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Original Article

Microbiology Section

Phenotypic and Genotypic Methods for Detection of Extended Spectrum β Lactamase Producing *Escherichia coli* and *Klebsiella pneumoniae* Isolated from Ventilator Associated Pneumonia

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ABSTRACT

Background: Ventilator Associated Pneumonia (VAP) is one of the common nosocomial infections associated with high morbidity due to multidrug resistant pathogens. Rapid spread of resistance to broad spectrum beta-lactams in pathogenic strains causes antibiotics ineffectiveness and increased severity of illness. The CTX-M is the most dominant Extended Spectrum β Lactamase (ESBL) among Enterobacteriaceae in many regions of the world. The aim of the study was to identify the occurrence of ESBL and detect the genes responsible for ESBL production by conventional Polymerase Chain Reaction (PCR) method.

Methods: This prospective study included patients, clinically diagnosed as VAP. Endotracheal aspirates (EA) were collected and cultured by quantitative method. The bacterial isolates were identified as per standard methods. Isolates resistant to 3rd generation cephalosporins were screened for ESBL production by disk approximation method and combination disc diffusion method. Isolates confirmed as ESBL producers were subjected to genotyping by conventional PCR. **Statistical Analysis:** Statistical analysis was done by using MS Excel sheet. Descriptive statistics like percentage was done in the study.

Results: Among the isolates from 428 patients who developed VAP, 144 isolates belonged to the Enterobacteriaceae family (*Klebsiella pneumoniae* 87 and *Escherichia coli* 57). A total of 66 isolates (28 *Klebsiella pneumoniae* and 38 *Escherichia coli*) were confirmed as ESBL producer by disc approximation method and 63 isolates by double disc combination method. In the present study by conventional PCR bla CTX-M was the common gene in 48.5% strains followed by 22.22% bla SHV and 14.81% bla TEM.

Conclusion: The genotypic methods using specific PCR amplification of resistance genes seems to have 100% specificity and sensitivity in detection of ESBL when compared to phenotypic methods which lacks the constant sensitivity.

Key words: blaTEM gene , blaSHV gene, blaCTX-M gene, Extended Spectrum Beta Lactamase, Polymerase Chain Reaction, Ventilator Associated Pneumonia

INTRODUCTION

Ventilator Associated Pneumonia (VAP) is one of the common nosocomial infections among patients admitted to the intensive care unit (ICU) [1] associated with high morbidity and mortality [2]. Risk of mortality and morbidity in VAP is increased due to wrong or delayed initial antibiotic therapy especially when VAP is caused by multidrug resistant pathogens (MDR) [3]. One of these resistant pathogens are the extended spectrum β- lactamase (ESBL) producing *Escherichia coli* and *Klebsiella pneumoniae* which have spread worldwide since their discovery in 1983 and pose a serious threat in healthcare associated infections [4]. ESBL producing strains often exhibit multidrug resistance, including resistance to aminoglycosides and fluoroquinolones, limiting the therapeutic options. The typical character of ESBL is their ability to hydrolyse oxyimino-cephalosporins and aztreonam while being inhibited by β-lactamase inhibitors [5]. ESBLs are often plasmid mediated and most are mutants of the classic TEM or SHV β-lactamase enzymes (class A), with one or more amino acid substitutions around the active site [6]. In recent years non TEM and non SHV plasmid mediated ESBL have been reported mainly of the CTX-M type. The CTX-M family first described in the early 1990s is the most dominant ESBL among Enterobacteriaceae and is recognized as a rapidly growing family of ESBLs. The CTX-M enzyme is the predominant type of ESBL found in many regions of the world, including Asia, South America, Europe and Africa. The CTX-M enzyme forms a rapidly growing family of over 69 enzymes currently [7]. This study has been undertaken to identify the occurrence of ESBL among *Escherichia coli* and *Klebsiella pneumoniae* from cases of VAP and detect the genes responsible for ESBL production by conventional PCR method.

MATERIAL AND METHODS

A prospective study was done over a period of 5 years from 2006 to 2010 in a urban city, Tumkur, Karnataka, India, which included patients who were clinically diagnosed as VAP [8]. Ethical clearance was obtained before collection of samples. Endotracheal Aspirates (EA) [9] were collected from patients fulfilling the clinical criteria for VAP. EA were cultured by quantitative method [10] and presence of >105cfu/ml was considered. The bacterial isolates were identified to species level by standard biochemical tests [11].

Antibiotic Susceptibility Test

Antibiotic susceptibility testing was done by Kirby Bauer Disk Diffusion method on Mueller Hinton Agar as per Clinical and Laboratory Standard Institute guidelines (CLSI) [12] to determine the resistance patterns of the isolates. *Escherichia coli* ATCC 25922 was used as the control. Samples showing an inhibition zone size of ≤ 22 mm with ceftazidime and ≤ 27 mm with cefotaxime were considered as potential ESBL producer and were further investigated for confirmation.

Detection of ESBL by Disk Approximation method [13]

Isolates that showed intermediate/resistance to 3rd generation cephalosporins were screened to detect ESBL production. A modified double disk synergy (Disk Approximation Test) was carried out on resistant isolates. Amoxicillin/clavulanic acid (20µg/10µg) disk was placed in the centre of the MHA plate on which a lawn culture of the test organism (turbidity matched to 0.5 McFarland turbidity) had been made, ceftazidime (Ce) (30µg) and cefotaxime (Ca) (30µg) were placed on either side at a distance of 15 mm centre to centre from the amoxicillin/clavulanic acid (Ac) disc. Plates were incubated at 35oC for 18-24 hrs and the pattern of zone of inhibition was noted. Isolates that exhibited a distinct potentiation towards amoxicillin + clavulanic acid disc were considered potential ESBL producer. *Escherichia coli* ATCC 25922 and *Klebsiella pneumoniae* ATCC 700603 were negative and positive controls respectively.

Combination Disc Diffusion Method [12]

A cefotaxime 30µg and a cefotaxime + clavulanic acid (30µg+10µg) discs (Hi-media, Mumbai) were placed at a distance of 25 mm on a Mueller-Hinton Agar plate incubated with a bacterial suspension of 0.5 McFarland turbidity standards and incubated overnight at 37°C. A ≥ 5mm increase in the diameter of inhibition zone for the combination disc versus ceftazidime disc confirmed ESBL production.

Genotyping [14]

ESBL producers among the isolates were subjected to PCR for gene detection. The Boiling method was used to extract DNA from Bacterial samples. TEM, SHV and CTX-M beta-lactamase genes were detected by PCR. PCR were carried out using thermal cycler (Eppendorf Mastercycler Gradient). The PCR mix was prepared in a volume of 20 µl containing 1 µl DNA template, 2 ml of 10 X PCR buffer (Sigma, Aldrich), 1.5 mM MgCl2 (Sigma, Aldrich), 0.6 µl 2 mM dNTPs (ferments), 1 U taq polymerase (Sigma, Aldrich), 1µl of 10 pmol of each three primers (Eurofins, Bangalore), volume made up to 20 µl with distilled water. The primer sequences and cycling conditions used and product size for three different PCR are shown in [Table/Fig-1 & 2]. The PCR products were separated by gel electrophoresis on 1% agarose gel. *Escherichia coli* Inhouse strains positive for bla TEM, bla SHV and bla CTX-M were used as positive controls and *Escherichia coli* ATCC 25922 were used as negative control. For all the PCR initial denaturation and final extension were 94°C for 4 min and 72°C for 8 min respectively.

RESULTS

In this prospective study, among the isolates from 428 patients who developed VAP, 144 isolates belonged to the Enterobacteriaceae family (*Klebsiella pneumoniae* 87 and *Escherichia coli* 57) 428 patients developed VAP. In this initial screening by standard disc diffusion method 86 isolates were identified as ESBL producers (59.72%). A total of 66 isolates (28 of *Klebsiella pneumoniae* and 38 of *Escherichia coli*) were confirmed as ESBL producer by disc approximation method and 63 isolates by double disc combination method [Table/Fig-3]. [Table/Fig -4] shows the distribution of ESBL.

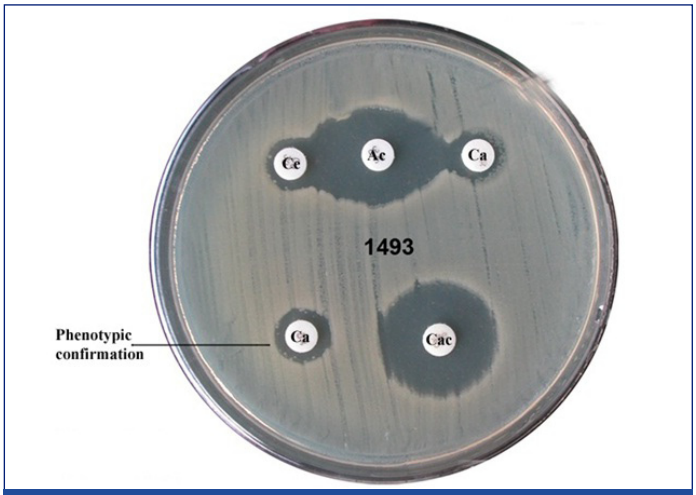
Among 38 *Escherichia coli*, 30 (78.95%) showed the presence of at least one ESBL genes. The distribution of more than one gene were seen in four isolates having a combination of genes bla TEM + bla CTX-M in two strains and bla SHV + bla CTX-M in two strains [Table/Fig-5, 6 & 7]. Among 28 *Klebsiella pneumoniae* phenotypically confirmed as ESBL producer only single gene was detected in 24 (85.71%) isolates. [Table/Fig-8] shows the distribution of ESBL genes.

Sl. No.	Gene Detected	Primer
1	bla TEM1[15]	F 5' ATAAAATTCTTGAAGACGAAA 3' R 5' GACAGTTACCAATGCTTAATCA 3'
2	bla SHV[15],[16]	F 5' GGGTAATTCTTATTTGTCGC 3' R 5' TTAGCGTTGCCAGTGCTC 3'
3	bla CTX-M[17]	F 5' TTTGCGATGCAGTACCAGTAA 3' R5'CGTATATCGTTGGTGGTGCCATA 3'

[Table/Fig-1]: Primers used for amplification of bla TEM, bla SHV, bla CTX-M genes

Gene Detected	Denaturation Time/temp	Annealing Time/temp	Extension Time/temp	No. of cycles	Product
bla TEM	94°C-1min	58°C -1 ½ min	72°C -1min	30	1100bp
bla SHV	94°C-1min	58°C -1 ½ min	72°C -1min	30	930bp
bla CTX-M	94°C-1min	58°C -1 ½ min	72°C -1min	30	544bp

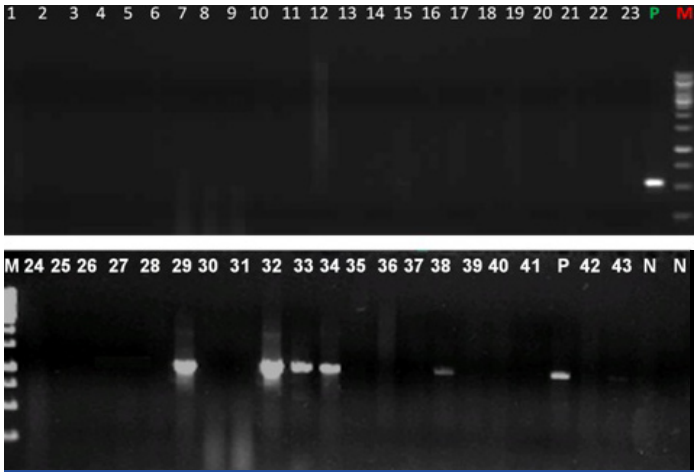
[Table/Fig-2]: PCR Conditions for amplification of bla TEM, bla SHV, bla CTX-M genes bp: base pair



[Table/Fig-3]: Disk Approximation method

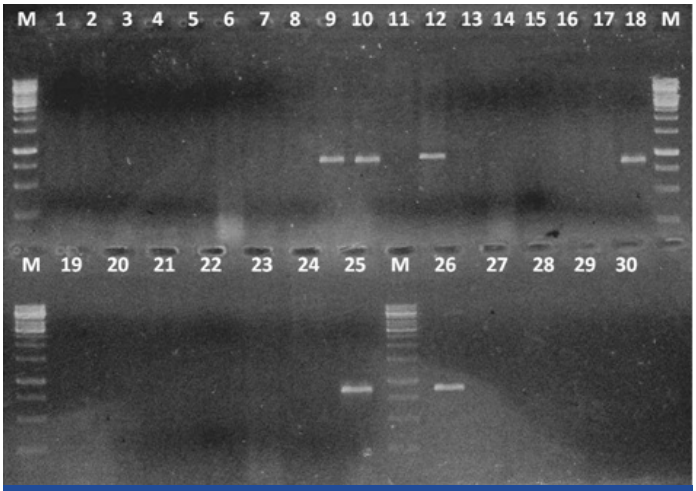
Sl. No	Isolates	No. isolated	ESBL		
			KB disc Diffusion Method	Disk Approximation Method	Combination Disc Method
1.	*Klebsiella pneumoniae*	87	37 (42.5%)	26 (29.8%)	28 (32.1%)
2.	*Escherichia coli*	57	49 (85.9%)	37 (64.9%)	38 (66.6%)
	Total	**144**	**86 (59.7%)**	**63 (43.7%)**	**66 (45.8%)**

[Table/Fig-4]: Distribution of ESBL

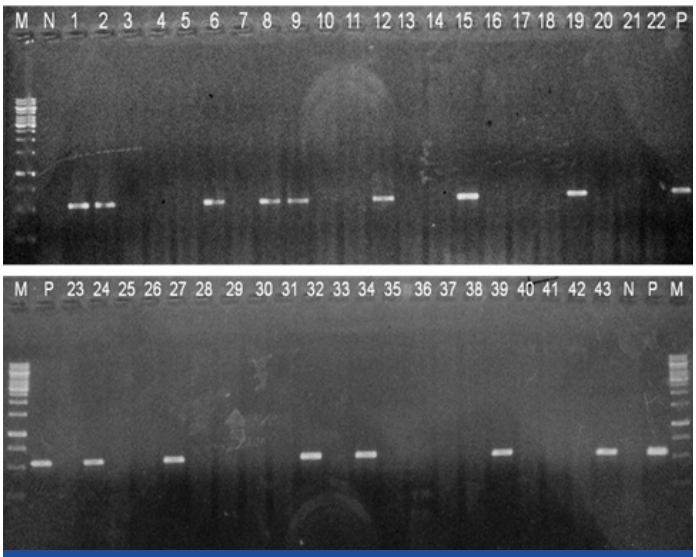


[Table/Fig-5]: PCR for bla TEM GENE

M - Marker 250bp-3000bp
No. 1-43 isolates lanes 4,5,8,10,11,18,19,25,26,28,29,33 *Klebsiella pneumoniae*
Lanes 1-3,12-17,21-24,30-32,34-43 *Escherichia coli*.



[Table/Fig-6]: PCR for bla SHV GENE
M - Marker 250bp-3000bp
No. 1-43 isolates lanes 4,5,8,10,11,18,19,25,26,28,29,33 *Klebsiella pneumoniae*
Lanes 1-3,12-17,21-24,30 *Escherichia coli*



[Table/Fig-7]: PCR for bla CTX-M GENE
M - Marker 250bp-3000bp
No. 1-43 isolates lanes 4,5,8,10,11,18,19,25,26,28,29,33 *Klebsiella pneumoniae*
Lanes 1-3,12-17,21-24,30-32,34-43, *Escherichia coli*

Sl. No.	Isolate	bla TEM	bla SHV	bla CTX-M	bla TEM+ bla CTX-M	bla SHV+ bla CTX-M
1.	*Klebsiella pneumoniae*	02	10	12	00	00
2.	*Escherichia coli*	04	02	20	02	02

[Table/Fig-8]: Distribution of ESBL genes

DISCUSSION

Increasing resistance to third generation cephalosporins has become a cause of concern especially among Enterobacteriaceae that cause nosocomial infections including VAP. The prevalence of ESBL among members of Enterobacteriaceae constitutes a serious threat to current β lactam therapy leading to treatment failure and consequent escalation of costs of hospital stay. Among the wide array of antibiotics, β lactam are the most widely used for over 50% of all the systemic infections [4].

In our study 46.15% ESBL producers belonged to Enterobacteriaceae family. There have been reports of ESBL's from major hospitals in India and some of them have recorded the incidence to be as high as 60-68% [18, 19]. The high incidence of ESBL is a cause of concern to regulators of hospital antibiotic policy. Over reliance on third generation cephalosporins to treat gram negative bacterial infections is one of the prime causes for increased resistance to this class of antibiotics.

The difference observed in detection of ESBL positive isolates by two different methods may be justified by the lower sensitivity of phenotypic method and the influence of environmental factors on the incidence of resistance [20]. The lack of constant sensitivity of different phenotypic methods has been emphasized by some studies [21]. In contrast, the genotypic method using specific PCR amplification of resistance genes seems to have 100% specificity and sensitivity. The cost of molecular method is particularly reduced for the bacteria belonging to enterobactericeae family as their DNA is easily extractable by boiling method, a quick and cost effective DNA extraction method [20].

The ESBL producers usually carry multiple resistant plasmid. In our study 4 isolates of *Escherichia coli* bla TEM + bla CTX-M and bla SHV+ bla CTX-M. Similar results have been reported by Yazdi M et al., and Yuan et al., [20, 22]. Most ESBLs are derived from plasmid mediated penicillinases belonging to the TEM and SHV families. Recently the CTX-M group with a typical ESBL resistant phenotype which does not originate from TEM and SHV families has been described. The CTX-M group is a new family of plasmid mediated ESBL they preferably hydrolyse cefotaxime have been recognized by Xiong et al., [23].

In the present study bla CTX-M was the common gene in 48.5% strains followed by 22.22% bla SHV and 14.81% bla TEM. Similar results have been obtained in many studies in India and other countries [7]. In a study conducted from 10 Indian sites in six Indian cities by Welsh et al., [24] bla CTX-M was the most common genotype isolated. Similar results have been documented in Europe, Latin America and other countries [25]. The CTX-M genotype, originating from chromosomally encoded enzymes of the Kluyvera spp, has risen in predominance especially in *Escherichia coli* and *Klebsiella pneumoniae* they have greater ability to spread and cause outbreaks [7].

The presence of more than one gene type in some of the isolates like bla TEM+ bla CTX-M means that the ESBL producing strains may be related to complex antimicrobial resistance. bla TEM producing TEM-1 is a broad spectrum β-lactamase that is always combined with CTX-M in the same plasmid [26]. It is important to continuously monitor resistance trends and enhancing the infection control of these pathogens in health care units.

CONCLUSIONS

Our findings suggest that the presence of bla CTX-M as the predominant genotype in *Klebsiella pneumoniae* and *Escherichia coli* isolates even in small town as ours. This study documents the presence of CTX-M type β lactamase even in hospitals of a small urban town in Karnataka. Incorrect identification of antibiotic resistance may lead to inappropriate antibiotic prescription, which in turn may direct bacteria to produce new resistance genes by selective pressure. The high incidence of ESBL is a cause of concern to regulators of hospital antibiotic policy. The genotypic method using specific PCR amplification of resistance genes seems to have 100% specificity and sensitivity. Therefore, the genotypic method is suggested to be used as the method of choice for detection of ESBL producing strains.

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