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Prevalence of Extended Spectrum Beta Lactamase Genotypes in *Klebsiella pneumoniae* from Respiratory Tract Infections at Tertiary Care Hospital

Shivali V. Gajul[1], Shivajirao T. Mohite[2], Kailash D. Datkhile[3], Satish V. Kakade[4], Smita S. Mangalagi[1], Sanjay M. Wavare[1]*

[1]Department of Microbiology, Shri B. M. Patil Medical College, BLDE (Deemed to be University), Vijayapura- 586103 (Karnataka) India, [2]Department of Microbiology, [3]Department of Molecular Biology and Genetics, [4]Department of Community Medicine, Krishna Institute of Medical Sciences, Krishna Institute of Medical Sciences "Deemed to be University", Karad-415110 (Maharashtra) India

Abstract:

Background: Extended Spectrum Beta Lactamases (ESBLs) are rapidly evolving group of β-lactamase enzymes that are of particular concern to clinicians and epidemiologists. Most ESBLs have been evolved by genetic mutation from *bla*TEM and *bla*SHV genes, and are well described in *Klebsiella pneumoniae. Aim and Objective:* To investigate the ESBL genotypes in *K. pneumoniae* isolates from Respiratory Tract Infections (RTIs). *Material and Methods:* Clinical isolates of *K. pneumoniae* were obtained from RTI -sputum samples. Antimicrobial susceptibility was determined by Kirby-Bauer disc diffusion method**.** ESBL was detected phenotypically and multiplex Polymerase Chain Reaction (PCR) specific for *bla*TEM, *bla*SHV and *bla*CTX-M genes was performed to identify genotypes. *Results:* During the 19 months period, a total of 212 of *K. pneumoniae* were found from RTIs. Of these 212 isolates, 60 isolates (28.3%) were ESBL producers by phenotypic method. Of these 212 isolates, 96 were randomly selected for multiplex PCR for *bla*TEM, *bla*SHV and *bla*CTX-M genes. The findings of multiplex PCR showed that 24 isolates (25%) possessed *bla*TEM gene and only 4 isolates (4.1%) possessed each *bla*SHV and *bla*CTX-M gene alone. Isolates having both *bla*TEM+*bla*SHV genes were 20 (20.8%), and both *bla*TEM+*bla*CTX-M genes were 12 (12.5%); and isolate possessing all three *bla*TEM+*bla*SHV+*bla*CTX-M genes were 20 (20.8%). The overall prevalence of *bla*TEM, *bla*SHV and *bla*CTX-M genes in this study was 79.1%, 45.8% and 37.5% respectively. Imipenem was most effective antibiotic. *Conclusion:* Spread of ESBL producing *K. pneumoniae* is a major concern, as it causes limitations to optimal treatment. Multiplex PCR can be used as a rapid method to identify ESBL genotypes in *K. pneumoniae.* It will prove valuable for surveillance and establishing the treatment line against drug resistant organisms, thus saving precious time and resources. In our study *bla*TEM genotype was most prevalent.

Keywords: *bla*TEM, *bla*SHV, *bla*CTX-M

Introduction:

Respiratory Tract Infection (RTI) is a growing threat to Public health and a leading cause of morbidity and mortality worldwide especially in developing countries [1-2]. *K. pneumoniae* has been familiar as a community-acquired pulmonary pathogen since it was discovered more than 100 years ago. Despite gaining popularity as an emerging pathogen in the community, the vast majority of *K. pneumoniae* infections are nosocomial and it is reported to be amongst the ten most common nosocomial pathogens [3].

K. pneumoniae is a well-known 'collector' of Multiple Drug Resistance (MDR) plasmids. MDR

bacteria are rapidly emerging across the world. They cause severe nosocomial and community acquired infections that are difficult to eradicate using available antibiotics. In recent days, *K. pneumoniae* shows greater resistance toward newer generation cephalosporins and other broad-spectrum antibiotics. Moreover widespread and non-specific use of broad-spectrum antibiotics in hospitalized patients has led to both increased carriage of *K. pneumoniae* and development of MDR isolates that produce Extended Spectrum Beta Lactamase (ESBL). The first ESBL was described in *K. pneumoniae* and *Serratia marcescens* in 1983 in Europe [4]. Most ESBLs are generally mutants of classical *bla*TEM and *bla*SHV genes [5]. ESBL producing *K. pneumoniae* have spread worldwide rapidly. Presently, it is the most common ESBL producing organism that is difficult to eradicate from high risk wards such as intensive care units. ESBLs have spread threateningly in many regions of the world and at present comprise over three hundred deviations (http://www.lahey.org/studies). ESBLs can be classified into three main genotypes, designated as *bla*TEM, *bla*SHV and *bla*CTX-M. All these three types are commonly found in *K. pneumoniae*.

However, information on these molecular types in *K. pneumoniae* is lacking from India. Thus, there is a need for geographical surveillance of ESBL production to guide appropriate therapy. Hence, we undertook this study to look for the *bla*TEM, *bla*SHV and *bla*CTX-M genes in *K. pneumoniae* isolated from the patients of RTIs admitted to a tertiary care hospital in North Karnataka. This study has helped to know antibacterial resistance pattern with respect to third generation cephalosporins among the ESBL producing isolates. This information would be helpful in establishing empiric therapy guidelines to prevent emergence of further resistance and to contribute data to larger and more extensive surveillance programs.

Material and Methods:

Bacterial Isolates:

The present prospective cross-sectional study was carried out from 3rd February 2017 to 30th August 2018 in the Department of Microbiology of BLDEU's Shri B. M. Patil Medical College, Hospital and Research Center, Vijayapura, India. A total of 212 clinical isolates of *K. pneumoniae* from sputum (n=2328) were included in the study.

Antimicrobial Susceptibility Testing:

Antimicrobial susceptibility testing was done by Kirby-Bauer's disk diffusion method in accordance with CLSI Guidelines against Ampicillin (10 µg), Cefotaxime (30 µg), Ceftriaxone (30 µg), Ceftazidime (30 µg), Cefixime (5 µg), Cefpodoxime (10 µg), Cefoperazone (75 µg), Cefepime (30 µg), Aztreonam (30 µg), Imipenem (10 µg), Amikacin (30 µg), Tetracycline (30 µg), Ciprofloxacin (5 µg), Co-trimoxazole (1.25 / 23.75 µg), and Chloramphenicol (30 µg).

Detection of ESBL:

All isolates were tested for ESBL production by CLSI Phenotypic Disk Confirmatory Test (CLSI-PDCT) by employing ceftazidime (30 µg) and ceftazidime-clavulanic acid (30/10 µg) as per CLSI Guidelines. *K. pneumoniae* ATCC 700603 was used as positive control and *Escherichia coli* ATCC 25922 was used as negative control (obtained from American Type Culture Collection, USA).

Molecular Analysis of ESBLs Genes:

Molecular analysis of the isolates was done in Molecular and Genetic Laboratory, in the Department of Molecular Biology, at Krishna

Institute of Medical Sciences "Deemed to be University" (KIMSDU), Karad, India. *K. pneumoniae* were cultured in Luria-Bertani (LB) broth at 37°C overnight, and then DNA was extracted as described by Jain *et al.* [6], and was further subjected to multiplex Polymerase Chain Reaction (PCR) for detection of ESBLs genes (*bla*TEM, *bla*SHV, *bla*CTX-M) as described previously by Dallenne *et al.* [7]. The primers and PCR cycling conditions used are given in the Tables 1, 2 and 3.

Table 1: Oligonucleotides Used for Amplification of ESBL Gene Fragments of *K. pneumoniae*

Target gene	Forward/ Reverse	Primers Sequences	Tm (^{0}C)	Amplicon size
*bla*TEM	20	5'- ATG AGT ATT CAA CAT TTC CG-3'	46	850 bp.
	20	5'- CCA ATG CTT AAT CAG TGA GG-3'	50	
*bla*SHV	17	5'-CAG CGA AAA ACA CCT TG-3'	45	471 bp.
	16	5'-CCG CAG ATA AAT CAC C-3'	44	
*bla*CTX-M	20	5'-GGT TAA AAA ATC ACT GCG TC-3'	48	717 bp.
	20	5'-TTG GTG ACG ATT TTA GCC GC-3'	52	

Table 2: Standardized Polymerase Chain Reaction Cycling Conditions

1. *bla*TEM (850 bp.)					
95^{0}C	95^{0}C	55^{0}C	72^{0}C	72^{0}C	4^{0}C
5 minutes	20 seconds	20 seconds	20 seconds	5 minutes	∞
30 cycles					
2. *bla*SHV (471 bp.)					
95^{0}C	95^{0}C	45^{0}C	72^{0}C	72^{0}C	4^{0}C
5 minutes	20 seconds	20 seconds	20 seconds	5 minutes	∞
30 cycles					
3. *bla*CTX-M (717 bp.)					
95^{0}C	95^{0}C	52^{0}C	72^{0}C	72^{0}C	4^{0}C
5 minutes	20 seconds	30 seconds	20 seconds	5 minutes	∞
30 cycles					

Table 3: Standardized Multiplexed PCR Cycling Conditions

1. *bla*TEM (850 bp.), 2. *bla*SHV (471 bp.), 3. *bla*CTX-M (717 bp.).					
95^{0}C	95^{0}C	52^{0}C	72^{0}C	72^{0}C	4^{0}C
5 minutes	20 seconds	30 seconds	30 seconds	5 minutes	∞
30 cycles					

Results:

During the 19 months period, total 212 *K. pneumoniae* isolates were obtained from RTIs. Of these 212 isolates, 60 (28.3%) were ESBL producers by CLSI-PDCT method and 152 (71.7%) were negative for ESBL production. Amongst ESBL producer *K. pneumoniae* (*n*=60), 93% isolates were resistant to ampicillin, ceftazidime, cefixime, and cefpodoxime (Table 4). Eighty to 86% isolates were resistant to cefotaxime, ceftriaxone, cefoperazone, cefepime, and aztreonam. Imipenem was found the most effective antibiotic (resistance 7%). Resistance to ciprofloxacin (73%) and co-trimoxazole (66%) was more among other antibiotics (Table 4).

A total 96 isolates were randomly selected to detect the presence *bla*TEM, *bla*SHV and *bla*CTX-M genes. Amplified product of *bla*TEM gene in *K. pneumoniae* clinical isolates had a fragment size of 850 bp. The amplified product of *bla*SHV gene had a fragment size of 471 bp. Amplified product of *bla*CTX-M gene had a fragment size of 717 bp. (Image 1). Twenty four (25%) isolates showed *bla*TEM gene corresponding to position 850 bp. and only 4 isolates (4.1%) possessed each *bla*SHV and *bla*CTX-M gene alone (Table 5). Isolates showing both *bla*TEM+*bla*SHV genes corresponding to position 850 bp. and 471 bp. respectively were 20 (20.8%), and 12 (12.5%)

Table 4: Antimicrobial Sensitivity Pattern of ESBLs Producing *K. pneumoniae* Isolates (n=60)

Antibiotics	**Resistance %**
Ampicillin	93
Cefotaxime	80
Ceftriaxone	86
Ceftazidime	93
Cefixime	93
Cefpodoxime	93
Cefoperazone	86
Cefepime	86
Aztreonam	80
Imipenem	07
Amikacin	47
Tetracycline	46
Ciprofloxacin	73
Co-trimoxazole	66
Chloramphenicol	27

Table 5: ESBL Genotypes in K. pneumoniae Isolates (n=96)

ESBL Genotype positive by Multiplex PCR	No. Amplified (%)
1. Single ESBL gene	
*bla*TEM only	24 (25)
*bla*SHV only	4 (4.1)
*bla*CTX-M only	4 (4.1)
2. Two or more ESBL genes	
*bla*TEM+*bla*SHV	20 (20.8)
*bla*SHV+*bla*CTX-M	0
*bla*TEM+*bla*CTX-M	12 (12.5)
*bla*TEM+*bla*SHV+*bla*CTX-M	20 (20.8)
3. Total Prevalence	
Total *bla*TEM	76 (79.1)
Total *bla*SHV	44 (45.8)
Total *bla*CTX-M	36 (37.5)

isolates showed amplified product of both *bla*TEM+*bla*CTX-M genes. Isolates possessing all three *bla*TEM+*bla*SHV+*bla*CTX-M genes were 20 (20.8%) (Table 5).

All together data revealed that, *bla*TEM gene was present in total 76 (79.1%) isolates irrespective of other two types of genes. Likewise *bla*SHV was present in 44 (45.8%) isolates and *bla*CTX-M was present in 36 (37.5%) isolates irrespective of other two types of genes (Table 5).

Discussion:

This study was carried out to examine the prevalence of ESBL genotypes in *K. pneumoniae* isolates obtained from sputum samples of RTIs. Infections caused by Gram-negative bacteria are a major concern. *K. pneumoniae* can cause a typical form of primary pneumonia. It is found in the oropharynx of a normal person. However, its prevalence rate is high in hospitalized patients [8]. In our study, the incidence of *K. pneumoniae* in RTIs was 9.1% that was less compared to other reports. In 2015, Vijay Kumar *et al.* from Andhra Pradesh reported 12% prevalence for *K. pneumoniae* recovered from RTIs [9]. Other studies also reported more prevalence of respiratory isolates of *K. pneumoniae.* It has been reported up to 35% in different parts of the country [10-12]. Difference in the prevalence rates may be due to geographical differences.

Nowadays, ESBLs are considered to be a major problem among hospitalized patients throughout the world. Patients suffering from infections caused by ESBL producing organisms are at increased risk of treatment failures with broad-spectrum β-lactams. Therefore, it is recommended that any organism confirmed for ESBL production be reported as resistant to the entire broad-spectrum β-lactam antibiotics, despite susceptibility results [13]. In our study, ESBL phenotypic method gave 28.3% positive results. Prevalence and phenotypic characteristics of ESBLs among clinical isolates is rapidly changing over time and it varies greatly worldwide. Similar to our study, Basu *et al.* and Yum *et al.* also reported similar prevalence rate [12, 14]. In 2013, Basu *et al.* from Andhra Pradesh reported 27% ESBL phenotype in *K. pneumoniae* respiratory isolates [12]. And Yum *et al.* in 2005 reported 28.4% ESBL prevalence in respiratory isolates of *K. pneumoniae* in Korea [14]. On the other hand, in 2010 Mehrgan *et al.* from Iran showed that ESBL production was seen more often (80.8%) in *K. pneumoniae* isolated from respiratory specimens [15].

In this study, susceptibility testing of *K. pneumoniae* isolates producing ESBL showed highest resistance rate (80 to 93%) for 3rd generation cephalosporins and aztreonam in our hospital (Table 4). This high resistance rate has alarmed to limit the usage of these drugs in future days in our hospital. Reason behind such higher resistance may be attributed to the frequent use of these drugs in hospital. This resistance is mainly associated with the production of enzymes *bla*TEM and *bla*SHV that are usually plasmid-mediated. The highest non-β lactam antibiotic resistance was seen for ciprofloxacin (73%) and co-trimoxazole (66%). Resistance to non-β-lactams is also a matter of concern. This co-resistance is usually transferred by the plasmids of ESBL that carry resistance to several agents [16]. This overall situation of soaring resistance to a large number of antimicrobials is a high time to re-think of curtailing the overuse of antimicrobials, otherwise days are not far when we are left with no effective antibiotics. Thus, it has become highly essential to be alert about the trend of susceptibility patterns of organisms to save the therapies. The resistance pattern of our study is in accordance with other studies reported by Gupta *et al*., Rampure *et al.*, and Sikarwar *et al.* [17-19]. In our study, the lowest rate of resistance (7%) was seen for imipenem. It was most effective antibiotic. Our results are in harmony with the finding of Gupta *et al*., and Ghafourian *et al.* [17, 20]. Gupta *et al*. from Uttar Pradesh reported lower resistance rate towards imipenem in respiratory isolates of *K. pneumoniae* [17]. Ghafourian *et al.* in Iran also showed all respiratory isolates of *K. pneumoniae* susceptible to imipenem [20]. Similarly, an Iranian survey done by Shahcheraghi *et al*. also reported almost all study isolates susceptible to imipenem [21]. Even though resistance for carbapenems is low, carbapenems should be used as little as possible.

The phenotypic expression of plasmid mediated ESBL producing isolates is resistant to 3rd generation cephalosporins. β-lactamase inhibitors such as clavulanic acid can restore susceptibility of inactive cephalosporin. The disk diffusion method does not allow routine differentiation of isolates producing ESBLs. Molecular methods, like PCR need to be used for differentiation of β-lactamase producing isolates [22]. In our study, a total 96 isolates were randomly selected to detect the presence *bla*TEM, *bla*SHV and *bla*CTX-M genes (Fig. 1).

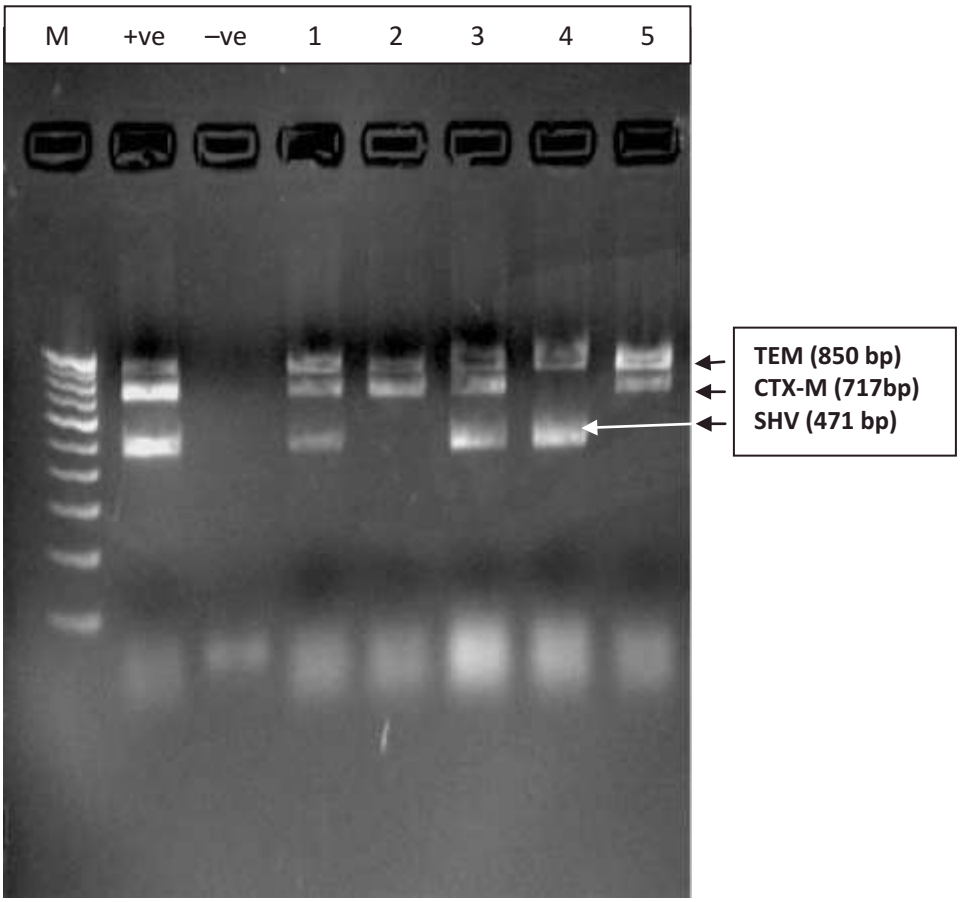

Fig. 1: Gel Image showing Multiplex Gene-Specific PCR Amplification: Multiplex Gene-Specific PCR amplification of 867 bp. fragment of TEM, 717 bp. fragment of CTX-M and 471 bp fragment of SHV genes. Lane M: 100 bp. DNA ladder, +ve: *K. pneumoniae* ATCC 700603 positive control, –ve: *E. coli* ATCC 25922 negative control and Lane 1-7: *K. pneumoniae* clinical isolates. Lane 1 and 3 showed amplified product of all three genes (TEM, CTX-M, and SHV). Lane 2 and 5 showed amplified product of TEM and CTX-M genes. Lane 4 showed amplified product of TEM and SHV genes.

In this study, ESBL phenotypic method gave only 28.3% positive results, on the other hand, highest ESBL positive genotype was present in 79.1% isolates (total *bla*TEM) (Table 5). Phenotypic test can only presumptively recognize the presence of an ESBL. These phenotypic tests are neither able to discriminate the specific genes responsible for ESBL production, nor they can detect low-level of resistance. The only advantage with them is that, they are very simple and easy to perform. The above disadvantages of phenotypic methods are replaced by genotypic techniques; moreover genotypic techniques can be done directly clinical specimens. However these techniques are cumbersome [23].

In multiplex PCR, we found the frequency of *bla*TEM was more than other two genotypes *bla*SHV and *bla*CTX-M (Table 5). If presence of

single gene is considered then, more isolates showed single gene presence of *bla*TEM only. It was seen in 25% isolates. And only 4.1% isolates showed *bla*SHV alone and *bla*CTX-M alone as separate gene (Table 5). Whilst if presence of combination genes was checked, then 20.8% isolates were found carrying both *bla*TEM+ *bla*SHV genes at a time, and only 12.5% isolates showed presence of both *bla*TEM+*bla*CTX-M genes. There was no isolate seen carrying combination of both *bla*SHV+*bla*CTX-M genes at a time. Moreover, isolates possessing all three genes at a time *(bla*TEM+*bla*SHV+*bla*CTX-M) were also seen in 20.8% (Table 5). All together data revealed that, proportion of total *bla*TEM gene was very much high than the total *bla*SHV and total *bla*CTX-M genes. This is the major finding of our study. *bla*TEM gene was present in totally 79.1% isolates irrespective of other two *bla*SHV and *bla*CTX-M genes (Table 5). Likewise *bla*SHV was seen in totally 45.8% isolates and *bla*CTX-M was carried in totally 37.5% isolates irrespective of the presence of other two genes. Our results revealed high prevalence of *bla*TEM and low frequency of *bla*SHV and *bla*CTX-M. Significantly, *bla*TEM was more responsible for ESBLs production.

There are very few studies on respiratory isolates of *K. pneumoniae* that have reported incredibly high *bla*TEM similar to our report. However, some studies have reported modest increase in frequency of *bla*TEM but that is much lesser than our prevalence rate. Lal *et al.,* Fouzia *et al.* and Goyal *et al.* reported increased prevalence of *bla*TEM and less for *bla*SHV and *bla*CTX-M in *K. pneumoniae* isolates recovered from diverse clinical specimens including respiratory specimens [22, 24, 25]. In 2007, Lal *et al.* from New Delhi reported more frequency of *bla*TEM and less of *bla*SHV, however their prevalence rate of *bla*TEM and *bla*SHV was lesser than our study [22]. They reported 20% *bla*TEM and 8% *bla*SHV and presence of both *bla*TEM+*bla*SHV genes together were shown in 67% isolates [22]. Fouzia *et al.* from Maharashtra showed more prevalence of *bla*TEM in 2015. They reported 60% *bla*TEM, 40% *bla*TEM+*bla*SHV and presence of only *bla*SHV was nil [24]. Goyal *et al.* from Lucknow showed 66% *K. pneumoniae* ESBL producers in 2009, and prevalence rate of only *bla*TEM, *bla*SHV and *bla*CTX-M was 19%, 14%, and 9% respectively. Moreover, *bla*TEM+CTXM were seen in 28%, *bla*SHV+*bla*CTX-M in 9%, and *bla*TEM+*bla*SHV+*bla*CTX-M were found in 19% isolates respectively [25].

K. pneumoniae has rapidly become the most common ESBL producing organism and trend of ESBL genotypes in *K. pneumoniae* circulating worldwide at a given time also varies greatly. Certain studies have reported contrast genotyping finding to our report [20, 26, 27]. Ghafourian *et al.* reported less frequency of *bla*TEM and highest of *bla*SHV than our study. They reported 59% ESBL production in *K. pneumoniae* respiratory isolates and prevalence of *bla*TEM, *bla*SHV, and *bla*CTX-M was 16%, 94% and 23% respectively [20]. Feizabadi *et al.,* in 2009 showed that 69.7% of *K. pneumoniae* isolates were ESBL producers in Tehran, Iran; and reported more prevalence for *bla*SHV. They showed prevalence of *bla*TEM, *bla*SHV, and *bla*CTX-M-I among these isolates was 54%, 67.4%, and 46.5% respectively [26]. Similar to above study and typically contrary to our findings, Kotekani *et al.* from similar geographical loci of our study (*i.e.* Karnataka) recently (2018) reported zero prevalence of *bla*TEM and

highest of *bla*CTX-M in *K. pneumoniae* isolates obtained from all clinical specimens including respiratory samples [27]. We have reported highest *bla*TEM (79.1%) and lowest *bla*CTX-M (37.5%) as a whole; whereas Kotekani *et al.* showed nil prevalence of *bla*TEM, 77% for *bla*SHV, 84% for *bla*CTX-M-1, 75% for *bla*CTX-M15, and 61% for *bla*SHV+*bla*CTX-M [27]. Disparity in the prevalence of ESBL genotypes exhibits extremely versatile trend of circulation of genotypes among clinical isolates at a given time, and it may be associated to population variations in different geographical areas and also usage of antibiotic and injectable formulations is generally high and irrepressible in many geographical regions.

Conclusion:

Incidence of RTIs caused by *K. pneumoniae* is a matter of concern worldwide. The inadvertent and indiscriminate usage of 3rd generation cephalosporins and other antibiotics has led to the emergence of MDR *K. pneumoniae* producing ESBLs. Our study showed a notable prevalence of *bla*TEM genotype in *K. pneumoniae* in RTIs during the study period. Thus high level of ESBLs among *K. pneumoniae* isolates is alarming and warrants special attention from clinicians and microbiologists. In most centers ESBLs is not regularly tested, that in due course results in dissemination of ESBL producing isolates in hospitals; and it remains undetected for longer periods. The elevated incidence of ESBL producing *K. pneumoniae* within healthcare center in the study area should be considered main public health concern both therapeutically and epidemiologically. As such, the identification, treatment, infection control and management of patients infected with these organisms are of prime necessity. Similar studies need to be done in various geographical regions of country to know the trend of circulating and prevalent genotypes for epidemiological purpose. Regular monitoring on the judicious use of antibiotics assists in conserving the effectiveness of sensitive antibiotics and prevent emergence of further resistance. The reliable and effective antimicrobial treatment for infections caused by this organism is imipenem as shown in this study. However, carbapenems are last resort drugs and should be used much more sparingly. In conditions, wherein the use of antibiotics is necessary, rotation of antibiotic regimens is suggested**.**

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[*] ***Author for Correspondence:*** *Dr. Shivali V. Gajul, Department of Microbiology, Shri B. M. Patil Medical College, BLDE (Deemed to be University), Vijayapura-586103, Karnataka Email: shivali.gajul@bldedu.ac.in Cell: 7411660842*