Molecular basis of quinolone resistance in clinical isolates of *Klebsiella pneumoniae* from Pakistan

Roman Farooq Alvi[1], Bilal Aslam[1], Naveed Shahzad[2], Muhammad Hidayat Rasool[1] and Muhammad Shafique[1]

[1]Department of Microbiology, Government College University, Faisalabad, Pakistan
[2]School of Biological Sciences, University of the Punjab, Lahore, Pakistan

Abstract: Antibiotic resistant *Klebsiella pneumoniae,* is associated with various nosocomial infections that are difficult to treat. This study is designed to find out the patterns of resistance against commonly used antibiotics in *K. pneumoniae* clinical isolates with special attention to fluoroquinolones. A total number of 200 *K. pneumoniae* clinical isolates collected from various tertiary care hospitals of Punjab, Pakistan for a span of 1 year were investigated. Isolates were identified biochemically and genetically using VITEK® system and species-specific PCR, respectively. Antibiogram of isolates was studied by using disc diffusion and broth micro-dilution assays. Highest infection of *K. pneumoniae* detected in urinary tract (43%) followed by respiratory tract (25.5%). Most of the isolates displayed strong resistance against ampicillin, cefotetan, tazobactam, cefuroxime, cefixime, ceftriaxone, ampicillin-sulbactam imipenem, meropenem, ciprofloxacin and moxifloxacin, while sensetive to cefotaxime. Chromosoaml mutation was deteted in *gyr*A gene, *gyr*A harbors a strong mutation which provides resistance against ciprofloxacin by substituting Ser83→Ile. However, no mutation was detected in *gyr*B gene. Moreover, *qnr*B1 plasmid born resistant gene was only detected among *qnr*A, *qnr*B and *qnr*S. The story depicts an alarming situation of antibiotic resistance among *K. pneumoniae* associated with various nosocomial infections.

Keywords: *Klebsiella pneumoniae*, antibiotic resistance, fluoroquinolone resistance, *gyr*A, *qnr*B1.

INTRODUCTION

Klebsiella pneumoniae, member of Enterobacteriaceae family is noxious pathogen also a natural dweller of human and animal gastrointestinal tract. It is an opportunistic pathogen associated with nosocomial infections ranging from urinary tract infection, pneumoniae, cystitis, septicemia, wounds and biliary tract infection to meningitis (Podschun *et al*., 1998; Struve and Krogfelt, 2004; Boll *et al*., 2012). This ubiquitous bacterium accounts for one third of all infections caused by Gram negative pathogens (Navon-Venezia *et al*., 2017).

Various antimicrobial agents tried against this pathogen is of less use due to numerous contributing factors including misuse of antibiotics, over-dosage, low-dose regime and substandard infection control measures that results into antimicrobial resistance (AMR) in developing countries (Shallcross and Davies, 2014). A decade long data from European Antimicrobial Resistance Surveillance Network, (http://atlas.ecdc.europa.eu/public/index.aspx? Instance = General Atlas) revealed that *K. pneumoniae* is resistant to four major antibiotic groups including aminoglycosides, 3rd generation cephalosporins, carbapenems and fluoroquinolones (Navon-Venezia *et al*., 2017).

Fluoroquinolones is a class of antimicrobial drugs, frequently used in antibiotic therapy against Gram-positive and Gram-negative bacteria, which specifically targets DNA gyrase. DNA gyrase also known as type II topoisomerase, a tetrameric protein comprised of subunit A and B, plays a significant role in DNA replication and transcription (Drlica and Zhao, 1997). Nowadays, Gram-negative bacteria developed resistance against fluoroquinolone by two known mechanisms of resistance namely chromosomal mutation in the *gyr*A and *gyr*B genes, and transferable plasmid mediated mechanism of resistance. Mutations in *gyr*A and *gyr*B genes are designated as QRDR (quinolone resistance-determining region) (Huang *et al*., 2015). The second mechanism is plasmid mediated resistance "*qnr*", first time reported in 1998 (Martinez-Martinez *et al*., 1998). The Qnr is a pentapeptide repeat protein which provides resistance against fluoroquinolone (Robicsek *et al*., 2006). At present there are three different types of *qnr* genes worldwide (Jacoby *et al*., 2008).

Keeping in view the burden of antibiotic resistance present study is designed to decipher different mechanism of fluoroquinolone resistance present in *Klebsiella pneumoniae* clinical isolates of Pakistan.

MATERIALS AND METHODS

Demographic and sample collection

A total number of 200 *Klebsiella pneumoniae* were isolated form patients hospitalized at various tertiary care

**Corresponding author:* e-mail: drbilalaslam@gcuf.edu.pk

hospitals of Punjab, Pakistan from June 2015 to May 2016. Samples were collected in sterile containers and transport swabs and transported to the Laboratory under aseptic conditions and stored at 4°C.

Isolation and biochemical identification of klebsiella pneumoniae

Samples were cultured on MacConkey and blood agar (Oxoid, UK). Initially samples were identified by using API® 20E kit (bioMérieux, France). For complete biochemical profiling and identification, the isolated bacteria were subjected to VITEK® microbial identification system (bioMérieux, France) by using GN ID Cards.

Molecular identification of K. pneumoniae

Biochemically characterized *K. pneumoniae* were cultured in nutrient broth (Oxoid, UK) and genomic DNA was extracted by using alkaline lysis method (Due *et al.*, 2014). Purity of DNA was monitored spectrophotometrically (λ=260nm). Purified DNA was subjected to PCR by using species specific primers (table 1). Amplified products were analyzed by ethidium bromide (Sigma-Aldrich®, USA) stained 2% agarose gel (Sigma-Aldrich®, USA).

Antibiotic susceptibility testing

Antibiogram of isolates were characterized by using Kirby-Bauer disc diffusion assay. Briefly, isolates were cultured on Mueller-Hinton Agar (Oxoid, UK). Antibiotics discs (Oxoid, UK) of following antibiotics; Cefotetan, Amikacin, Tazobactam, Imipenem, Meropenem, Cefuroxime, Cefixime, Ceftriaxone, Cefepime, Ciprofloxacin, Moxifloxacin, Sulfamethoxazole, Tigecycline, Aztreonam, Ampicillin, Ampicillin-Sulbactam, Chloramphenicol, Cefotaxime, Tetracycline were placed on agar plates. The results were interpreted as per guidelines of Clinical and Laboratory Standards Institute (CLSI). Moreover, *K. pneumoniae* isolates were classified into Multiple Drug Resistant (MDR), Extreme Drug Resistant (XDR) and Pandrug Resistant (PDR) as classified previously.

Ciprofloxacin susceptibility assay

Epsilometer test (Etest, bioMérieux, France) was performed for the susceptibility testing of ciprofloxacin and results were calculated according to the United States-Food and Drug Authority (US FDA). Micro-broth dilution assay was performed for the measurement of Minimum Inhibitory Concentration (MIC) of ciprofloxacin. Briefly, 0.5 McFarland of *K. pneumoniae* isolates were prepared and inoculated in 96 well plates containing various concentration of ciprofloxacin ranging from 0-1024 µg/ml. Bacterial growth was monitored spectrophotometrically (λ=600nm) and growth on nutrient agar (Oxoid, UK). Finally, results were recorded according to the recommendations of Clinical and Laboratory Standards Institute (CLSI).

Molecular characterization of gyrA and gyrB genes

Ciprofloxacin resistant *K. pneumoniae* clinical isolates were monitored for the presence of chromosomal QRDR (quinolone resistance determining region) responsible for Ciprofloxacin resistance. Briefly, genomic DNA of isolates was extracted, and *gyr*A and *gyr*B genes were amplified by using specific primers (table 1). The amplified products were analyzed on 1% ethidium bromide stained agarose gel. The amplicons were purified by using PCR purification Kit (Qiagen, Germany) and sequenced (Macrogen, Korea). Obtained sequences were analyzed by using MEGA7 (Molecular Evolutionary Genetics Analysis version 7.0) software package and online available BLAST NCBI program (Basic Local Alignment Search Tool, National Center for Biotechnology Information). The *gyr*A (GenBank accession number DQ673325) of *Klebsiella pneumoniae* strain ATCC 13883 was used as standard sequence for all performed analyses of gyrase A gene. Finally, sequences were submitted to GenBank NCBI for allotment of accession numbers.

Molecular characterization of qnrA, qnrB and qnrS Genes

Ciprofloxacin resistant isolates were analyzed for the existence of PMQR (plasmid-mediated quinolone resistances) responsible for ciprofloxacin resistance. Briefly, extracted DNA of isolates was subjected to PCR for the amplification of *qnr*A, *qnr*B and *qnr*S genes by using specific primers (table 1). The amplicons were visualized on 1% ethidium bromide stained agarose gel. The purified amplicons were dispatched to Macrogen, Korea for sequencing. Analysis of obtained sequences were accomplished by using MEGA7.0 software and online available BLAST NCBI program. The *qnr*A, *qnr*B and *qnr*S genes variant analysis was executed as per the guidelines of "*qnr* Numbering and Sequence" online available on Lahey clinic website (http://www.lahey.org/qnrstudies) (Jacoby *et al*., 2008). Finally, aligned sequences were submitted to GenBank NCBI for assigning of accession numbers.

RESULTS

Characterization and clinical distribution of K. pneumoniae

Clinical isolates were cultured on selective media and differential media (MacConkey and blood agar), followed by biochemical tests for the identification of *Klebsiella pneumoniae*. All the isolates hydrolyzed the urea and fermented the sucrose and lactose. Moreover, isolates were indole, Methyl red (MR), H_2S gas production negative while positive to Voges–Proskauer (VP) test. According to GNR ID cards isolates identified as *Klebsiella pneumoniae*, were subjected to molecular identification by using species specific primer (table 1). These primers amplified 126 bp product of 16S rDNA gene (fig. 1).

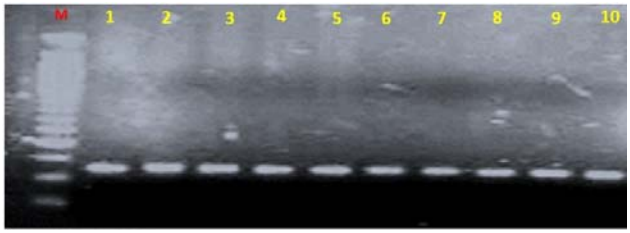

Fig. 1: PCR assay for identification of 16S rDNA K. pneumoniae. M: DNA size ladder 50bp, 1; Reference strain for 16s rDNA *K. pneumoniae* (126bp); 2-10; Positive samples for *K. pneumoniae*.

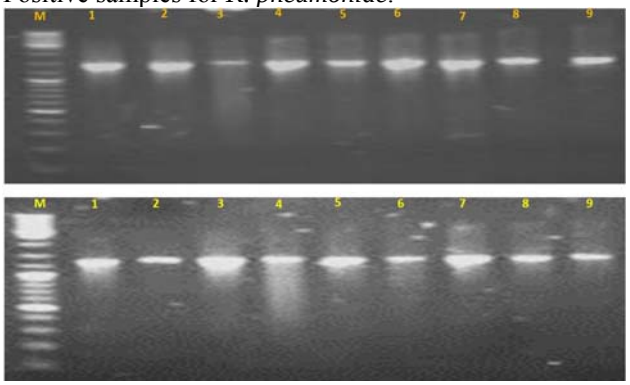

Fig. 2: Amplification of *gyr*A and *gyr*B genes: Single colony PCR amplification of 1270bp *gyr*A gene (top gel image) and 1300bp of *gyr*B gene (bottom gel image) from the clinical isolates of *Klebsiella pneumoniae* in 1.5% agarose gel.

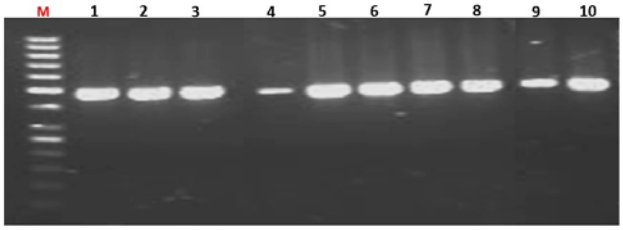

Fig. 3: Amplification of *qnr*B gene: DNA ladder Mix (M); 1-10 Clinical isolates of K. pneumoniae of 476 bp in 1.5% agarose gel.

Among 200 biochemically and genetically identified *K. pneumoniae* selected from both male and female patients of age ranging from 10-65 years with a median range of 35.5±2 years. The distribution of *K. pneumoniae* in clinical samples include urine (n=86, 43%), tracheal aspirates (n=51, 25.5%), purulent wounds (n=33, 16.5%) and blood (n=30, 15%). Out of 86 patients suffering from urinary tract infections (UTI), 24 (27.9%) were males while 62 (72.1%) were females.

Antibiotic suseptibility profiling

Kirby-Bauer agar diffusion assay was employed to study the antibiotic resistance profile of isoltaes. All isolates were resistance to ampicillin and sensetive to cefotaxime. More than 90% isolates were resistant to cefotetan, tazobactam, cefuroxime, cefixime, ceftriaxone and ampicillin-sulbactam. Furthermore, 89.5% isolates were resistant to imipenem, meropenem, ciprofloxacin and moxifloxacin. However, 81% isolates were sensitive to tigecycline, while 18% displayed intermediate resistance to tigecycline (table 2).

Fuloriqunolone resistance

Isolates resistant to fluoriqunolones were further studied and level of resistance was measured by broth micro-broth dilution assays. In general, all isolates showed that they may resist 16µg/ml of ciprofloxacin. However, 25% isolates had MIC value of 256µg/ml, while 16.64% isolates had MIC value more than 512 µg/ml. It displayed all isolates can strongly resist ciprofloxacin (table 3).

Genetic screening of fluoroquinolone resistance genes

PCR screening was carried out to identify possible genes that contributed to the resistance of each phenotype. Isolates resitant to ciprofloxacin were characterised at molecular level for the detection of chromosomal mutations in *gyr*A and *gyr*B genes. By using *gyr*A and *gyr*B Specific primers (table 1). Respective genes were amplified and product size ranging from 1200-1500bp was obtained (fig. 2). For the detection of plasmid borne *qnr* mediated resistance specific primers (table 1) were used to detect *qnr*A, *qnr*B and *qnr*S genes. Only *qnr*B gene was present in all isolates while *qnr*A and *qnr*S were not detected (fig. 3).

Sequence analysis and mutation detection

The amplified products of *gyr*A, *gyr*B and *qnr*B were sequenced and analyzed sequences were submitted to GenBank, NCBI with GenBank accession number MF953599 (for *gyr*A) and MF953600 (*qnr*B1). The *gyr*A harbors a strong mutation which provides resistance against ciprofloxacin. The Ser83→Ile substitution was observed in all ciprofloxacin (member of class fluoroquinolones) resistant *Klebsiella pneumoniae* clinical isolates, the 83-amino acid is isoleucine (ATC) instead of serine (TCC). All ciprofloxacin resistant *Klebsiella pneumoniae* clinical isolates also harbored the *qnr*B gene as PMQR (plasmid-mediated quinolone resistances). According to *qnr*B variant analysis, the isolated pathogens contained *qnr*B1 gene.

DISCUSSION

K. pneumoniae is an emerging MDR pathogen which is responsible for various infections and become a serious health concern for the healthcare systems of the developing countries like Pakistan (Ejaz *et al*., 2017). Emerging of MDR, XDR and PDR bacterial strains further worsen the situation in developing countries as these bacteria are responsible for increase morbidity and mortality rate in the clinical settings. Studies from Pakistan have been reported in the past about the emergence of MDR *K. pneumoniae* (Khan *et al*., 2010; Sattar *et al*., 2014), however genetic basis of antibiotic

Table 1: Prime list used for gene amplification

Sr. No.	Gene	Primer Sequence	Reference
1	16S rDNA	AGCACAGAGAGCTTGACTTTGGTCTTGCGAC	[20]
2	*gyr*A	GCGATGTCGGTCATTGTTGGCCGAACTGGTCACGGATCAG	[21]
3	*gyr*B	CTCCGTCTCCGTACAGGATGACTGTGATAGCGCAGTTTTATCC	[21]
4	*qnr*A	ATTTCTCACGCCAGGATTTGGATCGGCAAAGGTTAGGTCA	[22]
5	*qnr*B	GATCGTGAAAGCCAGAAAGGACGATGCCTGGTAGTTGTCC	[22]
6	*qnr*S	ACGACATTCGTCAACTGCAATAAATTGGCACCCTGTAGGC	[22]

Table 2: Resistance pattern of Klebsiella pneumoniae isolates (n=200) to various antimicrobial agents

Antibiotics	Sensitive	Intermediate	Resistant
	n (%)	n (%)	n (%)
Cefotetan	11 (5.5)	0 (0)	189 (94.5)
Amikacin	45 (22.5)	0 (0)	155 (77.5)
Tazobactam	3 (1.5)	0 (0)	197 (98.5)
Imipenem	10 (5)	11 (5.5)	179 (89.5)
Meropenem	10 (5)	11 (5.5)	179 (89.5)
Cefuroxime	3 (1.5)	0 (0)	197 (98.5)
Cefixime	3 (1.5)	0 (0)	197 (98.5)
Ceftriaxone	3 (1.5)	0 (0)	197 (98.5)
Cefepime	18 (9)	5 (5)	172 (86)
Ciprofloxacin	21 (10.5)	0 (0)	179 (89.5)
Moxifloxacin	21 (10.5)	0 (0)	179 (89.5)
Sulfamethoxazole	58 (29)	0 (0)	142 (71)
Tigecycline	162 (81)	36 (18)	2 (1)
Aztreonam	9 (4.5)	30 (15)	161 (80.5)
Ampicillin	0 (0)	0 (0)	200 (100)
Ampicillin-Sulbactam	3 (1.5)	0 (0)	197 (98.5)
Chloramphenicol	46 (23)	33 (16.5)	121 (60.5)
Cefotaxime	200 (100)	0 (0)	0 (0)
Tetracycline	43 (21.5)	9 (4.5)	148 (74)

Table 3: Distribution of MIC of Klebsiella pneumoniae isolates (n=179) to Ciprofloxacin

MIC (µg/ml)	N	%
8	15	8.38
16	8	4.27
32	15	8.38
64	30	16.76
128	37	20.67
256	45	25.14
512	15	8.38
1024	14	7.82

resistance of *K. pneumoniae* still need to be explored in Pakistan. From Pakistan it is the first study which reports the underline molecular determinants of quinolone resistance in the clinical isolates of *Klebsiella pneumoniae*.

All the samples used in the study were from clinical settings and highest isolation rate was observed from urine sample (n=86, 43%) followed by other sample sources, which signifying the true infection, it is significant because most of these samples were collected from the clinical patients, which probably driving these patients to the risk of *K. pneumoniae* associated infections.

The prevalence of MDR *Klebsiella pneumoniae* insists the past reports published from various parts of the country (Khan *et al*., 2010; Sattar *et al*., 2014) and in

addition to that in the present study we have added significant data regarding the prevalence of quinolone resistant *Klebsiella pneumoniae* in the clinical settings of Pakistan.

All isolates were found to resist 16µg/ml of ciprofloxacin. But, about 25% samples showed MIC of 256 µg/ml, whereas MIC of 16.64% was recorded at 512 µg/ml. taking together, findings of the current study showed the resistance to ciprofloxacin. About 85 % isolates were sensitive to tigecycline so in the current study none of the isolates was considered as Pan-drug resistance (PDR), therefore results suggested that the diversity in *Klebsiella pneumoniae* from different regions of Pakistan is possibly due to inconsistencies in the therapeutic regime against *Klebsiella pneumoniae* infections. Findings of the current study were partly alike with a study conducted in Iran (Rezazadeh *et al*., 2016), they found significant quinolone resistance among in enterobacteriacea. In their study 67.5% and 56% of isolates were found intermediate resistant to nalidixic acid and ciprofloxacin. Our findings were also moderately similar to the results of (Firoozeh *et al*., 2014) published from Iran and they found that 82.5% and 45% of urinary E. coli isolates showed resistance to ciprofloxacin. In China, the distribution of ciprofloxacin resistance among various members of enterobacteriacea particularly in E. coli was recorded about 59.4% (Shao *et al*., 2003). It appears that needless and extensive prescription of antibiotics are the most significant influencing factor that might ultimately steer the resistance in bacteria. Furthermore, the incidence rate of resistance estimated in the present study highlights the necessity for a national antibiotic resistance scrutiny system in hospital settings.

In the present study all the isolates were subjected to PCR screening and were characterized for the presence of chromosomal mutations in *gyr*A and *gyr*B genes, whereas PMQR (*qnr*A, *qnr*B, *qnr*S) were also studied. Among these plasmid mediated resistance all the isolates were positive for only *qnr*B whereas *qnr*A and *qnr*S were absent in these isolates. In contrast to the findings of the current study (Rezazadeh *et al*., 2016) which studied the enterobacteriacea and they reported a very low prevalence rate of 2.9% of qnrS1 in *E. coli* isolates they have not found qnrA and qnrB determinants. Singly, PMQR elements may not be able to cause resistance against quinolones but they may supplement various other mechanisms of quinolone resistance. PMQR are under extensive investigations around the world a recent study in China showed that aac-(6/)-Ib-cr are the dominant PMQR determinants in the clinical isolates of Enterobacteriaceae (Jiang *et al*., 2008). Present study showed similar results with this study, althugh they found the *qnr*S and *qnr*A as well, but they have also detected the *qnr*B (Hu *et al*., 2014). Substitution of Ser83 to Phe, Tyr, Ile and Leu confers higher levels of resistance against ciprofloxacin (Fu *et al*., 2008). It clearly represents like previous reports replacement of Ser83 by Ile is chromosomal QRDR (quinolone resistance determining region) (Fu *et al*., 2008). Moreover, various silent mutations at DNA levels were also observed, however these mutations did not alter the amino acid sequence (at positions 184, 233 and 242) (Weigel *et al*., 1998). The silent mutations are: 184 amino acid encoded as proline with codon CCG instead of CCA, 233 amino acid is encoded as tyrosine with codon TAC instead of TAT and 242 amino acid is encoded as isoleucine with codon ATT instead of ATC. However, DNA and amino acid sequence analyses of *gyr*B gene did not reveal presence of any mutation from 86 amino acid to 344 amino acid (Lindgren *et al*., 2003).Most abundant mutation detected from China (Fu *et al*., 2008) and Singapore (Schneiders *et al*., 2003) is Ser83→Leu substitution. However, in European continent Ser83→Tyr substitution of *gyr*A gene is most prevalent (Brisse *et al*., 1999; Gruteke *et al*., 2003; Martinez-Martinez *et al*., 2002). In contrast to well established studies, at present Ser83→Ile substitution was detected from *K. pneumoniae*.

CONCLUSION

As per our knowledge it is first comprehensive study designed in Pakistan to delineate the mechanism of fluoroquinolone resistance in *K. pneumoniae*. MDR *Klebsiella pneumoniae* a public health threat acquires resistance against not only the quinolones but various other effective antibiotics with very operative molecular mechanisms, so the findings of the current study suggest that strict control procedures must be initiated to curtail the potential threats of these emerging MDR pathogens across the country. Effective infection management guidelines should be implemented at national level to prevent the diseases associated with these MDR strains.

REFERENCES

Boll EJ, Nielsen LN, Krogfelt KA and Struve S (2012). Novel screening assay for *in vivo* selection of *Klebsiella pneumoniae* genes promoting gastrointestinal colonisation. *BMC Microbiol*., **12**: 201.

Brisse S, Milatovic D, Fluit AC, Verhoef J, Martin N, Scheuring S, Köhrer K and Schmitz FJ (1999). Comparative *in vitro* activities of ciprofloxacin, clinafloxacin, gatifloxacin, levofloxacin, moxifloxacin, and trovafloxacin against *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Enterobacter cloacae* and *Enterobacter aerogenes* clinical isolates with alterations in GyrA and ParC proteins. *Antimicrob Agents Chemother*., **43**(8): 2051-5.

Drlica K and Zhao X (1997). DNA gyrase, topoisomerase IV, and the 4-quinolones. *Microbiol. Mol. Biol. Rev*., **61**(3): 377-392.

Du J, Li P, Liu H, Lü D, Liang H and Dou Y (2014). Phenotypic and molecular characterization of multidrug resistant *Klebsiella pneumoniae* isolated from a University Teaching Hospital, China. *PloS One.,* **9**(4): 95181-95187.

Ejaz H, Wang N, Wilksch JJ, Page AJ, Cao H, Gujaran S, Keane JA, Lithgow T, Ul-Haq I, Dougan G, Strugnell RA and Heinz E (2017). Phylogenetic Analysis of *Klebsiella pneumoniae* from Hospitalized Children, Pakistan. *Emerg. Infect Dis*., **23**(11): 1872-1875.

Fu Y, Guo L, Xu Y, Zhang W, Gu J, Xu J, Chen X, Zhao Y, Ma J, Liu X and Zhang F (2008). Alteration of *gyrA* amino acid required for ciprofloxacin resistance in *Klebsiella pneumoniae* isolates in China. *Antimicrob. Agents Chemother*., **52**(8): 2980-3.

Gruteke P, Goessens W, Van Gils J, Peerbooms P, Lemmens-Den Toom N, Van Santen-Verheuvel M, Van Belkum A and Verbrugh H (2003). Patterns of resistance associated with integrons, the extended-spectrum beta-lactamase SHV-5 gene and a multidrug efflux pump of *Klebsiella pneumoniae* causing a nosocomial outbreak. *J. Clin. Microbiol*., **41**(3): 1161-1166.

Hou XH, Song XY, Ma XB, Zhang SY and Zhang JQ (2015). Molecular characterization of multidrug-resistant *Klebsiella pneumoniae* isolates. *Braz. J. Microbiol*., **46**(3): 759-768.

Hu L, Zhong Q, Shang Y, Wang H, Ning C, Li Y, Hang Y, Xiong J, Wang X, Xu Y, Qin Z, Parsons C, Wang L and Yu F (2014). The prevalence of carbapenemase genes and plasmid-mediated quinolone resistance determinants in carbapenem-resistant *Enterobacteriaceae* from five teaching hospitals in central China. *Epidemiol Infect*., **142**(9): 1972-7.

Huang Y, Ogutu JO, Gu J, Ding F, You Y, Huo Y, Zhao H, Li W, Zhang Z, Zhang W, Chen X, Fu Y and Zhang F (2015). Comparative analysis of quinolone resistance in clinical isolates of *Klebsiella pneumoniae* and Escherichia coli from Chinese children and adults. *Biomed. Res. Int*., 168292.

Jacoby G, Cattoir V, Hooper D, Martínez-Martínez L, Nordmann P, Pascual A, Poirel L and Wang M (2008). *qnr* Gene nomenclature. *Antimicrob Agents Chemother*., **52**(7): 2297-9.

Khan E, Schneiders T, Zafar A, Aziz E, Parekh A and Hasan R (2010). Emergence of CTX-M Group 1-ESBL producing *Klebsiella pneumonia* from a tertiary care centre in Karachi, Pakistan. *J. Infect Dev. Ctries*., **4**(8): 472-6.

Kurupati P, Chow C, Kumarasinghe G and Poh CL (2004). Rapid detection of *Klebsiella pneumoniae* from blood culture bottles by real-time PCR. *J. Clin. Microbiol*., **42**(3): 1337-1340.

Lindgren K, Karlsson PA and Hughes D (2003). Mutation rate and evolution of fluoroquinolone resistance in *Escherichia coli* isolates from patients with urinary tract infections. *Antimicrob Agents Chemother*., **47**(10): 3222-3232.

Mak JK, Kim MJ, Pham J, Tapsall J and White PA (2009). Antibiotic resistance determinants in nosocomial strains of multidrug-resistant *Acinetobacter baumannii*. *J. Antimicrob. Chemother*., **63**(1): 47-54.

Martinez-Martinez L, Pascual A and Jacoby GA (1998). Quinolone resistance from a transferable plasmid. *Lancet*., **351**(9105): 797-799.

Martínez-Martínez L, Pascual A, Conejo Mdel C, García I, Joyanes P, Doménech-Sánchez A and Benedí VJ (2002). Energy-dependent accumulation of norfloxacin and porin expression in clinical isolates of *Klebsiella pneumoniae* and relationship to extended-spectrum beta-lactamase production. *Antimicrob Agents Chemother*., **46**(12): 3926-32.

Navon-Venezia S, Kondratyeva K and Carattoli A (2017). *Klebsiella pneumoniae*: a major worldwide source and shuttle for antibiotic resistance. *FEMS Microbiol. Rev*., **41**(3): 252-275.

Podschun R and Ullmann U (1998). *Klebsiella* spp. as nosocomial pathogens: Epidemiology, taxonomy, typing methods, and pathogenicity factors. *Clin Microbiol Rev*., **11**(4): 589-603.

Robicsek A, Jacoby GA and Hooper DC (2006). The worldwide emergence of plasmid-mediated quinolone resistance. *Lancet Infect Dis*., **6**(10): 629-40.

Sambrook J and Russell DW (2001). Molecular cloning: a laboratory manual. Third ed.: Cold Pring Harbor Laboratory Press, New York, USA.

Sattar H, Toleman M, Nahid F and Zahra R (2016). Co-existence of *blaNDM-1* and *blaKPC-2* in clinical isolates of *Klebsiella pneumoniae* from Pakistan. *J Chemother*., **28**(4): 346-349.

Schneiders T, Amyes SG and Levy SB (2003). Role of *AcrR* and *ramA* in fluoroquinolone resistance in clinical *Klebsiella pneumoniae* isolates from Singapore. *Antimicrob Agents Chemother*., **47**(9): 2831-2837.

Shallcross LJ and Davies SC (2014). The World Health Assembly resolution on antimicrobial resistance. *J. Antimicrob. Chemother*., **69**(11): 2883-2885.

Struve C and Krogfelt KA (2004). Pathogenic potential of environmental *Klebsiella pneumoniae* isolates. *Environ. Microbiol*., **6**(6): 584-90.

Weigel LM, Steward CD and Tenover FC (1998). *gyrA* mutations associated with fluoroquinolone resistance in eight species of *Enterobacteriaceae*. *Antimicrob Agents Chemother.*, **42**(10): 2661-7.