Research article

Characterization of antibiotic resistance of isolates of *Klebsiella pneumoniae* causing health care-associated infections (HAIs) in hospitals in the Department of Nariño

Caracterización de la resistencia antibiótica de aislados de *Klebsiella pneumoniae* causante de Infecciones Asociadas a la Atención en Salud (IAAS) en hospitales del Departamento de Nariño

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Abstract

Introduction. *Klebsiella pneumoniae* is a critical priority pathogen that causes healthcare-associated infections (HAIs). The bacterium demonstrates intrinsic or acquired antimicrobial resistance (AMR). **Objective**. The resistance and genomic variability of *K.pneumoniae* in HAIs in the Department of Nariño (2021-2023) were analyzed. **Methodology**. 44 isolates were identified at the 16S rRNA gene level, which were evaluated for resistance to 13 antibiotics. Genetic variability was determined by in silico restriction enzyme cutting and with the BOX-A1R-based repetitive extragenic palindromic-PCR (BOX-PCR) and Enterobacterial Repetitive Intergenic Consensus (ERIC-PCR) markers. Subsequently, a presence-absence matrix was constructed using resistance data, microbiological data, and origin of infection. A dendrogram was constructed using the dice similarity coefficient. **Results**. 44 isolates were identified as *K.pneumoniae* (% identity>97%, e≈0, coverage>96%), 95% of these presented multidrug resistance, >80% were resistant to fluoroquinolones, beta-lactams and combination antibiotics. High genomic variability and a higher frequency of

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isolates from ICUs and blood samples were demonstrated; coinfection with COVID was observed in 20% of the samples. **Conclusion**: There is marked AMR and genomic variability among *K.pneumoniae*. This study corroborates the alarming challenges that public health may face due to AMR and reinforces the need to promote the implementation of a robust epidemiological surveillance system in the region to inform treatment decisions, guide policy recommendations, and assess the impact of resistance containment interventions.

Keywords: *Klebsiella pneumoniae*; drug resistance, bacterial, infection control, genetic variation.

Resumen

Introducción. *Klebsiella pneumoniae* es un patógeno de prioridad crítica causante de Infecciones Asociadas a la Atención en Salud (IAAS). La bacteria demuestra resistencia a los antimicrobianos (RAM) de forma intrínseca o adquirida. **Objetivo.** Se analizó la resistencia y la variabilidad genómica de *K.pneumoniae* en IAAS en el Departamento de Nariño (2021-2023). **Metodología**. Se identificaron 44 aislados a nivel del gen 16S rRNA, a los cuales se les evaluó la resistencia a 13 antibióticos. La variabilidad genética fue determinada por corte *in silico* con enzimas de restricción y con los marcadores BOX-A1R-based repetitive extragenic palindromic-PCR (BOX-PCR) y *Enterobacterial Repetitive Intergenic Consensus* (ERIC-PCR). Posteriormente, se construyó una matriz de presencia ausencia usando los datos de resistencia, datos microbiológicos y origen de infección. Un dendrograma fue construido mediante el coeficiente de similaridad de Dice. **Resultados**. 44 aislados se identificaron como *K.pneumoniae* (% identidad>97%, e≈0, cobertura>96%), 95% de estos presentaron multirresistencia, >80% fueron resistentes a fluoroquinolonas, betalactámicos y antibióticos combinados. Se demostró alta variabilidad genómica y mayor frecuencia de aislados procedentes de UCI y de muestras de sangre, se observó coinfección con COVID en el 20% de las muestras. **Conclusión**. Existe una marcada RAM y variabilidad genómica entre *K.pneumoniae*. Este estudio corrobora la alarmante problemática que puede enfrentar la salud pública por la RAM y refuerza la necesidad de incentivar la instalación de un buen sistema de vigilancia epidemiológica en la región, para informar las decisiones sobre el tratamiento, orientar las recomendaciones de políticas y evaluar el impacto de las intervenciones de contención de la resistencia.

Palabras clave: *klebsiella pneumoniae*; farmacorresistencia bacteriana; resistencia a múltiples medicamentos; control de infecciones; variación genética.

Introduction

Health care-associated infections (HAIs) are caused by the presence of one or more infectious agents or their toxins, which are not present upon admission to the health care center, causing increased morbidity, mortality, hospitalization time and excessive costs associated with their treatment (1, 2). The main concern with HAIs is the development of AMR because excessive or inappropriate use of antibiotics can promote the expression of resistance genes (3).

K. pneumoniae is one of the main causative agents of HAIs. This bacterium has demonstrated resistance to all beta-lactams through the production of extended-spectrum beta-lactamase (ESBL) enzymes and carbapenemase enzymes, increasing its resistance to antibiotic treatments (3, 4).

The high prevalence of carbapenem resistance in Colombia positions *K. pneumoniae* as an endemic pathogen in the country's hospitals (5, 6). Similarly, recent investigations on the genomic epidemiology of carbapenem-resistant *K. pneumoniae* demonstrated the complex and significant expansion of distinct clonal groups and, with these, the dissemination of HGT-related carbapenemase genes.

According to reports, in Nariño, *K. pneumoniae* is one of the most common pathogens causing HAIs, commonly reported in intensive care units (ICUs) and is resistant to different antibiotics; resistance mechanisms have been identified, such as the production of carbapenemase enzymes such as KPC-2, KPC-3 and NDM; the gene encoding extended-spectrum beta-lactamases (ESBL); and defects in noncarbapenemase porins (7,8).

Although preliminary resistance testing has been performed, epidemiological monitoring and surveillance of the infection caused by this bacterium have not been carried out. Thus, the present study contributes relevant information to monitor the AMR status and genomic variability in the department. Furthermore, this information can be analyzed in future studies aimed at decision-making and promoting local, national, and regional action on AMR, as recommended by PAHO. Hence, the objective of this study was to evaluate antimicrobial resistance and genomic variability in *K. pneumoniae* bacteria isolated from HAIs in hospitals in the Department of Nariño between 2021 and 2023.

Materials and methods

In figure 1 summarizes the methodology used for the development of this study.

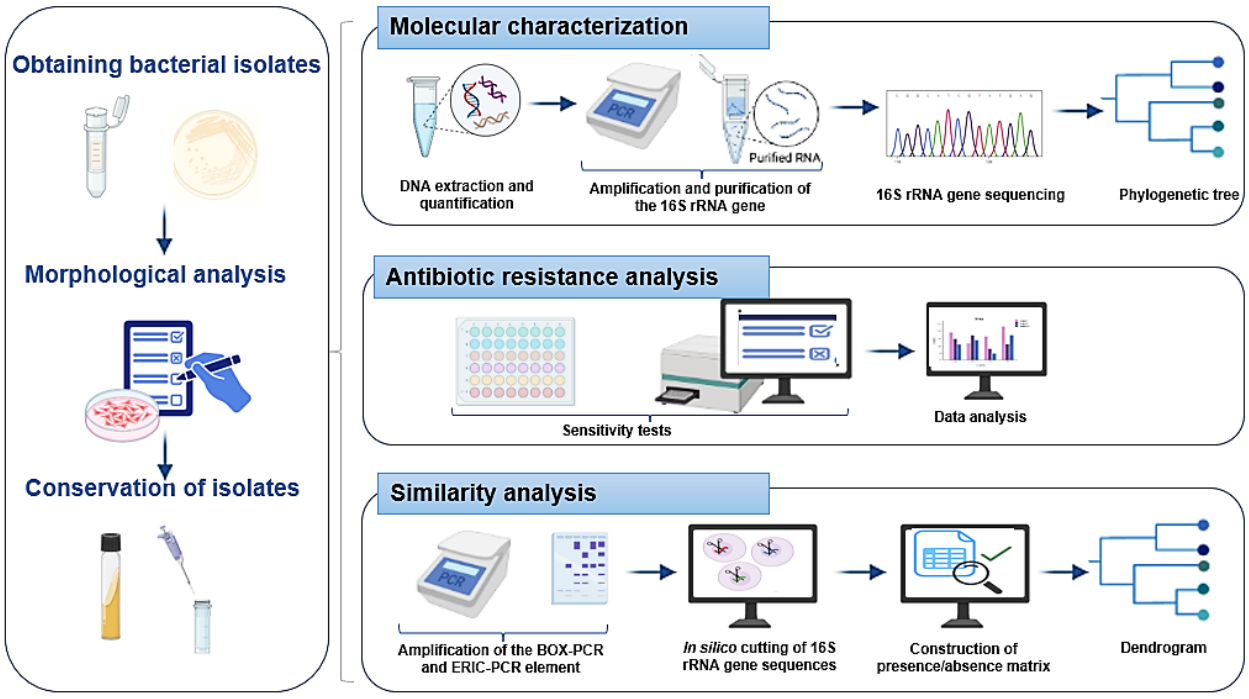


Figure 1. Flowchart of the methodology of this study. **Source:** authors.

Obtaining bacterial isolates

The 44 *K. pneumoniae* isolates from HAIs were supplied by the Clinical Microbiology Laboratory of the Departmental Health Institute of Nariño. The information available in the admission form for each sample was recorded, corresponding to sex, age, place of origin, service, patient diagnosis and origin of the sample.

Morphological analysis of bacterial isolates

Each sample was plated on MacConkey agar using the depletion method and incubated for 24 h at 37 °C. The attributes of the culture, such as shape, margin, elevation, size, and color, were described. A colony was removed and transferred using the same plating method onto Trypticase Soy Agar (TSA agar) and incubated for 24 h at 37 °C for further studies.

Conservation of isolates

In the Microbial Processes Laboratory of the University of Nariño, the complementary microbiological and molecular treatments for the analysis of the samples were conducted under biosafety conditions. For this purpose, a primary cell bank was built by conservation in 30% glycerol. Initially, a colony of *K. pneumoniae* was inoculated in Luria Bertani broth (LB broth) and incubated at 37 °C for 18 h. After the incubation 500 µL of the bacterial inoculum was transferred to a cryovial containing 500 µL of previously prepared 60% glycerol. This process was conducted in triplicate for each sample. The vials were then stored at -20 °C to preserve cell viability. The bacterial inoculum was removed with a sterile loop and seeded on the surface of LB agar in inclined glass tubes using the streak seeding technique. The tubes were incubated at 37 °C for 24 h and stored at room temperature. All the isolates were coded and entered a Microsoft Excel matrix to track the analyses performed in the laboratory.

Molecular characterization of K. pneumoniae isolates

Chromosomal DNA extraction and quantification

Bacterial chromosomal DNA extraction was conducted using the CTAB-Minimum Scale method following the protocol described by Burbano *et al.* (9). DNA resuspension was performed in 50 µL of molecular grade Milli-Q water. To check DNA integrity, 1.0% agarose gel electrophoresis was performed with the molecular size marker Lambda DNA/*Hind*lll (Promega-Madison, WI 53711 USA). The intercalant used was gel red (biotium). The run was programmed at 80 V for 1 h in a Cleaver Scientific Ltd. chamber, and after this time, the gel was visualized with a Smart Doc. Imaging Enclosure Benchmark Accuris E300 UV transilluminator. The DNA was subsequently quantified via a UV-Vis spectrophotometer (Thermo Scientific NanoDrop-One), and its quality was determined using the absorbance ratio (260/280).

Amplification of the 16S rRNA ribosomal gene

The primers for amplification of the 16S rRNA gene were 27F (5´ AGA GTT TGA TCM TGG CTC AG 3´) and 1041R (5´ CGG TGT GTA CAA GAC CC 3´) (Macrogen (Lio)) (10). The 25 µL reaction mixture (12.5 µL of Got Taq G2 Green Master Mix (REF: M7822 Promega), 1 µL of each primer (27F and 1041R), 1 µL of DNA, and 9.5 µL of water) was amplified in a thermal cycler (Bio-Rad T100 Thermal Cycler) with the following thermal cycler program: 95 °C for 2 min; 30 cycles of 94 °C for 2 min, 55 °C for 1 min, and 72 °C for 3 min; and a final extension for 10 min at 72 °C. The amplification product was verified by 1.0% agarose gel electrophoresis using the

1 kb DNA Ladder molecular size marker (Promega). The intercalant used was gel red (biotium). The run was performed in a Cleaver Scientific Ltd. chamber, which was programmed at 80 V for 1 h, and the gel was visualized with a Smart Doc. Imaging Enclosure Benchmark Accuris E300 UV transilluminator.

Purification of 16S rRNA gene amplicons

16S rRNA gene amplicons were purified using the "GFX PCR DNA and Gel Band Purification Kit" (Code 28-9034-71) from GE Healthcare, following the manufacturer's protocol. The product, which was suspended in 50 µL of solution (Elution buffer type 4), was verified by 1.0% agarose gel electrophoresis using the 1 kb DNA Ladder molecular size marker (Promega). The intercalant used was gel red (biotium). The run was performed in a Cleaver Scientific Ltd. chamber programmed at 80 V for 1 h, and the gel was viewed using the Smart Doc Imaging Enclosure Benchmark Accuris E300 UV transilluminator.

16S rRNA gene sequencing and sequence editing

Sanger-type sequencing of 16S rRNA gene amplicons was performed by Corpogen under service order No. 1 of April 10, 2024, Quotation 048S-2024-SEC. The sequence files were edited and aligned in the Geneious and BioEdit programs and then compared to sequences in the NCBI database using the Blast nucleotide tool. Identifications whose sequences showed a percentage greater than or equal to 97% identity, an e value close to zero and coverage greater than 96% were considered.

Phylogenetic analysis

The sequences edited in FASTA format were analyzed via the MEGA 11 program. Initially, a multiple alignment was run with the Clustal W tool, aligning the sequences of the 16S rRNA gene, an external group (*Rhodococcus* sp. (MT012184.1)) and three internal ATCC (American Type Culture Collection) roots of *K. pneumoniae* (ATCC 2146 (NR_OQ588741.1), ATCC 13883 (NR_119278.1) and ATCC 1705 (NR_OQ569537.1)). Once the sequences were aligned, the phylogenetic tree was constructed by the unweighted pair group method with the arithmetic mean (UPGMA), and bootstrap tests were performed with 1000 iterations with different substitution methods (Tamura-Nei model, maximum composite likelihood, Tamura-3 parameter model and p-distance).

Antibiotic resistance analysis

Sensitivity tests

The evaluation of antibiotic sensitivity was performed by the semiautomated method with MicroScan autoSCAN-4 System 4 equipment (Beckman Coulter). The

gram-negative NC94 panel was prepared by the PROMT method following the manufacturer's instructions.

Data analysis

The selected antibiotics are described in table 1. Microsoft Excel software was used to organize, summarize and schematize the data frequencies. Similarly, the percentages of resistance exhibited by *K. pneumoniae* isolates to the antibiotics selected for the analysis of this study were calculated and graphed.

Table 1. Antibiotics selected from the results provided by the MicroScan autoSCAN-4 System 4 equipment.

Type of antibiotic	Antibiotic	Abbreviation
Fluoroquinolones	Ciprofloxacin	CIP
	Levofloxacin	LVX
β-lactams	Aztreonam	ATM
	Amikacin	AMK
4th generation cephalosporin	Cefepime	FEP
2nd generation cephalosporin	Cefoxitin	FOX
3rd generation cephalosporin	Ceftazidime	CAZ
Aminoglycosides	Gentamicin	GEN
Carbapenems	Meropenem	MEM
	Ertapenem	ETP
	Imipenem	IPM
Compound antibiotic	Trimethoprim/Sulfamethoxazole	SMZ-TMP
	Piperacillin/Tazobactam	PIP-TZ

Similarity analysis

Genetic profile by amplification of the BOX-PCR element (BOX-A1RBased Repetitive Extragenic Palindromic)

A repetitive extragenic palindromic PCR based on the BOX-A1 genetic element (BOX-PCR) was performed using the BOX-A1 primer (5 'CTA CGG CAA GGC GAC GCT G 3') synthesized by Macrogen (11). A 25-µL reaction per sample (13.5 µL of Got Taq G2 Green Master Mix (Promega), 1 µL of Box-A1 primer [20 µM], 1 µL of DNA and 9.5 µL of water) was processed in a T100 Thermal Cycler (Bio-Rad), which was programmed as follows: 95 °C for 7 min; 30 cycles of 94 °C for 1 min, 53 °C for 1 min and 65 °C for 8 min; and a final extension period of 15 min at 65 °C. Amplicons were verified by 1.0% agarose gel electrophoresis using the 1 kb DNA Ladder (Promega) molecular size marker. The run was performed in a Cleaver Scientific Ltd. chamber set to 80 V for 1 h 30 min. The gel was treated with Gel Red® nucleic acid gel stain (Biotium) in 1× TAE buffer for 10 min and then viewed under a UV-Smart Doc. Imaging Enclosure Benchmark Accuris E300 UV transilluminator. The electrophoresis gel image was loaded

into Photocapt Mw version 10.01 (Copyright 1999–2001) software, where the genetic profiles were analyzed.

Genetic profile by amplification of the ERIC-PCR (Enterobacterial Repetitive Intergenic Consensus-PCR) element

ERIC-PCR (Enterobacterial Repetitive Intergenic Consensus-PCR) amplification was performed with the primers synthesized in Macrogen ERIC 1 (5' TGTAAGCTCCTGGGGAT3') and ERIC 2 (5'AAGTAAGTGACTGGGGGTGAGC 3') (11). A 25 µL reaction per sample (12.5 µL Got Taq G2 Green Master Mix, 1 µL of each primer: Eric1 [20 µM] and Eric2 [20 µM], 1 µL DNA and 9.5 µL water) was processed with the following thermocycling program: 95 °C for 7 min; 30 cycles of 94 °C for 1 min, 52 °C for 1 min and 65 °C for 8 min; and a final extension of 15 min at 65 °C.

Amplicons were verified in a 1.0% agarose gel using the 1 kb DNA Ladder molecular size marker (Promega). The running conditions were 80 V for 1 h. After this time, the gel was treated with Gel Red® nucleic acid gel stain solution (Biotium) in 1X TAE buffer for 10 min and then visualized via a UV-Smart Doc. Imaging Enclosure Benchmark Accuris E300 UV transilluminator. The electrophoresis gel image was analyzed into Photocapt Mw version 10.01 (Copyright 1999–2001) software, where the existing genetic profiles were analyzed.

In silico cutting of 16S rRNA gene sequences with restriction enzymes

Each 16S rRNA gene sequence was imported into the PDRAW32 program, where in silico cutting was performed with restriction enzymes. Ten enzymes that had between 3 and 10 cuts in the sequence were selected, and the sizes of each fragment were recorded in an Excel matrix.

Construction of a dendrogram

To find the similarity between the samples, a matrix was created that included the general information of each patient along with the results obtained. The matrix was then imported into the PAST4.03 program, where a dendrogram was constructed using the UPGMA algorithm and the Dice, Jaccard and correlation similarity indices were applied.

Ethical considerations

This project was endorsed by the Research Ethics Committee of the University of Nariño through Approval Act No. 006 of twenty-two (22) April two thousand twenty-four (2024). In the development of this study, the principle of confidentiality was considered through the coding of the isolates. In addition, the handling of the information was agreed upon in accordance with the confidentiality commitment document and nondisclosure of information reserved for third parties issued by the IDSN Public Health Laboratory.

Results

Morphological analysis of bacterial isolates

During the morphological observations of the colonies, they presented pink coloration, indicating lactose fermentation; the shape was defined with rounded edges, a smooth surface, convex elevation, a moist appearance, and creamy consistency. The growth of these strains on TSA agar was homogeneous, confirming that they were axenic, as seen in figure 2.

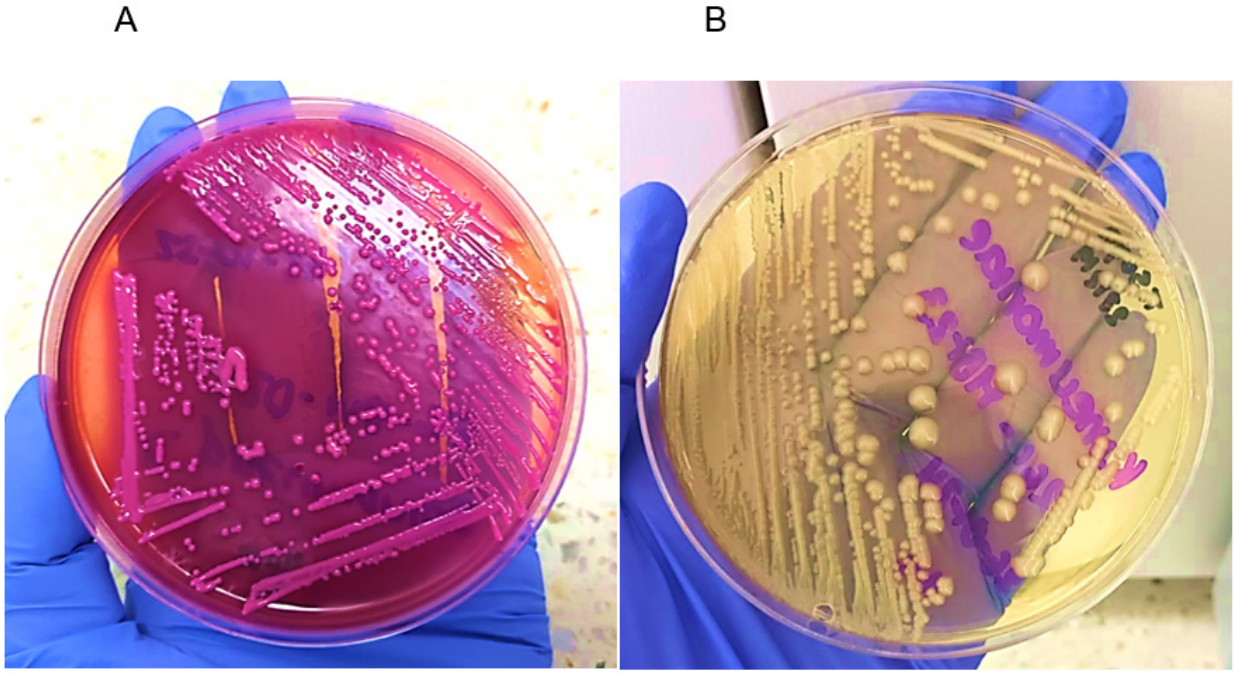


Figure 2. *K. pneumoniae* isolated from HAIs. A. Growth of *K. pneumoniae* on MacConkey agar. B. Growth of *K. pneumoniae* on TSA.

Molecular characterization of K. pneumoniae isolates

Extraction of bacterial chromosomal DNA

Figure 3 shows the DNA obtained by the CTAB-Minimum Scale method. DNA was of excellent quality, with well-defined bands of good concentration and an absorbance ratio (A260/280) ranging between 1.8–2.0.

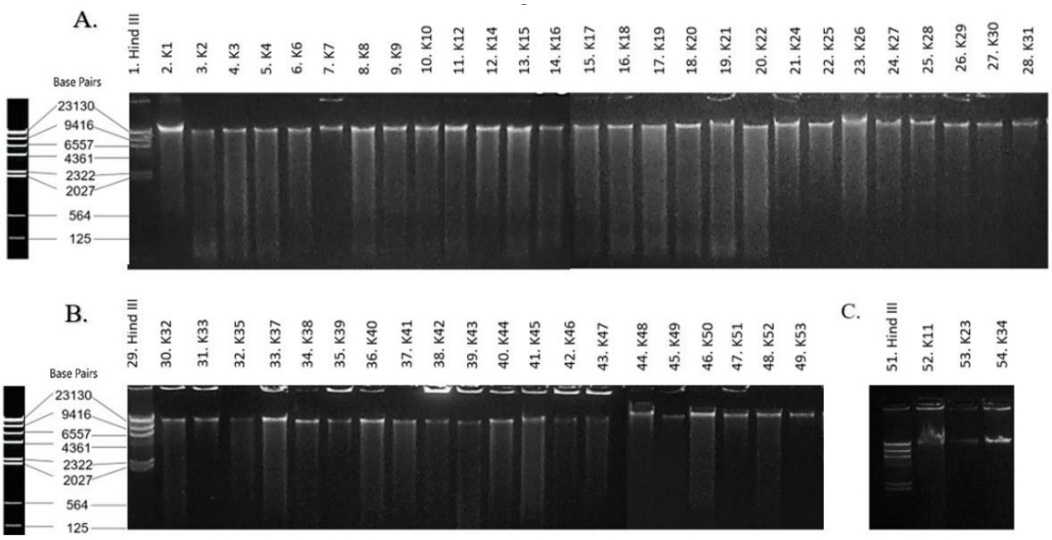


Figure 3. 1.0% agarose gel electrophoresis of bacterial DNA obtained via the CTAB-Minimum Scale method. Wells 1A, 29B and 51C: lambda DNA/*Hind*III marker (Promega); Wells 2A-28A; Wells 30B-49B; and Wells 52C-54C: DNA of bacterial isolates.

Amplification of the 16S rRNA subunit

As a result of amplification of the 16S rRNA gene, an amplicon of approximately 1500 bp was obtained, as seen in figure 4.

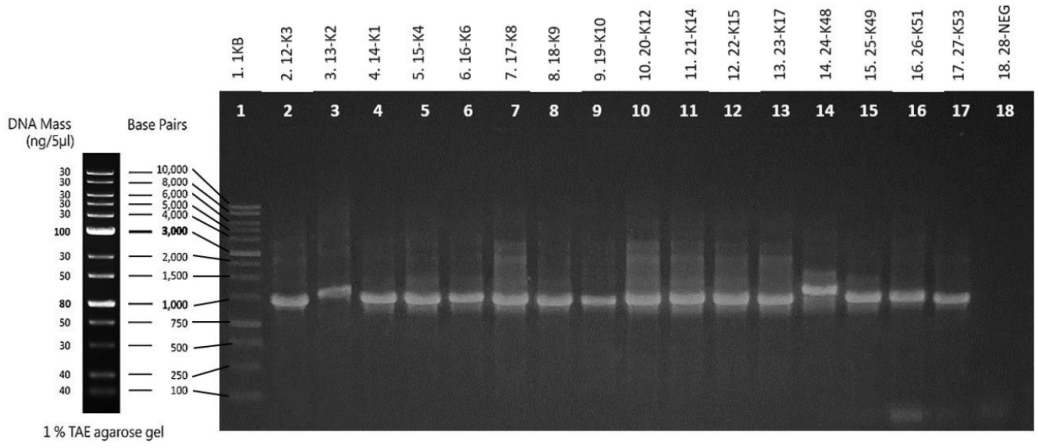


Figure 4. 1.0% agarose gel electrophoresis for verifying 16S rRNA gene amplification products without purification. Well 1: 1 kb marker (Promega); Wells 2–2 to 17: 16S rRNA amplicon from *K. pneumoniae* isolates; and Well 18: negative control (PCR mix without DNA sample).

Since the amplicons for the sequencing process should have a purification protocol, all samples were subjected to treatment with the "GFX PCR DNA and Gel Band Purification Kit" (Code 28-9034-71) from the GE Healthcare brand; products were obtained without scanning, as seen in figure 5, the concentrations ranging from 20.6–6 to 57.6 ng/µL, which were required and verified by the sequencing company Corpogen

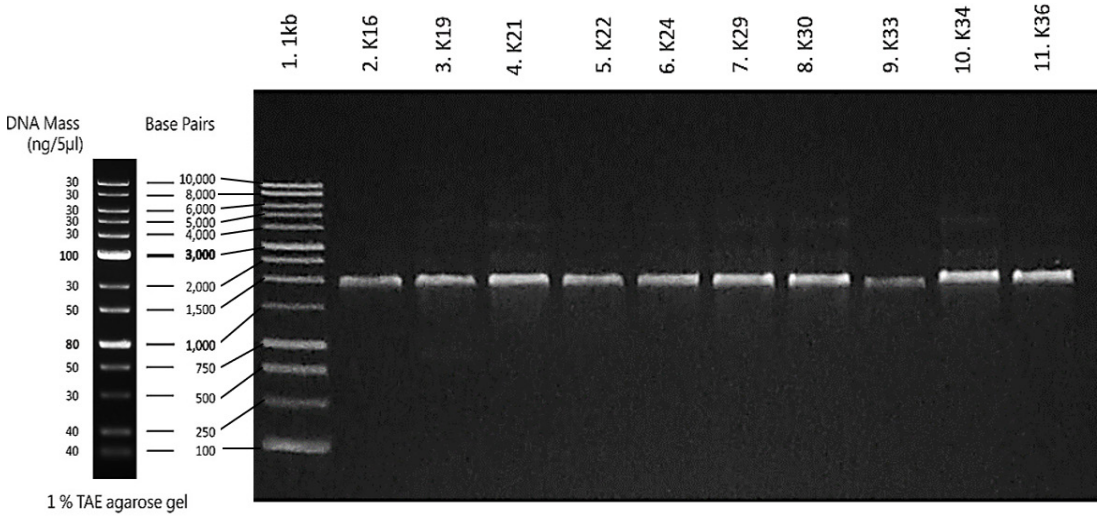


Figure 5. 1.0% agarose gel electrophoresis for checking 16S rRNA gene purification products. Well 1: 1 kb marker (Promega), and Wells 2–2 to 11: purified amplicons.

Sequence analysis

The chromatogram was evaluated in abi format, and the sequences were edited via the BioEdit and Geneious programs. The sequences corresponding to K5, K28, K31, K46, K47 and K48 were eliminated from the analysis since they did not present defined peaks and the determined base was of low quality.

Sequence identity analysis

The aligned sequences were analyzed using the NCBI BLAST nucleotide tool. All the sequences presented an identity percentage greater than 97% for *K. pneumoniae*, with an e value equal to or remarkably close to zero and a coverage greater than 96%.

The sequences were deposited in the NCBI database with the following accession codes: PP838948, PP838949, PP838950, PP838951, PP838952, PP838953, PP838954, PP838955, PP838956, PP838957, PP851004, PP851005, PP838958, PP838959, PP838960, PP838961, PP838962, PP838963, PP838964, PP838965, PP838966, PP838967, PP838968, PP838969, PP838970, PP838971, PP838972, PP838973, PP838974, PP838975, PP838976, PP838977, PP838978, PP838979, PP838980, PP838981, PP838982, PP838983, PP838984, PP838985, PP838986, PP838987, PP838988, and PP838989.

Phylogenetic tree analysis

Phylogenetic relationships between *K. pneumoniae* samples were inferred using the UPGMA method. Figure 6 shows the grouping of the *K. pneumoniae* sequences resulting from this study together with the ATCC reference bacteria forming the first cluster, demonstrating that all of them are closely related; the second cluster formed by the sequence of the external group showed that there are evolutionary differences with the rest.

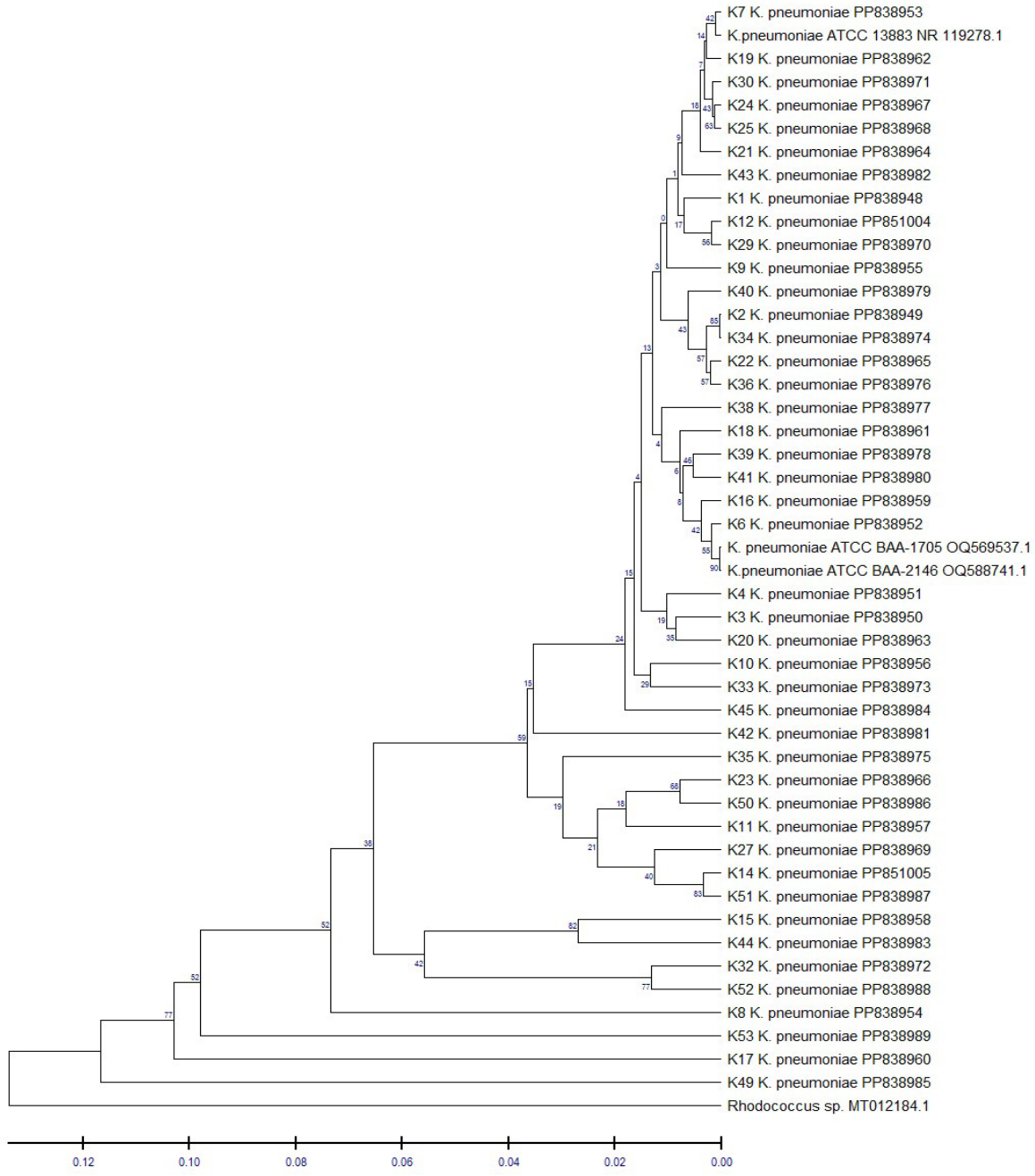


Figure 6. Phylogenetic tree of *K. pneumoniae* isolates constructed with the UPGMA algorithm in the MEGA 11 program.

Analysis of the frequencies of HAIs

Figure 7 indicates the frequency of isolates according to the place of origin, the highest percentage of samples belonged to isolates from the San Pedro Hospital Foundation (34.1%), followed by the Departmental University Hospital of Nariño, and less frequently from the Civil Hospital of Ipiales, the Pabón Clinic, Medgroup Laboratory and Proinsalud SA, the Clinizad Laboratory, Glicol Laboratory and the Los Ángeles Children's Hospital.

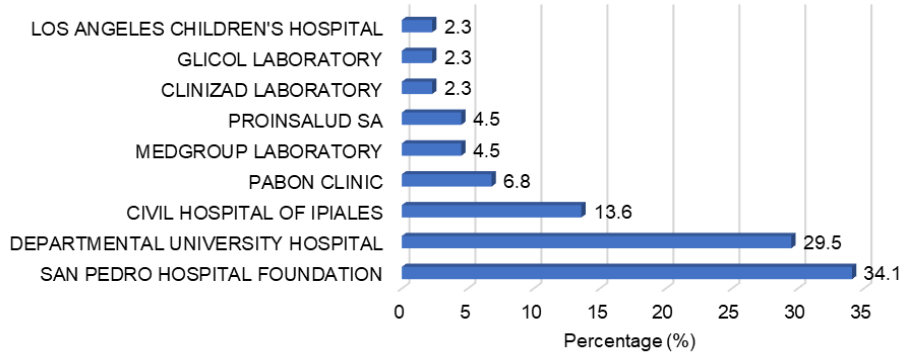


Figure 7. Percentages of *K. pneumoniae* isolates from HAIs according to their place of origin.

According to the age and sex of the patients, as seen in figure 8, a greater number of isolates were observed from male patients than from female patients, with 27 and 17 isolates, respectively. In addition, an increase in the total number of cases was observed after the age of 50 years; however, this increase was more relevant in males, who presented the greatest number of isolates between 50–59 years of age.

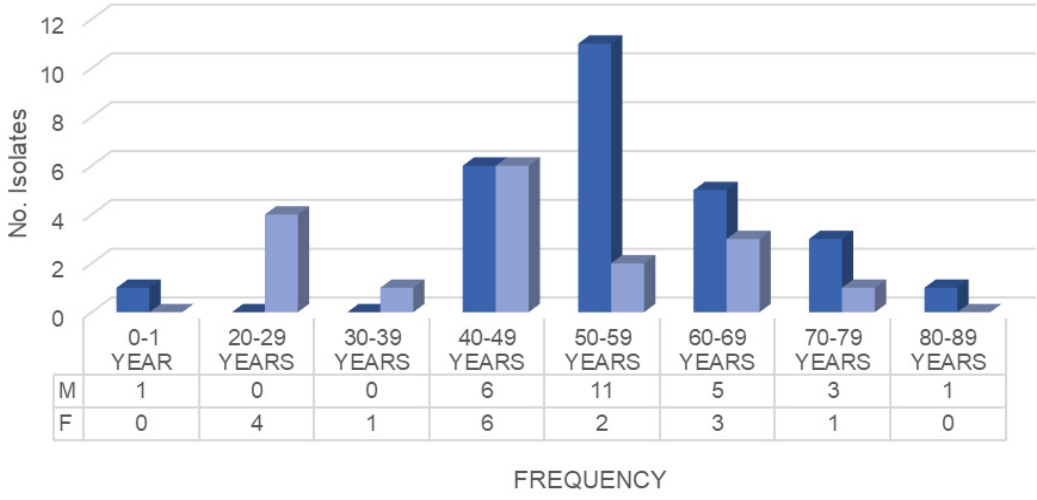


Figure 8. Distribution of the frequency of *K. pneumoniae* isolates from HAIs according to the age range and sex of the patients.

As seen in figure 9, more than half of the isolates were obtained from blood (55%), followed by urine obtained via a catheter (23%), peritoneal fluid (7%), unspecified culture (5%), tracheal secretion (5%), tissue (2%), urine (2%) and tracheal spirate (2%).

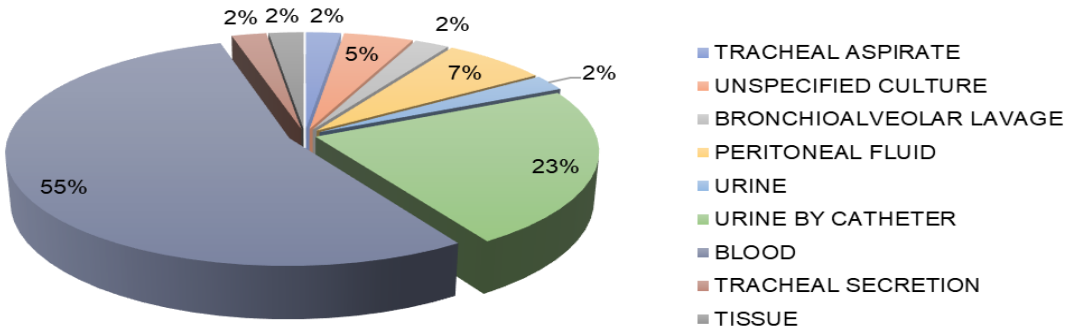


Figure 9. Percentage of *K. pneumoniae* isolates from HAIs according to the origin of the sample.

According to the hospital service where the sample was obtained, figure 10 shows that the intensive care unit (ICU) presented 57% of the isolates, followed by internal medicine, emergency, particular care unit (SCU) and surgical.

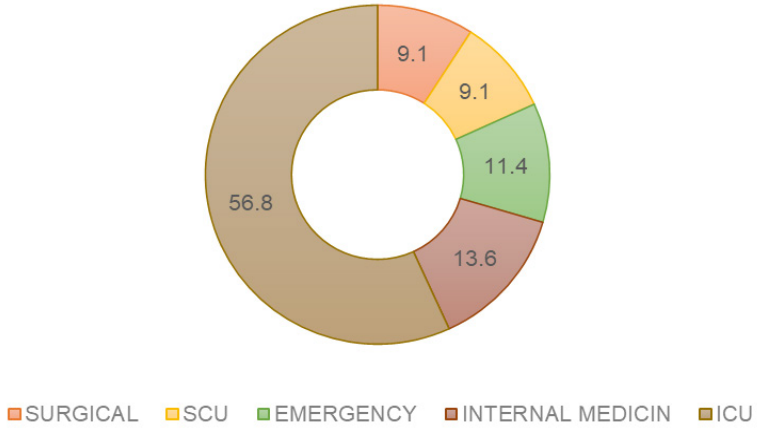
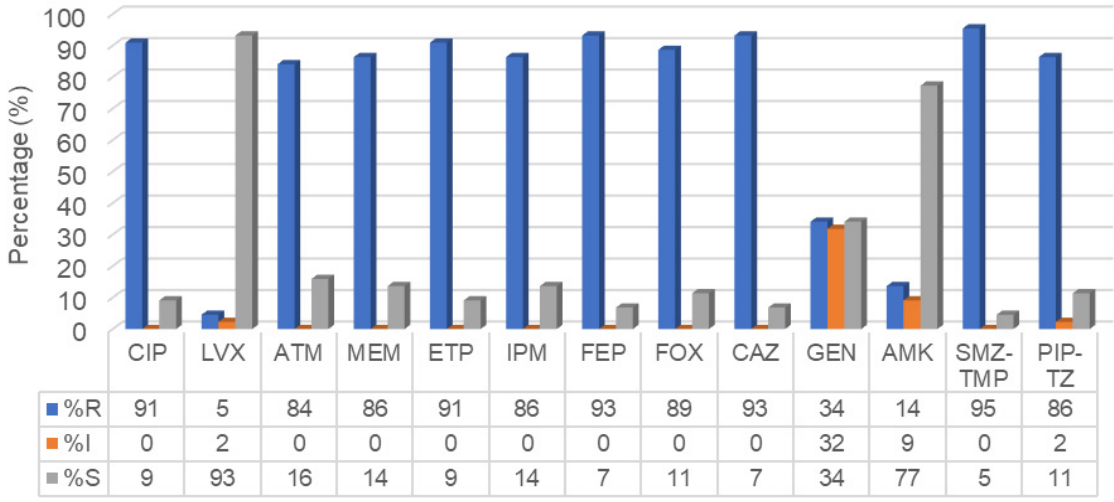


Figure 10. Percentage of *K. pneumoniae* isolates from HAIs according to the hospital service where the sample was taken.

Determination of antibiotic resistance

As seen in figure 11, 95% of the isolates were multi-resistant. (resistance to more than four antibiotics). In addition, a high percentage (over 80%) of resistance was observed to the following antibiotics: ciprofloxacin, aztreonam, meropenem, ertapenem, imipenem, cefepime, cefotaxime, and ceftazidime, as well as resistance to

antibiotics such as trimethoprim/sulfamethoxazole and piperacillin/tazobactam. On the other hand, the percentages of isolates resistant to gentamicin, amikacin and levofloxacin were lower, at 34%, 14% and 5%, respectively. In addition, gentamicin, amikacin, cefepime and levofloxacin showed intermediate resistance (Figure 10).



	CIP	LVX	ATM	MEM	ETP	IPM	FEP	FOX	CAZ	GEN	AMK	SMZ-TMP	PIP-TZ
%R	91	5	84	86	91	86	93	89	93	34	14	95	86
%I	0	2	0	0	0	0	0	0	0	32	9	0	2
%S	9	93	16	14	9	14	7	11	7	34	77	5	11

Figure 11. Antimicrobial resistance of *K. pneumoniae* isolates. Antibiotics evaluated included fluoroquinolones, including ciprofloxacin (CIP) and levofloxacin (LVX); β-lactams, such as monobactam Aztreonam (ATM); carbapenems, such as meropenem (MEM), ertapenem (ETP), imipenem (IPM) and 4th generation cephalosporins, such as cefepime (FEP); 2nd generation, such as cefoxitin (FOX); 3rd generation, such as ceftazidime (CAZ); aminoglycosides, such as amikacin (AMK) and gentamicin (GEN); and antibiotics, such as trimethoprim/sulfamethoxazole (SMZ-TMP) and piperacillin/tazobactam (PIP-TZ).

Analysis of genetic variability among bacterial isolates

In Silico Restriction Enzyme Cutting

16S rRNA sequences were subjected to in silico restriction enzyme cutting using the PDRAW32 program. The enzymes selected were *Bcc*I, *Bis*I, *Bls*I, *Bs*lI, *CviK*I-1, *FaiI*, *Fnu4*HI, *HpyA*V, *Mn*lI, and *Sau*96I; eight fragments shared among all samples were detected using the following enzymes: *Bcc*I with 2 and 97 bp, *Bs*lI with 58 bp, *CviK*I-1 with 45 and 85 bp, *FaiI* with 39 bp, *Fnu4HI* with 249 bp and *Mn*lI with 89 bp.

Molecular genotyping

The sequences of the BOX-PCR and ERIC-PCR genetic elements were amplified. For the first element, 19 distinct genetic profiles (P1–P19) were identified with fragments between 500–5300 bp, as seen in figure 12. Figure 13 shows the second intergenic sequence marker, 38 genetic profiles (P1–P38) were distinguished with fragments ranging from 27–6439 bp, which revealed high genetic variability between the samples.

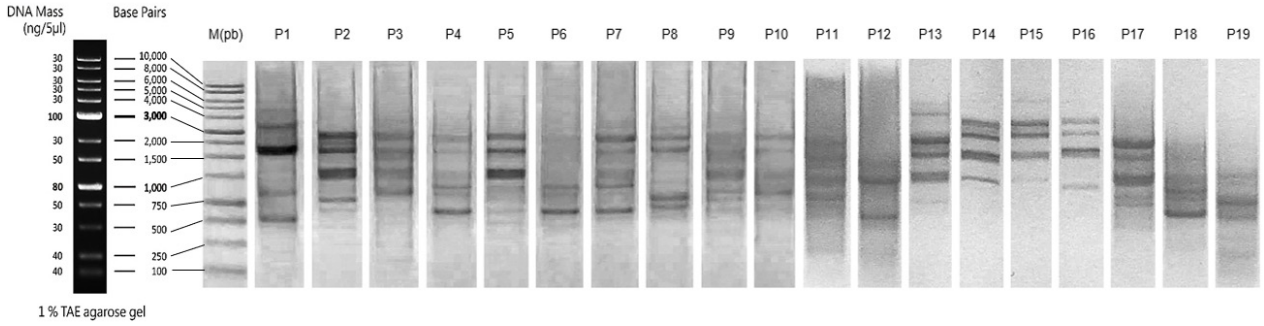


Figure 12. 1.0% agarose gel electrophoresis for verification of BOX-PCR products. Well 1: 1 kb marker (Promega), and Wells 2– to 20: genetic profiles (P1–P19) found in *K. pneumoniae* isolates.

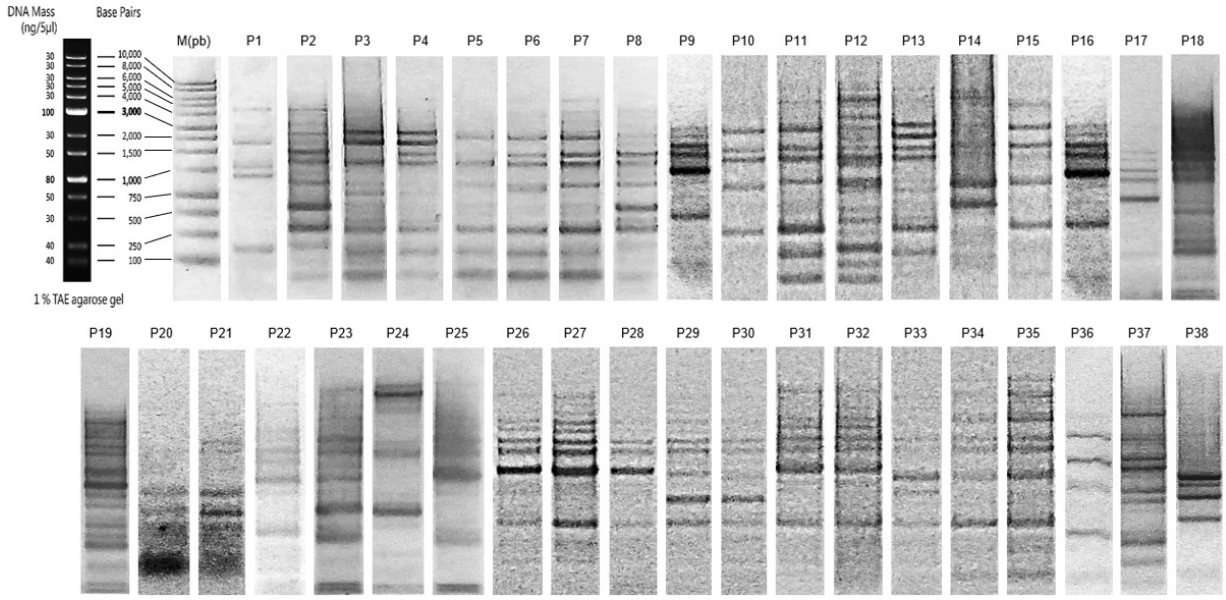


Figure 13. 1.0% agarose gel electrophoresis for verification of ERIC-PCR products. Well 1: 1 kb marker (Promega), Wells 2– to 39: genetic profiles (P1–P38) found in *K. pneumoniae* isolates.

Similarity analysis

Figure 14 shows the similarity dendrogram constructed using the Dice similarity index. It was found a variable range of similarity between the samples (between 37% and 96%), with K32 being the most different isolate, which forms the first cluster (I), with approximately 37% shared information; the second cluster (II), consisting of isolate K15, which shares 45% similarity; and the third cluster (III), where the remaining isolates with more than 45% similarity are grouped.

Within the latter, subclusters with a similarity percentage greater than 70% can be observed in the following order: subcluster 1 groups

isolates K19 and K49, with 74% similarity, which come from urinary tract infection and malignant tumors, respectively, and share resistance to ciprofloxacin and trimethoprim/sulfamethoxazole; in the second subcluster are isolates K49 and K1, with approximately 77% similarity, taken from patients with septic shock and pneumonia, and with shared resistance to 6 of the 13 antibiotics. Subcluster 3 comprises K8, K10, K22, K35, K29, K42, K3, K2, K43, K4, K30, K44, K24, K25, K36, K39, K40, K16, K34, K18, K20, K8, K14, K51, K33, K38, K45, K41, K11 and K27; various pathologies are found in this group, as are multiresistance in all its isolates. In addition, isolates K8 and K10 presented the highest percentage of similarity, at approximately 96%.

Finally, subcluster four, with 76% shared information, included isolates K7, K12 and K9, which were all resistant to trimethoprim/sulfamethoxazole and originated from patients with respiratory disorder pathologies, acute appendicitis, and urinary tract infection, respectively.

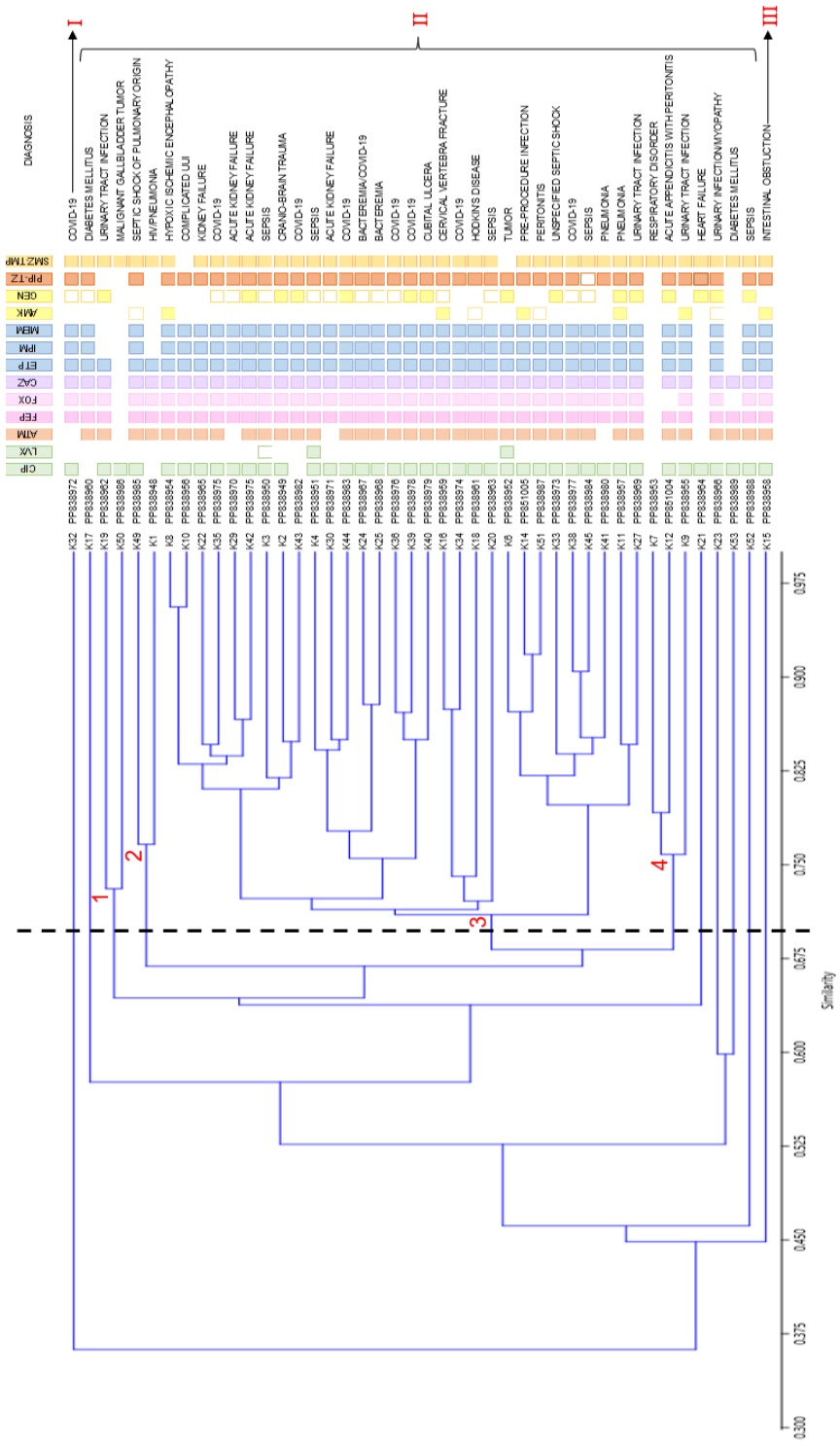


Figure 14. Dendrogram for assessing similarity between *K. pneumoniae* isolates based on patient information, resistance results, in silico restriction enzyme cleavage sizes, and the sizes of each genetic profiles found by BOX-PCR and ERIC-PCR. The dendrogram was constructed via the PAST4.03 program using the UPGMA algorithm. Resistance is coded by filled squares, intermediate resistance is denoted by unfilled squares, and each color corresponds to a type of antibiotic: fluoroquinolones, monobactam, 4th generation cephalosporins, 2nd generation cephalosporins, 3rd generation cephalosporins, aminoglycosides, carbapenems and combined antibiotics. Dice similarity index - Cophenetic correlation coefficient = 0.8212.

Discussion

K. pneumoniae is an important nosocomial pathogen that causes multiple acute infections and is notable for its resistance to most antibiotics (2).

This study evaluated the AMR of bacterial isolates of *K. pneumoniae* from HAIs in the Department of Nariño. The results showed the morphological, biochemical, and molecular identification of *K. pneumoniae*. In the hospital environment, there was a higher frequency of isolation from ICUs and blood samples. All the isolates showed resistance to antibiotics, especially beta-lactams and combined antibiotics, and significant genetic variability was clear among the isolates. The growth (Figure 2) of the forty-four isolates presented morphological characteristics like those reported in earlier studies (12,13,14). DNA extraction was evaluated with agarose gel electrophoresis (Figure 3), DNA quantification and measurement of the absorbance ratio (260/280). The data confirmed that the extraction method using CTAB was low cost and highly effective (15). The 16S rRNA gene amplification results (Figure 4, Figure 5 and results consistent with the NCBI database) complement the morphological and biochemical identification (MicroScan autoSCAN-4 System 4) used for the presumptive *K. pneumoniae* isolates.

The analysis of the phylogenetic tree (Figure 6) allowed us to observe the grouping of the bacterial isolates; however, in the first cluster, differences in distance were observed, which suggests the existence of genetic diversity, which may be influenced by different factors, including mutations, genetic recombination, adaptations to the environment through selective pressure and HGT events, given the high capacity of *K. pneumoniae* to rapidly acquire and transfer mobile elements (7,16).

With respect to HAI analysis, the San Pedro Hospital Foundation and the Departmental University Hospital were identified as the institutions with the highest frequency of isolates (Figure 7). This may be linked to the complexity of health facilities, which creates conditions and risk factors for colonization and infection by microorganisms (17). With respect to sex (Figure 8), the effect of *K. pneumoniae* is not a determining factor in the acquisition of HAIs since it involves a combination of other factors, such as biological, behavioral, social and hospitalization systems (18). The frequency with respect to the origin of the sample (Figure 9) was related to the most important typologies associated with invasive procedures or surgical sites in the development of HAIs (1), with the Pili of the bacteria being an important virulence factor in the adhesion and formation of biofilms on

surfaces such as blood and urinary catheters (19,20). Likewise, the ICU service (Figure 10) represents a substantial risk of acquiring HAIs because of its relationship with the use of vascular access, urinary catheters, and endotracheal tubes (21).

The results obtained provide evidence of the importance of monitoring multi-resistant bacteria. Studies show that *K. pneumoniae* is one of the enterobacteria with the highest antimicrobial resistance and incidence at the intrahospital level, and they recommend IMP and MEM as the most efficient antibiotics against beta-lactamase enzymes (22). However, these antibiotics have started to lose their efficacy; this is reflected in the high resistance found in the isolates, leading to inefficacy and few treatment alternatives.

Compared to other countries, such as Egypt, where resistance rates higher than 65% have been reported with ceftazidime/ampicillin, cephalosporins (cefotaxime, cefepime), fluoroquinolones (ciprofloxacin, norfloxacin, and ofloxacin), low resistance rates to carbapenems and combined antibiotics have been reported (23). Similarly, countries in Latin America such as Argentina, Bolivia, El Salvador, Brazil, and Ecuador recorded relatively low percentages for carbapenem and cephalosporin families (less than 40%), which are therapeutic alternatives. However, in this study, the percentage of resistant isolates exceeds 80% (22), which coincides with findings that classify Colombia as endemic for carbapenem production in Latin America (6).

These figures become more alarming when contrasted with earlier studies (24,25), which show that the percentage of antibiotic resistance did not exceed 40% in the antibiotics evaluated, proving the accelerated increase in resistance in recent years.

Antibiotic resistance was shown in each antibiotic family (Figure 11). In the case of fluoroquinolones, the antibiotic ciprofloxacin exhibited resistance in 91% of the isolates; this multifactorial property may be due to one or a combination of genetic mutations at the target site, increased production of multidrug resistance (MDR) efflux pumps, modifying enzymes, and/or target protective proteins (26). Scientific documentation indicates that quinolone resistance in *K. pneumoniae* may be mediated by plasmids (PMQRs), which may involve genes such as *qnr (qnrB, qnrS, qnrC and qnrD)*, which encode proteins that confer resistance to these antibiotics and spontaneous mutations in the quinolone resistance determining regions (QRDRs) of the *gyr*A, *par*B, *par*C, and *par*E genes (27, 28). All these mechanisms can influence the innate or acquired resistance of these pathogens in a hospital environment. In Colombia, ciprofloxacin is the most prescribed drug, which explains the greater degree of resistance, in contrast to levofloxacin, which is prescribed less of-

ten because of its excessive cost (Figure 11) (29). The beta-lactam antibiotics evaluated correspond to different classes: Aztreonam belongs to the monobactam class; carbapenem classes, which include meropenem, imipenem and ertapenem; and cefoxitin, ceftazidime and cefepime are part of the 2nd, 3rd, and 4th generation cephalosporins, respectively. In addition, among the combined antibiotics, piperacillin-tazobactam is a class of penicillinase-resistant penicillin (30). Different authors describe the production of beta-lactamases as the main resistance mechanism in *K. pneumoniae* and state that the use and abuse of antibiotics has favored the evolution of these enzymes, increasing their spectrum of action, such that they are capable of hydrolyzing cephalosporins, antibiotics prescribed to treat multiresistant bacteria, and the monobactam Aztreonam, a broad-spectrum antibiotic against gram-negative bacteria (31, 32).

Resistance to carbapenems can be generated by several mechanisms, but the most frequent and relevant mechanism is the production of carbapenemases, which are broad-spectrum enzymes that limit therapeutic alternatives (7). In Colombia, the genes *bla*KPC, *bla*NDM and *bla*VIM, which encode these enzymes, have been reported for *K. pneumoniae,* and in Nariño, the variants *bla*KPC-2, *bla*KPC-3 and *bla*NDM-1 have been found, as well as the coproduction of *bla*KPC−2+NDM−1 (7, 32). Given the wide distribution of resistance to carbapenems, these findings could explain the high percentages of resistant isolates in this study.

In terms of aminoglycoside resistance, *K. pneumoniae* has acquired resistance to this group of antibiotics because it is typically used to treat serious infections, and although it is less resistant than the other strains, a significant percentage of *K. pneumoniae* strains are resistant to gentamicin, in addition to intermediate resistance, indicating an increase in AMR. In bacteria, the combination of resistance mechanisms enhances their action, giving rise to MDR strains, in such a way that plasmids encode resistance genes to beta-lactams and carry genes for resistance to aminoglycosides (33). Amikacin presents a lower percentage of resistance, so it is considered effective, but expressing intermediate resistance creates bias, and given its adverse effects, it could limit its clinical application. The combination of antibiotics, on the other hand, resulted in high resistance rates. In the case of trimethoprim/sulfamethoxazole, resistance can be caused by mobile elements that transmit various mechanisms, such as modifications in the efflux pumps, inhibition of porins and mutations in the genes encoding the target enzymes; in the case of resistance to trimethoprim, changes in the *dhfr* gene encoding the enzyme DHFR (dihydrofolate reductase) can alter the affinity with the antibiotic, and resistance to sulfamethoxazole is mediated by the sul1 and sul2 genes, causing conformational changes in the

structure of the enzyme DHPS (dihydropteroate synthetase) (34).

The bacterial isolates exhibited genetic variability, which was evidenced by in silico mapping with restriction enzymes along the 16S rRNA sequence. According to De la Cruz (2), the 16S rRNA gene, with a size of approximately 1500 bp, is suitable for ribotyping, where restriction enzymes act to obtain fragments and generate patterns or ribotypes. In addition, in silico assays allow highly similar and accurate simulations that are useful for strain differentiation and the selection of enzymes for in vitro studies. The effectiveness of *in silico* assays was confirmed by the variety in the size and number of fragments, and eight cutting patterns based on size were established.

Genotyping of the isolates using the BOX-PCR genetic elements and ERIC-PCR (Figure 12 and Figure 13) support the effectiveness of these techniques for molecular typing and demonstrate the different patterns in the bands of the genetic variability of the *K. pneumoniae* isolates, which agrees with the findings of Rihab *et al.* (35) and Sedighi *et al.* (36). In relation to the above findings, the genetic variability among *K. pneumoniae* isolates is consistent with its genetically diverse nature. In addition, the importance of implementing molecular tools for the typing and epidemiological study of pathogens causing HAIs is highlighted.

On the other hand, the genetic similarity analysis (Figure 14) associated the results of the three markers used—BOX-PCR, ERIC-PCR and RFLP (restriction fragment length polymorphism)—through *in silico* enzymatic restriction analysis. The fidelity of the dendrogram was evaluated by the cophenetic correlation coefficient, which can range between 0.6–0.95, with the highest values being understood as indicators of good correspondence between the original matrix and its representation (37). The Jaccard and Dice indices obtained the highest values (Jaccard: 0.9195 and Dice: 0.9236), indicating high correspondence of the data; however, the dendrogram applying the Dice index was selected since, in this case, the shared information is closer to the results observed when the samples were analyzed with the different methods (Figure 14) (38). On the other hand, the analysis results of low similarity agreed with the genetic variability evidenced in the simulation of enzymatic restriction and in the genetic profiles detected with BOX-PCR and ERIC-PCR, showing the genetic heterogeneity presented by *K. pneumoniae* (35).

The K11 isolate (subcluster 3) exhibited resistance to 12 of the 13 antibiotics evaluated (susceptible to LVX), bacteria isolated from a patient with pneumonia and admitted to the ICU, confirming that the main risk factors for acquiring HAIs by *K. pneumoniae* in patients under invasive mechanical ventilation are those related to preexisting di-

seases, bacterial resistance and invasive and surgical procedures (36).

An important event considered in this study is the COVID-19 pandemic. Several authors have observed an increase in antimicrobial resistance after the pandemic, linked to high antimicrobial prescriptions and more cases of carbapenem-resistant Enterobacteria (37-41). The widespread and inappropriate use of antibiotics during this period generated selective pressure on this bacterium, reflected in the resistance patterns observed, in addition to the appearance of coinfections with *K. pneumoniae*, which was resistant to all beta-lactams in 20% of patients.

The high resistance observed in this study in *K. pneumoniae* isolates from hospital environments confirms the need for continuous monitoring of HAIs to detect and control outbreaks of resistant bacteria, protect public health, and ensure effective treatments.

Infections caused by multidrug-resistant bacteria increase morbidity, mortality, and healthcare costs, and pose treatment challenges. Understanding the factors associated with multidrug-resistant bacterial infection is key to adequate surveillance and control, allowing for improved patient care in hospitals. Due to the high bacterial concentration and constant use of antibiotics, the hospital environment becomes a breeding ground for the emergence of resistance. Therefore, it is not uncommon to find endemic multidrug-resistant bacteria in hospitals, which increase morbidity and mortality (42).

Studying genomic variability in resistant bacteria allows us to decide whether a given infection is caused by a single pathogen or by genomic variants circulating in the environment. Genomic variability is a key factor in the spread of multidrug-resistant bacteria. Variability allows bacteria to rapidly develop new traits, such as antibiotic resistance, expression of virulence genes, enzyme production, protein secretion to evade the immune system, biofilm formation, adaptation to adverse environments, etc. (43).

If there is marked genomic and phenotypic variability at the hospital level, the treatments adopted at the hospital level will become more complex. Patients with infections caused by multidrug-resistant bacteria are at greater risk of adverse clinical outcomes and death.

Conclusions

Most *K. pneumoniae* isolates from healthcare-associated infections are multi-resistant, showing significant resistance to beta-lactams and combination antibiotics.

There is high genetic variability among the isolates, suggesting multiple mechanisms and genetic elements contributing to antimicrobial resistance.

Molecular and *in silico* techniques are effective, but it is recommended to use at least three markers to obtain more accurate results.

Proposed research involves conducting complete genomic sequencing studies to identify new resistance genes and virulence mechanisms. Additionally, implementing molecular epidemiology studies to track the spread of resistant strains and analyze the impact of infection control programs and hygiene practices on the prevalence of resistant *K. pneumoniae*.

As a surveillance strategy, it is proposed to establish a robust epidemiological surveillance system that includes continuous monitoring of antibiotic resistance and genomic variability of *K. pneumoniae*.

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Declaration of conflicts of interest

There are no conflicts of interest.

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