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Analysis of beta-lactamases, bla_{NDM-1} phylogeny & plasmid replicons in multidrug-resistant *Klebsiella* spp. from a tertiary care centre in south India

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***Background & objectives*: β-lactamases play a predominant role in drug-resistance amongst *Enterobacteriaceae*. Presence of genes on transferable plasmids encoding these enzymes favours their dissemination across species and genera within and outside geographical boundaries. This study was aimed to understand the presence of β-lactamases and transferable plasmids in clinical isolates of *Klebsiella* spp. which can contribute to the spread of resistance determinants.**

***Methods*: A total of 41 clinical isolates of *Klebsiella* spp., collected from a tertiary care centre in Kerala, India, were checked for antibiotic sensitivity and the presence of plasmids. The ability to produce extended-spectrum β-lactamases (ESBLs) and metallo-β-lactamases (MBLs) was screened for and confirmed in 29 plasmid-harbouring isolates. bla_{NDM-1}-specific primers were used for polymerase chain reaction amplification with plasmid DNA as template to determine episomal prevalence of this gene and its sequence-based phylogeny employing similar sequences from GenBank. Plasmid replicon typing was also carried out to determine the presence of transferable plasmids.**

***Results*: Our results showed a high degree of multidrug-resistant (MDR) pathogens with ESBL production confirmed in 52 per cent, MBL in 31 per cent and co-production of both enzymes in seven per cent of the plasmid-bearing isolates. Plasmid DNA from 14 per cent of the isolates produced bla_{NDM-1}-specific amplicons which showed sequence homology with those from bacteria of different genera and geographical areas. The predominant replicon type was found to be that of conjugative plasmids belonging to the incompatibility group - $IncFII_K$.**

***Interpretation & conclusions*: This study provides insight into the predominance of various β-lactamases and potent gene-disseminating agents in *Klebsiella* spp. and emphasizes the need for constant surveillance of these pathogens to determine appropriate treatment strategies.**

Key words bla_{NDM-1} - conjugative plasmids - extended-spectrum β-lactamases – metallo-β-lactamase - multidrug-resistance - replicon-typing

Emergence of multidrug resistant (MDR) Gram-negative bacteria continues to be a major health concern worldwide[1,2]. During the past decades, a significant rise has been observed in the dissemination of multidrug and pandrug-resistant *Enterobacteriaceae*[3]. *Klebsiella* spp. represent one of the predominant nosocomial pathogens of this family[2,4,5]. These pathogens are a cause of concern due to their ability to produce various β-lactamases such as extended-spectrum β-lactamases (ESBLs), metallo-β-lactamases (MBLs) and carbapenemases, which make them resistant to even carbapenems with therapeutical options possibly remaining limited mostly to tigecycline and fosfomycin. Hence, adoption of effective treatment strategies and efficient health management becomes increasingly difficult[6-8]. Amongst the ESBL genes, $bla_{\text{CTX-M15}}$ is the most widespread type reported from *Enterobacteriaceae* in India[9]. One of the latest MBLs identified in Gram-negative bacteria[10], the bla_{NDM} type carbapenemases, was identified first in India[11]. Moreover, since the genes encoding these enzymes are frequently disseminated through transferrable plasmids, this has also contributed to a faster spread of resistance across species and genera[12]. Hence, the alternate name plasmid-encoding carbapenem-resistant MBL has been suggested to be a more appropriate one compared to the earlier controversial designation bla_{NDM}[13].

The gene bla_{NDM} has been reported both on narrow host-range plasmids belonging to the incompatibility group IncF, as well as other wide host-range plasmids such as IncA/C, IncL/M, IncH and IncN[10,14,15]. IncF comprises conjugative, low copy number plasmids capable of multireplicon existence, which facilitate their occurrence in a wide host range. Along with bla_{NDM}, the IncF plasmids frequently carry genes for ESBL of clinical importance such as $bla_{\text{CTX-M15}}$[6,14,16]. In addition, IncF plasmids are also known to carry genes encoding resistance to aminoglycosides, which confer multidrug resistance to the host organism [6,14,15].

In this study, clinical isolates of *Klebsiella* spp. were screened for ESBL and MBL production. Presence of $bla_{\text{NDM-1}}$ gene on plasmid DNA was determined and the amplicons obtained were checked for maximal homology against sequences in databases reported from elsewhere. A polymerase chain reaction (PCR)-based replicon typing of plasmid DNA was also performed to identify the predominant type of plasmid replicons present in these pathogens.

Material & Methods

The study was conducted in the recombinant DNA laboratory, department of Biotechnology, University of Calicut, Thenhipalam, India. Bacteria originally isolated from clinical sources including urine, catheter tip, pus and sputum, during a two-year period from August 2011 to August 2013, were transported in pure line form to our laboratory from the Microbiology Division of Little Flower Hospital, Angamaly, Ernakulam district, Kerala. A selective sampling was performed, in which bacterial isolates resistant to more than one antibiotic were selected. The sample size of these select clinical isolates was 100 which comprised six genera including species of *Klebsiella, Escherichia, Pseudomonas, Acinetobacter, Enterococcus* and *Staphylococcus*. Of these, 41 *Klebsiella* spp. were subjected to ribotyping, using *Klebsiella* MTCC3384 as a reference strain, and plasmid isolation by alkaline lysis method[17]. Only those isolates which were found to harbour plasmid DNA, as evidenced by electrophoretic profiles (n=29), were included in the study.

Antibiotic sensitivity: The sensitivity of the isolates against 11 antibiotics belonging to five different classes was assayed by disc diffusion method[18] as per Clinical Laboratory Standards Institute (CLSI) guidelines[19]. The antibiotic discs (Hi-Media, Mumbai) used in this study were - ampicillin (AMP) - 10 µg, ceftazidime (CAZ) - 30 µg, cefotaxime (CTX) - 30 µg, aztreonam (AT) - 30 µg, piperacillin/tazobactam (PIT) - 100/10 µg, azithromycin (AZM) - 15 µg, gentamicin (GEN) - 10 µg, nalidixic acid (NA) - 30 µg, ciprofloxacin (CIP) - 5 µg, meropenem (MRP) - 10 µg and chloramphenicol (C)-30 µg.

Phenotypic detection of extended-spectrum β-lactamases (ESBL) and metallo-β-lactamase (MBL) production: The ability of the isolates to produce ESBL was detected in accordance with CLSI guidelines[19]. CTX, CAZ and AT discs were used for ESBL screening and CTX/CAZ-clavulanic acid combination [cefotaxime-clavulanic acid (CEC) and ceftazidime-clavulanic acid (CAC)] were used for ESBL confirmation. MRP and MRP-EDTA discs were used for disc potentiation test to detect MBL production[20].

PCR-based screening for $bla_{\text{NDM-1}}$ gene sequences: A PCR-based screening was conducted to detect the presence of $bla_{\text{NDM-1}}$ gene on plasmid DNA from the isolates, using primers NDM-F -

5'GGTTTGGCGATCTGGTTTTC 3' and NDM-R - 5'CGGAATGGCTCATCACGATC 3'[6,21]. The PCR reactions were performed in a MiniCycler™ (MJ Research, USA) in 25 µl reaction volume containing 1x PCR buffer, 1.5 mM $MgCl_2$, 200 µM each of dNTPs, 2 U of Taq DNA polymerase, 0.5 µM of each primer and 100 ng template DNA. All reagents were purchased from Bangalore Genei, India. NDM-F/R primers (HPLC Purified) were obtained from Sigma-Aldrich Chemicals Pvt. Ltd. (Bengaluru). The programming of PCR cycle was as follows: Initial denaturation for 10 min at 94°C followed by 30 cycles of denaturation, annealing and extension at 94°C for one minute, 60°C for one minute and 72°C for two minutes respectively. After 30 cycles, a final extension was carried out for 10 min at 72°C. Amplicons were loaded on one per cent (w/v) agarose gels and the images were captured on an AlphaImager™ 2200 (Alpha Innotech Corporation, USA).

The PCR products were sequenced at a commercial facility (Xcelris Labs Limited, Ahmedabad). The nucleotide sequences and deduced protein sequences were analyzed with BLAST and FASTA programmes of NCBI (*www.ncbi.nlm.nih.gov*). Presence of ORFs and conserved regions were checked by ORF finder (*www.ncbi.nlm.nih.gov/gorf/gorf.html*).

Phylogenetic analysis of bla_{NDM-1} sequences: Phylogenetic analysis was performed with 4 bla_{NDM-1} sequences obtained in the present study (GenBank Accession numbers KX090027, KX090028, KX090029 and KX090030) and five similar sequences previously reported from different geographical areas showing highest query coverage and maximum identity with our sequence in BLASTN analysis. To determine the nearest phylogenetic neighbours, each sequence was subjected to the nucleotide sequence homology searches using BLAST homology search tool. All the sequences were aligned using default configuration of multiple sequence alignment tool 'MUSCLE' embedded in MEGA 5 software (*http://www.megasoftware.net*). The phylogenetic tree was constructed by neighbour-joining method with 1000 heuristic bootstrap replicates and substitution model as 'p distance'[22].

PCR-based replicon typing of plasmid DNA: Plasmids were typed into various incompatibility groups as described previously[23] using PBRT kit purchased from Diatheva, Fano, Italy and the amplicons were analyzed with respect to controls provided according to the manufacturer's instructions.

Statistical analysis: A significant difference between two proportions was checked by *Z*-test using Statistica software (Statsoft, India) version 5.0. A two-tailed $P<0.01$ was considered significant.

Results

Ribotyping of the 41 clinical isolates along with the control strain, *Klebsiella* MTCC3384, confirmed the isolate identity, as these were found to display similar amplicons with identical molecular weight of 1.3 kb. The sequence identity of the amplicon was confirmed to be 16s ribosomal RNA of *Klebsiella* spp. by DNA sequencing. Of the 41 isolates, 29 plasmid-bearing isolates, designated as K1 to K29, were subjected to further study.

Antibiotic resistance of the isolates: All isolates were found to be resistant to AMP, β-lactam/β-lactam inhibitor combination - PIT, first- and second-generation quinolones (NA, CIP and AT). Resistance to third-generation cephalosporins (CTX and CAZ) and AZM was also found to be high with 96.5 per cent of the isolates showing resistant phenotype. However, comparatively decreased resistance was observed in isolates against GEN (86%), MRP (79%) and C (76%). The antibiotic resistance phenotype of each of the 29 plasmid-bearing isolates is given in Table I.

ESBL and MBL production: ESBL production was confirmed in 15 of the 29 plasmid-bearing isolates (52%) as per CLSI (2014) criteria – (*i*) zone of inhibition ≤22, ≤27 and ≤27 mm for CAZ, AT and CTX, respectively, and (*ii*) a>5 mm enhancement in the zone of inhibition around clavulanic acid-containing discs. The latter criterion was observed only with CAC but not with CEC discs. Only nine isolates (31%) were found to produce MBL as evidenced by a >7 mm enhanced zone of inhibition around EDTA-containing discs. Two isolates (7%) co-produced both ESBL and MBL.

Phylogenetic analysis of bla_{NDM-1} gene sequences: Plasmid DNA from the isolates was employed as template for amplification by PCR using bla_{NDM-1}-specific primers. Amplicons were observed in only four (14%) (Table I) isolates which were found to vary in size from 580 to 605 bp (Fig. 1). The DNA sequence identity was confirmed using BLASTN analysis. These four DNA sequences together with five other similar sequences, retrieved from NCBI Genbank database, based on the criteria mentioned

Table I. Analysis of β-lactamases and resistance phenotypes in 29 plasmid-bearing clinical isolates of *Klebsiella* spp.

Isolate designation	Antibiotic resistance phenotype	ESBL	MBL	bla_{NDM-1}
K1	AMP PIT NA CIP AT CAZ CTX MRP C	+	+	+
K2	AMP PIT NA CIP AT CAZ CTX AZM GEN MRP C	-	-	-
K3	AMP PIT NA CIP AT CAZ CTX AZM GEN MRP C	+	-	-
K4	AMP PIT NA CIP AT CAZ CTX AZM GEN MRP C	-	-	-
K5	AMP PIT NA CIP AT CAZ CTX AZM GEN MRP C	-	-	-
K6	AMP PIT NA CIP AT CAZ CTX AZM GEN MRP C	-	+	-
K7	AMP PIT NA CIP AT CAZ CTX AZM GEN MRP C	+	-	-
K8	AMP PIT NA CIP AT CAZ CTX AZM GEN MRP C	-	-	-
K9	AMP PIT NA CIP AT CAZ CTX AZM GEN MRP C	-	-	-
K10	AMP PIT NA CIP AT CAZ CTX AZM GEN C	+	-	-
K11	AMP PIT NA CIP AT CAZ CTX AZM GEN MRP C	-	+	-
K12	AMP PIT NA CIP AT CAZ CTX AZM MRP C	+	+	-
K13	AMP PIT NA CIP AT CAZ CTX AZM GEN	-	+	+
K14	AMP PIT NA CIP AT CAZ CTX AZM GEN C	+	-	-
K15	AMP PIT NA CIP AT CAZ CTX AZM GEN MRP C	-	-	-
K16	AMP PIT NA CIP AT CAZ CTX AZM GEN MRP C	-	+	-
K17	AMP PIT NA CIP AT CAZ CTX AZM GEN MRP C	-	-	-
K18	AMP PIT NA CIP AT CAZ CTX AZM GEN MRP C	+	-	-
K19	AMP PIT NA CIP AT CAZ CTX AZM C	+	-	-
K20	AMP PIT NA CIP AT CAZ CTX AZM GEN	+	-	-
K21	AMP PIT NA CIP AT AZM GEN	+	-	-
K22	AMP PIT NA CIP AT CAZ CTX AZM GEN MRP	-	+	-
K23	AMP PIT NA CIP AT CAZ CTX AZM GEN MRP	+	-	-
K24	AMP PIT NA CIP AT CAZ CTX AZM GEN MRP C	+	-	-
K25	AMP PIT NA CIP AT CAZ CTX AZM GEN MRP	+	-	-
K26	AMP PIT NA CIP AT CAZ CTX AZM GEN MRP C	+	-	-
K27	AMP PIT NA CIP AT CAZ CTX AZM MRP	-	+	+
K28	AMP PIT NA CIP AT CAZ CTX AZM GEN MRP C	-	+	+
K29	AMP PIT NA CIP AT CAZ CTX AZM GEN MRP C	+	-	-

" + and - "represents beta-lactamase producers and non-beta-lactamase producers, respectively
ESBL, extended-spectrum β-lactamase; MBL, metallo-β-lactamase; AMP, ampicillin; PIT, piperacillin/tazobactam; NA, nalidixic acid; CIP, ciprofloxacin; AT, aztreonam; CAZ, ceftazidime; CTX, cefotaxime; AZM, azithromycin; GEN, gentamicin; MRP, meropenem; C, chloramphenicol

above, were used to construct the phylogenetic tree to understand the nearest neighbour of the study sequences. The genetic divergence and homogeneity of the sequences are apparent in the phylogenetic tree (Fig. 2). Amongst the DNA sequences obtained from our study, those from isolates K1 and K28 were found to form distinct lineages, while those from K27 and K13 shared similarity and were found to be closely related to the sequences reported earlier from Tamil Nadu (Accession no. 817735963) and New Delhi (Accession no. 749446381 and 749446385). Interestingly, the two sequences reported from Korea (Accession no. 937297641) and Japan (Accession no. KP347609.1) fell under a separate clade, and their genetic similarity with other sequences was also clearly discernible in the phylogenetic tree.

PCR-based replicon typing of plasmid DNA: Of the 29 plasmid-bearing isolates, plasmids from 22 isolates were typed, by PCR, into ten different incompatibility groups, namely, IncFIA, IncFIB-M, IncFII, $IncFII_K$, $IncFII_S$, IncHIB-M, IncA/C, $IncX_2$, IncK and IncR

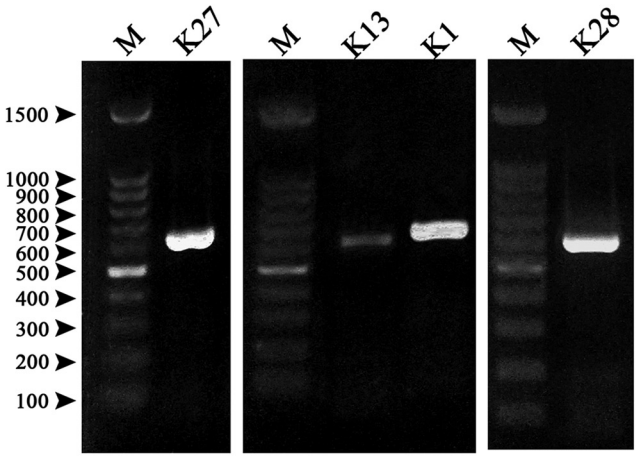

Fig. 1. Agarose gel (1.0%) showing bla_{NDM-1} amplicons from plasmid DNA of four *Klebsiella* isolates: Lane M:100bp DNA ladder. The other four lanes show bla_{NDM-1} amplicons from isolates - K1, K13, K27 and K28.

(Fig. 3A). The remaining isolates failed to produce a specific amplicon. All the replicons, except $IncX_2$, were found to exist as a combination of different replicons perhaps due to the presence of multiple plasmids in a cell or occurrence of more than one replicon on individual plasmids. $IncFII_K$ was found to be the most prevalent one followed by IncR (Fig. 3B). Of the 10 IncR plasmids obtained, nine were found to coexist with $IncFII_K$ (P=0.0003) and one with IncFIA. The next predominant replicon type, $IncX_2$, was found to exist both as a single replicon (50%) as well as a multireplicon plasmid (50%) (Table II). All of the $IncX_2$ harbouring isolates were found to be resistant to MRP with 87.5 per cent resistant to all antibiotics tested (P=0.002). Likewise, 90 per cent of the IncR-bearing isolates were found to be resistant to MRP and AZM (P=0.0003). IncFIA was found to coexist either with IncR (17%) or with $IncFII_K$ alone (33%) or in combination with IncR and $IncFII_K$ (50%). Three isolates were found to share an association of IncR, $IncFII_K$, IncHIB-M and IncFIB-M replicons (Table II), two of which, K1 and K12, co-produced ESBL and MBL.

Table II. Replicon types of plasmids from 22 *Klebsiella* spp.

Isolate	Replicons
K1	IncR, IncFIIK, IncHIB-M, IncFIB-M
K2	IncX2
K3	IncR, IncFIIK, IncHIB-M, IncFIB-M
K4	IncR, IncFIIK, IncX2
K5	IncX2
K6	IncX2
K9	IncFIA, IncFIIK
K10	IncFIIK, IncFII
K11	IncFIB-M, IncX2
K12	IncR, IncFIIK, IncHIB-M, IncFIB-M
K13	IncFIA, IncFIIK, IncA/C
K14	IncFIIS, IncFII, IncFIIK
K16	IncX2
K18	IncFIA, IncR, IncFIIK, IncK
K19	IncR, IncFIIK, IncK
K23	IncFIA, IncR, IncFIIK
K24	IncR, IncFIIK
K25	IncFII, IncX2, IncK
K26	IncFII, IncX2, IncK
K27	IncFIA, IncR, IncFIIK
K28	IncFIIK, IncK
K29	IncFIA, IncR

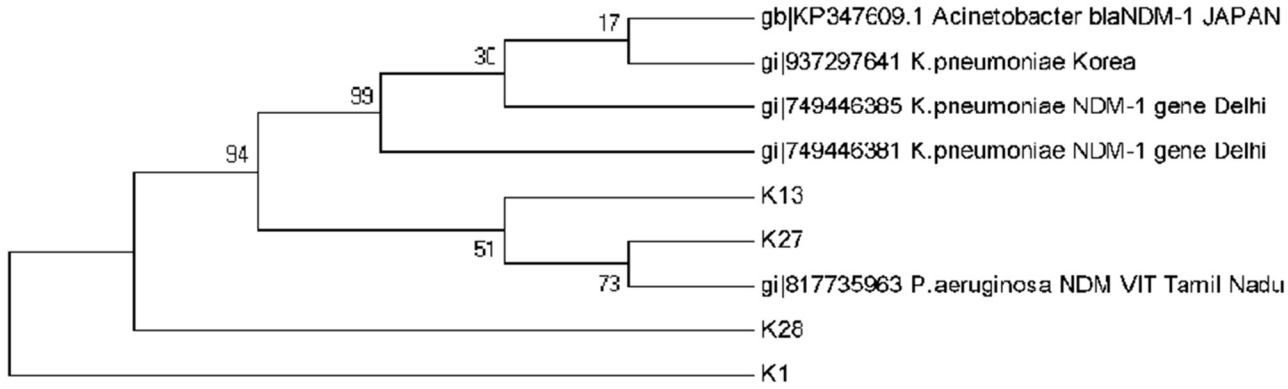

Fig. 2. Phylogenetic analysis based on bla_{NDM-1} gene sequences obtained from the four *Klebsiella* spp. in this study and five sequences retrieved from GenBank database (NCBI). Numbers on nodes represent bootstrap support values.

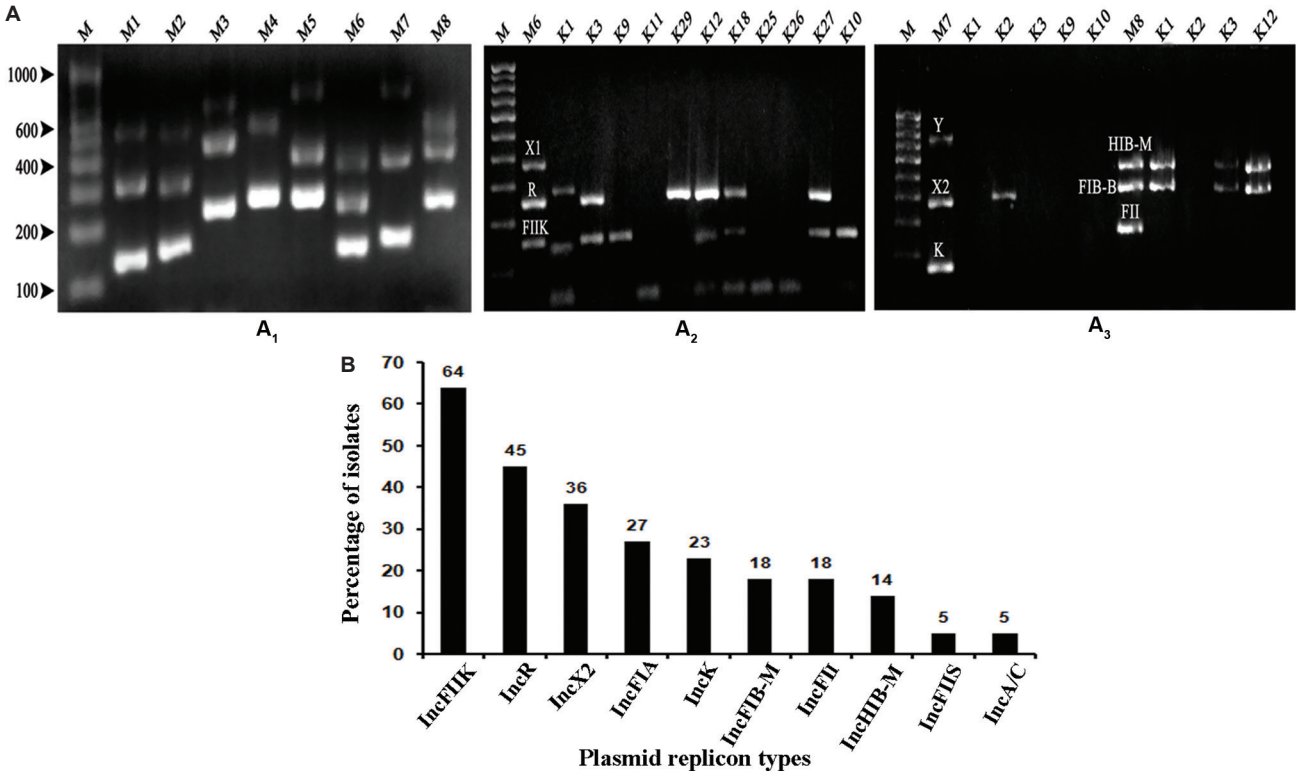

Fig. 3. (**A**) 1.5 per cent agarose gels showing plasmid replicon-specific amplicons – A1 represents control lanes, M1-M8 showing amplicons obtained from control DNA supplied with PBRT kit using Master Mixes with replicon-specific primers; A2 represents control M6 and 11 representative isolates amplified with M6 master mix; A3 represents controls M7 and M8 and 5 representative isolates amplified with M7 and M8 master mix, respectively. Lane M represents molecular weight marker 100bp DNA ladder in all the gels. (**B**) Percentage distribution of ten different plasmid replicons in 22 multidrug-resistant isolates of *Klebsiella* spp.

Discussion

β-lactam antibiotics are currently in use for treating infections with *Enterobacteriaceae* including *Klebsiella* spp., in which β-lactam resistance is mainly mediated by production of β-lactamases such as AmpC β-lactamases, ESBLs, MBL and other carbapenemases. MDR bacteria producing each individual type of β-lactamase alone or in combination continue to emerge as a worrisome challenge for antimicrobial therapy[9,24]. Occurrence of ESBL and MBL producers observed in this study and the observation of co-production of these two enzymes by two isolates was in agreement with the findings of the above-mentioned studies. ESBL producers are reported to be often resistant to multiple antibiotics[16,24]. In our study also 60 per cent of the isolates were found to be resistant to all of the antibiotics tested.

Following its first identification from India[11], bla_{NDM-1}-producing Gram-negative bacteria have become a worrisome issue in the clinical field[9,25]. In our study, bla_{NDM-1} gene was amplified from plasmid DNA of four isolates (14%). Although these four DNA sequences exhibited individual distinctness, they also shared similarities. Close relatives of the sequences obtained in the present study were found to be those reported from two other States in India - Tamil Nadu and New Delhi, in earlier studies (Accession nos. gi|817735963, gi|749446381 and gi|749446385). Homology was also evident with the sequences deposited from other countries – Korea[26] and Japan[27] (Accession nos. gi|937297641, gb|KP347609.1). Further studies are required to unravel the clinical implications of these plasmid harbouring strains. Widespread dissemination of pathogens carrying antibiotic resistance genes breaching geographical boundaries due to increased human mobility undoubtedly play a pivotal role contributing to such a situation[12,28].

In this study, $IncFII_K$ plasmids were prevalent followed by that belonging to the IncR group. Up to 90 per cent of the IncR plasmids were found to coexist with $IncFII_K$. This situation is capable of imparting wide host range and self-transferring capacity to these plasmids if existing in a multireplicon state, in spite of the immobility of IncR[29] and narrow host range of IncF plasmids. Moreover, all of the IncF type plasmids,

namely, IncFIA, IncFIB-M, IncFII, $IncFII_S$ and $IncFII_K$ observed in this study were found to exist as part of multireplicon plasmids which affirms observations reported earlier[23]. A prominent feature of $IncX_2$-bearing isolates noticed in this study was resistance shown by 87.5 per cent isolates against all antibiotics tested, and the enhanced resistance to MRP observed amongst IncR-bearing isolates was in agreement with the earlier reports on these plasmid-borne resistance genes[29,30]. Bonnin *et al*[10], speculated that IncFII, plasmid replicons endemic to Indian subcontinent, might play a major role in the dissemination of bla_{NDM} gene. This narrow host range, multireplicon plasmid, was also reported to be a frequent carrier of ESBLs, especially bla_{CTX-M}[14,16,23,31]. Interestingly, the prevalent plasmid in bla_{NDM-1}-producing isolates in this region was also found to be a variant of IncFII, $IncFII_K$. Interestingly, the fact that these plasmids have been associated with several addiction systems[10,23,32], combined with the unique feature of bla_{NDM-1} gene's presence on plasmids, ensures stable maintenance of this MBL gene during host replication.

In conclusion, our findings showed a normal β-lactamase production and a higher occurrence of $IncFII_K$ type plasmid replicons in MDR *Klebsiella* spp. from Kerala compared to similar investigations[5,7,16,31]. The same replicon was also found to be the predominant one amongst bla_{NDM-1}-carrying isolates of this species. The observation of the coexistence of $IncFII_K$ with wide host-range replicons of IncR type and phylogenetic studies of bla_{NDM-1} sequences provide indication of the challenging situation of horizontal gene transfer occurring amongst these potential pathogens. This study mainly showcases the threats to healthcare in a major tertiary care centre in Kerala. However, this limitation can be overcome by conducting elaborate and detailed molecular investigations on a larger scale to better understand the current scenario of pathogenic bacterial drug resistance in the country.

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***Conflicts of Interest*:** None.

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