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CLINICAL MICROBIOLOGY - RESEARCH PAPER

Phenotypic and genetic screening of *Klebsiella pneumoniae* isolates from human UTI patients for beta-lactamases and their genetic diversity analysis by ERIC and REP PCRs

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Abstract

Klebsiella pneumoniae is one of the major nosocomial pathogens responsible for pneumoniae, septicaemia, liver abscesses, and urinary tract infections. Coordinated efforts by antibiotic stewardship and clinicians are underway to curtail the emergence of antibiotic-resistant strains. The objective of the present study is to characterize *K. pneumoniae* strains through antibiotic resistance screening for production of beta-lactamases (β-lactamases) such as extended spectrum beta lactamases (ESBLs), AmpC β-lactamases, and carbapenemases by phenotypic and genotypic methods and genetic fingerprinting by enterobacterial repetitive intergenic consensus-polymerase chain reaction (ERIC-PCR) and repetitive element palindromic PCR (REP-PCR). A total of 85 *K. pneumoniae* strains isolated from 504 human urinary tract infections (UTI) were used in this study. Only 76 isolates showed positive in phenotypic screening test (PST), while combination disc method (CDM) as phenotypic confirmatory test (PCT) confirmed 72 isolates as ESBL producers. One or more β-lactamase genes were detected by PCR in 66 isolates (91.66%, 66/72) with bla_{TEM} gene being the most predominant (75.75%, 50/66). AmpC genes could be detected in 21 isolates (31.8%, 21/66) with FOX gene being the predominant (24.24%, 16/66), whereas NDM-I was detected in a single strain (1.51%, 1/66). Genetic fingerprinting using ERIC-PCR and REP-PCR revealed wide heterogeneity among β-lactamase producing isolates with discriminatory power of 0.9995 and 1, respectively.

Keywords *Klebsiella pneumonia* · ESBL · AmpC β-lactamases · NDM-1 · ERIC-PCR · REP-PCR

Introduction

Klebsiella pneumoniae is one of most ubiquitous and serious nosocomial pathogens of the Enterobacteriaceae family which is frequently responsible for infections involving the gastrointestinal, respiratory and urinary tracts of humans [1–3]. Patients with long-term catheterization and spinal cord injuries are at high risk of urinary tract infections caused by *K. pneumoniae* [4]. In spite of being a common inhabitant on mucosal surfaces of animals, soil, and environment, the organism sprung to limelight with the discovery of strains producing extended spectrum beta-lactamases (ESBLs) in early 1980s in Europe. Since then, the timeline of resistance showed an upward trend with identification of similar strains in the other continents. ESBLs are plasmid-borne enzymes which are primarily produced by members of Enterobacteriaceae family, in particular *Klebsiella pneumoniae*, *K. oxytoca*, and *Escherichia coli*. ESBL enzymes confer the microorganism with resistance

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to third- and fourth-generation cephalosporins and monobactams [5]. AmpC β-lactamases operate by chromosomal mediated mechanism and are commonly identified in members of Enterobacteriaceae [6]. *Klebsiella pneumoniae* is also known for its notorious resistance to Carbapenem antibiotics which often serve as the last line of treatment in many clinical conditions [7]. The mechanism is often due to plasmid-mediated *Klebsiella pneumoniae* carbapenemase (KPC) and rarely by chromosomally mediated elements. Prevalence of Carbapenem resistant *Klebsiella pneumoniae* (CRKP) often limits the choice of treatment to handful of drugs such as polymyxins, tigecycline, aminoglycosides, and Fosfomycin, and KPC infections are associated with high mortality rate [8]. Dissemination of ESBL, AmpC, and carbapenemase producing *K. pneumoniae* strains and their infections in humans and animals is one of the greatest threats to global health.

New Delhi metallo-β-lactamase-I (NDM-I) is a carbapenemase enzyme that makes bacteria resistant to broad range of beta-lactam antibiotics. Bacteria that produce NDM-I have emerged as serious public health issues because infections from these bacteria are very difficult to treat. NDM-I was first identified in *Klebsiella pneumoniae* isolated from a Swedish patient of Indian origin in the year 2008. Since then, reports were available from different organisms and from different locations predominantly in the Indian subcontinent. The organisms which are capable of producing NDM-I include *K. pneumoniae*, *E. coli*, *Citrobacter freundii*, *Enterobacter* spp., *Providencia* spp., *Morganella morganii*, and *Acinetobacter baumanii*. NDM-I was also identified in members of *Salmonella* [9].

Understanding genetic characteristics and diversity of microorganism is often necessary to understand the transmission and evolution of epidemic strains, planning patient care, designing control measures, and their dissemination control. This is achieved using techniques based on the amplification of non-coding repetitive elements which are often interspersed between the coding regions. Two commonly exploited elements include enterobacterial repetitive intergenic consensus (ERIC) sequences and repetitive extragenic palindromic (REP) elements. ERIC sequences are 127 bp imperfect palindromes, conserved, noncoding, and repetitive elements [10]. REP sequences are repetitive, palindromic variable length (21–65 bp) extragenic sequences present in certain bacterial genomes [11]. ERIC and REP-PCRs are preferred over other typing methods due to their rapidity, sensitivity, and consistency in analysing bacterial diversity and cost-effectiveness [10].

The purpose of this study is to investigate the presence of ESBLs, AmpC β-lactamases, and carbapenemase genes from *K. pneumoniae* strains isolated from human UTI patients. Furthermore, the genetic diversity of the isolates was analysed by ERIC and REP-PCR methods. Early detection of antibiotic resistance patterns in specific geographical location will help in appropriate patient care, infection control, and planning preventive measures [12].

Materials and methods

Materials, bacterial strains, and oligonucleotides

Dehydrated culture media, antibiotic discs, and supplements were procured from HiMedia laboratories, Mumbai, India. The American Type Culture Collection (ATCC) reference strain of *Klebsiella pneumoniae* ATCC 700603 was taken as a reference strain and was procured from Microbiologics, Minnesota, USA. The oligonucleotides used for multiplex PCRs were synthesized and procured from Bioserve Biotechnologies India Pvt. Ltd., Hyderabad.

K. pneumoniae isolates

A total of 85 *K. pneumoniae* isolates recovered from human urine samples of UTI patients and characterized by biochemical and molecular methods in our previous study were chosen and evaluated in this study [2]. The isolation and characterization of isolates were undertaken during the period from April 2017 to February 2020. Institutional Biosafety Committee of VFSTR (Deemed to be University) approved the study and protocols employed. *K. pneumoniae* strains stored at −80 °C ultralow freezer were revived by streaking on MacConkey agar and incubated at 37 °C for 24 h to ascertain the viability of cultures. A single isolated colony of *K. pneumoniae* from MacConkey plates were further subcultured into BHI agar and BHI broth for undertaking further studies.

Phenotypic tests for identification of β-lactamase production

ESBL detection has become a major challenge for clinical laboratories. The standard disk diffusion tests are effective and still recommended for ESBL diagnosis routinely in most of the clinical practice. However, a combination of standard disk diffusion tests and other approaches is suggested. In this study, an initial phenotypic screening test (PST) by Kirby-Bauer disc diffusion followed by phenotypic confirmatory test (PCT) by combination disc method (CDM) was followed. The combination disk method (CDM) measures zones of inhibition surrounding a cephalosporin and a second disc containing same cephalosporin and clavulanate (beta-lactamase inhibitor). A difference of ≥ 5 mm between the two zone diameters are indicative of ESBL producers [13].

Antibiogram of confirmed *K. pneumoniae* isolates was performed on Mueller-Hinton agar by Kirby-Bauer disc diffusion protocol [14] and interpretations were based on the criteria defined by Clinical and Laboratory Standards Institute (CLSI) guidelines [15]. *Klebsiella pneumoniae* antibiotic susceptibility patterns were confirmed based on production of ESBLs which was measured as diameter of zone of inhibition around antibiotic discs. Initially, antibiotic discs containing third-generation cephalosporins namely cefotaxime (CTX, 30µg), ceftazidime (CAZ, 30 µg) ceftriaxone (CTR, 30 µg), and a monobactam namely aztreonam (AT, 30 µg) were used for the phenotypic screening test (PST) as recommended by CLSI. Hexadisc containing all the four above mentioned antibiotics along with cefpodoxime (CPD) and cefpodoxime with clavulanic acid were used for PST. For phenotypic confirmation test (PCT), hexadisc containing the antibiotics cefpodoxime, cefpodoxime with clavulanic acid, cefotaxime, cefotaxime with clavulanic acid, ceftazidime, and ceftazidime with clavulanic acid were used by combination disc method (CDM). An Ezy MIC Strip (HiMedia, Mumbai, India) was employed to phenotypically identify the presence of Metallo-β-lactamase (MBL).

Identification of ESBL genes by multiplex PCR

Genomic DNA was isolated by boiling and chilling method [2, 16] using the inoculum from overnight cultures. DNA samples from the PCT positive strains were subjected to two multiplex PCR reactions [17] to identify various ESBL genes (Table 1). PCR assays were optimized for a 25-µl reaction constituted by 2 µl of template DNA, 12.5 µl of 2X Master mix (GoTaq Promega Green Master Mix), and 0.5 µl each of forward and reverse primers (10 pmol/µl), and the rest of the volume is made by adding nuclease free water. The PCR conditions were as follows: initial denaturation at 94 °C for 10 min followed by 30 cycles of denaturation at 94 °C for 40 s, annealing at 60 °C for 40 s, extension at 72 °C for 1 min, and a final extension at 72 °C for 7 min. Amplified

Table 1 List of oligonucleotides used in the present study

Resistance gene groups targeted	Primer name	Coding genes	Primer sequence (5′–3′)	Amplicon size (bp)
ESBLs (Multiplex PCR 1)	MultiTSO-T	*bla*TEM gene	CATTTCCGTGTCGCCCTTATTC	800
			CGTTCATCCATAGTTGCCTGAC	
	MultiTSO-S	*bla*SHV gene	AGCCGCTTGAGCAAATTAAAC	713
			ATCCCGCAGATAAATCACCAC	
	MultiTSO-O	*bla*OXA gene	GGCACCAGATTCAACTTTCAAG	564
			GACCCCAAGTTTCCTGTAAGTG	
ESBLs (Multiplex PCR 2)	MultiCTXM-Gp1	*bla*CTX-M group 1 gene	TTAGGAAATGTGCCGCTGTA	688
			CGATATCGTTGGTGGTACCAT	
	MultiCTXM- Gp2	*bla*CTX-M group 2 gene	CGTTAACGGCACGATGAC	404
			CGATATCGTTGGTGGTACCAT	
	MultiCTXM- Gp9	*bla*CTX-M group 9 gene	TCAAGCCTGCCGATCTGGT	561
			TGATTCTCGCCGCTAAG	
AmpC β-lactamase genes	CITMF	*LAT-1 to LAT-4, CMY-2 to CMY-7, BIL-1*	TGGCCAGAACTGACAGGCAAA	462
	CITMR		TTTCTCCTGAACGTGGCTGGC	
	DHAMF	*DHA-1, DHA-2*	AACTTTCACAGGTGTGCTGGGT	405
	DHAMR		CCGTACGCATACTGGCTTTGC	
	ACCMF	*ACC*	AACAGCCTCAGCAGCCGGTTA	346
	ACCMR		TTCGCCGCAATCATCCCTAGC	
	EBCMF	*MIR-IT ACT-1*	TCGGTAAAGCCGATGTTGCGG	302
	EBCMR		CTTCCACTGCGGCTGCCAGTT	
	FOXMF	*FOX-1 to FOX-5b*	AAC ATG GGG TAT CAG GGA GAT G	190
	FOXMR		CAA AGC GCG TAA CCG GAT TGG	
Carbapenemase genes	MultiVIM-F	VIM gene	GATGGTGTTTGGTCGCATA	390
	MultiVIM-R		CGAATGCGCAGCACCAG	
	NDM-Fm	bla_{NDM-1} gene	GGTTTGGCGATCTGGTTTTC	621
	NDM-Rm		CGGAATGGCTCATCACGATC	

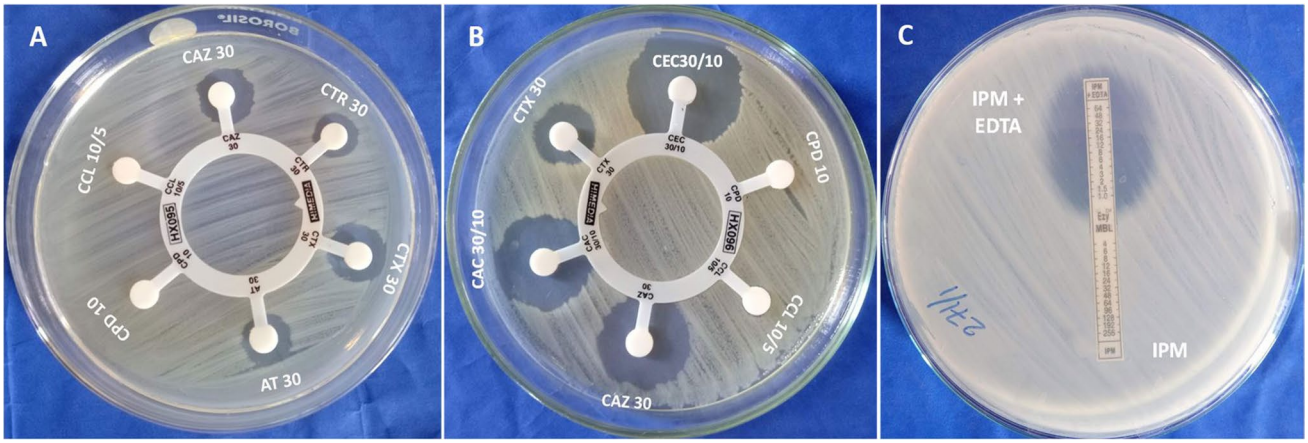


Fig. 1 **A** Phenotypic screening test for detection of ESBL production. **B** Phenotypic confirmation test for confirmation of ESBL production. **C** Ezy strip test for detection of metallo-β-lactamases

PCR products were resolved on 2% agarose gel stained with 0.5 µg/ml of ethidium bromide. The gel images were captured using Bio-Rad gel documentation system.

Identification of AmpC genes by multiplex PCR

All isolates were subjected to multiplex PCR for detection of AmpC group of genes. The oligonucleotides used for detection of AmpC genes is provided in Table 1 [18]. PCR reaction was optimized for a final volume of 25 µl. PCR reaction comprised 12.5 µl of 2× master mix (GoTaq Promega Green Master Mix), 2 µl of template DNA, and 0.5 µl each of forward and reverse primers, and the final volume of 25 µl was reached by adding nuclease free water. PCR program consisted of initial denaturation at 95 °C for 2 min followed by 30 cycles of denaturation at 94 °C for 45 s, annealing at 62 °C for 45 s, extension at 72 °C for 1 min. After last cycle, a final extension at 72 °C for 5 min was observed. The amplified PCR products were resolved on 2% agarose gels at constant voltage of 100 V. The gel images were captured using Bio-Rad gel documentation system.

Identification of carbapenemase genes

All isolates were subjected to monoplex PCR reactions for amplification of VIM [17] and NDM-I genes [19]. Oligonucleotide sequences for amplification of VIM and NDM-I genes are provided in Table 1. The PCR reaction was optimized in a 25-µl reaction. PCR program consisted of an initial denaturation at 94 °C for 4 min followed by 30 cycles of denaturation at 94 °C for 40 s, annealing at 55 °C for 40 s, extension at 72 °C for 1 min, and a final extension at 72 °C for 7 min. PCR amplified products were visualized after resolving them in 1.5% agarose gel stained with 0.5 µg/ml ethidium bromide at 100 V constant voltage. Gel images were captured in Bio-Rad gel documentation system.

Assessment of genetic diversity by ERIC-PCR and REP-PCR

All β-lactamase producing isolates were subjected to genetic fingerprinting by repetitive element-based ERIC-PCR and REP-PCR. ERIC-PCR and REP-PCR were optimized in 25 µl reactions. ERIC-PCR was performed using the primer pairs ERIC-1-FOR (5′-ATGTAAGCTCCTGGGGATTCAC-3′) and ERIC-2-REV (5-AAGTAAGTGACTGGGGTG AGCG-3′) [20]. PCR reaction is composed of 12.5 µl of 2× PCR master mix, 2 µl of genomic DNA, and 0.5 µl each of forward and reverse primers, and the remaining volume is composed nuclease free water. The following PCR programme was followed: initial denaturation at 95 °C for 7 min followed by 30 cycles of 94 °C for 30 s, annealing at 50 °C for 1 min, extension at 65 °C for 8 min, and final extension at 65 °C for 16 min was observed.

REP-PCR was performed using $(GTG)_5$ primer [21]. PCR reaction consisted of 12.5 µl of 2× PCR master mix, 2 µl of genomic DNA, and 1 µl of primer, and the final

Table 2 Isolates with positive phenotypic screening test for production of ESBLs

Antibiotics	No. of resistant isolates	Percent of resistant isolates
CAZ	30	35.29
CTR	45	52.94
CTX	36	42.35
AT	27	31.76

Table 3 Phenotypic confirmation test for ESBL production

Combination discs that detected ESBL production	Number of isolates confirmed for ESBL production
Cefpodoxime (CPD) and Cefpodoxime/Clavulanic acid (CCL)	7
Cefotaxime (CTX) and Cefotaxime/Clavulanic acid (CEC)	25
Ceftazidime (CAZ) and Ceftazidime/Clavulanic acid (CAC)	20
Both CPD-CCL and CTX-CEC	6
Both CPD-CCL and CAZ-CAC	3
Both CTX-CEC and CAZ-CAC	11
All three combinations CPD, CCL; CTX, CEC; CAZ, CAC	0
Total	72

Taken together, CPD-CCL combination detected ESBL production in a total of 16 isolates (i.e., 7 + 6 + 3 + 0). CTX-CEC combination detected ESBL production in a total of 42 isolates (i.e., 25 + 6 + 11 + 0). CAZ-CAC combination detected ESBL production in a total of 34 isolates (i.e., 20 + 3 + 11 + 0)

volume was adjusted with nuclease free water. PCR program comprised 95 °C for 7 min followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 40 °C for 1 min, and extension at 65 °C for 8 min, and a final extension at 65 °C for 16 min was observed. In both ERIC and REP-PCRs, a negative control (PCR reaction devoid of genomic DNA) and a positive control (*K. pneumoniae* ATCC 700603) were included. PCR products were resolved on 1.5% agarose gel using 0.5 × TBE buffer. The gel patterns were converted into binary matrix, and the data was analysed by DOLLOP program of PHYLIP version 3.6 software, and dendrograms were constructed (http://evolution.gs.washington.edu/phylip.html). Numerical index of discrimination was calculated by Simpson's index of diversity [22] using the formula

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{s} x_j\left(x_j - 1\right)$$

where D is Simpson's index of diversity (discriminatory power), N is total number of strains in the sample population, x_j is the number of strains belonging to the jth type, and s is the total number of types defined.

Results

Phenotypic identification of β-lactamase producers

Resistance to any one of the four β-lactam indicator antibiotics was considered positive in phenotypic screening test (PST) for ESBL production. Out of the 85 isolates of *K. pneumoniae* taken for this study, only 76 (89.41%) isolates showed positive reaction in PST. Of the four antibiotics, resistance to Ceftriaxone (CTR) was found to be highest showing resistance in 45 (52.94%) isolates. Least resistance was observed against aztreonam (AT) which is 27 isolates (31.7%) (Fig. 1A; Table 2). Furthermore, PCT by combination disc method (Fig. 1B) was performed on the PST positive isolates and ESBL production was confirmed in 72 PST positive isolates (94.73%, 72/76). Overall, the percentage rate of ESBL production among the *K. pneumoniae* isolates identified by phenotypic methods was found to be 84.7% (72/85). Combination of Cefotaxime (CTX) and Cefotaxime/clavulanic acid (CEC) showed highest resistance with a total of 25 isolates. Combination of cefpodoxime (CPD) and cefpodoxime/clavulanic acid (CCL) showed least resistance with a total of 7 isolates. The resistance patterns of higher (2 or more) antibiotic combinations are given in Table 2. Among the combinations, cefotaxime (CTX) and cefotaxime with clavulanic acid (CEC) was found to be most sensitive as it could detect the ESBL production in 42 isolates compared to the other two combinations (Table 3). Phenotypically, metallo-β-lactamase was identified in only one isolate (Fig. 1C).

Identification of ESBL genes by mPCR

All the 72 isolates positive for PCT were subjected to β-lactamase gene detection by multiplex PCR, and at least one β-lactamase gene was discovered in 66 isolates (91.66%, 66/72) (Fig. 2A and B). Similar findings were reported by Purighalla et al. in *K. pneumoniae* (84.78%) isolated from nosocomial infections [23]. In this study, bla_{TEM} was identified as the most predominant ESBL gene (75.76%, 50/66) present either alone (54.55%, 36/66) or in combination (21.22%, 14/66) with other genes (Fig. 2C). These observations were in agreement with the findings of Bora et al. [24] and Pishtiwan and Khadija [25]. However, contrasting reports were given by Maleki et al. [26] and Lim et al. [27] who reported *bla*CTX and *bla*SHV as predominant over *bla*TEM gene. Furthermore, *bla*OXA (27.27%, 18/66) and *bla*CTX-M-1 (12.12%, 8/66) were the next predominant genes present. CTX-M-2 gene was

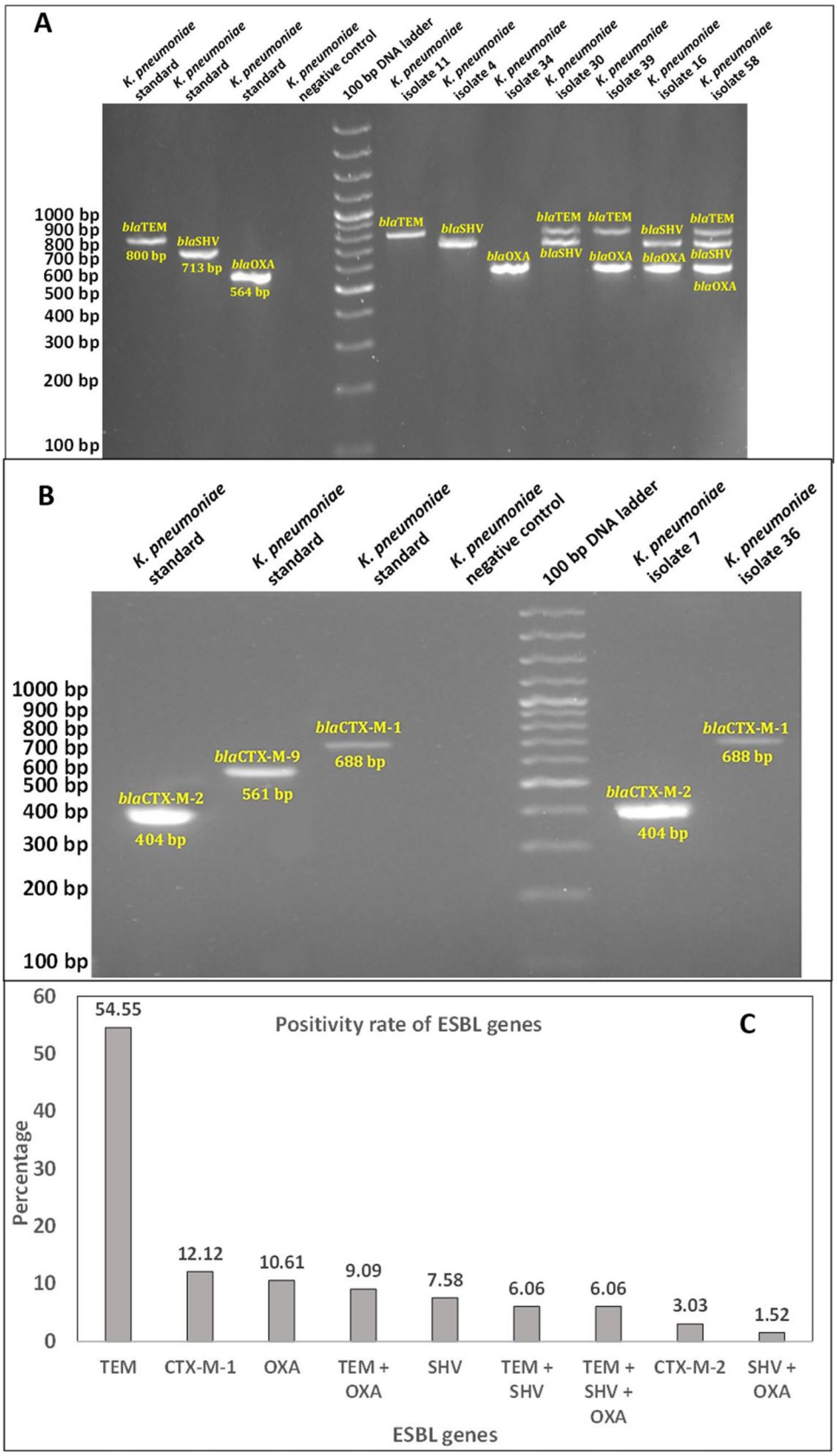


Fig. 2 **A** Molecular detection of ESBL genes (*bla*TEM, *bla*SHV, and *bla*OXA) in *Klebsiella pneumoniae*. **B** Molecular detection of ESBL genes (*bla*CTX-M1, *bla*-CTXM2, and *bla*CTX-M9) in *Klebsiella pneumoniae*. **C** Positive rate of ESBL (*bla*TEM, *bla*SHV, *bla*OXA genes and *bla*CTX-M group genes) genes in percentage. The number of isolates detected positive for ESBL genes by PCR are as follows: TEM — 36/66; CTX-M1 — 8/66; OXA — 7/66; TEM and OXA — 6/66; SHV — 5/66; TEM and SHV — 4/66; TEM, SHV, and OXA — 4/66; CTX-M2 — 2/66; SHV and OXA — 1/66

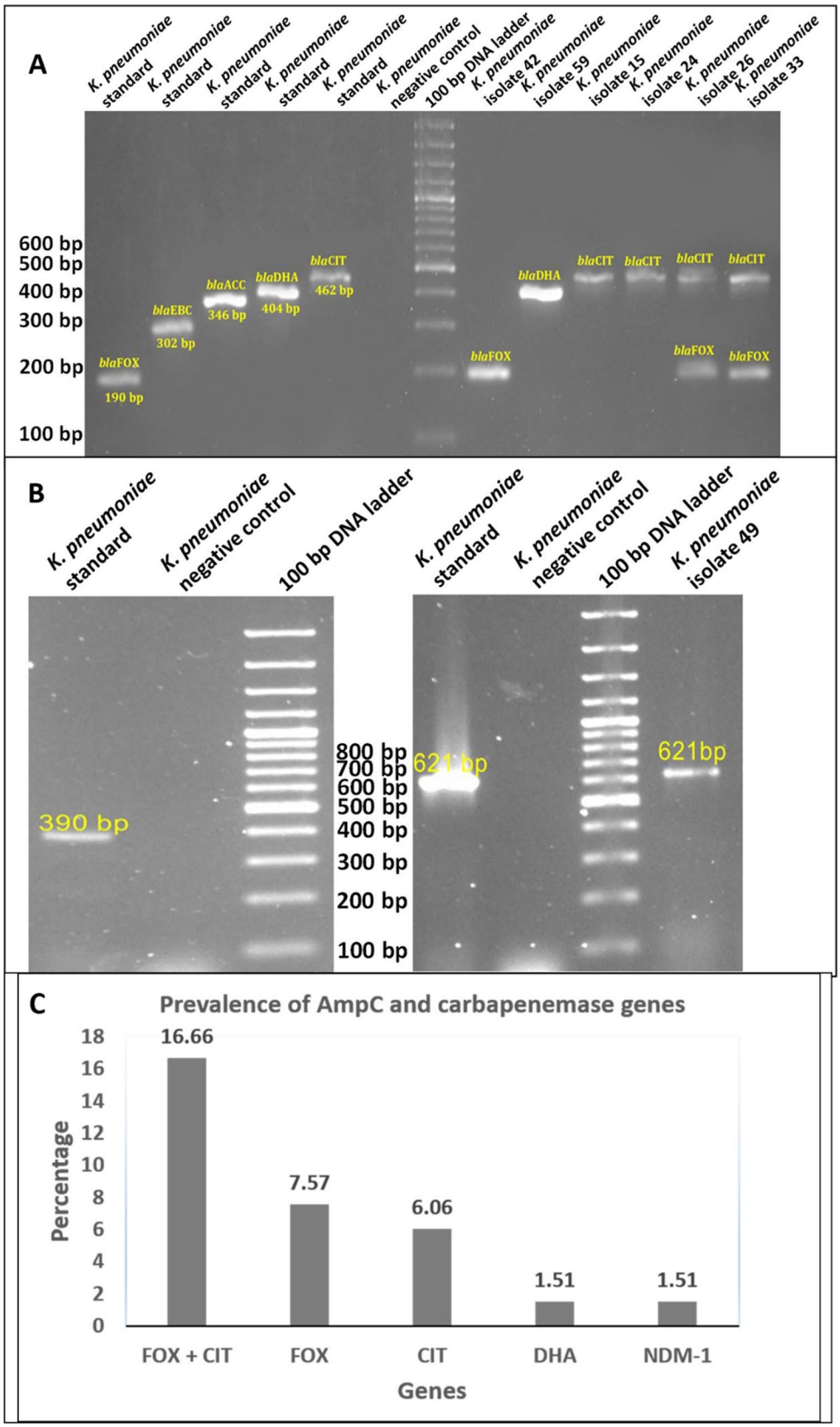
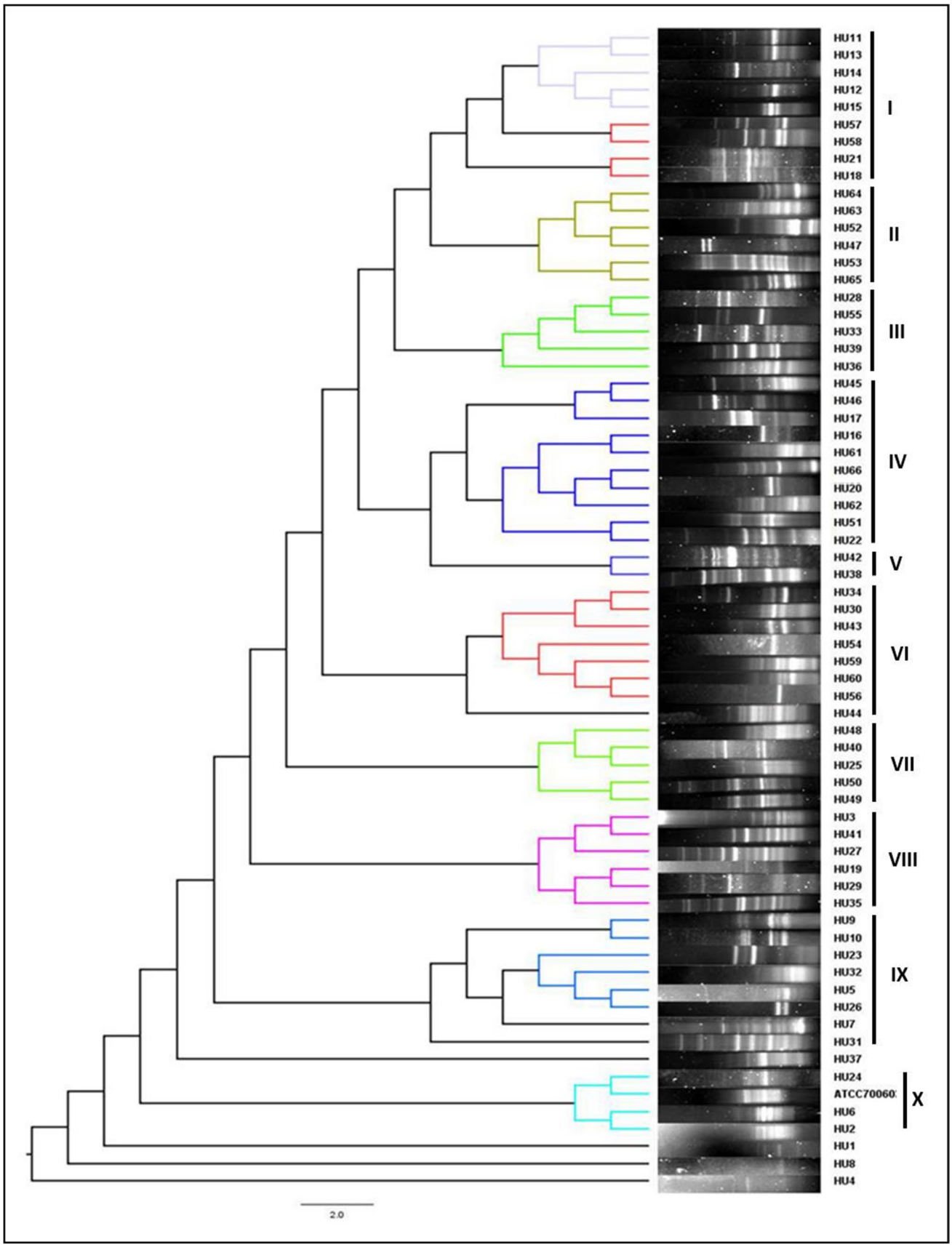


Fig. 3 **A** Molecular detection of AmpC genes (FOX, EBC, ACC, DHA, and CIT) in *Klebsiella pneumoniae*. **B** Molecular detection of VIM gene (390 bp) and NDM-1 gene (621 bp). **C** Positive rate of AmpC and carbapenemase genes in percentage. The number of isolates detected positive for AmpC beta lactamase genes by PCR are as follows: FOX and CIT — 11/66; FOX — 5/66; CIT — 4/66; DHA — 1/66; NDM-1 — 1/66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HU37
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◂**Fig. 4** DNA fingerprints of ESBL producing *K. pneumoniae* isolates generated by REP-PCR. Dendrogram resulting from analysis of REP-PCR profiles from 66 *K. pneumoniae* isolates. Neighbour joining (NJ) tree was constructed using PHYLIP version 3.6 program. Strains shown in same colour are isolates having similar band patterns and thus cluster together in phylogenetic tree

discovered in only 1 isolate (3.03%). CTX-M-9 group of genes was not detected in any isolate. The percentage prevalence of other ESBL genes by mPCR is depicted in Fig. 2C. Bora et al. reported the presence of more than one ESBL genes in 47.33% of the *Klebsiella pneumoniae* isolates recovered from human urine samples [24]. Bora et al. also detected one or more ESBL genes in all the PCT positive isolates of *K. pneumoniae* [24]. The presence of multiple ESBL genes in *K. pneumoniae* was also reported by Romero et al. [28], Livermore et al. [29], and Lim et al. [27]. In this study, the targeted ESBL genes could not be detected in 10 phenotypically confirmed isolates. The reason may be attributed the non-inclusion of ESBL genes which were not targeted in this study.

Identification of AmpC genes by mPCR

AmpC β-lactamases were previously believed to be chromosomally mediated. However, plasmid-mediated AmpC β-lactamases were identified in *K. pneumoniae* in South Korea in 1989. In our study, among the 5 tested AmpC β-lactamase genes, *bla*AmpC genes were detected in 21 isolates (31.8%, 21/66) out of which FOX group of genes was the most prevalent (24.24%, 16/66) followed by CIT genes (22.72%, 15/66). FOX gene was detected either alone or in combination with CIT gene. Similarly, CIT gene was detected either alone or in combination with FOX gene. The DHA gene was present in only a small percentage of isolates (1.51%, 1/66). The remaining two genes namely ACC and EBC were not detected in any of the 85 isolates. After FOX gene, the CIT gene was more prevalent. However, various authors have reported the prevalence of diverse families of AmpC genes in their studies.

Identification of carbapenemase genes

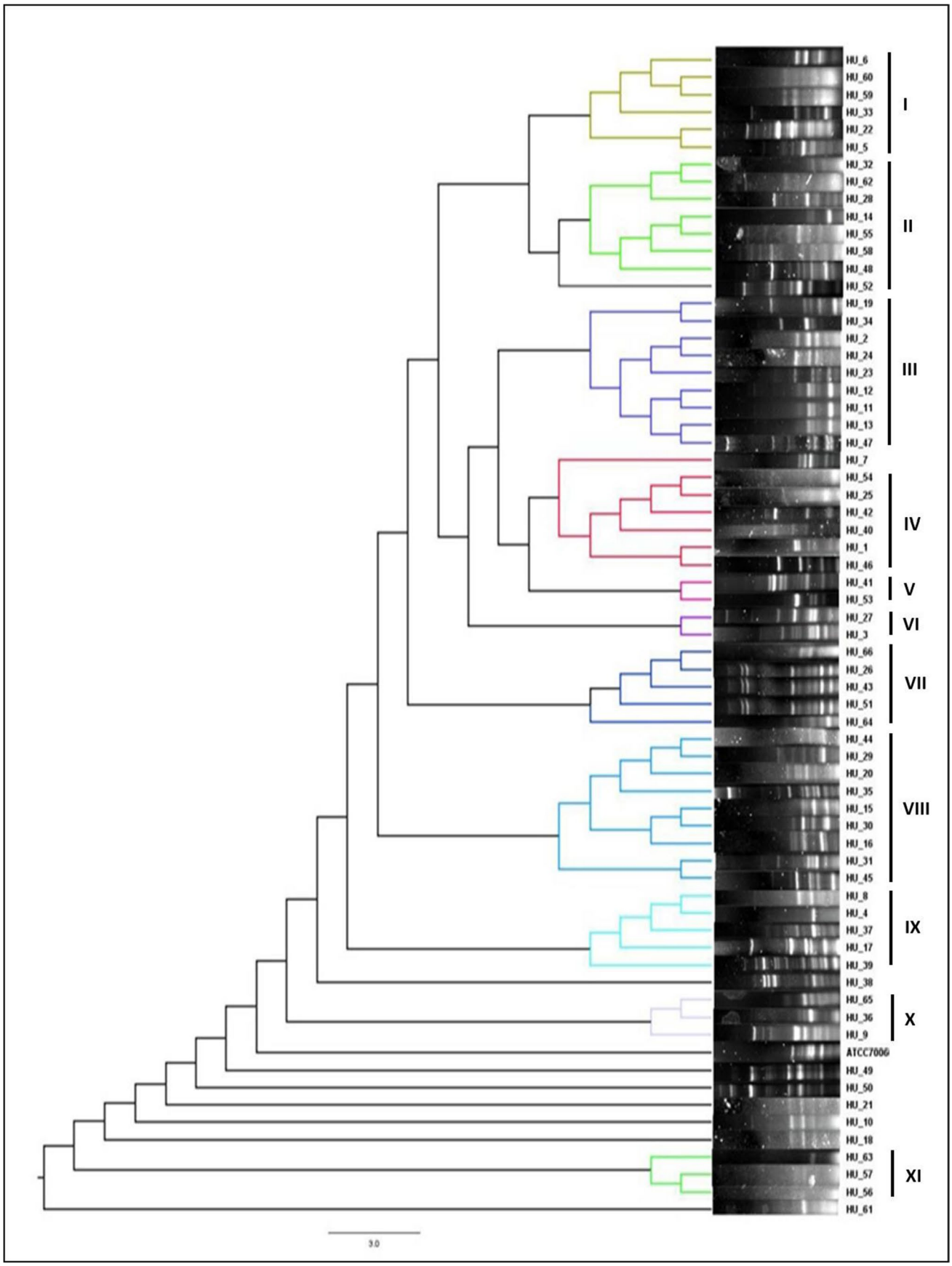
Carbapenemases are an emerging threat in the subcontinent due to its potential to make most of the antibiotics used in clinical setup ineffective. There is an upsurge of carbapenemases in clinical isolates of Enterobacteriaceae whose infections are very difficult for treatment. Among the two carbapenemases tested by PCR, $bla_{\text{VIM-I}}$ was not detected in any *K. pneumoniae* isolate (Fig. 3B). However, only one isolate carrying the $bla_{\text{NDM-I}}$ gene was detected by PCR (Fig. 3C) which gave positive phenotypically. Cabral et al. also reported similar observations wherein they were also not able to detect $bla_{\text{VIM-I}}$ gene in *Klebsiella pneumoniae* isolates [30]. Plasmids carrying $bla_{\text{NDM-I}}$ gene also carry a number of other genes conferring resistance to aminoglycosides and macrolides.

Assessment of genetic diversity by ERIC and REP PCR

REP-fingerprinting of all 66 β-lactamase producing *K. pneumoniae* isolates revealed 66 REP-profiles (Fig. 4) with a discriminatory power of 1. A total of 10 clusters were observed at a genetic similarity of 80% (Table 4; Fig. 4). A wide genetic diversity was observed between the isolates. Four isolates did not fit into any of the ten clusters. Similarly, ERIC-PCR displayed excellent discriminatory power on 66 *K. pneumoniae* isolates. Of these two isolates namely HU56 and HU57 isolated from same geographical location produced similar ERIC profile but these isolated were discriminated into different genotypes by REP-PCR. ERIC PCR showed a high genetic heterogeneity with a total of

Table 4 Cluster analysis of REP-PCR fingerprints of ESBL producing *K. pneumoniae* isolates

Cluster	Subcluster	Number of isolates	Isolate number
I	Ia	2	HU11, HU13
	Ib	3	HU12, HU14, HU15
	Ic	2	HU57, HU58
	Id	2	HU18, HU21
II	IIa	2	HU63, HU64
	IIb	2	HU47, HU52
	IIc	2	HU53, HU65
III	IIIa	4	HU28, HU33, HU39, HU55
	IIIb	1	HU36
IV	Iva	3	HU17, HU45, HU46
	IVb	2	HU16, HU61
	IVc	3	HU20, HU62, HU66
	IVd	2	HU22, HU51
V		2	HU38, HU42
VI	Via	3	HU30, HU34, HU43
	VIb	4	HU54, HU56, HU59, HU60
	Vic	1	HU44
VII	VIIa	3	HU25, HU40, HU48
	VIIb	2	HU49, HU50
VIII	VIIIa	3	HU3, HU27, HU41
	VIIIb	3	HU19, HU29, HU35
IX	IXa	2	HU9, HU10
	IXb	4	HU5, HU23, HU26, HU32
	IXc	1	HU7
	IXd	1	HU31
X		4	HU2, HU6, HU24, ATCC
Unclustered		4	HU1, HU4, HU8, HU37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III
HU_7
HU_54
HU_25
HU_42
HU_40
HU_1
IV
HU_46
HU_41
HU_53
V
HU_27
HU_3
VI
HU_66
HU_26
HU_43
HU_51
HU_64
VII
HU_44
HU_29
HU_20
HU_35
HU_15
HU_30
HU_16
HU_31
HU_45
VIII
HU_8
HU_4
HU_37
HU_17
HU_39
HU_38
IX
HU_65
HU_36
HU_9
X
ATCC7000
HU_49
HU_50
HU_21
HU_10
HU_18
HU_63
HU_57
HU_56
HU_61
XI
3.0

◂**Fig. 5** DNA fingerprints of ESBL producing *K. pneumoniae* isolates generated by ERIC-PCR. Dendrogram resulting from analysis of ERIC-PCR profiles from 66 *K. pneumoniae* isolates. Neighbour joining (NJ) tree was constructed using PHYLIP version 3.6 program. Strains shown in same colour are isolates having similar band patterns and thus cluster together in phylogenetic tree

11 clusters (Fig. 5; Table 5). A total of 9 isolates did not cluster with any of the genetic groups. Wide heterogeneity observed in ERIC and REP fingerprinting methods was expected as the isolates were from different geographical locations. Similar heterogeneity among *K. pneumoniae* isolates were reported by Ben-Hamouda et al. [31], Pai et al. [32] and Lim et al. [27]. However, no association between geographical location, the presence of β-lactamase genes, and the clustering of isolates could be observed which was consistent with the findings of Lim et al. [27].

Discussion

Klebsiella pneumoniae is an important nosocomial pathogen and one of the top three priority pathogens of international concern to public health as documented by World Health Organization. *Klebsiella pneumoniae* is the second most common UTI pathogen isolated in India and some of the isolates carry antibiotic resistant mechanisms such as ESBLs, AmpC β-lactamases, and carbapenemase phenotypes. Spread of ESBL pathogens is a serious concern around the world since they are also resistant to several other antibiotic classes such as penicillins and cephalosporins, thereby severely limiting the choice of antibiotics available for treatment and simultaneously increasing the patients mortality [33, 34]. CTX-M type enzymes are a rapidly growing group of class A ESBLs that have now spread rapidly and could be detected in several bacteria of clinical importance especially among Enterobacteriaceae members [35]. Plasmid-mediated AmpC β-lactamases first reported in 1988 are mobile derivatives of inducible chromosomal cephalosporinases which confer resistance to cephalothin, cefazolin, cefoxitin, most penicillins, and β-lactam/β-lactam inhibitor combinations [18]. AmpC β-lactamases are cause of concern due to the fact that their mobility permits them to move with in genus, species or between different groups of microorganisms. Carbapenems were considered to be the last resort of treatment for ESBL producing enterobacterial members [7]. Due to unrestricted usage of these antimicrobials, rapid emergence and wide dissemination of carbapenem (imipenem and meropenem) resistance among enterobacterial members have conferred them with resistance to carbapenems, cephalosporins, fluoroquinolones, aminoglycosides, and co-trimoxazole [36]. Early detection of these three antibiotic resistance groups will aid in planning proper treatment course, prevention, and infection control measures by mitigating their further spread. In the present study, we have undertaken the phenotypic and molecular profiling of ESBL producing *K. pneumoniae* isolated from UTI patients in Krishna district of Andhra Pradesh, India.

In our study, highest and lowest resistance to β-lactam indicator antibiotics was observed against ceftriaxone and aztreonam respectively by PST. The PST percentage (76/85, 89%) towards β-lactam antibiotics seen in this study is comparatively greater than 30.10%, 39.00%, and 41.17% as reported by Ejaz et al. [37], Sarojamma and Ramakrishna [38], and Shakya et al. [39] respectively. In CDM, the agreement between PST and PCT was found to be 94.7% (72/76). This corroborates with the finding of Ejaz et al. [37] who reported 99.5% ESBL confirmation levels. However, Shakya et al. [39] and Sarojamma and Ramakrishna [38] reported lower values of 42.7% and 43.58% respectively. The positivity of ESBL production among the *K. pneumoniae* isolates identified by phenotypic methods was found to be 84.7% (72/85) which is more than the values reported by Cabral et al. (37.51%) [30], Kumar et al. (49.20%) [40] and Lim et al. (47.05%) [27]. In CDM, resistance to cefotaxime and cefotaxime/clavulanic acid alone or with other combination was found in a total of 42 isolates. Of the 72 PCT positive isolates, 66 isolates showed one or more of the limited β-lactamase genes screened in this study. Only 6 isolates did not show any of the tested β-lactamase genes. This may mean that these isolates which are positive in PCT may be carrying a different β-lactamase genes which were not analysed by this study. Among the tested ESBL genes, the *K. pneumoniae* UTI isolates showed predominance of bla_{TEM} genes. Similarly, FOX group of genes were predominant for AmpC β-lactamase genes. Carbapenems are the antibiotics of choice when the members of Enterobacteriaceae show resistance to ESBLs and AmpC β-lactamases. Due to increased used of carbapenems, resistant strains have emerged which are associated with high morbidity and mortality. Such microorganisms pose serious challenges in treatment of pandrug-resistant infections as these are most tedious to manage and control in ICUs. Among the screened carbapenemase, only one isolated showed positive for NDM-1 gene. Treatment outcomes can be improved if the carbapenemase-resistance phenotype is discovered early and effective antimicrobial against carbapenemase producing *K. pneumoniae* such as colistin is prescribed. Colonization of hospitals and ICUs with metallo-β-lactamases is a complex phenomenon involving varied mechanisms of dissemination of resistant determinant genes. Outbreaks can be controlled by isolation and careful fumigation of existing ICU facilities and transfer of patients having $bla_{\text{NDM-1}}$ genotypes to a prefumigated facilities under strict aseptic conditions and thorough disinfection protocols.

Genetic fingerprinting of clinical isolates is important in epidemiological studies and provides information about

Table 5 Clusters of *K. pneumoniae* isolates based on ERIC-PCR

Cluster	Subcluster	Number of isolates	Isolate number
I	Ia	3	HU6, HU59, HU60
	Ib	1	HU33
	Ic	2	HU5, HU22
II	IIa	3	HU28, HU32, HU62
	IIb	4	HU14, HU48, HU55, HU58
	IIc	1	HU52
III	IIIa	2	HU19, HU34
	IIIb	3	HU2, HU23, HU24
	IIIc	4	HU11, HU12, HU13, HU47
IV	Iva	4	HU25, HU40, HU42, HU54
	IVb	2	HU1, HU46
V		2	HU41, HU53
VI		2	HU3, HU27
VII		5	HU26, HU43, HU51, HU64, HU66
VIII	VIIIa	4	HU20, HU29, HU35, HU44
	VIIIb	3	HU15, HU16, HU30
	VIIIc	2	HU31, HU45
IX		5	HU4, HU8, HU17, HU37, HU39
X		3	HU9, HU36, HU65
XI		3	HU56, HU57, HU63
Unclustered		9	HU7, HU10, HU18, HU21, HU38, HU49, HU50, HU61, ATCC

sources and nature of infection and possible control of nosocomial infections. Typing methods such as pulsed-field gel electrophoresis, ribotyping, and multilocus sequence typing (MLST) have higher discriminatory power but are very expensive and time consuming. ERIC and REP-PCRs are quick, reliable, and cost-effective procedures for genotyping of Enterobacteriaceae members [10, 41, 42]. DNA fingerprinting of all ESBL positive *K. pneumoniae* strains isolated from UTI patients by ERIC and REP-PCRs differentiated all 66 strains and revealed a discriminatory power of 1. Dendrogram analysis revealed wide diversity among isolates taken for study in the given region. $(GTG)_5$ PCR showed a higher discriminatory power than ERIC PCR as it generated more clusters and two isolates which are similar in ERIC were discriminated by $(GTG)_5$ PCR. As per our study, there was no significant correlation between members of ERIC and REP groups with their susceptibility patterns. These observations are consistent with findings of other studies [43, 44] where in genetically diverse *K. pneumoniae* isolates display diverse antibiotic resistance patterns and therefore are difficult to treat in hospitals. Proper hygienic practices by individuals and strict disinfection protocols for all medical equipment and aids by hospital staff is crucial in preventing entry of *K. pneumoniae* into patients.

Conclusion

In conclusion, β-lactamase producing *K. pneumoniae* proves to be a major challenge in health care settings and the prevalence of ESBL, AmpC β-lactamases, and NDM genes highlights its importance. This study also highlighted the wide genetic diversity prevalent among clinical isolates of β-lactamase producing *K. pneumoniae* isolates. Early diagnosis of resistant phenotypes and genotypes and strict preventive measures will avoid serious disasters. The need of the hour is to enact surveillance and antibiotic stewardship to prevent the emergence and mitigate the spread of resistant strains.

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Author contribution SB maintained the isolates and performed the experiments. MNB helped in analysing the data and revising of the manuscript. BKC performed the final revision of the manuscript and approved the final version. PNR is involved in analysing the data, preparation, and final approval of manuscript. SK helped write the initial draft of the manuscript and analyse the data partially.

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Data availability All data generated or analysed during this study are included in manuscript.

Declarations

Consent to participate All authors gave formal consent to participate in the study

Consent for publication All the authors read and approved the manuscript in its present format

Competing interests The authors declare no competing interests

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