High Prevalence and Significant Association of ESBL and QNR Genes in Pathogenic *Klebsiella pneumoniae* Isolates of Patients from Kolkata, India

Anusri Tripathi · Sudip Kumar Dutta ·
Monalisa Majumdar · Lena Dhara ·
Debolina Banerjee · Krishnangshu Roy

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Abstract Pathogenic *Klebsiella pneumoniae*, resistant to beta-lactam and quinolone drugs, is widely recognized as important bacteria causing array of diseases. The resistance property is obtained by acquisition of plasmid encoded bla_{TEM}, bla_{SHV}, bla_{CTX-M}, QNRA, QNRB and QNRS genes. The aim of this study was to document the prevalence and association of these resistant genes in *K. pneumoniae* infecting patients in India. Approximately 97 and 76.7 % of the 73 *K. pneumoniae* isolates showed resistance towards beta-lactam and quinolone drugs respectively. *Bla* genes were detected in 74 % of *K. pneumoniae* isolates; with prevalence in the following order: bla_{TEM} > bla_{SHV} > bla_{CTX-M}. QNR genes were detected in 67 % samples. Chi-square analysis revealed significant association between presence of *bla* and *qnr* genes in our study (*P* value = 0.000125). Sequence analysis of some bla_{TEM}, bla_{SHV}, bla_{CTX-M} and QNRB PCR products revealed presence of bla_{TEM1} (GenBank accession: JN193522), bla_{TEM116} (JN193523 and JN193524), bla_{SHV11}, bla_{CTX-M72} variants (JF523199) and QNRB1 (JN193526 and JN193527) in our samples.

Keywords *Klebsiella pneumoniae* · beta-Lactamase · Quinolone · Resistance · India

Introduction

Multi-drug resistant *Klebsiella pneumoniae* is one of the important nosocomial and community acquired opportunistic bacteria causing urinary tract infection, pneumonia, septicemia, soft tissue infection etc. [1]. Resistance against beta-lactam and quinolone group of drugs, widely used for their treatment is mainly acquired by intra or inter species exchange of transferable plasmid encoded antibiotic resistant genes (viz bla_{TEM}, bla_{SHV}, bla_{CTX-M}, QNR A, QNR B, and QNR S) [2]. Currently ≥150 TEM, ≥88 SHV, ≥69 CTX-M types variants has been described [3]. Six QNR A (QNR A1–QNR A6), 20 QNR B (QNR B1–QNR B20) and 3 QNR S variants (QNR S1–QNR S3) have been identified worldwide. Antibiotic sensitivity assays have revealed high frequencies of quinolone drug resistance in extended spectrum beta lactamase (ESBL) producing clinical isolates [4]. However, genetic evidence of this association is limiting and prevalence of these resistance genes in *K. pneumoniae* infecting Indian patients is not known [5–7].

This study demonstrated prevalence of both *bla* and *qnr* genes circulating in pathogenic *K. pneumoniae* of Indian origin. The occurrence of bla_{TEM} and QNR B genes was observed in 52 %, bla_{CTX-M} and QNR A genes in 37 % and bla_{SHV} in 42 % samples individually. We hereby report significant genotypic association between these two groups of resistance genes (*bla* and QNR) in our samples as evidenced by Chi-square analysis. Sequence analysis of some of the genes indicated presence of bla_{TEM1}, bla_{TEM116}, bla_{SHV11}, bla_{CTX-M72} variant and QNRB1 circulating in pathogenic *Klebsiella*. Identification of these variants

Anusri Tripathi and Sudip Kumar Dutta contributed equally to this study.

A. Tripathi (✉) · S. K. Dutta · L. Dhara · D. Banerjee
Department of Biochemistry and Medical Biotechnology,
Calcutta School of Tropical Medicine, 108 C.R. Avenue,
Kolkata 700073, India
e-mail: anusri.stm@gmail.com

M. Majumdar
Department of Microbiology, Calcutta School of Tropical
Medicine, Kolkata 700073, India

K. Roy
Department of Pharmacology, Calcutta School of Tropical
Medicine, Kolkata 700073, India

circulating in pathogenic bacteria might be helpful in proper therapeutic management of infected patients.

Materials and Methods

Bacterial Isolates

Klebsiella pneumoniae specimens ($n = 73$) were collected from clinical isolates of patients visiting Calcutta School of Tropical Medicine, Kolkata for 1 year (Table 1). Among the 73 isolates, 53 were isolated from urine, 10 from blood, 8 from throat swab and 2 from wound pus (data not shown). Forty-one of the patients with *Klebsiella* infection were female and the rest were male. Samples were processed for gram-stain and culture. Inoculation was done on nutrient agar, blood agar and Mac Conkey's agar media and incubated overnight at 37 °C. Identification of *K. pneumoniae* isolates was done by standard biochemical methods [8].

Susceptibility Assay for beta-Lactam and Quinolone Antibiotics

Antibiotic susceptibility of these *Klebsiella* isolates was determined according to Kirby–Bauer disc diffusion method and results were interpreted according to guidelines of Clinical and Laboratory Standard Institute (CLSI; former NCCLS, 1993) [9]. The following beta-lactam and quinolone antibiotics were used (disc: 6 mm: µg): ceftazidim (30), ceftazidim and clavulenic acid (30/10), cefotaxim (30), cefotaxim and clavulenic acid (30/10), cefpodoxim (10), nalidixic acid (30), ciprofloxacin (5), prulifloxacin (10) and levofloxacin (5) (HiMedia Lab Ltd., India). *K. pneumoniae* ATCC 700603 and *E. coli* ATCC 25922 were used as positive and negative controls respectively in each phenotypic ESBL test [10]. *K. pneumoniae* B1 was used as quinolone drug resistant positive control [11].

Preparation of Plasmid DNA

Plasmid DNA was extracted from the *K. pneumoniae* isolates by alkaline lysis method [12]. Briefly, 3 ml overnight culture of patient-sample isolated *K. pneumoniae* was lysozyme and RNase treated followed by alkaline lysis, phenol/chloroform extraction and isopropanol precipitation of the plasmid DNA. Integrity of the plasmids was checked by agarose gel electrophoresis.

PCR Amplification of bla_{TEM}, bla_{SHV}, bla_{CTX-M}, QnrA, QnrB and QnrS Genes

Amplification of bla_{TEM}, bla_{SHV}, bla_{CTX-M}, QNR A, QNR B and QNR S genes were performed in thermal cycler

(Applied Biosystems, USA) using primers previously mentioned [13, 14]. Briefly each reaction was carried out in 20 µl reaction volume using 1x PCR buffer (Fermentus, USA), 20 pmol of primers (Integrated DNA Technologies, USA), 1 mM of each dNTPs, 1 unit of Taq polymerase (Fermentus, USA) and 100 ng plasmid DNA. The MgCl₂ concentration varied between 1 and 2 mM. Thermocycling parameters were as follows: an initial denaturation of 94 °C for 60 s, 30 cycles of denaturation at 94 °C for 30 s, primer annealing between 50 and 60 °C for 30 s, and extension at 72 °C for 60 s. PCR products were separated on 1.2 % agarose gels and visualized under UV transilluminator (UVP, USA). Some randomly chosen PCR products of bla_{TEM} (TS, SP old, JBC6), bla_{SHV} (HB), bla_{CTX-M} (B06 and B271) and QNR B (K13 and HB) genes were purified with a QIAquick PCR purification kit (Qiagen, USA) and sequenced using automated DNA sequencer (ABI Prism 377; Applied Biosystems, USA). The nucleotide sequences and deduced protein sequences were analyzed by the programs BLAST (<http://www.ncbi.nlm.nih.gov/>) and Clustal W of the European Bioinformatics Institute (<http://www.cbi.ac.uk/>). The sequences were deposited in GenBank-Database. Chromatograms were visually inspected for double peaks as signs of presence of different variants of bla/QNR genes in the same PCR product.

Statistical Analysis

Chi-square analysis was performed to determine association between presence of bla/QNR genes with antibiotic resistance-pattern and also between presence of bla genes with that of QNR genes. Probability values of $P < 0.05$ was considered statistically significant.

Results

In order to get overviews of antibiotic resistance pattern of pathogenic *Klebsiella* infecting Indian patients and their genetic background, 73 *K. pneumoniae* isolates were obtained from Calcutta School of Tropical Medicine (CSTM), Kolkata, India (Table 1). Male: female ratio among the patients was 1:1.28. Fifty eight samples were collected from patients undergoing treatment in inpatient department, whereas 15 isolates were collected from patients visiting outpatient department of CSTM.

Antibiotic sensitivity of the samples was mentioned in Fig. 1. Approximately 97 % of them (71/73) showed resistance to at least one cephalosporin drugs. Frequency of resistance towards 3rd generation cephalosporin drugs were as follows: cefpodoxim (97 %), ceftazidime (89 %), ceftazidime and clavulenic acid (73 %), cefotaxime (85 %),

Table 1 Antibiotic sensitivity and resistant gene status of *K. pneumoniae* isolates

Patient's ID	blaTEM	blaSHIV	blaCTX-M	QNR A	QNR B	QNR S	ESBL/non ESBL	Quinolone resistance	Cephalosporin resistance
PP13	–	–	–	+	–	–	+	+	+
U195/Dy8	–	–	–	–	–	–	+	+	+
B39	–	–	–	+	–	–	+	+	+
B40	+	–	+	+	+	–	+	+	+
JG	+	–	–	–	–	–	+	+	+
SZ	+	+	–	+	+	–	–	+	+
IM	+	+	–	+	+	–	–	–	+
U492(AP-1)	+	–	–	–	+	–	–	+	+
PP12	+	+	–	+	+	–	+	+	+
Dy5	–	–	–	–	+	–	–	+	+
UCS(PP15)	+	+	+	+	+	–	+	+	+
Dy2	–	–	–	–	–	–	–	+	+
TS(JBC-6)	+	+	–	–	–	–	–	+	+
NBC-4	+	+	–	+	+	–	+	+	+
Hch-8	–	+	–	–	+	–	+	–	+
SZ2	–	–	–	–	+	–	–	–	+
AA	+	–	–	+	+	–	–	+	+
Dy-7	+	+	–	+	+	–	–	+	–
Dp-1	–	–	–	–	–	–	+	+	+
Dy-3	+	+	–	–	+	–	+	+	+
PP16	+	–	+	–	+	–	–	–	–
SP-O	+	+	–	+	–	–	+	–	–
Lu-5K	–	–	–	–	–	–	+	+	+
T-216	+	–	–	–	+	–	–	+	+
B271	+	–	+	+	–	–	+	+	+
Lc-8	–	–	–	–	–	–	+	+	+
AP-10	+	+	+	+	+	–	–	+	+
SD	+	+	–	+	+	–	+	+	+
JBC-12	+	+	+	+	+	–	+	+	+
B06	+	–	+	+	+	–	–	+	+
HAD-1	+	+	+	+	–	–	–	+	+
S.PATRA(NEW)	+	+	+	+	+	–	–	+	+
JBC-6	+	+	–	+	+	–	–	+	+
B-88	+	–	–	+	+	–	+	+	+
SC1	+	+	–	+	+	–	+	–	+
B86A	+	–	–	–	+	–	+	–	+
JNC-14	–	–	–	–	–	–	–	+	+
SP1	–	–	–	–	–	–	+	–	+
B06 (2)	–	–	+	–	+	–	+	+	+
LU-6	–	+	–	–	+	–	–	–	+
AP-9	–	–	–	–	–	–	+	+	+
SC2	+	+	–	–	+	–	+	–	+
SC3	+	–	–	–	+	–	+	+	+
PM	–	–	–	–	–	–	+	+	+
DY-5	–	–	+	–	–	–	–	+	+
JBC-6(NEW)	–	+	–	+	–	–	+	–	+
HAD-2	–	–	–	–	–	–	–	+	+
SM	–	–	–	+	+	–	+	+	+

Table 1 continued

Patient's ID	blaTEM	blaSHV	blaCTX-M	QNR A	QNR B	QNR S	ESBL/non ESBL	Quinolone resistance	Cephalosporin resistance
LU-10	–	–	–	–	–	–	–	+	+
K17	–	+	–	+	–	–	+	+	+
DY-4	–	+	+	–	+	–	–	+	+
JNC-7	–	+	+	+	+	–	–	+	+
Lu-5	–	–	–	–	+	–	+	+	+
Lcab-5	–	+	+	–	–	–	+	–	+
TS	+	+	+	–	–	–	+	–	+
DY-10	–	+	+	–	–	–	–	+	+
JNC-7	+	+	+	–	–	–	+	–	+
HAD-2	+	+	+	–	–	–	+	+	+
K9	–	–	+	–	–	–	+	+	+
B01	–	–	+	–	–	–	+	+	+
BA	+	–	+	–	–	–	+	+	+
AP-5	+	–	+	–	+	–	+	+	+
HAD-1	–	–	–	–	–	–	–	–	+
BO5	–	–	+	–	–	–	+	+	+
K13	+	+	+	+	+	–	+	+	+
HB	–	+	–	–	+	–	+	–	+
PP4	+	–	+	–	–	–	–	+	+
TM	–	–	–	–	–	–	–	+	+
LD	–	–	–	–	–	–	–	+	+
HAD-1	–	–	–	–	–	–	–	–	+
BH	+	+	+	–	+	–	–	+	+
DY-2	+	+	–	–	+	–	–	+	+
K-CABIN	+	–	–	–	+	–	–	+	+

+: Presence of the specific parameters, –: absence of the specific parameters

cefotaxime and clavulenic acid (80 %). Out of the 73 isolates, 43 (58.9 %) were ESBL as evidenced by their antibiotic sensitivity assay against 3rd generation cephalosporin alone and in combination with clavulenic acid. Resistance to at least one quinolone group of drugs was found in 76.71 % isolates (56/73). Resistance rate to quinolone drugs was as follows: nalidixic acid (85 %), ciprofloxacin (85 %), prulifloxacin (80 %) and levofloxacin (77.6 %). 75.34 % (55/73) of *K. pneumoniae* showed resistance to both beta-lactam and quinolone group of antibiotics (at least one in each group) whereas two samples (#Hch8 and PP16) showed susceptibility towards all drugs studied.

Genes *bla*_{TEM} were detected in 52 % (38/73), *bla*_{SHV} in 45 % (33/73) and *bla*_{CTX-M} in 37 % (27/73) of the *Klebsiella* isolates (Fig. 2, Table 1). Overall 74 % (54/73) samples harbored at least one *bla* genes studied. Ten samples, mostly of UTI origin (9/10), showed presence of all the three *bla* genes (# PP15, AP10, JBC12, HAD1, SP/new, TS, JNC7, HAD2, K13 and BH). Genes QNRA were detected in 37 % (27/73), QNRB in 56 % (41/73) of the

Klebsiella isolates. QNRS was not present in any of the samples. Both QNRA and QNRB genes were detected in 19 of them. All the three *bla* genes and two QNR genes were detected in five samples (viz. # PP15, AP10, JBC12, SP/new and K13).

The analysis of the nucleotide sequences of three *bla*_{TEM} PCR products revealed presence of two types of *bla*_{TEM} genes—*bla*_{TEM-1} and *bla*_{TEM-116}, in both the samples TS (GenBank accession: JN193523) and SP/old (GenBank accession: JN193524)—as evidenced by presence of double peaks in their discriminatory regions; whereas JBC6 (GenBank accession: JN193522) harbored only *bla*_{TEM1}. The sequence of *bla*_{CTX-M} PCR products of B06 and B271 (both these *K. pneumoniae* were isolated from blood of two unrelated patients suffering from septicemia—GenBank accession: JF523199) were similar to *bla*_{CTX-M72} (GenBank accession: AY847148.1)—except in three positions—at codon 167 (GGT → CGT), at codon 242 (GAC → GGC) and at codon 259 (one C deletion resulting in single frame shift and premature termination at codon 260)

Fig. 1 Percentage of β -lactam and quinolone drug sensitivity of *K. pneumoniae* isolates. Antibiotics in mg; cefotaxime (30), cefotaxime/clavulanic acid (30/10), ceftazidime (30), ceftazidime/clavulanic acid (30/10), and cefpodoxime (10), nalidixic acid (30), ciprofloxacin (5), prulifloxacin (10) and levofloxacin (5); *black bars* indicate % of *K. pneumoniae* resistant to the antibiotic, *gray bars* indicate % of *K. pneumoniae* sensitive to the antibiotic. *R* resistance to that antibiotic, *S* sensitive to that antibiotic

Fig. 2 Representative photograph of *bla*_{TEM} (a), *bla*_{SHV} (b), *bla*_{CTX-M} (c), QNRA (d) and QNRB (e) genes in pathogenic *K. pneumoniae* isolates

Fig. 3 Partial chromatogram of bla_{CTX-M} in B271 sample

Fig. 4 Location of mutations in CTX-M72 predicted protein structure. Amino acid substitutions are indicated in black. The region marked grey after codon 259 was absent. The diagram was drawn using UCSF CHIMERA (43)

Table 2 Chi-square analysis of presence of *bla* and *qnr* genes among *K. pneumoniae* isolates

<i>K. pneumoniae</i>	<i>Bla</i> gene ⁺	<i>Bla</i> gene [−]
<i>QNR</i> gene ⁺	43	6
<i>QNR</i> gene [−]	11	13

(Figs. 3 and 4). The sequences of QNRB PCR products [K13 (GenBank accession: JN193526) and HB (GenBank accession: JN193527)] revealed presence of QNRB1 among them.

Chi-square analyses among different genotypic and phenotypic characteristics revealed significant association between presence of *bla* genes to that of QNR genes in the *Klebsiella* samples studied (P value = 0.000125) (Table 2). No significant association was found among other characteristics of the *Klebsiella* isolates.

Discussion

Multidrug-resistant *K. pneumoniae* is the most common species of pathogenic *Enterobacteriaceae* in many parts of the world [17, 18]. During our one year study, most of the *K. pneumoniae* was isolated from patients with urinary tract infection at inpatient department. The prevalence of *Klebsiella* among hospitalized patients has been reported by other investigators from different countries including France, Turkey, Brazil and India [2, 3, 5, 19]. Similar to our study (58.9 % ESBL), reports from other parts of India and other Asian countries have demonstrated comparable prevalence of ESBL *K. pneumoniae* (40–83.3 and 60 %

respectively) whereas much lower prevalence is noted in western countries [5–7, 20]. Unlike our report of 76.71 % quinolone resistance, it has been reported in lower frequency (4.9–11.9 %) among *Klebsiella* isolates of other countries [16, 19, 21]. Comparatively higher percentage of antibiotic resistance in Indian patients indicated the importance of antibiotic resistance in Indian health care system. Hence correct identification of resistant genes circulating among pathogenic bacteria is required for proper surveillance of their transmission.

Few studies have been done in India regarding the occurrence of *bla* and QNR genes in pathogenic *K. pneumoniae* [5, 6]. In our samples, occurrence of *bla* genes was observed in the following order: *bla*_{TEM} > *bla*_{SHV} > *bla*_{CTX-M}. Similar to our report QNR genes were less prevalent than *bla* genes among pathogenic bacteria in other countries [4, 22, 23]. Significant association between the presence of *bla* and QNR genes (as observed in the study) is of serious concern since quinolone resistant ESBL bacteria has limited treatment options. Higher resistance against Ciprofloxacin in ESBL *Klebsiella* has been previously reported by Paterson et al. and Lautenbach et al. [17, 24].

The sequences of our *bla*_{TEM1} PCR products were identical to that of *bla*_{TEM1} (FJ668715.1) previously reported by Leinberger et al. in *K. pneumoniae* from Germany [15]. Our *bla*_{TEM116} sequences were identical to that isolated from *K. pneumoniae* and *K. oxytoca* in Australia (AY265882.1). Our *bla*_{SHV11} was identical to that isolated from *K. pneumoniae* in China by Zhou et al. (AF187732.1). The *bla*_{CTX-M} PCR products had novel sequence that closely matched with *bla*_{CTX-M-72} reported from China by Cheng et al. (AY847148.1), except at three positions—codons 167, 242 and 259 of *bla*_{CTX-M-72} [25]. Mutation at codon 167 resulted in Glycine → Arginine substitution in the constituent omega loop (161 to 179 codon), situated at the floor of catalytic active site of CTX-M enzyme [26]. Both codons 242 and 259 encode amino acids constituent of α/β domain of CTX-M protein. Mutation at codon 242 resulted in Aspartic acid → Glycine substitution at the wall of its catalytic active site. The C deletion at codon 259 resulted in single frame shift mutation (CCG → CGC) of Proline → Arginine at this codon, followed by a premature termination (TGA) at codon 260. Such premature truncation of antibiotic resistant genes was previously reported in case of AmpD beta lactamase [27]. However, more resistant genes need to be genotyped in order to identify the strain variants circulating in our patient population. This study highlights the potential risk of spread of multidrug resistant bacteria among Indian patients—thus demanding their pre-screening before treatment and development of new drugs for effective treatment of these resistant pathogenic bacteria.

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