Original Research Article **Microbiology**

International Journal of Pharma and Bio Sciences

ISSN 0975-6299

PHENOTYPIC AND GENOTYPIC CHARACTERISATION OF EXTENDED SPECTRUM BETA-LACTAMASES (ESBLS) AND AMPC BETA- LACTAMASES AMONG *Klebsiella pneumoniae*

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ABSTRACT

Klebsiella pneumoniae, is an important pathogen causing community acquired and nosocomial infections. Production of AmpC and ESBLs confer multidrug resistance in them. The spread of ESBLs and AmpC beta- lactamase producing *Klebsiella pneumoniae* is a major health concern globally. Most of the clinical laboratories have difficulty in detecting ESBLs and AmpCs, because of their co-existence and their ability to mask each other. Failure to detect these enzymes can result in their rapid spread and therapeutic failure. This study was done to detect the presence of ESBLs and AmpC by phenotypic and molecular methods in clinical isolates of *Klebsiella pneumoniae*. A total of 370 isolates of *Klebsiella pneumoniae*, isolated from various clinical samples over a period of one year was included in this study. Antibiotic susceptibility testing for various classes of antimicrobials was done as per CLSI 2015 guidelines. Presence of ESBLs (TEM,SHV,CTX-M) and plasmid mediated AmpCs (MOX, CIT, DHA, ACC, EBC, FOX) in clinical isolates were detected by both phenotypic and genotypic methods. Among 370 isolates 58.91% were resistant to any one of the third generation cephalosporins.Phenotypic test detected 37.02 % isolates as ESBL producers. Co-existence of all three genes (TEM,SHV,CTX-M) were seen in 47.29% in this study. In 79 isolates, by PCR ESBL genes (one or more) were detected though the phenotypic test was negative in them. Cefoxitin resistance was exhibited by 118 isolates of which 34 harboured AmpC genes. The most prevalent AmpC gene in this study was DHA (n=29) followed by EBC (n=3) and CIT (n=2). Phenotypic test for ESBLs and AmpC can give false positive or negative result. Detection of the resistance mechanism by molecular methods will help to prevent therapeutic failure and the spread of multi drug resistant *Klebsiella pneumoniae*.

KEY WORDS: ***Klebsiella pneumoniae*, ESBLs, AmpC, Phenotypic test, PCR.**

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Received on: 12-Dec-17
Revised and Accepted on: 25-Jan-18
DOI: http://dx.doi.org/10.22376/ijpbs.2018.9.1.b341-347

INTRODUCTION

Klebsiella pneumoniae, a nosocomial pathogen, causes various infections such as pneumonia, soft tissue infections, septicaemia, and urinary tract infections.[1] Extended spectrum beta lactamases (ESBLs) are plasmid mediated enzymes that hydrolyse third generation cephalosporins (3GC) and monobactams like aztreonam.They are inhibited by clavulanic acid and are susceptible to cephamycins (cefoxitin and cefotetan).[2] The most common ESBLs belong to SHV, TEM and CTX-M families and are found usually in *Klebsiella* spp, *Escherichia coli* and other gram negative bacteria.[3] AmpC beta lactamases belongs to the ambler class C, exhibit resistance to cephalosporins and cephamycins, and are not inhibited by all beta lactamase inhibitors. AmpC beta lactamases are found in the chromosome of *Citrobacter freundii, Enterobacter cloacae, Morganella morgani, Hafnia alvei* and *Serratia marcescens. Escherichia coli* expresses chromosomal AmpC at low levels. Plasmid mediated AmpCs that occur in *Klebsiellasps, Proteus mirabilis, and Salmonella spp* have been found worldwide in nosocomial and community settings.[4-5] Plasmid AmpC mediated resistance is less common than resistance due to ESBLs. They are often difficult to detect in the laboratory, especially when they coexist together.[6] This study aimed to detect ESBLs and AmpC by phenotypic and molecular method, and also to determine the prevalence of these beta lactamases in *Klebsiella pneumoniae* isolated both from the hospitalised and non-hospitalised patients. Knowing their prevalence in hospital settings helps to formulate a policy of empirical therapy.

MATERIALS AND METHODS

Bacterial Isolates

The study was conducted in a University teaching hospital from September 2014 to August 2015.A total number of 370 clinically significant, consecutive, non-duplicate isolates of *Klebsiella pneumoniae* were included in this study. The isolates were identified up to species level by automated system (VITEK2 GN-card; Bio Merieux, Brussels, Belgium) and standard biochemical tests.[7] The isolates were obtained from, urine (170), exudative specimens (132), respiratory secretions such as bronchial wash, endotracheal secretion, broncho alveolar lavage, pleural fluid (38) and blood (30).

Antibiotic Susceptibility Testing

Antibiotic susceptibility testing was done by Kirby-Bauer disc diffusion method as per CLSI guidelines.[8] The antibiotics tested were, cefotaxime (30µg), ceftazidime (30µg), cefoxitin (30 µg), amikacin (30µg), ciprofloxacin (5µg), piperacillin/ tazobactam (100µg/ (10µg) and imipenem (10µg) [Himedia laboratories, Mumbai].ATCC *Escherichia coli* 25922 was used as control strain.

Phenotypic tests for ESBL confirmation

Isolates were tested for ESBL production as per CLSI guidelines.[8] Combined disk diffusion test was performed using both cefotaxime and ceftazidime (30µg) alone and in combination with clavulanic acid. An increase in the diameter 5mm or above, when compared to cephalosporins alone was interpreted as ESBL. ATCC *Escherichia coli* 25922 and *Klebsiella pneumoniae* ATCC 700603 were used as control strain.

Phenotypic tests for AmpC beta lactamase

AmpC production was tested by using inhibitor based method. Phenyl boronic acid (benzeneboronic acid; Sigma-Aldrich, India.) 120 µg was dissolved in 3ml of dimethyl sulfoxideand 3ml of sterile distilled water. Twenty microliters of stock solution was added onto 30 µg of cefoxitin disk.[9] The test organism was inoculated on a Mueller Hinton Agar (MHA) [Himedia laboratories, Mumbai]. Disk containing cefoxitin and cefoxitin impregnated with 400 µg of phenyl boronic acid were placed on the plate. The plates were incubated overnight, and an increase in the zone diameter of ≥5mm for cefoxitin with boronic acid compared with cefoxitin alone was considered as indicative of AmpC production.[9-10]

Preparation of template DNA

A single colony of each organism from a Mac conkey agar plate, inoculated in to 1.5ml of Luria-Bertani broth (Himedia laboratories, Mumbai.),and incubated for overnight at 37°C.Cells from the overnight culture were centrifuged at 10000rpm for 10min.Supernatent was decanted and the pellet was resuspended in 250µl of distilled water. The cells were lysed by heating at 100°C for 10 min.2 µl of the supernatant of the total sample, was used as the source of the template for amplification.[11]

Detection of ESBL genes by multiplex PCR

All the isolates were tested for the presence of SHV, TEM, and CTX-M genes by multiplex PCR using the earlier described primers.[12] The primers used are listed in Table1. PCR was performed with a final volume of 25 µl. Each reaction contained 10 pmol of each primer (Sigma-Aldrich, India), 10mM of dNTP mixture (Takara,India), 5U Taq polymerase (Takara, India) in 2.5µl of 10X Taq polymerase buffer(Mg2+plus).Two µl of template DNA was added to 23 µl of the master mix. Negative control was the PCR mixture with water instead of template DNA, and a positive control was also included in every PCR run. Amplification reactions were performed under the following conditions: initial denaturation at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds with an extension at 72°C for 50 seconds, and a final extension for one cycle at 72°C for 5 minutes. The PCR product was then run on a 1.5 % agarose gel for detection of the amplified fragment.

Detection of AmpC genes by multiplex PCR

All the isolated organisms were tested for plasmid mediated AmpC genes by previously described primers.[11] Primers used were listed in Table 2.

Table 1
List of primers used for PCR of ESBL genes

Gene	Primer	Amplicon
SHV	SHV-F: CGCCTGTGTATTATCTCCCT SHV-R: CGAGTAGTCCACCAGATCCT	294bp
TEM	TEM-F: TTTCGTGTCGCCCTTATTCC TEM-R:ATCGTTGTCAGAAGTAAGTTGG	404bp
CTX-M	CTX-M-F: CGCTGTTGTTAGGAAGTGTG CTX-M-R:GGCTGGGTGAAGTAAGTGAC	754bp

Table 2
Primers used for amplification of plasmid mediated AmpC genes

Target(s)	Primer	Sequences-5'-3'	Amplicon
MOX-1,MOX-2, CMY-1, CMY-8 to CMY-11	MOXMF MOXMR	GCTGCTCAAGGAGCACAGGAT CACATTGACATAGGTGTGGTGC	520bp
LAT-1 to LAT-4, CMY-2 to CMY-7, BIL-1	CITMF CITMR	TGGCCAGAACTGACAGGCAAA TTTCTCCTGAACGTGGCTGGC	462bp
DHA-1, DHA-2	DHAMF DHAMR	AACTTTCACAGGTGTGCTGGGT CCGTACGCATACTGGCTTTGC	405bp
ACC	ACCMF ACCMR	AACAGCCTCAGCAGCCGGTTA	346bp
MIR-1T ,ACT-1	EBCMF EBCMR	TCGGTAAAGCCGATGTTGCGG CTTCCACTGCGGCTGCCAGTT	302bp
FOX-1to FOX-5b	FOXMF FOXMR	AACATGGGGTATCAGGGAGATG CAAAGCGCGTAACCGGATTGG	190bp

DNA Sequencing

PCR positive amplicons were purified and sequenced by big dye 3.1 cycle sequencing kit using the Sanger AB13730 XL DNA analysing instrument (Sci genome, India).The nucleotide sequences analysed are compared with the sequence available at the National Centre for Biotechnology information website (www.ncbi.nIm.nih.gov).Sequences obtained in this study was submitted to gen bank with following accession number, KY115614, KY115613, KY115615, KY115612, KY115610, KY11561.

RESULTS

Antibiotic Susceptibility Testing

The susceptibility of the study isolates to various antimicrobial agents is as follows, imipenem (96.75%), amikacin (82.16%), piperacillin/ tazobactam (80.27%), cefoxitin (68.10%), ciprofloxacin (62.97%), ceftazidime (50.54%), and cefotaxime (41.35%).

ESBL Confirmatory Test and Genotypic Test

Among 370 isolates, 218 (58.91%) were resistant to any one of the third generation cephalosporins (Cefotaxime, Ceftazidime).Of the total isolates eight showed susceptibility to 3GC but were resistant to cefoxitin by disc diffusion. ESBL screening by combined disc diffusion method was positive in 137(37.02%) isolates. Distribution of the three ESBL genes looked for isolates is shown in Table 3. Gel picture of the amplified genes are shown in Figure 1.

Amp C beta lactamase detection by Phenotypic and Genotypic Methods

Of the 370 study isolates 118 were resistant to cefoxitin. Among the 118 cefoxitin resistant isolates inhibitor based method using phenyl boronic acid was positive in 34.Among the 34 PBA positive isolates 26 harboured any one of the AmpC gene in them, remaining 8 isolates were negative for the gene. Eight cefoxitin resistant isolates showed negative for screening test by PBA, carried AmpC genes. Distribution of plasmid mediated AmpC genes and its co carriage of ESBLs is shown in Table 4. Multiple AmpC genes were not encountered in single isolates. Gel picture of the detected AmpC genes are shown in Figure 2. Both ESBL and AmpC genes were present in 33 out of 370 isolates.

Table 3
Distribution of ESBL genes detected in total isolates.

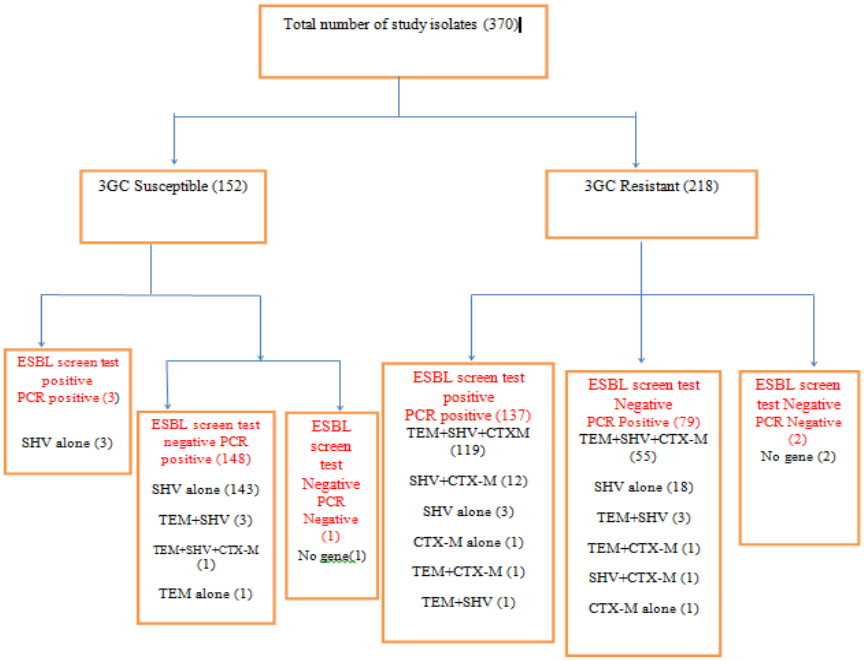

Table 4
Distribution of AmpC genes and its coexistence with ESBLs

AmpC genes obtained in this study	Number of AmpC genes alone (n=34)	Co-existence AmpC along with other ESBL genes (n=33)
DHA	29	DHA+TEM+SHV+CTX-M (3) DHA+TEM+SHV (2) DHA+SHV (24)
EBC	3	EBC+TEM+SHV+CTX-M (1) EBC+SHV+CTX-M (1) EBC alone(no ESBL genes)
CIT	2	CIT+TEM+SHV+CTX-M (2)

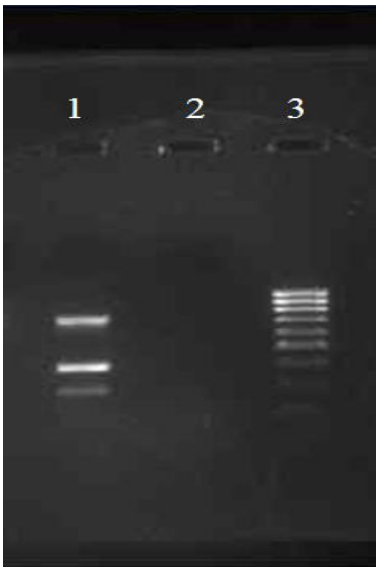

Figure 1
Gel picture of ESBL genes

[Lane1-Multiplex of CTX-M, TEM, SHV], [Lane2-Negative control], [Lane3-100bp ladder

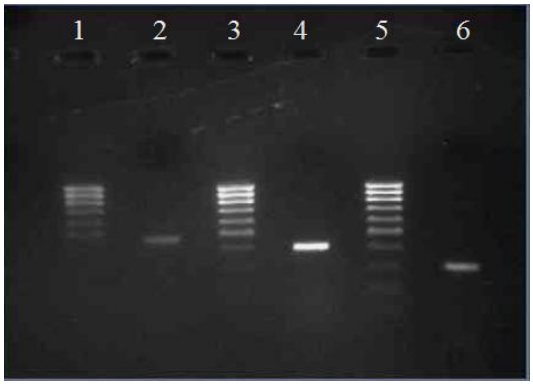

Figure 2
Gel picture of amplified AmpC genes

[Lane 1,3,5-100bp Ladder], [Lane 2-CIT,Lane 4-DHA,Lane 6-EBC]

DISCUSSION

ESBLs are one of the most common antibiotic resistance mechanisms among Enterobacteriaceae.In 1983 ESBL producing *Klebsiella pneumoniae* was first reported from Germany. Since then they have spread worldwide and reached an alarming rate.[13] The prevalences varies greatly from country to country and among the institutions within the country. This fact strongly supported by previous reports.[14-18] In the present study, 58.91% of *Klebsiella pneumoniae* were resistant to any one of the 3GC tested. A study from Warangal in India reported that out of 200 isolates, 36% were resistant to 3GC.[19] Another study conducted in Puducherry, 76.6% of *Klebsiella pneumoniae* exhibited resistance to any one of the 3GC .[20]A recent study from Egypt, reported that, of the 100 isolates tested 49% were resistant to Cefotaxime and Ceftriaxone and 40% was resistant to Ceftazidime.[21] This reflects a high prevalence of resistance to commonly used class of antibiotics. In this study 3 isolates that were 3GC susceptible carried SHV genes in them. Since the study used a consensus primer for detection of SHV genes, the PCR positivity was due to the presence of SHV-1 as confirmed by sequencing of the PCR product. Of the remaining 149 3GC susceptible isolates,148 carried one or more genes looked for in the study and they were ESBL screen test negative.TEM or SHV or both were detected in 147 isolates .PCR positivity in these isolates (147) was due to the presence of SHV-1 and or TEM-1 as confirmed by DNA sequencing. One isolatealone harboured CTX-M along with TEM and SHV. This isolates exhibited susceptibility to 3GC and was ESBL screen test negative but carried an AmpC gene EBC. Of the 218, 3GC resistant isolates, all except two carried one or more ESBL encoding genes looked for in this study. Presence of multiple genes in a single isolate was reported in many studies conducted worldwide including India.[16-17,20-22] Coexistence of TEM, SHV, and CTX-M was seen in a higher percentage of isolates (47.29%) in this study. In previous studies from Puducherry and Amritsar, co-existence of all the above was reported in 29.5% and 6.45% respectively.[20,23] Concurrent presence of all three genes was not observed by Savitha Rani *et al* and Trupti Bajpai*et al*.[16-17] Of the 218, 3GC resistant isolates 137 were ESBL screen test positive and harboured one or more ESBL genes tested in this study. Among 81 isolates, that were ESBL screen test negative, by PCR it showed 79 carried one or more ESBL gene in them. Of the remaining two isolates, one carried the EBC type AmpC and the other did not harbour any AmpC gene though being cefoxitin resisted. It can be reasonably presumed that this isolate could have harboured the AmpC genes that were not included in the multiplex PCR.It is well documented that the ESBL screen test could be false negative in the presence of other beta lactamase like AmpC or carbapenemases. This is evident by the fact 20 of the ESBL positive isolates co harboured AmpC genes and exhibited resistance to cefoxitin. The remaining 59 isolates that carrying the ESBL gene possibly harboured a carbapenemase encoding gene or other AmpC. False susceptibility to 3GC was detected in eight isolates in this study, as they actually carried AmpC genes, with them, strongly supporting the earlier statement, that detection of plasmid mediated AmpC is challenging in laboratories, since they may appear susceptible to 3GC initially. This may lead to inappropriate selection of antibiotics and therapeutic failure.[24-25] Carriage of ESBL and/or plasmid mediated AmpC is often associated with resistance to other class of antimicrobials.[26] Among the 118 cefoxitin resistant isolates, only 34 were positive for plasmid AmpC genes by PCR.This is in agreement with the earlier studies that, not all cefoxitin resistant isolates carry an AmpC gene.[27-29] In this study, 8 cefoxitin resistant isolates that were AmpC screen test positive using PBA harboured ESBL genes and not the AmpC genes that were looked for. In contrast some of the isolates (n=8) harboured AmpC gene in spite of being AmpC screen test negative. This further strengthens the fact that ESBL and AmpC mask each other in phenotypic tests.[30] Most predominant AmpC gene detected in our study was DHA (n=29), followed by EBC (n=3) and CIT (n=2). Sequencing of the amplicons of AmpC showed similarity with DHA-9, MIR-15, and CMY-4. An earlier study conducted in the same centre detected other than DHA, CIT and EBC, ACC and multiple genes in a single isolate which was not detected in the present study.[5] Another study by Manoharan*et al* reported that EBC (16.6%) was the most prevalent gene in *Klebsiella pneumoniae*.[31] A study from Puducherry, reported that DHA (54.5%) was the most common gene detected followed by CIT (40%), MOX (3.6%) and ACC (1.8%) among 55 isolates.[20]

Anitha *et al* from Kerala reported among 130 cefoxitin resistant enterobacteriaceae, five *Klebsiella pneumoniae* harbouring CIT alone.[32] Studies from Netherland have found DHA and CIT in *Klebsiella pneumoniae* elsewhere in China DHA was the only AmpC gene present in *Klebsiella pneumoniae*.[33-34] Based on data of this study, we suggest PCR remains gold standard for the detection of ESBLs and AmpC in clinical microbiology laboratories.

CONCLUSION

Prevalence of ESBLs in this study among 3GC resistant *Klebsiella pneumoniae* was 58.91% and AmpC was 9.1% as by PCR.Phenotypic test for both ESBLs and AmpC can give false positive or negative results. It is necessary to detect the resistant mechanisms by using molecular techniques to prevent the spread of resistance and achieve therapeutic success.

FUNDING ACKNOWLEDGEMENT

We acknowledge the resources and financial support provided by the Sri Ramachandra University (Deemed to be) as SRU-Chancellor Fellowship for this study.

CONFLICT OF INTEREST

Conflict of interest declared none.

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