements such as plasmids, which carry resistance genes and virulence factors. These elements contribute to the production of extended-spectrum β-lactamases (ESBL) and carbapenemases, which further complicates treatment. Despite the high prevalence of MDR *K. pneumoniae* in Peruvian hospitals, the genomic characterization of these strains remains limited. This study investigated the phenotypic and molecular identification of extended-spectrum β-lactamases (ESBLs), carbapenemases, and virulence factors in 91 MDR *K. pneumoniae* strains collected from three hospitals between 2022 and 2023. Phenotypic detection of ESBLs was performed using the Jarlier method, while carbapenemases were identified via double-disk synergy testing with boronic acid, EDTA, and Carba NP test. The positive isolates were further analyzed for resistance genes *(blaCTX-M, blaTEM, blaSHV, blaKPC, blaNDM, blaIMP, and blaVIM)*. Four isolates were subjected to whole-genome sequencing (WGS) for further characterization. All multidrug-resistant *K. pneumoniae* strains (100%) were ESBL-positive, with 14.3% producing carbapenemases, primarily KPC-type and metallo-β-lactamases (MBLs). The virulence factor analyses revealed that only 7.7% exhibited hypermucoviscosity. Protease activity was detected in 19.8% of the strains, and lipase activity in 1.1%. Regarding biofilm formation, 85.7% of the strains showed moderate adherence. Molecular analysis identified ESBL (*blaCTX-M*, 78%; *blaTEM*, 71.4%; *blaSHV*, 82.4%) and carbapenemase genes (*blaKPC* 7.7%, *blaNDM* 4.4%). Genomic analysis revealed various antimicrobial resistance mechanisms, including porin-coding gene mutations, aminoglycoside resistance linked to fluoroquinolone resistance, and multidrug efflux pump regulators. Sequence typing has identified high-risk clones (ST147, ST629, and ST37) associated with hospital outbreaks globally. These findings underscore the considerable concern of MDR and hypervirulent *K. pneumoniae* in Peruvian hospitals. These findings emphasize the pressing need for sustained genomic surveillance, enhanced infection control measures, and strategies to address the expanding problem of *MDR K. pneumoniae*.

Keywords *Klebsiella pneumoniae*, Multidrug-resistant, ESBL-type β-lactamases, Carbapenemases, Virulence factors

Enterobacterales have rapidly developed multidrug resistance, with *Klebsiella pneumoniae (K. pneumoniae)* being one of the most clinically significant pathogens[1,2]. This bacterium is a major pathogen responsible for nosocomial infections, such as septicemia and infections of soft tissues, as well as urinary, digestive, and respiratory tracts, particularly in immunosuppressed patients, those with mechanical ventilation, and patients

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in intensive care units[3,4]. Due to the pathogenicity of *K. pneumoniae* infections are commonly treated with antibiotics, particularly β-lactams, including extended-spectrum β-lactams[5,6]. However, the acquisition of various mobile genetic elements, including plasmids, facilitates the expression of resistance genes and virulence factors in *K. pneumoniae*[7,8]. As the primary resistance mechanism, MDR *K. pneumoniae* produces β-lactamase enzymes including carbapenemases, which inhibit antibacterial activity by breaking the β-lactam ring structure of antibiotics[6,9,10].

Additionally, *K. pneumoniae* harbors resistance genes encoding Class A serine β-lactamases (e.g., *blaKPC*)[3], Class D serine β-lactamases (e.g., *blaOXA-48*)[7], and Class B metallo-β-lactamases (MBLs) (e.g., *blaNDM*, *blaIMP*, and *blaVIM*)[11,12] with the latter two being predominantly prevalent in Asia and Europe[12,13]. Furthermore, ESBL-type β-lactamase genes, including *blaCTX-M*, *blaSHV*[9], and *blaTEM*[14], have been identified as endemic to Latin America[15].

In Peru, MDR *K. pneumoniae* is the most frequently isolated Enterobacterales species across 12 hospitals nationwide[16–18]. Resistance genes such as *blaTEM*, *blaKPC*, *blaNDM*, and *blaIMP* have been widely reported[16,19], with the co-existence of *blaTEM* and *blaCTX-M* genes documented in cities including Lima, Junín, Arequipa, and Trujillo[20]. In Cusco, however, only *blaNDM* has been genotypically reported to date[21]. The lack of reports on virulence factors and resistance genes in these isolates highlights the need for further research to better understand the molecular and genetic mechanisms, as well as their phylogenetic relationships. This will be crucial for elucidating the spread of genetic components and virulence factors, providing valuable insights into the *MDR K. pneumoniae* population in Peru.

In this context, the present study employed a combination of phenotypic and molecular techniques to identify the presence of ESBL-type β-lactamases, carbapenemases, and associated virulence factors. In addition, genomic analysis was performed on *K. pneumoniae* strains supplied by three hospitals in the city of Cusco.

Results

Bacterial isolates

Biochemical identification revealed that 87.9% (80/91) of the isolates corresponded to *Klebsiella pneumoniae subsp. pneumoniae*, and 12.1% (11/91) to *Klebsiella pneumoniae subsp. ozaenae*.These findings confirm that 100% of the strains belong to *Klebsiella pneumoniae*, as detailed in Supplementary Table 4.

Antimicrobial susceptibility profile of *K. pneumoniae* strains

The isolates showed high resistance to Ceftriaxone and Aztreonam 100%, Ceftazidime 94,5%, and Cefepime 78%. Additionally, 60.4% of the isolates were resistant to Amoxicillin/Clavulanic acid. Regarding carbapenems, 14,3% of the strains were resistant to Meropenem, while 12,1% were resistant to Imipenem, as outlined in Table 1.

Phenotypic identification of ESBL and carbapenemases

All *K. pneumoniae strains* 100% were phenotypically positive for ESBL, with 14.3% also producing carbapenemases, primarily KPC-type and Metallo-β-lactamases (MBLs) as presented in Fig. 1. Additionally, the Carba NP test showed that 13.2% of the strains were carbapenemase as detailed in Supplementary Table 5.

Pathogenic analysis

The 91 strains of *K. pneumoniae* included in this study were analyzed using various methods to detect virulence factors, as shown in Table 2 and Fig. 2. The presence of a capsule was detected in some strains, along with hypermucoviscosity, protease, and lipase activity. Additionally, certain strains exhibited lipase production. Biofilm formation, observed in some strains, enhances their ability to evade the host immune system and, in some cases, allows them to persist within the host.

Molecular characterization of ESBL β-lactam and Carbapenem resistance genes

We observed a high prevalence of ESBL genes, with *blaCTX-M* present in 78% of isolates, *blaTEM* in 71.4%, and *blaSHV* in 82.4%. In contrast, carbapenem resistance genes were less prevalent, with *blaKPC* detected in 7.7% and *blaNDM* in 4.4% of isolates, while *blaIMP, blaOXA-48,* and *blaVIM* were not observed. The strains

	S		I		R		(SDD)	
Antibiotic	**N°**	**%**	**N°**	**%**	**N°**	**%**	**N°**	**%**
Ceftriaxone (CRO)	0	0	0	0	91	100	0	0
Ceftazidime (CAZ)	0	0	5	5.5	86	94.5	0	0
Cefepime (CEF)	5	5.5	-	-	71	78.0	15	16.5
Aztreonam (ATM)	0	0	0	0	91	100	0	0
Amoxicillin/clavulanic acid (AMC)	1	1.1	35	38.5	55	60.4	0	0
Meropenem (MEN)	77	84.6	1	1.1	13	14.3	0	0
Imipenem (IMP)	79	86.8	1	1.1	11	12.1	0	0

Table 1. Antimicrobial susceptibility profile of the *K. pneumoniae* strains isolated in this study. The grey box illustrates the high percentage of resistance to the antibiotic in question. S: Susceptible; I: Intermediate; R: Resistant and SSD: Susceptible-dose dependent.

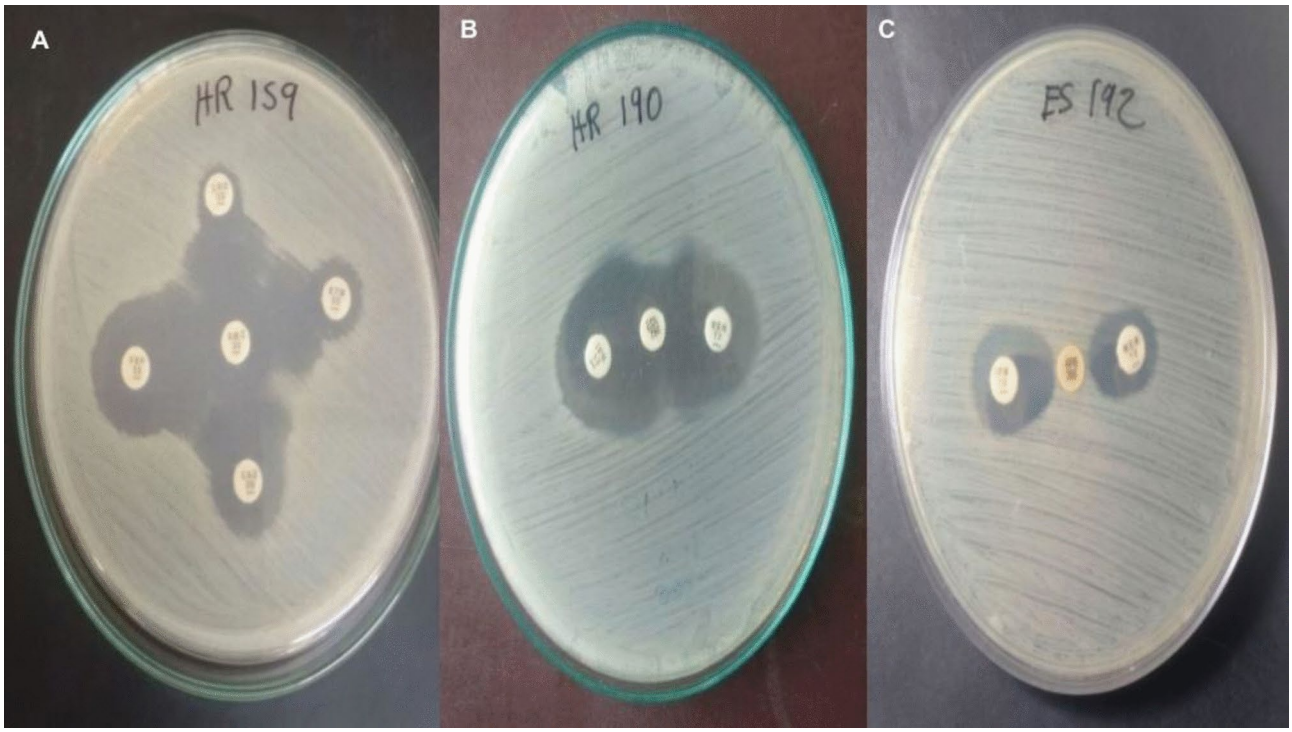

Fig. 1. Phenotypic determination of ESBL and carbapenems. (**A**) ESBL, (**B**) MBL type carbapenemase, (**C**) KPC type carbapenemase.

Factor	Strain kpn N°	%
Capsule	91	100
Hemolysis	0	0
Hypermucoviscosity [Hv]	7	7.7
Proteases	18	19.8
Gelatinases	0	0
Lecithinases	0	0
Lipases	1	1.1
Non-adherent biofilm	6	6.6
Adherent biofilm	7	7.7
Biofilm Moderately adherent	78	85.7
Biofilm Strongly adherent	0	0

Table 2. Phenotypic characterization of virulence factors.

were positive for four genes in 9.9% of cases, for three genes in 47.2% of cases, for two genes in 23.1%, for one gene in 16.5%, and no genes in 3.3% of cases. These findings suggest a higher prevalence of ESBL genes than carbapenemase genes in the study area, as observed in Fig. 3. The presence of these genes was confirmed and visualized using agarose gel electrophoresis (Fig. 4).

Whole genome sequencing and related information analysis

Although only four strains from different hospitals in Cusco were sequenced (ES221, HAL79, HR192, and HR200), important findings were evident. Strains ES221, HAL79, and HR200 were isolated from urine samples, while HR192 was obtained from blood, as detailed in Table 3. In terms of sequence typing (ST), strains ES221 and HR200 were classified as ST629, HAL79 as ST147, and HR192 as ST37. Additionally, Fig. 5 presents the STs identified in this study alongside those previously reported by other authors[4,17,22].

Analysis of the capsular loci (K-locus) and lipopolysaccharide loci (O-locus) revealed that ES221 and HR200 share the capsular locus 107-D1, while HAL79 also presents locus 107-D1, and HR192 shows locus K15. Regarding the O-locus, ES221 and HR200 exhibited O3/O3a, HAL79 showed O1/O2v1, and HR192 displayed O4. Additionally, several plasmids associated with resistance factors were identified, including IncFIB(K), which was present in all strains, as well as IncFII and IncHI1B. Common virulence genes such as *fimH*, *iutA*, *mrkA*, *traT*, and *nlpI* were detected across all strains, with *nlpI* found in the majority suggesting their involvement in

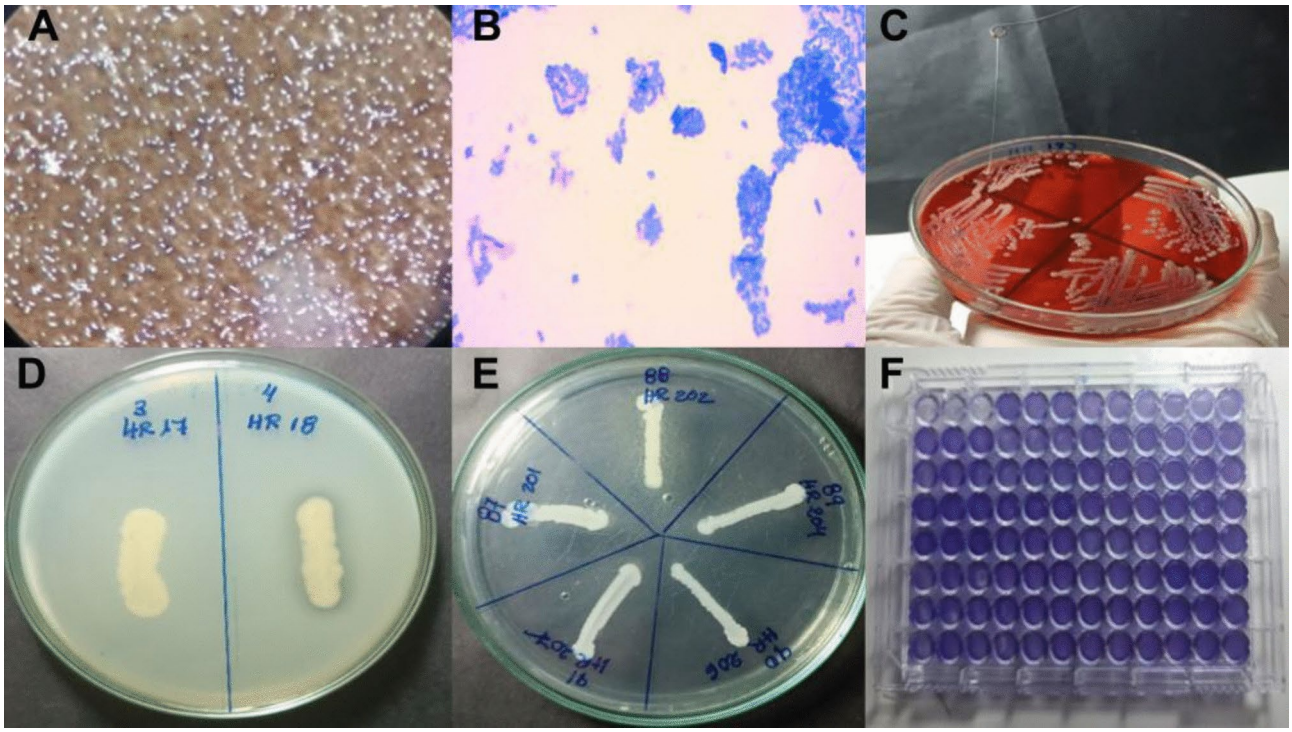

Fig. 2. Screening of virulence factors in *K. pneumoniae* strains. (**A**) Capsule detection using India ink, (**B**) Capsule detection with methylene blue, (**C**) Hypervirulent phenotype identified by the string test, (**D**) Lipase activity detected using Tween 80, (**E**) Protease activity positive, (**F**) Biofilm formation.

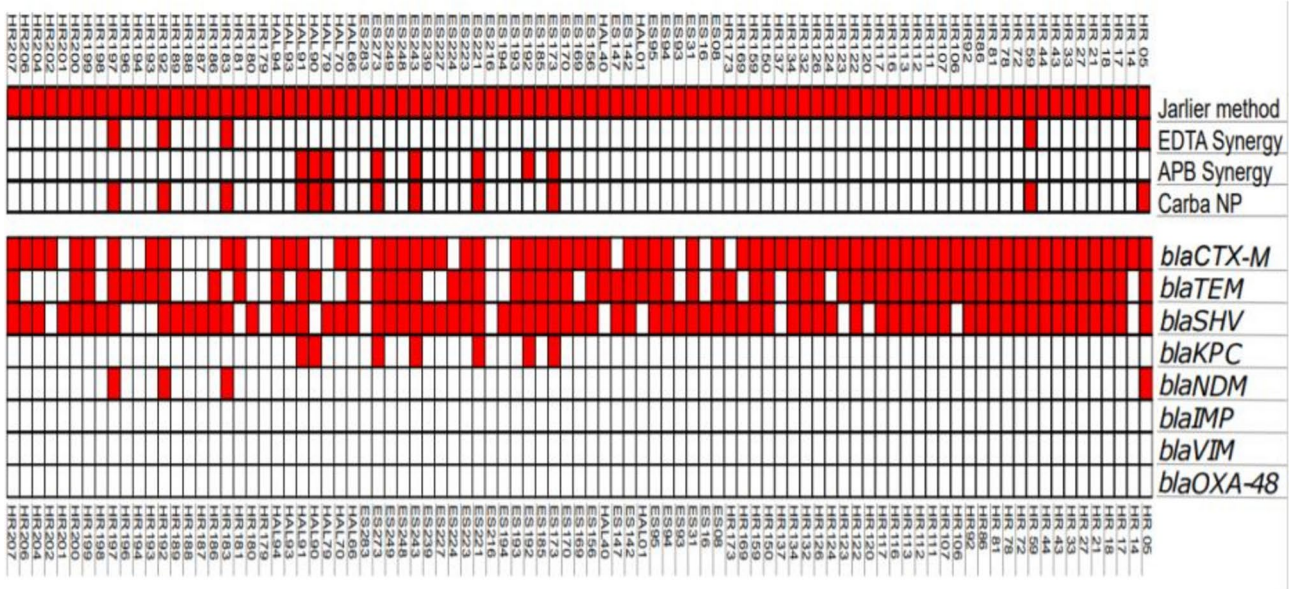

Fig. 3. Molecular and phenotypic characterization of ESBL and carbapenem resistance in *K. pneumoniae* strains. The figure shows the presence (red) and absence (white) of phenotypic and genotypic markers evaluated in the MDR *K. pneumoniae* strains.

the pathogenic potential of these strains, particularly in urinary tract infections and bacteremia. Despite being limited to four sequenced samples, this genomic analysis underscores significant genetic diversity among the strains, which may have important clinical and epidemiological implications.

The resistomes of four bacterial isolates (ES221, HAL79, HR192, and HR200) were sequenced and analyzed using advanced bioinformatics tools, as shown in Fig. 6. The results revealed a wide spectrum of antimicrobial

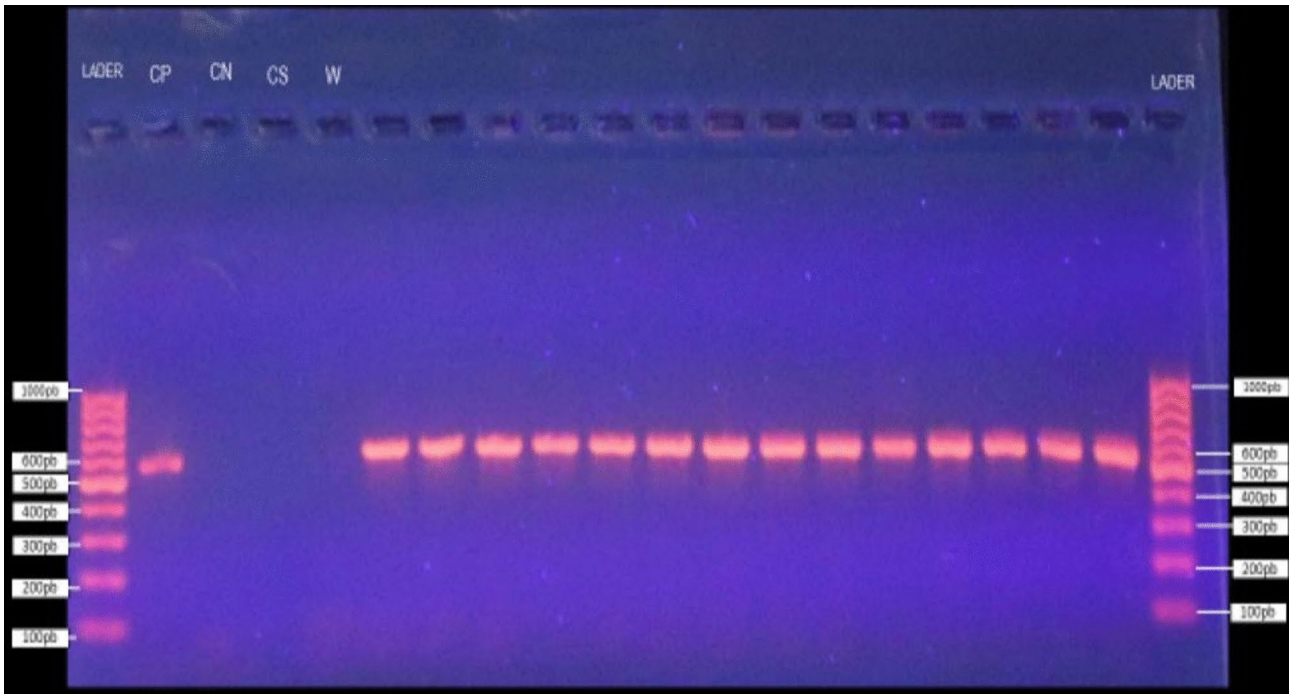

Fig. 4. Electrophoresis of *blaCTX-M* gene. LADER: LADDER (100 bp), CP: Positive control (*E. coli* NCTC 13,353), CN: Negative control (*E. coli* ATCC 25,922), CS: PCR control, W: PCR blank, and lanes 1 to 14 correspond to *K. pneumoniae* strains for the *blaCTX-M* gene.

resistance mechanisms present in these strains. Key findings include the detection of β-lactamase genes (*blaKPC-2, blaNDM-1*), mutations in porin-coding genes (such as *ompK36* variants), and the presence of aminoglycoside resistance genes *(aadA1, aph(3")-Ib*). Additionally, mutations linked to fluoroquinolone resistance (*gyrA(S83I)*) were identified, alongside alterations in regulators of multidrug efflux pumps, including *acrR*. These findings reflect the complex antimicrobial resistance landscape observed in the hospital settings of Cusco, highlighting the urgent need for ongoing surveillance genomic to track the emergence and spread of resistance mechanisms.

Figure 7 illustrates the distribution of metal resistance genes in our four clinical isolates of *Klebsiella pneumoniae* obtained from Cusco (ES221, HAL79, HR192, and HR200). The presence of genes associated with arsenic resistance (*arsD, arsR, arsA, arsB, arsC*) indicates that this resistance mechanism is widely conserved in these strains. Similarly, genes from the *pco* operon, which are linked to copper resistance (*pcoE, pcoS, pcoR, pcoD, pcoC, pcoB, pcoA*), highlight these microorganisms' ability to withstand this toxic metal. Regarding silver resistance, the *sil* genes (*silP, silA, silB, silF, silC, silR, silS, silE*) were also identified.

However, there are some discrepancies in their distribution pattern, which could be indicative of variations in environmental or clinical selective pressure. Furthermore, additional resistance genes were identified, including *fieF*, which is involved in iron homeostasis, and the ter operon, which is responsible for tellurium resistance. The distribution of these genes among the isolates was found to vary. It is noteworthy that genes associated with antimicrobial resistance, including *emrD* (multidrug efflux pumps) and qacE (resistance to quaternary ammonium compounds), were identified. This suggests that these isolates possess mechanisms enabling them to evade the effects of certain disinfectants and antibiotics.

Phylogenomics

The phylogenetic analysis of *K. pneumoniae* strains isolated in Cusco (Fig. 8) reveals that strains ES221, HAL79, HR192, and HR200 exhibit diversity in sequence types (ST), with one identified as ST147, two as ST629, and another as ST37. This study also compares with other strains from South America, including those isolated in Peru and from the Lima region, highlighting the southward expansion of ST147. Furthermore, these strains share significant resistance plasmids such as IncFIB(K), IncFII(K), and IncR, along with virulence loci like fimH, mrkD, and iutA, (Table 3) suggesting a high virulence profile capable of persisting in various clinical and environmental settings. Detecting genes conferring resistance to heavy metals and biocides underscores their potential adaptability and persistence in diverse environments. This broad geographic distribution and adaptability highlight the importance of monitoring these strains to better understand their spread and resistance.

Discussion

The rising global prevalence of hypervirulent *Klebsiella pneumoniae* (hvKp) strains, as highlighted by the World Health Organization[25], represents a significant public health threat. Despite this increase, many regions, including Peru, lack structured surveillance programs to effectively track and identify hvKp strains[25]. In Peru, the situation is particularly concerning due to the absence of robust genomic surveillance systems, which limits the ability

Characteristic	Strain ID			
	ES221	HAL79	HR192	HR200
BioSample accessions	SAMN42151949	SAMN42151950	SAMN42151951	SAMN42151952
Specie	*K. pneumoniae*	*K. pneumoniae*	*K. pneumoniae*	*K. pneumoniae*
Isolation year	2022	2022	2022	2022
SRA ID	*SRR29637058*	*SRR29637057*	*SRR29637056*	*SRR29637055*
Ward	Adolfo Guevara Velasco Hospital	Antonio Lorena Hospital	Regional Hospital of Cusco	Regional Hospital of Cusco
Isolation site	urine	urine	blood	urine
Length (bp)	5,766,021	5,760,432	5,621,675	5,767,786
N° of contigs	140	131	303	127
N50	258,277	290,549	373,405	5,767,786
Largest_contig	611,857	991,976	766,313	916,990
GC(%)	56.93	56.68	57.09	56.93
ST	629	147	37	629
wzi	485	64	50	485
K_locus	107-D1	107-D1	15	107-D1
O_locus	O3/O3a	O1/O2v1	O4	O3/O3a
Plasmids	IncFIB(K) IncFIB(pKPHS1) IncFII(K) IncFII(pCRY)	IncFIB(pNDM-Mar) IncFII IncHI1B(pNDM-MAR)	IncFIB(K) IncFII(K) IncR repB(R1701)	IncFIB(pKPHS1) IncFIB(K) IncFII(K)
Virulence factor	fimH iutA mrkA nlpI traT	fimH iutA mrkA nlpI terC traT	fimH iutA mrkA traT	fimH iutA mrkA nlpI traT
IS	ISKpn1 ISKpn27 ISEcl1 ISSen4 IS903 ISKpn19 ISKpn28 Tn5403 IS6100 IS5075 ISEc9 ISKpn26	ISKpn38 ISKox3 IS903 IS903 ISVsa5 ISEsa1 ISKpn11 ISKpn12 ISKpn43 IS5075 ISSen4 ISKpn1 ISKpn8 ISEcl1 ISKpn34 IS26	IS903 ISKpn42 ISKox1 ISEc33 IS6100 ISKpn14 ISKpn19 ISEc15 ISEc59 ISKpn47 IS903 ISVsa3 IS5075 IS26 cn_2119_IS903 cn_2303_IS903	ISKpn1 ISKpn27 ISEcl1 ISSen4 IS903 ISKpn19 ISKpn28 Tn5403 IS6100 IS5075 ISEc9 ISKpn26

Table 3. Genomic Analysis of four *MDR K. pneumoniae* strains isolated from clinical samples in Hospitals of Cusco, Peru.

to monitor the spread of hvKp strains carrying carbapenemase genes[26]. In Cusco, this challenge is especially concerning. Ministry of Public Health (MINSA) and Essalud hospitals have limited diagnostic capabilities, despite the critical need for molecular evaluation to detect resistant strains. The growing number of reports identifying *K. pneumoniae* as a leading cause of life-threatening hospital-acquired infections[2,19,21] underscores the urgent necessity for a genomic surveillance system in Peru. Such a system would facilitate more accurate tracking of multidrug-resistant (MDR) bacteria, providing critical insights into their spread and supporting more informed public health strategies[27].

In this study, 91 strains of *K. pneumoniae* were analyzed through phenotypic characterization, with 100% of strains identified as extended-spectrum β-lactamases (ESBLs) producers. The Jarlier method was found to be effective and specific for detecting ESBL-producing bacteria[19,21,28]. Among these strains, carbapenemases were detected in 13 isolates. The ethylenediaminetetraacetic acid (EDTA) test identified 38.5% of these as class B carbapenemases (metallo-β-lactamases, MBLs), while the double-disk synergy method with boronic acid (APB) detected 61.5% as class A carbapenemases[29,30]. Notably, none of the strains exhibited characteristics of both classes simultaneously. The Carba NP test demonstrated high sensitivity, detecting 92.3% of carbapenemases overall (classes A and B)[29,31]. The observed increase in class A KPC-type carbapenemases in this study may be attributed to co-infections occurring in the post-COVID-19 pandemic period, contrasting with findings from other studies[19,32,33] (Supplementary Figs. 9, 10, 11).

The findings of this study underscore the effectiveness of the phenotypic methods employed, which allowed for a comprehensive assessment of all isolates to identify various virulence factors. Remarkably, every analyzed strain (100%) exhibited a polysaccharide capsule, aligning with previous research findings[8]. This capsule, as the outermost structure of bacteria, is crucial for mediating the initial interaction with the host, facilitating adherence and immune system evasion, underscoring its pivotal role in bacterial pathogenicity[34].

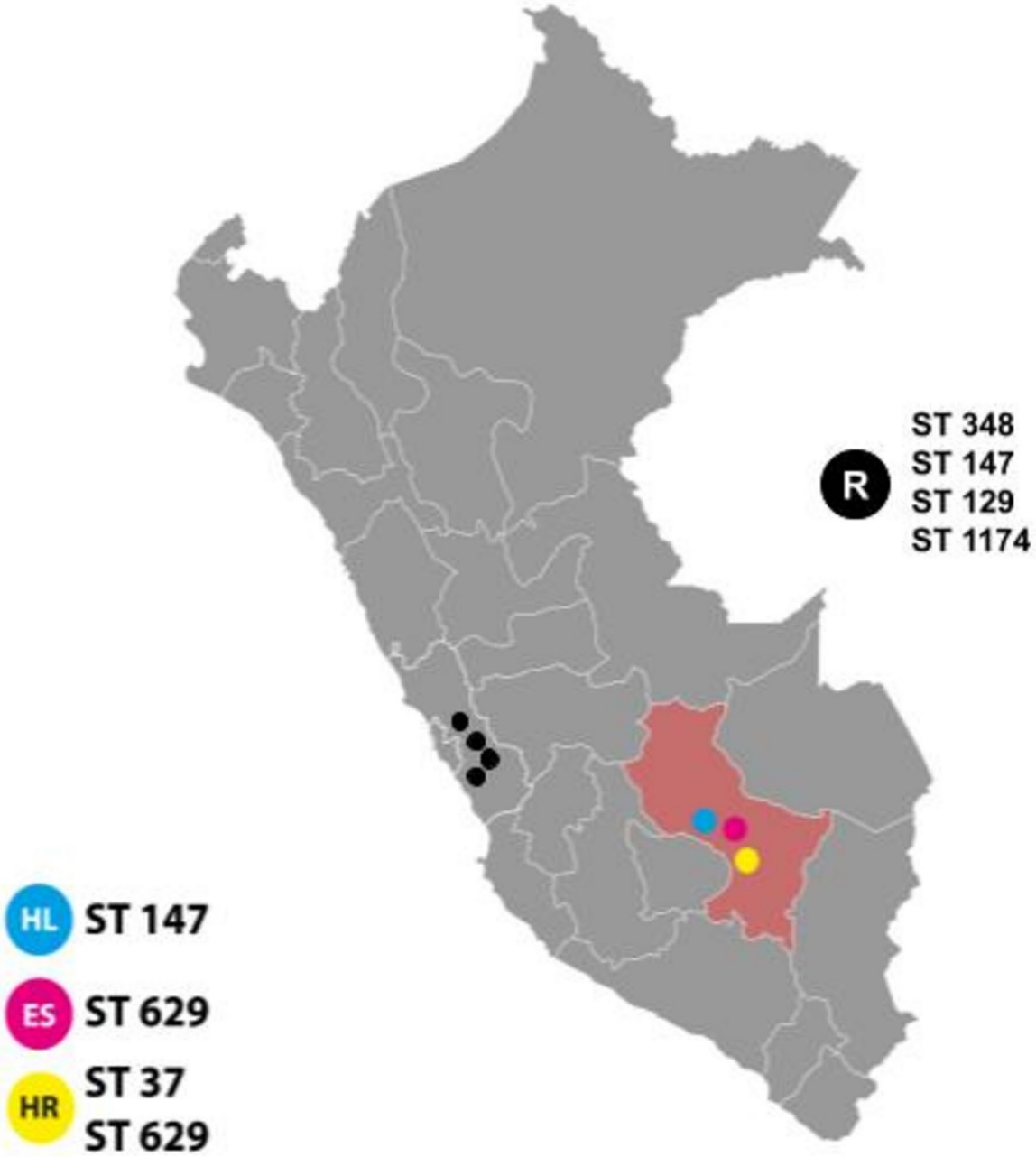

Fig. 5. The sequence types (STs) identified in the present study across three hospitals in Cusco: HL (Antonio Lorena Hospital), ES (Adolfo Guevara Velasco Hospital), and HR (Regional Hospital of Cusco). Additionally, it includes previously reported sequence types for Peru (R).[23] (https://estadist.inei.gob.pe/map),[24] QGIS software (https://qgis.org version 3.16) and Google Slides (https://docs.google.com/presentation/create?hl=en).

Furthermore, a significant proportion of the strains (93.4%) demonstrated a notable ability to develop both adherent and moderately adherent biofilms. Biofilms are complex structures that not only protect bacteria from host immune responses but also significantly increase their resistance to antibiotics, complicating treatments and heightening the clinical relevance of these microorganisms[35–37]. This capability to form robust biofilms highlights the need for therapeutic strategies that effectively interfere with the formation or stability of these structures.

In addition to biofilm formation, another key virulence factor was the hypermucoviscous phenotype, which is directly associated with increased resistance to complement-mediated killing and phagocytosis[8,38]. This phenotype enhances the virulence of *K. pneumoniae* by boosting its ability to survive and proliferate in the hostile host environment, making these strains particularly difficult to eradicate. Together, these findings reinforce the importance of monitoring and understanding the virulence mechanisms of *K. pneumoniae*, as they provide valuable insights into potential pathways for the development of more effective therapeutic interventions. The

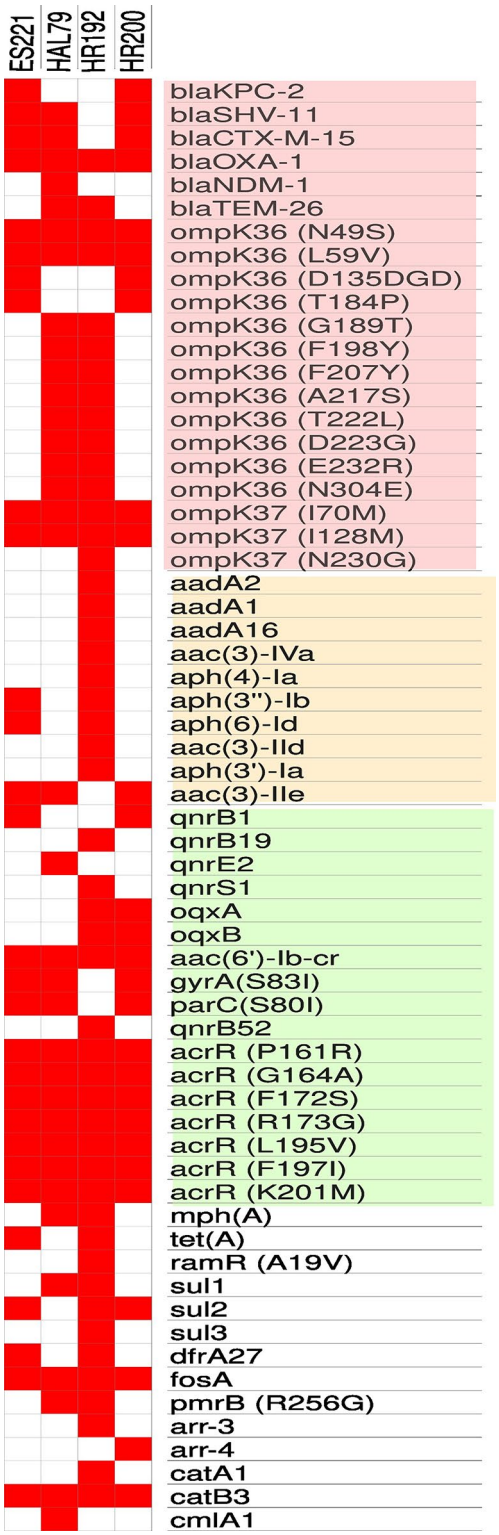

Fig. 6. Heatmap of the resistome of MDR *K. pneumoniae* strains. Mutations or resistance genes are shown in red, indicating their presence, while white indicates wild-type regions without mutations.

persistence of these virulent features underscores the urgent need for innovative approaches in the prevention and treatment of infections caused by resistant strains.

Identifying hvKp strains is crucial for managing infections in vulnerable patients and those at risk of multidrug-resistant (MDR) infections[25,26,39]. As these strains are now globally widespread, our findings provide valuable insights into their national distribution and transmission pathways, underscoring the need for enhanced surveillance and control measures to address hvKp strains resistant to last-line antibiotics[40].

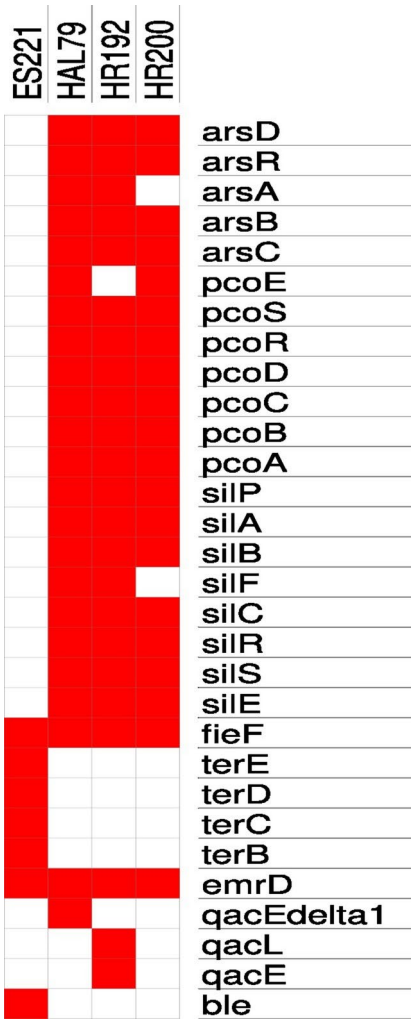

Fig. 7. Profile of genetic resistance to heavy metals and biocides in clinical isolates of *K. pneumoniae*. The color red indicates the presence of the gene, while white indicates its absence.

The molecular profile indicated the presence of ESBL genes (*blaSHV, blaCTX-M,* and *blaTEM*) in *K. pneumoniae* strains, consistent with findings of the authors[19,22] in Lima. However, in the Northern Andean region of the country, particularly in Cajamarca, a higher prevalence of ESBL genes (90%) was found, as reported by Cornejo[41]. In the present study, the strains were sourced from three primary hospitals in the Cusco region, highlighting the notable dispersion of extended-spectrum ESBL-type genes. This phenomenon is of particular concern because the production of β-lactamases continues to be the predominant resistance mechanism among Gram-negative enterobacteria[6].

Mayta 2021, marks the first genotypic detection of the *blaNDM* gene in the Cusco region[21]. The prevalence of carbapenemase genes (*blaNDM* and *blaKPC*) was lower than that of ESBL-type genes[16,22,42]. Notably, our molecular analysis did not detect *blaOXA-48*, *blaVIM*, or *blaIMP* genes in *K. pneumoniae* strains, aligning with previous findings[21,22]. In contrast, Sacsaquispe (2017) reported a 1.2% prevalence of the *blaIMP* in Peru[16], suggesting that resistance linked to this gene has not significantly expanded in *K. pneumoniae* over time. This could indicate that the gene is less prevalent in the Cusco region or that its prevalence has declined. Nevertheless, studies have shown that carbapenemase genes are widely distributed across Peru[16,22], and variations in reported prevalence may reflect differences in antibiotic resistance profiles and gene distribution patterns[43]. This study is the first report of *blaKPC* in the Cusco region, offering valuable insights into the epidemiology of carbapenem resistance and the dissemination of resistance genes in Peru.

Only 3.3% of the analyzed strains lacked the targeted resistance genes in this study. However, all of these strains exhibited a phenotypic profile indicative of ESBL and/or carbapenemase production. A similar observation was reported by Caparachin[19], in which 2% of the analyzed strains did not amplify the assessed genes. This suggests the potential presence of other ESBL genes, such as *blaPER*, and less common carbapenemase genes, such as *blaIMI*, in these strains[36,44]. While genomic analysis of the selected strains confirmed the absence of these specific genes, this does not conclusively rule out their presence in the region, as not all strains were sequenced.

The resistome analysis of *K. pneumoniae* clinical isolates from the Cusco region revealed a significant diversity of genes that confer resistance to antibiotics and heavy metals. This highlights the potential for these

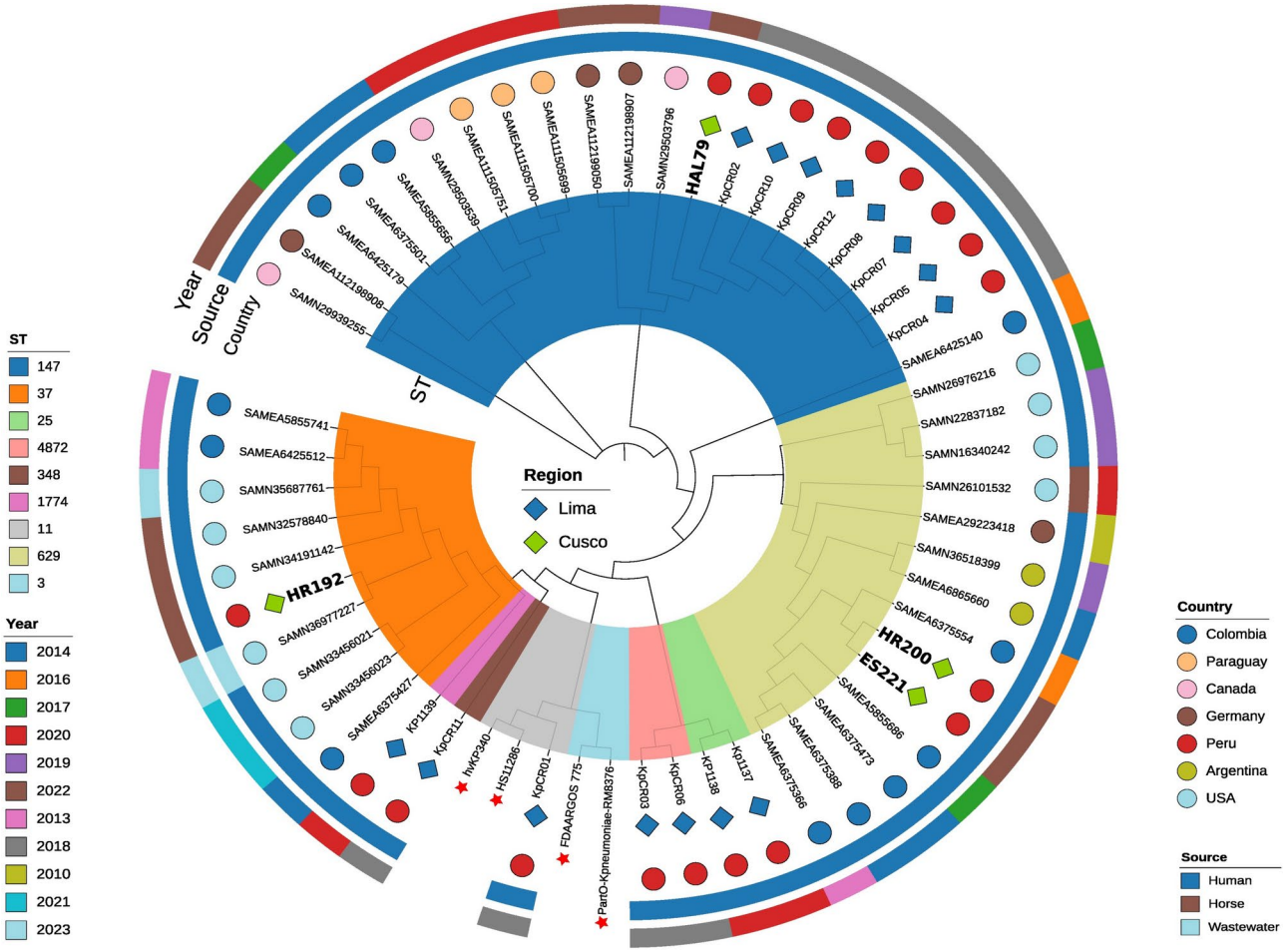

Fig. 8. Core-Genome Phylogenetic Analysis of *K. pneumoniae* strains isolated from Cusco Reference genomes are indicated with red stars.

pathogens to persist in hospital environments and cause infections that are challenging to treat. Among the antibiotic resistance genes, we were able to identify *blaKPC-2*, *blaCTX-M-15*, *blaNDM-1*, and *blaSHV-11*, which are associated with resistance to carbapenems and third-generation cephalosporins. This finding is consistent with observations reported by Carvalho, who detected carbapenemase-producing strains of *K. pneumoniae*[45].

Additionally, we report mutations in the porins ompK36 and ompK37, which suggest reduced bacterial membrane permeability, contributing to increased resistance to β-lactam antibiotics. This is consistent with results from previous studies[46] that reported similar mutations. The presence of mobile genetic elements; such as plasmids, insertion sequences (IS), and transposons in our isolates; supports the hypothesis that these strains have a high capacity to acquire and disseminate resistance genes, potentially facilitating horizontal transmission in clinical environments. Insertion elements such as ISKpn, coupled with the presence of broad-spectrum conjugative plasmids, pose a serious public health risk by enabling the rapid spread of resistance genes among various pathogenic bacteria[47].

However, the identification of genes related to resistance to heavy metals, such as *ars, pco,* and *sil*, suggests that these isolates may be exposed to environments contaminated with metals. This co-selection of resistance to antibiotics and heavy metals has been documented in several studies, which have shown that metals can act as selective pressure factors, facilitating the retention of mobile genetic elements carrying antibiotic-resistance genes[48]. The detection of *pco* and *sil* genes, which confer resistance to copper and silver, respectively, suggests the presence of these metals as possible selection agents in hospital or industrial environments, where both metals are used for bacterial control[49]. These findings reinforce the urgency of implementing more rigorous infection control measures, as well as monitoring the spread of resistance genes in hospital environments and surrounding ecosystems, given that the presence of heavy metals could contribute to the evolution of multiresistant bacteria.

Analysis of *K. pneumoniae* sequence types (ST) from three hospital isolates (four strains) revealed notable genetic diversity, highlighting the circulation of clinically relevant lineages. The ST147, ST37, and ST629 lineages present characteristics that favor their dissemination in hospital environments while acquiring resistance and virulence mechanisms that make them particularly dangerous. The ST147 has been identified as a high-risk clone worldwide (pandemic), associated with resistance to carbapenems and the production of extended-spectrum β-lactamases (ESBL). In the Peruvian context, this lineage has been documented in previous research by the

authors[4,17]. Recent studies have implicated ST147 in hospital outbreaks of multidrug-resistant *K. pneumoniae*, particularly in critically ill patients, such as those with COVID-19-related infections. This underscores its public health relevance, given its high mortality rates and increasing resistance to conventional antimicrobial treatments[50,51].

In our study, the HAL79 strain, which belongs to this sequence type (ST), exhibited a clear pattern of carbapenem resistance, consistent with reports in the literature regarding ST37. This sequence type is also associated with severe infections and multidrug resistance, with its ability to disseminate genes such as *blaKPC* being one of the major therapeutic challenges[52]. This lineage has been reported across various regions globally, and its persistence suggests a remarkable capacity to adapt to diverse clinical environments, emphasizing the need for enhanced epidemiological surveillance.

Regarding ST629, although it is less prevalent in clinical isolates, it has been documented in non-hospital environments, including wild birds and wastewater[53,54]. The presence of ST629 in sour isolates may support the hypothesis that strains adapted to animals can also interact with humans[55]. Its identification in hospital settings points to a recent clonal spread, potentially facilitated by local factors and an increased ability to colonize different hospital niches. Furthermore, ST629 is related to the risk of nosocomial infections, which complicates the treatment of nosocomial infections and limits the available therapeutic options. These findings highlight the importance of genomic surveillance in tracking the circulation of these high-risk lineages.

Conclusion

Notably, this study is the first to report the presence of *blaKPC*, *blaSHV*, *blaTEM*, and *blACTX-M* in *Klebsiella pneumoniae* strains from the Cusco region. Strains from the three hospital centers exhibited a higher prevalence of extended-spectrum β-lactamases (ESBLs) than carbapenemases, along with a significant number of heavy metal resistance genes, resistance to other antibiotics, and virulence factors. Furthermore, this study describes the inaugural detection of the high-risk clone ST147 in Cusco, marking one of the initial reports for Peru. The genomic analyses conducted in this study represent a critical first step toward the establishment of urgent molecular surveillance in Peru. These findings have important implications and offer a clearer understanding of antibiotic resistance in Peru. They also highlight the need to develop genomic surveillance systems for pathogens, which are essential for tracking the spread and virulence of these organisms in both hospital environments and the wider community.

Methods

Reagents

Amoxicillin/Clavulanic Acid (AMC) (20/10 µg) was purchased from Oxoid, USA. Cefotaxime (CTX) (30 µg), Cefepime (FEP) (30 µg), Ceftazidime (CAZ) (30 µg), Aztreonam (ATM) (30 µg), Ceftriaxone (CRO) (30 µg), Meropenem (MEM) (10 µg), Imipenem (IMP) (10 µg), Boronic Acid (APB) (300 µg), and Ethylenediaminetetraacetic Acid (EDTA) (750 µg) were all purchased from Bioanalyse (Turkey) and Taq DNA polymerase brand Gotaq Flexi DNA from Promega-USA.

Bacterial isolates

The hospitals provided 91 presumptive strains of *K. pneumoniae* along with the associated data for each strain. These strains exhibited profiles suggestive of extended-spectrum β-lactamase (ESBL) and carbapenemase production. Hospitals provided the strains. Antonio Lorena Hospital (9 strains), Regional Hospital (55 strains), and Adolfo Guevara Velasco National Hospital—EsSalud (27 strains) during the years 2022 and 2023. All Klebsiella strains were transported in Amies transport medium with charcoal to the Institutional Laboratory of Microbiology and Immunology at the Faculty of Biological Sciences, Universidad Nacional San Antonio Abad del Cusco. All procedures were performed following the relevant guidelines and regulations and with the approval of the Institutional BioEthics Committee of Universidad Nacional San Antonio Abad del Cusco with protocol N° CBI-UNSAAC-2022-002 and Universidad Mayor de San Marcos N° 017-2022-CB-FCB-UNMSM and with each hospital's authorization: Cusco Healthcare Network Management Resolution No.230-GRACU-ESSALUD-2022, Proveido N°98-2022-GORE/DIRESA/HRC/DE, and letter N° 008-2022-GRC-DIRESA-OISC.

Furthermore, the strains were identified by growth on MacConkey agar, incubated at 37 °C for 24 h to facilitate visualization and verification of the morphological characteristics of *Klebsiella* spp. Biochemical identification was performed using the following biochemical tests: Lysine Iron Agar (LIA), Triple Sugar Iron Agar (TSI), Simmons Citrate, Urea, MIO (motility, indole, and ornithine), Voges-Proskauer (VP), Methyl Red, and Malonate[56,57]. Subsequently, the strains were cryopreserved in trypticase soy broth (TSB) with 20% glycerol at − 80 °C[56] for further analysis. Reference strains were used for the subsequent analyses, as described in Supplementary Table 1.

Antimicrobial susceptibility profile to β-lactams

To perform antimicrobial susceptibility, *K. pneumoniae* strains were cultured in TSB broth and adjusted to a 0.5 McFarland scale[58]. Disks containing Ceftriaxone (CTX, 30 µg), Ceftazidime (CAZ, 30 µg), Cefepime (FEP, 30 µg), Amoxicillin/Clavulanic Acid (AMC, 20/10 µg) (Oxoid), Aztreonam (ATM, 30 µg), Ceftriaxone (CRO, 30 µg), Meropenem (MEM, 10 µg), and Imipenem (IMP, 10 µg) were used by Kirby-Bauer method and results were interpreted according to CLSI guidelines[59].

Phenotypic identification of ESBL and carbapenemases

All isolates were reactivated in TSB and cultured on MacConkey agar and Trypticase Soy Agar (TSA) following the methodology described[60]. The characterization of extended-spectrum β-lactamases (ESBL) was performed

according to the Jarlier method[28,61]. For carbapenemase detection, the double-disk synergy method with Boronic Acid (APB) was used[42], as well as the synergy method with the Ethylenediaminetetraacetic Acid (EDTA) test[29,59]. The Carba NP test was conducted according to the protocol outlined in[31]. The control strains employed included *Escherichia coli* ATCC BAA-2523 *blaOXA-48* (positive control), *Escherichia coli* NCTC 13,476 (carbapenemase positive control), and *Escherichia coli* ATCC 25,922 (negative control).

Pathogenic analysis

All strains underwent phenotypic screening for various virulence factors, including:

The capsule determination was performed by placing a colony of *K. pneumoniae* on a slide and stained with methylene blue[62] and India ink[63] for 2 min at room temperature. The slide was then rinsed and observed under a microscope. Hypermucoviscosity (Hv) was determined by inoculating the *K. pneumoniae* isolates were inoculated isolates on 5% sheep blood agar, incubating at 37 °C for 24 h, and performing the string test, where a mucosal string > 5 mm indicated positive Hv[64]. Biofilm formation was assessed by transferring a 200 µl sample of the bacterial culture were transferred to a 96-well microtiter plate and incubated overnight in LB, following the methodology of O'Toole[65]. The supernatant was removed, and the biofilm was stained with 1% crystal violet. Readings were quantified by spectrometry at 595 nm. All assays were conducted in triplicate, as described[66]. Hemolysis was determined by inoculating colonies on 5% sheep blood agar and incubating at 37 °C for 24 h to determine hemolysis by observing clear zones around the colonies[67].

Casein hydrolysis was evaluated by inoculating strains onto 1% skim milk agar (Oxoid) and incubated at 37 °C for 72 h. A clear zone around the colonies indicated protease production[8]. For gelatin hydrolysis, plates with nutrient agar containing 3% gelatin were inoculated with Kpn colonies and incubated for 16 h at 37 °C. The plates were then refrigerated for 5 h at 4 °C, followed by the addition of mercury chloride as a developing solution[68]. Lipase and lecithinase activities were determined by inoculating isolates onto blood agar base supplemented with egg yolk[69], incubating at 37 °C for 24 h, and applying 1 M copper sulfate as a developer for 20 min; for lipase determination, Tween 80 was added to the egg yolk agar, and the same incubation and development conditions were applied[8].

Molecular characterisation of ESBL-type β-lactamases and carbapenemases

The boiling-colony method[70] was used to obtain genetic material[42,71]. The microcentrifuge tube was homogenized, and 1 µL of the lysate was used as a template in a PCR master mix, with a final reaction volume of 25 µL (containing 1X GoTaq Buffer, 2.5 mM $MgCl_2$, 0.6 mM dNTPs, 0.5 µM primers (Supplementary Table 2), and 1.0 U GoTaq® Flexi DNA polymerase. Each gene was amplified in individual assays, as described in Supplementary Table 3. All PCRs were performed in a thermocycler (PROFLEX-THERMAL CYCLER, Applied Biosystems) with specific amplification programs for each gene, based on the protocol provided by the GoTaq® Flexi DNA polymerase insert, which had been previously standardized in the laboratory. Finally, the PCR products were visualized via agarose gel electrophoresis and photodocumented under UV light.

Whole genome sequencing and related information analysis

Four strains resistant to carbapenemases and β-lactamases, identified as ES221, HAL79, HR192, and HR200, were selected for whole genome sequencing. Genomic DNA was extracted using the Presto™ Mini gDNA Bacteria Kit (Geneaid) according to the manufacturer's instructions, and purity was assessed via fluorometry using the Quantus™ Fluorometer (Promega). The sequencing library was prepared with the Hieff NGS® MaxUp II DNA Library Prep Kit for Illumina® (Shanghai Yisheng Biotechnology Co., Ltd.), and sequencing was performed by Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China, https://www.sangon.com/techService) on the MGI DNBSEQ-T7 platform (https://en.mgi-tech.com/products/instruments_info/5/) with a read length of 2 × 150 bp paired-end.

The sequencing reads were visualized using the FastQC program (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/)[72] to filter high-quality sequences. Adapters and low-quality sequences were removed using Trimmomatic[73]. The genome assembly was carried out using SPAdes v3.15.3[74], and the assembly quality was evaluated with QUAST v5.2.0[75]. Contigs were annotated using the RAST web server interface[76] and the Prokka program[77]. Furthermore, the antimicrobial resistance genes and associated mutations were identified using the ARIBA[78], ResFinder v4.4.2[79], and AMRFinderPlus[80] databases. Pathogenwatch (https://pathogen.watch/)[81] was also used. Data was tabulated in binary format, and a heat map was generated using the Morpheus program (https://software.broadinstitute.org/morpheus). Virulence genes were identified by evaluating the assembled contigs on two platforms: VirulenceFinder[82] and the Virulence Factor Database (VFDB)[83,84]. Additionally, the PlasmidFinder database[85] was used to identify potential plasmid sequences.

Geographic distribution of the strains

To illustrate the geographical distribution of the *Klebsiella pneumoniae* strains found in our study, a base map from the INEI[23], available at https://estadist.inei.gob.pe/map, was first used. This map was projected using QGIS software (https://qgis.org) version 3.16[24], using the WGS 84 geodetic system for geographic projection, and adding the locations of the strains. Final details and annotations were added using Google Slides (https://docs.google.com/presentation/create?hl=en), improving the data's clarity and visualization.

Phylogenomics

The relationship between the sequenced genomes and reference genomes was determined by identifying single nucleotide polymorphisms (SNPs) using the Snippy program[86] and comparing them with the Klebsiella reference genome. Recombinant regions were identified and removed, and the resulting alignment was used as

input in the IQ-TREE program, with 1000 bootstrap replicates. A phylogenetic tree was then constructed and annotated using the iTOL software.

Data availability

Illumina raw reads of isolates have been submitted to NCBI under BioProject PRJNA1129429. SRR-NCBI accession numbers for sequence data are SAMN42151949, SAMN42151950, SAMN42151951, SAMN42151952.

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AA-EG Conceived the project, Conceptualization, Methodology, Data Curation, Supervision, Project administration, Writing—review and editing. MMC Research, Experimentation, Formal analysis, Writing: original draft, Review and editing. CP-LI, Experimentation, Formal analysis, Review, and edition. MDG Review and edition. AE-C Data curation, Formal analysis, Methodology, Writing—review and editing of manuscript. All authors reviewed the manuscript.

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Declarations

Ethical standards

In this study, we used only clinical samples provided by the Regional Hospital, Es Salud and Antonio Lorena of Cusco, Peru; therefore, informed consent was not required from the patients.

Competing interests

The authors declare no competing interests.

Additional information

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