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Molecular Identification of Co-Existence of Carbapenemase and Extended-Spectrum *β*-Lactamase Genes in *Klebsiella pneumoniae* Clinical Isolates, and Their Phylogenetic Patterns in Kenya

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Abstract

The increasing incidence of multidrug-resistant *Klebsiella pneumoniae* strains has become a serious global healthcare problem. Additionally, the carriage of both extended-spectrum ß-lactamase and carbapenemase genes on plasmid and genomic DNA in *K. pneumoniae* clinical isolates has not been documented in Kenya. This study aimed to assess the presence of extended spectrum *β*-lactamase (ESBL) and carbapenemase genes on genomic and plasmid DNA in *K. pneumoniae*, and classify these super-bug clinical isolates based on their phylogenetic patterns. The identification of *Klebsiella*-like clinical isolates (n = 20) collected from Kenyatta National Hospital in Nairobi was performed using API 20E Kit. Screening and confirmation for ESBL and carbapenemase phenotypes were conducted using Kirby-Bauer disk diffusion susceptibility test protocol. Conventional PCR technique was used to characterize ESBL and carbapenemase resistant genes on both genomic and plasmid DNA. Subsequently, 16S rRNA gene amplification and sequencing were performed. The 16S rRNA gene contiguous sequences of the bacterial isolates were analyzed using the ChromasPro. The gene sequence was compared with the sequences in GenBank database, using the BLAST program of NCBI to obtain the nearest phylogenetic neighbours from the databases. Then, the sequences of MDR *K. pneumoniae* and its relatives were aligned using ClustalW. The evolutionary history was inferred by using the maximum like-

lihood algorithm in MEGA MX. The phenotypic data of antibiotic susceptibility testing revealed that 2/20 (10%) clinical isolates were resistant both to imipenem and meropenem and producers of carbapenemase. These isolates were carbapenemase producers but not extended β-lactamases. However, 3/20 (15%) isolates that co-harboured blaNDM-1, blaIMP, blaTEM, and bla-OXA were identified during genotypic analysis. The positive control used separately yielded the expected band sizes for blaIMP (275 bp), blaOXA-48 (438 bp), and BlaKPC (798). The phylogenetic analysis showed the dual ESBL and carbapenemase producing *Klebsiella pneumoniae* could be classified as *K. pneumoniae* strain DSM 30104 and *K. pneumonia subsp. pneumoniae* strain GMH1080. This study confirmed the co-existence of ESBL and carbapenemase genes in *Klebsiella pneumoniae* on both bacterial genomic and Plasmid DNA, and demonstrated that the isolates are evolutionarily distinct. These findings raise a concern about the genotypic diversity of antibiotic resistance genes in bacterial isolates and their location. We, therefore, recommend an alternative management approach to combat these MDR bacterial isolates as well as frequent molecular surveillance programs to support antimicrobial stewardship.

Keywords

MDR, *Klebsiella pneumoniae*, Plasmid, Genomic DNA, Extended Spectrum β-Lactamase, Carbapenem, 16S rRNA

1. Introduction

The wide and long-term clinical application of broad-spectrum antibacterial drugs has resulted in the selection of *K. pneumoniae* strains producing extended-spectrum beta-lactamases (ESBLs) and carbapenemases, whose continual emergence now poses a major challenge in clinical settings [1]. *K. pneumoniae* is one of the top three bacteria of international concern in the World Health Organization report on the global status of antibacterial resistance because a number of antibiotics in use to control bacterial pathogens are becoming ineffective [2]. In addition, *K. pneumoniae* strains have become the second multi-drug resistant bacteria among the top ten multi-drug resistant (MDR) bacteria in intensive care units [3]. This phenomenon of resistance to antibiotics in *K. pneumoniae* is mostly due to the production of carbapenemases, which are β-lactamase enzymes with a capacity to hydrolyze not only the carbapenems but also all the other β-lactam agents [4]. The most common carbapenemases are veronica integron metallo-β-lactamases types (VIM), imipenemase (IMP) types, *Klebsiella pneumoniae* carbapenemase (KPC), oxacillinase-48 (OXA-48), and New Delhi metallo-beta-lactamase-1 (NDM-1) encoded by beta-lactamase genes (bla) such as bla-VIM, bla-IMP, bla-KPC, bla-OXA-48 and bla-NDM, respectively [5] [6]. Globally, the high prevalence of carbapenem-resistant *Klebsiella pneumoniae* isolates in healthcare units, community, and animals represents a huge burden to the healthcare system [7]. In the East African region, CR *Klebsiella pneumo-*

niae are the most prevalent among CR *Enterobacteriaceae* isolates [4]. The carriage of genes conferring resistance to carbapenems in *K. pneumoniae* has also been reported in Kenya [8] [9]. The carbapenemase genes are primarily mediated by the expression of chromosomally-encoded or plasmid-encoded carbapenemases [10]. However, the carriage of CR genes was mostly reported to be present either on bacterial genomic DNA or plasmid DNA in some published data [11] [12]. Furthermore, there is a scarcity of data on the concomitant carriage of β-lactamase and carbapenemase genes in Enterobacteriaceae species in African countries though it was reported that the carriage rate of both ESBL and carbapenemase genes was on rise in East African region, and had been detected in Uganda and Tanzania but not in Kenya [13]. This poses a public health concern in the region because the genotypic diversity of the AMR genes among bacterial isolates might suggest a flow of genes among different strains due to transfer by mobile genetic elements or multiple sources of resistance bacteria [13]. Therefore, there is need to investigate the phenotypic and genotypic diversity of AMR bacterial strains in Kenya. Our study aimed to assess the presence of extended spectrum β-lactamase (ESBL) and carbapenemase genes on genomic and plasmid DNA in *K. pneumoniae* clinical isolates, in Kenya, and classify these super-bug clinical isolates based on the sequence analysis of 16S rRNA gene.

2. Methods

2.1. Sample Collection and Phenotypic Identification of Clinical Isolates Species

A total of twenty (n = 20) multidrug resistant *Klebsiella-like* clinical isolates were collected from the culture collection bank of the Microbiology laboratory of Kenyatta National Hospital (KNH). These isolates were sourced from various clinical specimens including tracheal aspirate, blood, urine and pus swab. Bacterial clinical isolates were subcultured on MacConkey media. Then, pure colonies were picked from the overnight culture plate based on the morphological characteristics of *Klebsiella* species on MacConkey followed by oxidase reaction. Another pure colony was picked from the same culture plate and inoculated into tryptic soy broth (TSB) in order to obtain only pure bacterial colonies and re-identify the *Klebsiella* clinical isolates using the analytical profile index (API) 20E Kit (BioMerieux, Inc., Hazelwood, MO). API is a biochemical panel from BioMerieux, Inc. for the identification and differentiation of Gram-negative bacteria of the family *Enterobacteriaceae*. Briefly, each well of the API strip was filled up with bacterial suspension to its neck, except the wells labelled as citrate (CIT), voges-porskauer (VP), and charcoal gelatin (GEL). The wells labelled as lysine decarboxylase (LDC), ornithine decarboxylase (ODC), arginine decarboxylase (ADH), Na-thiosulfate (H2S), and urea (URE) were filled up to the top with bacterial suspension and sterile mineral oil, respectively. After 18 - 24-hour incubation period,

Kovac's reagent, Barritt's reagents and ferric chloride ($FeCl_3$) were added the

wells labelled as indole (IND), VP and tryptophan deaminase (TDA), respectively prior to the interpretation of the results. The reactions of three tests were added together at a time to obtain a 7-digit number, which was then looked up in the API WEB software for identification of the bacterial species and/or subspecies. The NCTC 13438 *K. pneumoniae* and sterile saline solution served as positive and negative control samples, respectively.

2.2. Antibiotic Susceptibility Testing and Detection of Extended *β*-Lactamases (ESBL) and Carbapenemase

The multidrug-resistant profile of the bacterial clinical isolates was reconfirmed by checking the susceptibility profile of the isolates to Ceftazidime (CAZ, 30 µg), Cefotaxime (CTX, 30 µg), Cefoxitin (FOX, 30 µg), Cefepime (CPM, 30 µg), Ampicillin (AMP, 2 µg), Imipenem (IPM, 10 µg), Meropenem (MER, 10 µg), Colistin (CT, 10 µg) (HiMedia Laboratories Pvt. Limited, Mumbai, India). The isolates were further processed for detection of extended spectrum *beta*-lactamases (ESBL) and carbapenemase production. The antibiotic susceptibility testing was performed by following and Kirby-Bauer disk diffusion susceptibility test protocol [14]. Each bacterial suspension was adjusted to a 0.5 McFarland standard and used within fifteen minutes. A disk of augmentin (20 µg of amoxicillin plus 10 µg of clavulanic acid) was placed at the center of the culture plate, and a disk of Ceftriaxone (CTX) and Ceftazidime (CAZ) 30 mm apart (center to center) in order to analyze the production of Extended *β*-lactamases and carbapenemases (ESBL). Once all disks were placed, the lid was replaced and the plate was inverted, and incubated at 37˚C in Wised Cube for 18 - 22 hours. The production of carbapenemase was also analysed in bacterial clinical isolates, which showed resistant to imipenem and/or meropenem by following the guidelines of clinical and laboratory standards institute (CLSI)s [15]. Briefly, a disk of imipenem (10 µg) or meropenem (10 µg) was applied on the culture plate of *E. coli* ATCC® 25922™ at its center. Using a 10-µl sterile inoculating loop, 2 to 3 colonies of each clinical isolate was inoculated in a straight line at least 20 to 25 mm in length out from the edge of the disk by streaking. The plate was closed, inverted, and incubated at 37˚C in Wised Cube for 16 to 18 hours. For quality control test, *Escherichia coli* ATCC® 25922™ was used as negative control sample whereas *Escherichia coli* ATCC® 35218™ and *K. pneumoniae* NCTC 13438 served as positive control samples to validate the production of extended spectrum *beta*-lactamases and carbapenemase, respectively. All the results were interpreted in accordance with the Performance Standards for Antimicrobial Disk Susceptibility tests of CLSI and European Committee on Antimicrobial Susceptibility Testing [15] [16].

2.3. Extraction of Total Bacterial DNA

Bacterial genomic DNA and plasmid DNA were extracted from all clinical isolates and ATCC strains by using Quick-DNA™ Fungal/Bacterial Miniprep Kit (Zymo Research, California, USA) and the Zyppy™ Plasmid Miniprep Kit (Zymo

Research, California, USA), respectively. Approximately 60 mg of wet colonies was resuspended in 200 µl sterile Phosphate-buffered saline in a ZR Bashing-Bead™ Lysis Tube for extraction of genomic DNA. Regarding the Plasmid DNA extraction, pellet approach was considered over the broth medium approach. All the bacterial samples including the NCTC 13438 *K. pneumoniae* were processed according to the manufacturers' instructions. The genomic DNA and plasmid DNA extracts were then separated by electrophoresis on a 1% agarose gel (AmpliSize; Bio-Rad Laboratories) for 40 minutes at 70 volts using 1× TAE Buffer containing GelRed Nucleic Acid and visualized by UV transillumination. O'GeneRuler 1 kb DNA Ladder, 0.1 µg/µL (Thermo Fisher Scientific, California, USA) was used as a marker for the amplicons' product sizes. The mixture of the master mix and sterile saline solution was used as negative control. The quantity and quality of genomic plasmid DNA extracts were also determined using Nanodrop. The genomic and plasmid DNA extracts were stored at -20˚C for use in PCR assay.

2.4. PCR Protocols for Amplification of Carbapenemase, ESBL and 16S rRNA Genes

Bacterial genomic DNA and plasmid DNA extracts were used as templates during the PCR reactions. All the clinical isolates (21) including the positive control were screened for the presence of blaNDM-1, blaKPC, blaOXA-48 and blaIMP genes using PCR 5x Master Mix (New England BioLabs, Massachusetts, USA) and the published primers shown in Table 1 [17] [18].

Table 1. Primer sequences and their annealing temperatures.

Primer	Gene	Sequence	Annealing Temperature	Expected amplicon size (bp)
16S rRNA	27F	AGAGTTTGATCCTGGCTCAG	51˚C	1500
	1492R	TACGGTTACCTTGTTACGACTT		
Bla_{KPC}	KPC-F	CGTCTAGTTCTGCTGTCTTG	54˚C	798
	KPC-R	CTTGTCATCCTTGTTAGGCG		
Bla_{OXA-48}	OXA-F	GCGTGGTTAAGGATGAACAC	51˚C	438
	OXA-R	CATCAAGTTCAACCCAACCG		
Bla_{NDM}	NDM-F	GGTTTGGCGATCTGGTTTTC	52˚C	621
	NDM-R	CGGAATGGCTCATCACGATC		
Bla_{IMP}	IMP-F	GGAATAGAGTGGCTTAATTCTC	45˚C	232
	IMP-R	GGTTTAATAAAACAACCACC		
Bla_{TEM}	TEM-F	GCGGAACCCCTATTTG	51˚C	1000
	TEM-F	TCTAAAGTATATATGAGTAAACTTGGTCTGAC		
Bla_{OXA}	OXA-F	ATGAAAAACACAATACATATCAACTTCGC	57˚C	750
	OXA-F	GTGTGTTTAGAATGGTGATCCATT		

The 16S rRNA gene amplification was also performed. The mix for the detection of each gene contained 11.5 µl of nuclease free buffer, 3.75 µl of each primer, 5 µl of Multiplex PCR 5× Master Mix (New England BioLabs, Massachusetts, USA) 1 µl of template (genomic DNA). The total volume was 25 µl for the reaction. Amplification was carried out with the following thermal cycling conditions: 1 min at 95˚C for initial denaturation and 35 cycles of amplification consisting of 20 s at 95˚C, 1 min s at 51˚C for annealing, 1 minute at 68˚C, and 5 min at 72˚C for the final extension. The PCR products were separated by electrophoresis at 70 volts for 50 minutes on a 1% agarose gel (AmpliSize; Bio-Rad Laboratories) using 1× TAE Buffer containing GelRed Nucleic Acid and visualized under UV transillumination. O'GeneRuler 1 kb DNA Ladder, 0.1 µg/µL (Thermo Fisher Scientific, California, USA) was used as a marker for the amplicons' product sizes. The clinical, which carried carbapenemase genes were also screened for the presence of ESBL genes such as blaTEM and blaOXA. DNA extracts of *K. pneumoniae* NCTC 13438 and *Escherichia coli* ATCC® 35218™ represented the control positive samples whereas the master mix plus blank control sample without DNA served as positive and negative control samples, respectively. The quantity and quality of PCR products were determined using the Nanodrop. The PCR reaction was performed in duplicate to ensure reproducibility.

2.5. Sequence Analysis of the 16S rRNA Gene

The sequence analysis was performed on 16s rRNA sequences from the clinical isolate forward and reverse sequences of each sample were assembled using ChormasPro software version 2.3.1 (Technelysium Pty. Ltd., http://www.technelysium.com.au). A copy of revised sequences can be viewed on GenBank (https://submit.ncbi.nlm.nih.gov/subs/?search=SUB10035180). Briefly, the forward and the reverse sequences of each sample were imported in the programme by opening new sequencing project the "file" tab, and then adding the files in FASTA format. The wrong bases were automatically trimmed out from the raw sequences. The reference sequence, *Klebsiella pneumoniae subsp. pneumoniae* NTUH-K2044 (NC_012731.1) was uploaded in FASTA format and the corrected sequences were assembled and a contiguous sequence was generated. The contiguous sequence was edited by making base call while checking the quality of each base and the right peak of the chromatogram. Each corrected contiguous sequence was blasted in order to sort out the closed relative species. The best hits were selected based on the species names, the E-values and the percentage of identity. The sequences of the closed relative species to *Klebsiella* species and the most distant ones were considered for pairwise and global alignment using ClustalW algorithm in Molecular Evolutionary Genetics Analysis software (MEGA MX, Version 10.2.3). *Yersinia enterocolitica* an *E. coli* species were taken as outgroups. The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and

BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 17 nucleotide sequences. Codon positions included were 1^{st} + 2^{nd} + 3^{rd} + Noncoding. There were a total of 1091 positions in the final dataset. Evolutionary analyses were conducted in MEGA X.

3. Results

3.1. Phenotypic Identification of Clinical Isolates Species, Antibiotic Susceptibility Testing and Detection of Carbapenemase

All the twenty-one (21) isolates including the *K. pneumoniae* NCTC 13438 were pink (lactose fermenter), mucoid on MacConkey and oxidase-negative. The API 20 E analysis showed that all the clinical isolates did not consist of only *Klebsiella pneumoniae* species. The taxon of two isolates was *Klebsiella oxytoca* and the isolates were positive for indole and negative for Voges-Proskauer (VP). The isolates classified as *Kluyver spp*. were both positive for indole and VP. The other isolates (17) including the positive control were negative for indole and positive for VP, and were identified as *Klebsiella pneumoniae subsp. pneumoniae* (Table 2).

Table 2. Biochemical characteristics of the clinical isolates.

Biochemical test	Results for 11 isolates										
	K1	K2	K3	K4	K7	K10	K11	K13	K16	K20	Positive Control NTCT 13438
ONPG	+	+	+	+	+	+	+	+	+	+	+
ADH	−	−	−	−	−	−	−	−	−	−	−
LDC	+	+	+	+	+	−	+/−	−	+	+	+
ODC	−	−	−	−	−	−	−	−	−	−	−
CIT	+	+	+	+	+	+	+	+	+	+	+
H_2S	−	−	−	−	−	−	−	−	−	−	−
URE	+	+	+	+	+	+	+	+	+	+	+
TDA	−	−	−	−	−	−	−	−	−	−	−
IND	−	−	+	+	−	−	−	−	−	−	−
VP	+	+	+	−	+	−	+	+	+	+	+
GEL	−	−	−	−	−	−	−	−	−	−	−
GLU	+	+	+	+	+	+	+	+	+	+	+
MAN	+	+	+	+	+	+	+	+	+	+	+

Continued

INO	+	+	+	−	+	+	+	+	+	+	+
SOR	+	+	+	+	+	+	+	+	+	+	+
RHA	+	+	+	−	+	+	+	+	+	+	+
SAC	+	+	+	−	+	+	+	+	+	+	−
MEL	+	+	+	+	+	+	+	+	+	+	+
AMY	+	+	+	+	+	+	+	+	+	+	+
ARA	+	+	+	+	+	+	+	+	+	+	+
OX	−	−	−	−	−	−	−	−	−	−	−
API Identity	*K pneu-moniae ssp. pneu-moniae*	*K pneu-moniae ssp. pneu-moniae*	*K oxy-toca*	*Kluy-vera spp*	*K pneu-moniae ssp. pneu-moniae*	*K pneu-moniae ssp. pneu-moniae*	*K pneu-moniae ssp. pneu-moniae*	*K pneu-moniae ssp. pneu-moniae*	*K pneu-moniae ssp. pneu-moniae*	*K pneu-moniae ssp. pneu-moniae*	*K pneumoniae ssp. pneumoniae*

ONPG = Ortho-nitrophenyl-beta-D-galactopyranoside, ADH = Arginine dihydolase, LDC = Lysine decarboxylase, ODC = Ornithine decarboxylase, CIT = Citrate, H_2S = Hydrogen sulfide, URE = Urea; TDA = Tryptophan deaminase, IND = Indole, VP = Voges-Porskauer (Na pyruvate), GEL = Charcoal gelatin, GLU = Glucose, MAN = Manitol, INO = Inositol, SOR = Sorbitol, RHA = Rhamnose, SAC = Sucrose, MEL = Melibiose, AMY = Amygdalin, ARA = Arabinose, OX = Oxidase.

The susceptibility profiles of all the isolates to the antibiotics used in our study revealed that two (2) out of the 20 clinical isolates were resistant both to imipenem and meropenem (Table 3).

Table 3. Antibiotic susceptibility test results of clinical bacterial isolates on MHA versus NA using CLSI & EUCAST zone size interpretative chart.

Antibiotic disc	**Inhibition Diameter (mm), resistance Profile to imipenem and meropenem only, production of ESBL and Carbapenemase, results for 10 clinical isolates**													
	K1	**K2**	**K3**	**K4**	**K7**	**K10**	**K11**	**K13**	**K14**	**K15**	**K16**	**K20**	***Kp* NCTC 13438**	**ATCC 25922**
IMP, 10 µg	22	21	21	21	22	23	22	26	23	23	9	12	9	26
	I	I	I	I	I	S	I	S	S	S	R	R	R	S
MER, 10 µg	25	25	26	23	25	25	26	28	26	26	6	6	6	30
	S	S	S	S	S	S	S	S	S	S	R	R	R	S
ESBL (SYNERGISTIC ACTION)	+	+	+	+	−	+	+	+	+	+	−	−	−	−
CP	−	−	−	−	−	−	−	−	−	−	+	+	+	−

CAZ = Ceftazidime, CTX = Cefotaxime, FOX = Cefoxitin, CPM = Cefepime, AMP = Ampicillin, IMP = Imipenem, MER = Meropenem, CT = Colistin AMC = Amoxicillin-clavulanic acid, ESBL = Extended β-lactamases, CP = Carbapenemase, R = Resistant, S = Susceptible, I = Intermediate.

These isolates were carbapenemase producers (Figure 1) but not extended β-lactamases.

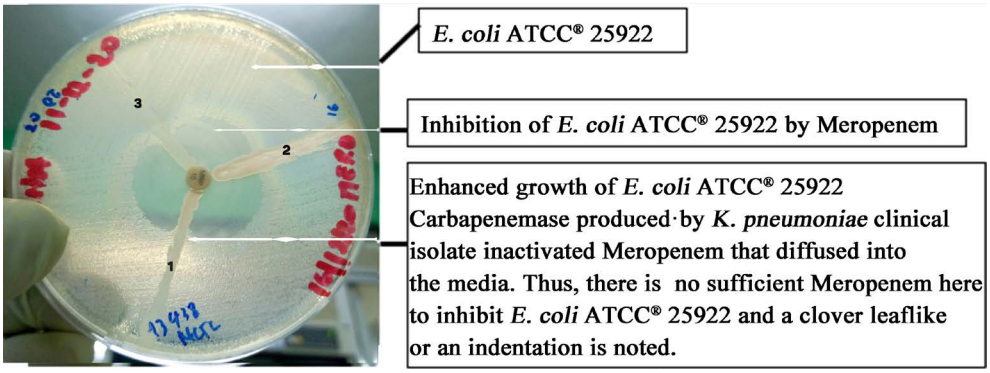


Figure 1. Production of Carbapenemase by *K pneumoniae subsp pneumoniae*. (1) *K. pneumoniae* NCTC 13438, positive result; (2) *K. pneumoniae* clinical isolate 16, positive result; (3) *K. pneumoniae* clinical isolate 20, positive result.

The other clinical isolates (17) were ESBL producers (Figure 2), except for one isolate.

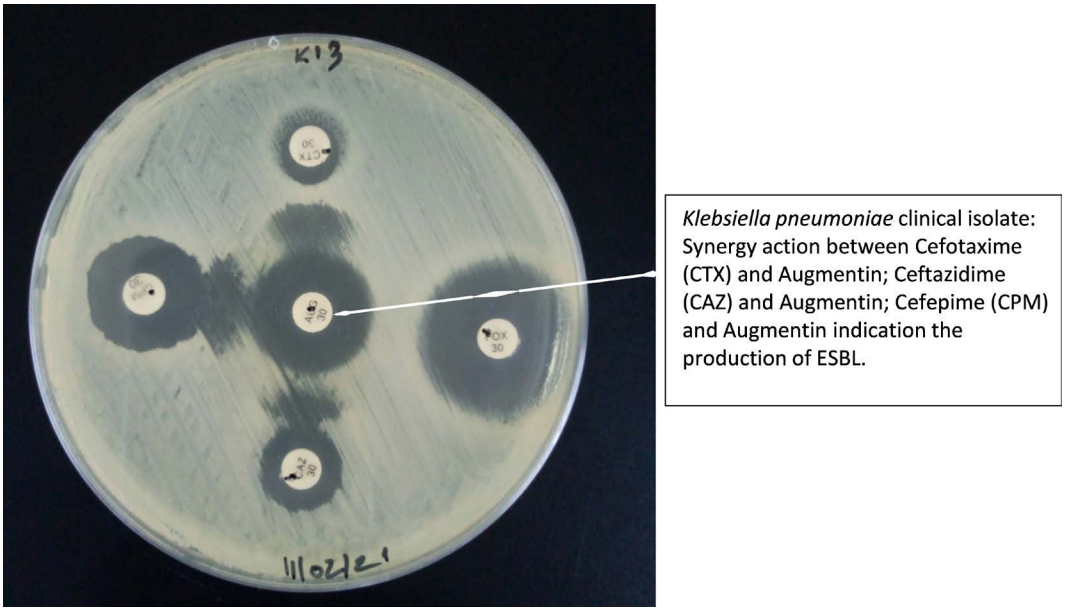


Figure 2. Phenotypic confirmation test for ESBL-producing *Klebsiella pneumoniae* strain.

3.2. Genotypic Characterization of Carbapenemase and ESBL Genes

The genomic and plasmid DNA extracts differed from each other based on the thickness of their bands (Figure 3(a)).

Out of the twenty *Klebsiella* clinical isolates screened for presence of four carbapenemase genes including blaNDM-1, blaKPC, blaOXA-48 and blaIMP, it appeared that three (3) isolates harboured blaNDM-1 and blaIMP as shown in Figure 4 (Figure 4(b) and Figure 4(c)) below. The positive control used sepa-

rately yielded the expected band sizes for blaOXA-48 (438 bp) and BlaKPC (798). However, different band size was observed for blaIMP (275 bp). BlaOXA-48 was found in one clinical isolate on the plasmid DNA. Furthermore, all these three *Klebsiella* clinical isolates carried extended spectrum *beta*-lactamases: blaTEM and bla-OXA (Figure 4(d) and Figure 4(e)). The resistant genes were both found on the genomic and plasmid DNA as described in Table 4. The band sizes of amplicons for blaTEM and BlaOXA were 1000 and 750 bp, respectively.

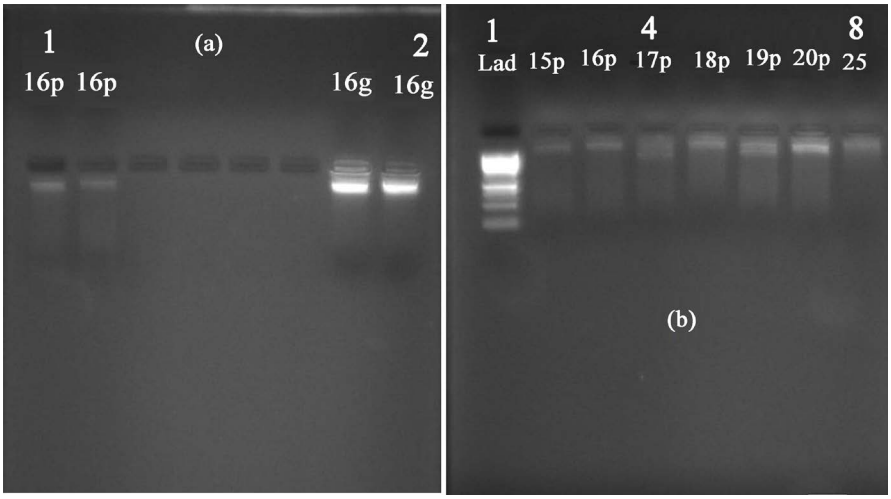
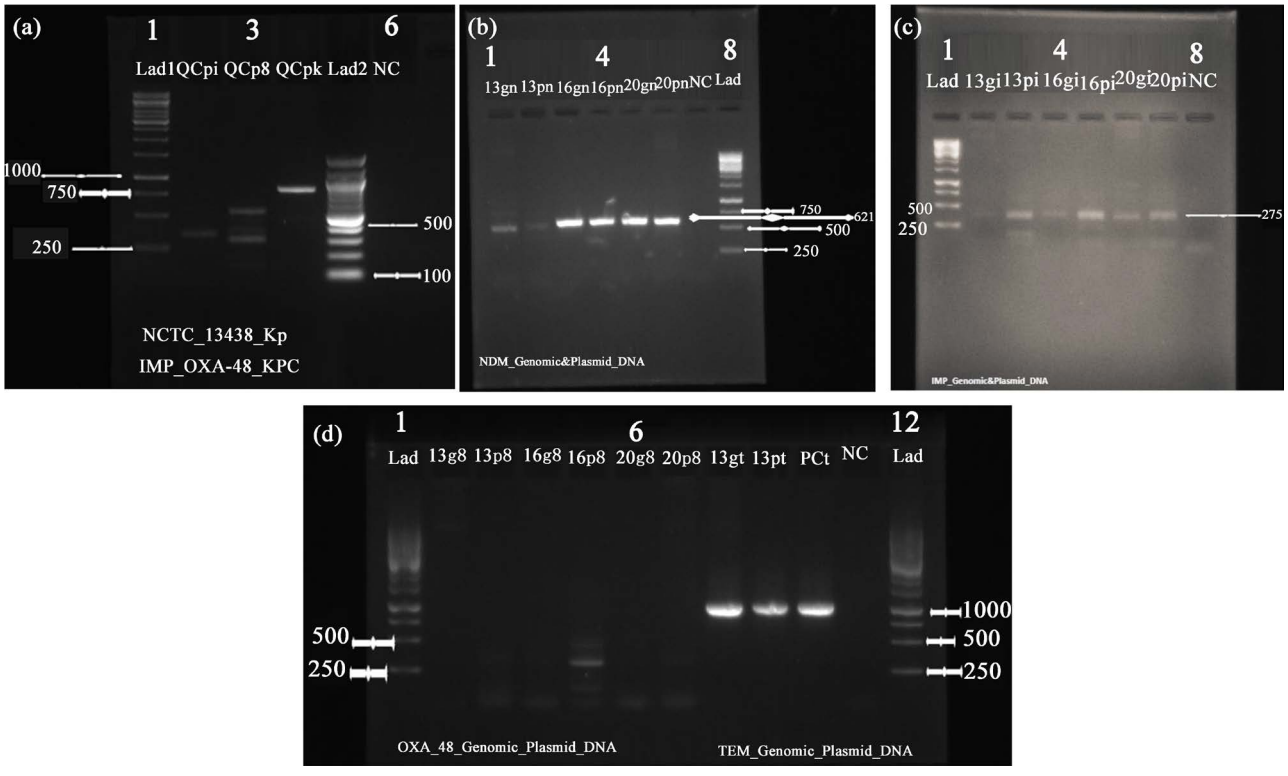


Figure 3. Gel agarose images of bacterial genomic and plasmid DNA where p stands for plasmid and g for genomic. Image (a) shows the successful optimization of plasmid and genomic DNA extraction step and the different bands of the plasmid are shown on image. (b) The band of genomic DNA was thicker than the one of plasmid DNA.



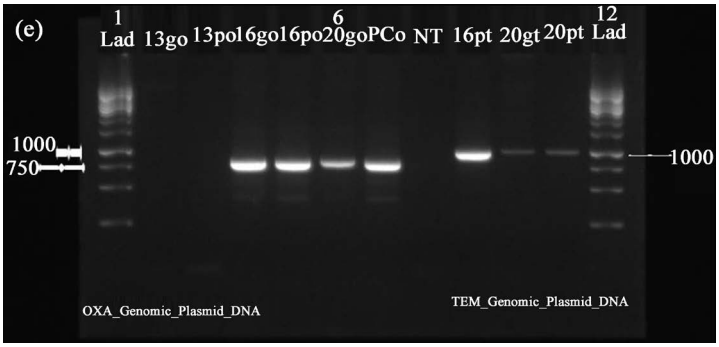


Figure 4. Images of agarose gel electrophoresis used for separation of the PCR products of the carbapenemase and extended spectrum *beta*-lactamases genes. The letter **g** corresponds to genomic DNA extract whereas **p** stands for plasmid DNA extract. NC and PC represent the negative control and positive control samples, respectively. The lanes labeled "Lad" correspond to the 1 kb DNA ladder and the size of each amplicon/fragment is indicated on the side of the molecular marker. (a) Representing the quality control (QC) sample for detection of blaIMP (QCpi) blaOXA-48 (QCp8) and BlaKPC (QCpk). (b) Detection of BlaNDM gene on both plasmid and genomic DNA in Klebsiella clinical isolates with 621 bp as amplicon size. "n" was the short form of NDM. (c) Detection of BlaIMP gene on both plasmid and genomic DNA in Klebsiella clinical isolates with 275 bp as amplicon size. (d) Detection of BlaOXA-48 and BlaTEM gene on both plasmid and genomic DNA in *Klebsiella* clinical isolates. There was presence of BlaOXA-48 in only isolate K16. "8" was the short form of OXA-48. (e) Detection of BlaTEM and BlaOXA gene on both plasmid and genomic DNA in *Klebsiella* clinical isolates. "t" and "o" were the abbreviated of TEM and OXA, respectively.

Table 4. Antibiotic resistance pattern according to detected associated CP and/or ESBLs genes.

Associated gene	Antibiotic resistance pattern										
	N (%)	AMC n (%)	CAZ n (%)	CTX n (%)	FOX n (%)	CPM n (%)	AMP n (%)	IMP n (%)	MER n (%)	CT n (%)	MDR n (%)
BlaTEM only	19 (95)	19 (95)	16 (80)	17 (85)	1 (5)	18 (90)	20 (100)	2 (10)	2 (10)	0	19 (95)
BlaOXA only	19 (95)	19 (95)	16 (80)	17 (85)	1 (5)	18 (90)	20 (100)	2 (10)	2 (10)	0	19 (95)
BlaNDM-1 only	3 (15)	19 (95)	16 (80)	17 (85)	1 (5)	18 (90)	20 (100)	2 (10)	2 (10)	0	19 (95)
BlaIMP only	3 (15)	19 (95)	16 (80)	17 (85)	1 (5)	18 (90)	20 (100)	2 (10)	2 (10)	0	19 (95)
BlaOXA-48 only	1 (10)	19 (95)	16 (80)	17 (85)	1 (5)	18 (90)	20 (100)	2 (10)	2 (10)	0	19 (95)
BlaTEM + BlaOXA	19 (95)	19 (95)	16 (80)	17 (85)	1 (5)	18 (90)	20 (100)	2 (10)	2 (10)	0	19 (95)
BlaNDM-1 + BlaIMP	2 (10)	19 (95)	16 (80)	17 (85)	1 (5)	18 (90)	20 (100)	2 (10)	2 (10)	0	19 (95)
blaNDM1 + blaIMP + blaOXA-48	1 (5)	19 (95)	16 (80)	17 (85)	1 (5)	18 (90)	20 (100)	2 (10)	2 (10)	0	19 (95)
(blaNDM1 + blaIMP) + (BlaTEM + BlaOXA)	3 (15)	19 (95)	16 (80)	17 (85)	1 (5)	18 (90)	20 (100)	2 (10)	2 (10)	0	19 (95)
(blaNDM-1 + blaIMP + blaOXA-48) + (BlaTEM + BlaOXA)	1 (5)	19 (95)	16 (80)	17 (85)	1 (5)	18 (90)	20 (100)	2 (10)	2 (10)	0	19 (95)

N = Total number of clinical isolates carrying each resistance gene; n = Number of clinical isolate resistant to each antibiotic in association with resistance gene(s) carriage.

3.3. Evolutionary Study of Carbapenem Resistant *Klebsiella* Species Based 16S rRNA Gene

The band sizes of the 16S rRNA amplicons were about 1500 bp (**Figure 5**). The

similarity analysis of carbapenem-resistant bacterial clinical isolates 16S rRNA gene sequences and other *Klebsiella* isolates showed that the percentage of identities between the K13, K16, K20 and *Klebsiella pneumoniae* strain DSM 30104 were 99.58%, 99.50% and 97.29%, respectively. Their E-values were all zero and the length of contigs ranged from 1422 to 1450 base pairs. Based on the ruling concept, strains having 16S rRNA similarity less than 97.5% most likely belong to different species whereas 16S rRNA similarity values upper than this threshold require more investigation to determine the taxonomic status [19].

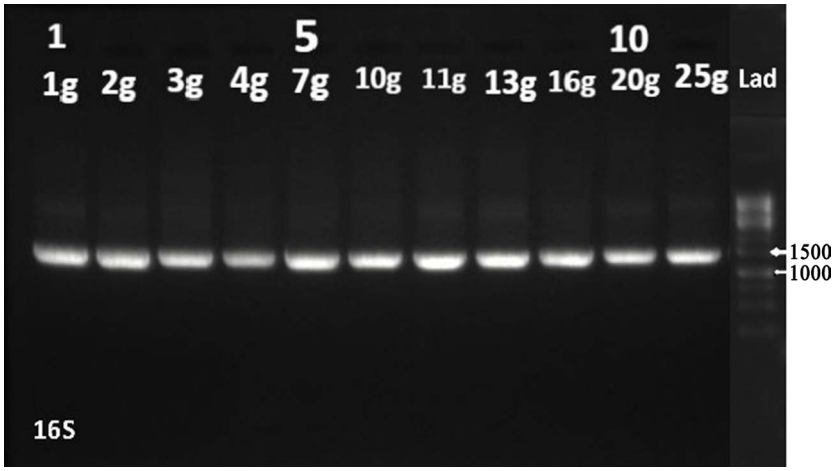


Figure 5. Gel electrophoresis image of 16S rRNA gene amplicons.

4. Discussion

The purpose of this study was to highlight the dual carriage of ESBL and carbapenemase genes on both bacterial genomic and plasmid DNA in *Klebsiella pneumoniae* clinical isolates and further trace the evolutionary history of these MDR bacterial isolates. Thus, this study provided data on phenotypic and genotypic detection of ESBL, carbapenemase and their resistant genes in clinical isolate of *K. pneumoniae*, especially resistant profile of bacterial strains to meropenem and imipenem. Most importantly, it demonstrated that non-carbapenemase producing *Klebsiella strains* could carry carbapenem resistant genes. The frequency of extended spectrum β-lactamase producing *K. pneumoniae* was high (95%) in our study supporting the data published previously in Kenya [20] [21] [22]. Out of the twenty clinical isolates of multi-drug resistant *Klebsiella* species, two (10.0%) of them were carbapenemase producers. However, the genotype test revealed that three of the isolates (15%) carried blaNDM-1 and blaIMP. Indeed, the presence of CP genes in carbapenemase non-producing *K. pneumoniae* was not reported in previous study. It was reported that a number of CAR genes including blaIMP, bla-VIM-1 blaSPM-l, blaNDM-1, blaOXA-23 blaOXA-24, blaOXA-58 and blaKPC have been identified, mostly isolated from carbapenemase-producing clinical isolates in hospital settings in East African region including Kenya [4]. Some factors contribute to the greater risk of the spread of carbapenemase genes in those clinical settings, such as the frequent use of invasive devices, more hos-

pital-acquired infections, long duration of hospitalization and more inadequate use of third and fourth line antibiotic drugs [23].

The current study described the co-existence of CP resistant genes (blaNDM-1, blaIMP) with ESBL genes (blaTEM and bla-OXA) in *Klebsiella pneumoniae* clinical. This is in contrast to the previous studies, which did not report the presence of imipenemase gene co-existing with ESBL genes. In Kenya, Musila *et al.*, 2021 reported the presence of carbapenem MDR genes in gram negative bacteria of clinical relevance including the detection of *bla*OXA-181, *bla*NDM-1 and ESBL genes in *K. pneumoniae* [10].

A study conducted in a Moroccan university hospital also revealed the occurrence of CP genes (blaOXA-48 and blaNDM-1) and ESBL genes in clinical isolate of *K. pneumoniae* [24]. This could be explained by the instability of blaIMP gene in bacterial genome. It was reported that the dual carriage of these genes was associated with the presence of blaNDM-1 gene [4]. Indeed, the blaNDM-1 gene encoding a metallo-β-lactamase (MBL) is plasmid borne and was first identified in *Escherichia coli* and *Klebsiella pneumoniae* in Sweden in 2008 from a patient transferred from India, and was also reported in Kenya [8] [25]. This enzyme hydrolyses all β-lactams except aztreonam and has been recognized as the main source for the transmission of carbapenemases in blaESBLs and blaMBLs [25] [26].

Furthermore, this study pointed out, for the first time in Kenyan isolates, the presence of ESBL and carbapenemase genes on both bacterial genomic and plasmid DNA (Confer gel images). Generally, many of the carbapenem-resistant genes in *Enterobacteriaceae* are known to be plasmid-mediated. Nevertheless, the occurrence of resistance to carbapenems may also be of intrinsic nature. Indeed, opportunistic and environmental pathogenic bacteria carried chromosomal metallo-β-lactamase enzymes (MBLs), which are not transferrable, from the groups which had a serine-based hydrolytic mechanism of action [27].

Finally, 16S ribosomal RNA sequence analysis was used for the first time to investigate the evolutionary history of ESBL and carbapenemase resistant *Klebsiella pneumoniae* strains in Kenya. The three carbapenem resistant clinical isolates were initially identified as *K. pneumoniae subsp. pneumoniae* in accordance with API 20E system (**Table 2**). Subsequent phylogenetic analysis of 16S rRNA gene of the clinical isolates showed that the three ESBL and carbapenemase resistant bacterial isolates, which have been highlighted in red in the above phylogenetic tree, formed a robust clade with the strains of *Klebsiella pneumoniae* indicated in green (**Figure 6**). The isolate K13 could be classified as *Klebsiella pneumoniae* strain JCM1662, whereas the isolates K16 and K20 as *Klebsiella pneumonia subsp. pneumoniae* strain GMH1080 or *Klebsiella pneumoniae* strain DSM 30104. *Klebsiella pneumoniae* strain JCM1662 was reported as *Klebsiella pneumoniae subsp. Pneumoniae* [28]. However, the bacterial isolate K20 evolved faster than the other strains and this could be due to the rate of mutation that occurred in its genome.

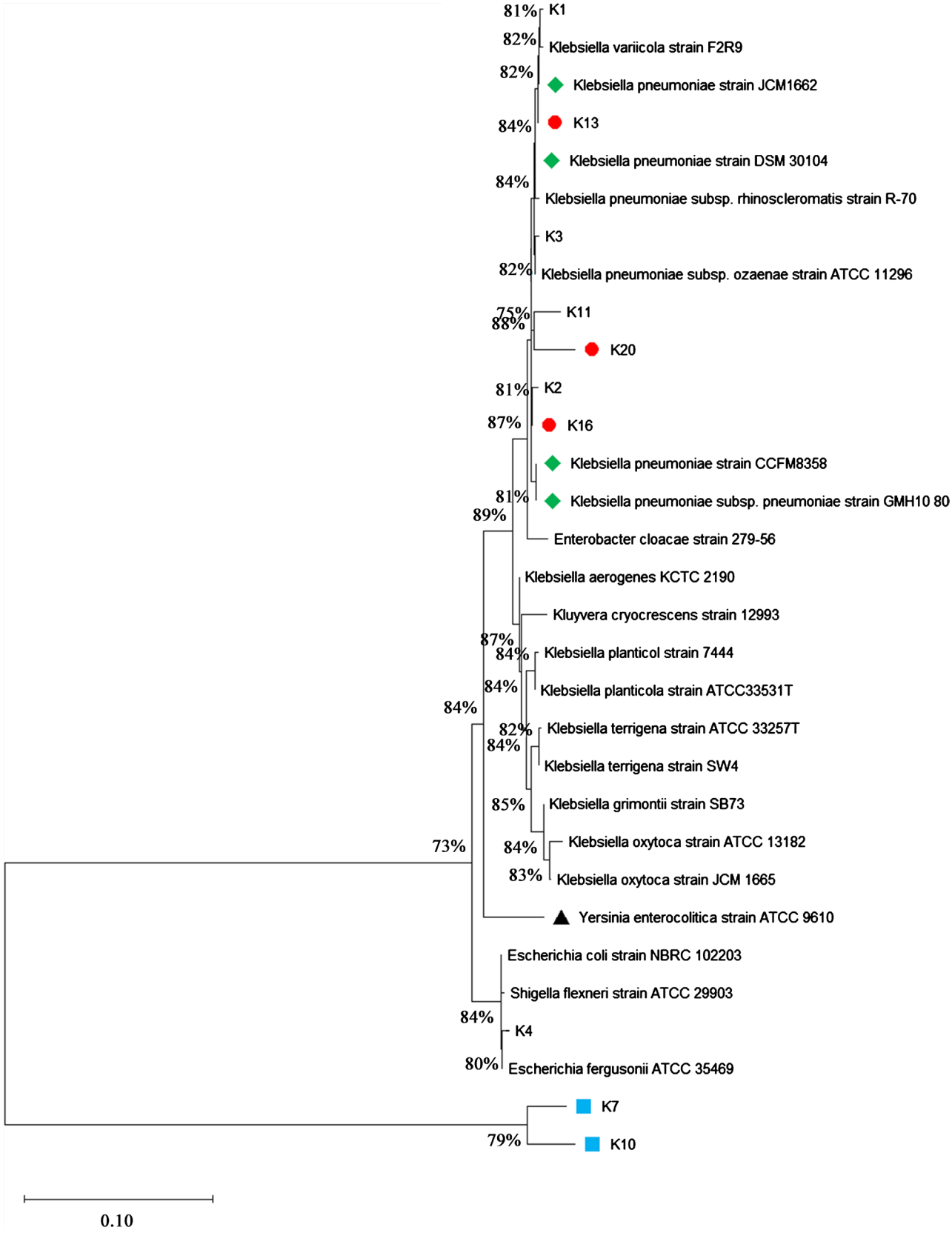


Figure 6. Evolutionary analysis by Maximum Likelihood method based on the 16S rRNA sequences, showing the evolutionary relatedness of the three carbapenem-resistant *Klebsiella* species clinical isolates (K13, K16 and K20).

5. Conclusion

This current study showed that carbapenemase non-producing Klebsiella strain could carry carbapenemase genes. It also outlined the dual presence of extended-spectrum *β*-lactamase and carbapenemase genes in *Klebsiella pneumoniae*, in addition, the carriage of ESBL and carbapenem-resistant genes on both bacterial genomic and Plasmid DNA. In this study, 16S ribosomal RNA approach was used to understand the evolutionary history of ESBL carbapenemase-producing *Klebsiella pneumoniae* for the first time in Kenya and indicated that the CR clinical isolates of *K. pneumoniae* are different strains. These findings raise a concern about the genotypic diversity of antibiotic resistance genes in bacterial isolates and their location. We, therefore, recommend an alternative management approach to combat these MDR bacterial isolates as well as frequent molecular surveillance programs to support antimicrobial stewardship.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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