Int J Pharm Bio Sci 2012 Oct; 3(4): (B) 782 - 788

Research Article **Microbiology**

International Journal of Pharma and Bio Sciences

ISSN 0975-6299

OCCURRENCE OF CTX-M AND SHV GENES IN ESBL PRODUCING GRAM NEGATIVE ORGANISMS CAUSING PYOGENIC INFECTIONS IN A TERTIARY CARE HOSPITAL IN PUDUCHERRY.

DHANDAPANY SENTHIL PRAGASH*[*1], MANGAIYARKARASI[2], LATHA RAGUNATHAN[3], SAGEERA BANOO[3], VIJAYA RAYAPU[1] AND IVVALA ANAND SHAKER[4]

[1]Department of Microbiology, Melmaruvathur Adhiparasakthi Institute of Medical Sciences,Melmaruvathur, Tamilnadu, India.
[2]Department of Microbiology, Sri ManakulaVinayagar Medical College, Pondicherry, India.
[3]Department of Microbiology, Aarupadai Veedu Medical College, Pondicherry, India.
[4]Department of Biochemistry, Melmaruvathur Adhiparasakthi Institute of Medical Sciences, Melmaruvathur, Tamilnadu, India.

ABSTRACT

An extended spectrum beta lactamase (ESBL) production among gram-negative bacilli has emerged as a significant mechanism of resistance to oxyimino-cephalosporin antibiotics during the last 3 decades. Detection of common ESBL genes such as CTX-M and SHV by molecular methods in ESBL-producing bacteria and their pattern of antimicrobial resistance can provide useful information about its epidemiology and aid in rational antimicrobial therapy. A total of 138 multi drug resistant clinical isolates of *Enterobacteriaceae* family obtained from pyogenic lesions were included in the study. Antibiotic susceptibility test, Phenotypic confirmatory methods, Minimum inhibitory concentration and detection of CTX-M and SHV genes by Polymerase chain reaction were performed. Out of 138 samples, 84 were ESBL producers. Among the 84 isolates CTX-M gene was seen in 55 (65.5%) isolates, SHV was seen in 16 (19%) isolates and both CTX-M and SHV was seen in 14 (16.7%). A strict monitoring mechanism of antimicrobial resistance is necessary because of the high rate of prevalence of CTX-M and SHV genes among gram negative organisms causing clinically significant infections.

KEY WORDS: CTX-M, SHV, Extended spectrum beta lactamase, ESBL, Polymerase chain reaction.

DHANDAPANY SENTHIL PRAGASH
Department of Microbiology, Melmaruvathur Adhiparasakthi Institute of Medical Sciences, Melmaruvathur, Tamilnadu, India.

INTRODUCTION

Extended spectrum beta lactamases (ESBLs) are the rapidly evolving group of beta lactamase enzymes produced by the Gram negative bacteria, which have the ability to hydrolyse all cephalosporins and aztreonam but are inhibited by clavulanic acid[1]. Most ESBLs are generally mutants of classical *TEM* and *SHV* genes[2]. During the last 3 decades, extended spectrum beta lactamases found in gram-negative bacilli have emerged as a significant mechanism of resistance to oxyimino-cephalosporin antibiotics.[3] The increased use of extended spectrum cephalosporins has led to the emergence of resistant strains and outbreaks due to these organisms have been associated with higher morbidity and mortality[4].

Mostly, ESBL-encoding genes are located within transposons or integrons, which strongly facilitates antibiotic-resistant gene transfer between bacterial species resulting in cross-transmission, thereby spreading resistance among related and unrelated gram-negative bacteria[5]. Several molecular methods are available for research and epidemiological studies of CTX-M and SHV derivatives, including polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis, oligotyping, PCR single-strand conformational polymorphism and ligase chain reaction.[6] Detection of common ESBL genes such as CTX-M and SHV by molecular methods in ESBL-producing bacteria and their pattern of antimicrobial resistance can provide useful information about its epidemiology and aid in rational antimicrobial therapy.[7]

Hence, the present study was undertaken to determine the prevalence of CTX-M and SHV genes responsible for ESBL production amongst gram negative organisms causing pyogenic infections in a tertiary care hospital in Puducherry.

MATERIALS AND METHODS

This study was conducted in the Department of Microbiology, Aarupadai Veedu Medical College, Puducherry over a period of 18 months (October 2008 to April 2010). Institutional ethical committee permission was sought for the study.

A total of 138 multi drug resistant clinical isolates of *Enterobacteriaceae* members obtained from pyogenic lesions were included in the study. These were identified based on colony morphology and by standard biochemical tests.[8] Antibiotic susceptibility test was performed by disc diffusion technique, using Ampicillin 10 µg, Amikacin 30 µg, Gentamicin 10 µg, Piperacilin 100 µg, Piperacilin/Tazobactam 100/10 µg, Ceftazidime 30 µg, Ceftriaxone 30 µg, Ciprofloxacin 5 µg, Imipenem 10 µg, Cefoxitin 30 µg (HiMedia, Mumbai), as per standard CLSI guidelines.[9] For confirmation of ESBL production from these samples, two methods, namely Double Disc Synergy Test (DDST)[10] and Phenotypic confirmatory disc diffusion test (PCDDT)[11] were used. Minimum inhibitory concentration was determined by agar dilution method by incorporating serial two fold dilutions of Ceftazidime (1 µg/ml to 256 µg/ml) according to CLSI guidelines.[11]

Genotypic detection methods

For the detection of CTX-M and SHV genes PCR (Polymerase chain reaction) was done to all the 84 isolates.

Plasmid extraction was done according to the procedure of Subhash Chandra Parija**:**[12] Inoculate single colony of ESBL strain into 2ml Luria broth (HiMedia, Mumbai), incubate overnight. Take 1.5ml of the broth into microcentrifuge tube, centrifuge at 12,000g for 2 minutes. Discard supernant. Suspend pellet

in 100µL of ice cold solution A (50mM glucose, 25mM trischloride, 10mM EDTA) and mix. Add 200 µL solution B (0.2N NaOH, 1%SDS) and mix by inverting tube rapidly. Add 150 µL if ice cold solution C (5M potassium acetate, Glacial acetic acid, distilled water) and mix by inverting tube. Store the tube in ice for 5 minutes. Centrifuge at 12,000g for 5 minutes at 45^{o}C. Transfer the supernant to fresh tube. Precipitate the DNA with 2 vol of 100% ethanol at room temperature. Mix and allow standing for 2minutes. Centrifuge at 12,000g for 5 minutes at 4^{o}C and remove supernant. Allow air dry. Suspend the pellet in 50 µL of 1X TE (pH8) containing RNAsae.

PCR: amplification for CTX-M and SHV gene was carried out with specific primers procured commercially (Medox Biotech India Pvt Ltd, Chennai), using specific parameters as shown in Table 1.

Table 1
Primers and PCR conditions used for CTX-M and SHV.

PCR Target	Primer name	Oligonucleotide sequence	Reaction parameters
CTX-M[13] (according to SA Jemima)[13]	CTX-M (F)	5' ATG TGC AGY ACC AGT AAR GTK ATG GC 3'	Initial denaturation at 97^{o}C for 7 minutes. Denaturation at 94^{o}C for 50 seconds.
	CTX-M (R)	5' TGG GTR AAR TAR GTS ACC AGA AYC AGC GC 3'	Annealing at 50^{o}C for 40 seconds. Elongation at 72^{o}C for 60 seconds. Repeated for 35 cycles. Final extension at 72^{o}C for 5 minutes.
SHV[14] (According to Ju-Hsin Chia)[14]	SHV (F)	5' AAC GGA ACT GAA TGA GGC GCT 3'	Initial denaturation at 94^{o}C for 2 minutes. Denaturation at 94^{o}C for 60 seconds.
	SHV (R)	5' TCC ACC ATC CAC TGC AGC AGC T 3'	Annealing at 62^{o}C for 60 seconds. Elongation at 72^{o}C for 1 minute. Repeated for 35 cycles. Final extension at 72^{o}C for 10 minutes.

Table 1 shows the PCR targets, oligonucleotide sequences for the primers and their reaction parameters during thermocycling.

In a PCR tube using thermocycler, amplification was done in 25µL volume containing master mix 5 µL, template DNA 2 µL, forward primer (CTX-M / SHV) 1 µL.

Gel electrophoresis: after amplification, the resulting PCR products were run in 1.2% agarose gel containing 1X TAE and 0.5 µg of Ethidium bromide/ml and visualized under UV

transilluminator. The PCR band was visualized at 543 bp for CTX-M and 141 bp for SHV gene.

SHV gene: Gene bank accession No. AF124984.

Nucleotide positions: 486-506 (SHV-F) and 626-605 (SHV-R).

RESULTS

Out of 138 isolates tested for ESBL production by both methods, 84 (60.9%) were ESBL producers. All the 84 ESBL producers were preceded with determination of MIC for Ceftazidime and detection of CTX-M and SHV gene. Minimum inhibitory concentration for Ceftazidime among ESBL producing Enterobacteriaceae is shown in Table 2.

Table 2
Minimum inhibitory concentration for Ceftazidime among ESBL producing Enterobacteriaceae.

S.No.	Concentration	Number	Percentage
1	256	80	95.2 %
2	>256	4	4.8 %
	Total	84	100 %

Table 2 shows number of organisms showing minimum inhibitory concentration of more than 256 and their percentages for Ceftazidime. Total number of ESBL producing organisms were 84.

Distribution of CTX-M and SHV gene among 84 isolates

Among the 84 isolates CTX-M gene was seen in 55 (65.5%) isolates. In Escherichia coli 28 out of 38 were positive, accounting for 73.7%. In Klebsiella species 23 out of 31 were positive (74.2%). In Proteus and Citrobacter only 2 isolates each were positive.

SHV gene was seen commonly in Klebsiella species, 14 isolates were positive, accounting for 45.2%. Only 2 isolates of Escherichia coli was positive but none of the Proteus and Citrobacter species were positive. The distribution of CTX-M and SHV is shown in Table 3.

Table 3
Distribution of CTX-M and SHV.

S. No.	Organism	Number (n)	CTX-M		SHV		Both CTX-M and SHV	
			+ ve	%	+ ve	%	+ ve	%
1	Escherichia coli	38	28	73.7	2	5.3	2	5.3
2	Klebsiella spp	31	23	74.2	14	45.2	12	38.7
3	Proteus spp	9	2	22.2	0	0	0	0
4	Citrobacter spp	6	2	33.3	0	0	0	0
	Total	84	55	65.5	16	19	14	16.7

Table 3 showing the Distribution of CTX-M and SHV among various organisms and their individual percentage positivity for CTX-M, SHV and both CTX-M & SHV.

Both CTX-M and SHV genes were seen in 14 (16.7%) isolates. Out of which 12 (38.7%) were Klebsiella species and 2(5.3%) was Escherichia coli.

Statistical analysis of Data:
The results were analysed statically using Chi-square test at appropriate places.

DISCUSSION

CTX-M type ESBLs are a global concern for infectious disease clinicians and clinical microbiologists. Over the past decade, these enzymes have become the mast common ESBLs in many widely dispersed geographical areas. Recently, a number of molecular biological methods have been proposed for the identification of CTX-M and SHV derivatives. The disadvantages of these molecular biology based methods, which include labour expenditure, cost and lack of general applicability, have outweighted their advantages and have so far prevented their broad acceptance. PCR allows the non-laborious, reliable detection and quantification of most nucleic acid target sequences.[6]

Among the newer ESBL families, the CTX-M type MLSBs have become widely dispersed in many parts of the world.[15] PCR for the 84 ESBL positive revealed CTX-M was demonstrated in 55 (65.5%). S.A. Jemima *et al*[13] showed only 15.83% positive. In another study by S Baby Padmini *et al* out of 23 isolates 19 were positive.[15] 87.3% were encoding CTX-M in Taiwan showing the high endemicity in that country.[17] Our study has documented the presence of CTX-M beta lactamase as predominant ESBLs in isolates of Escherichia coli and Klebsiella spp.

SHV gene was demonstrated in 16 isolates (19%). S.A. Jemima *et al*[13] detected in 41 of 200 isolates (20.5%). A study by Chia *et al*[14] in Taiwan harbor 108 of 142 (76%), Drik *et al*[16] detected 76% CTX-M, 22% SHV and 2% of both gene in UK. In another study by Jain *et al*[7] SHV alone was seen in 20.3%. A study by James *et al*[18] out of 64 isolates, 17 harbored SHV alone and both CTX-M and SHV gene.

In the present study, CTX-M gene was seen in 73.3%, among the ESBL positive Escherichia coli and 74.2% among Klebsiella species. Where as in Taiwan it was 99.6% and 92.2% respectively.[17]

Out of 84 isolates tested, both CTX-M and SHV gene was seen in 14 (16.7%). Whereas in a study in Taiwan by Ju-Hsin Chia *et al*[14] 17 out of 199 isolates harbored both CTX-M and SHV. SHV gene is frequently found in 7 out of 16 Klebsiella species isolated accounting for 43.8%. In a study by Elif *et al* SHV is found in 21.87%, CTX-M in 17.18%.[19]

CTX-M and SHV beta lactamases are mainly found in *E.coli* and *Klebsiella*

pneumoniae, but can occur in other members of the family *Enterobacteriaceae.* Because these strains become resistant to available antibiotics and they can pass the gene to other clinical strains, the quick detection of these strains in microbiology laboratories is very important. Molecular typing would determine which types of ESBL are present in each isolate. Molecular detection and identification of beta lactamases would be essential for a reliable epidemiological investigation of antimicrobial resistance. ESBL producing organisms should be identified quickly so that appropriate antibiotic usage and infection control measures can be implemented.[19]

CONCLUSION

This study has revealed a higher percentage of CTX-M and SHV genes among gram negative organisms, the design of rational infection control measures followed in this medical institution requires the adoption of new antibiotic policies in addition to improving hospital hygiene. Knowledge of epidemiological and anti-microbial susceptibility pattern of common pathogens in a given area helps to inform the choice of antibiotics. Latest epidemiological surveillance studies are required to provide useful information base to guide practice and policies on rational use of anti-infective agents and to eradicate the source of environmental reservoir.

Simple hygienic measures, such as hand washing practices, the use of sterile equipment (particularly for intravenous access) and patient cohorting (i.e., grouping patients with similar infections in the same location) can help prevent the further spread of these resistance traits. We suggest strict monitoring mechanisms of antimicrobial resistance as well as comprehensively evaluating the recently introduced antimicrobial agents for their *in vitro* activity.

REFERENCES

1. Kapil A. The challenge of antibiotic resistance: need to contemplate. Indian J Med Res, 121: 83 - 91, (2005).
2. Prabha Lal, Arti Kapil, Bimal K. Das & Seema Sood. Occurrence of TEM & SHV gene in extended spectrum b-lactamases (ESBLs) producing Klebsiella sp. Isolated from a tertiary care hospital. Indian J Med Res, 125: 173-178, (2007).
3. David L. Paterson, Kristine M. Hujer, Andrea M. Hujer, Bethany Yeiser, Michael D. Bonomo, Louis B. Rice, Robert A. Bonomo and the International *Klebsiella* Study Group. Extended-Spectrum β-Lactamases in Klebsiella pneumoniae Bloodstream Isolates from Seven Countries: Dominance and Widespread Prevalence of SHV- and CTX-M-Type β-Lactamases. Antimicrob Agents Chemother, 47(11): 3554 - 3560, (2003).
4. L B Rice, S H Willey, G A Papanicolaou, A A Medeiros, G M Eliopoulos, R C Moellering Jr and G A Jacoby. Outbreak of ceftazidime resistance caused by extended spectrum β –lactamases at a Massachusetts chronic care facility. Antimicrob Agents Chemother, 34: 2193 - 99, (1990).
5. Gniadkowski M. Evolution and epidemiology of extended-spectrum β –lactamases (ESBLs) and ESBL producing microorganisms. Clin Microbiol Infect, 7: 597-608, (2001).

6. MS Alfaresi, AA Elkoush. Real-time polymerase chain reaction for rapid detection of genes encoding SHV extended spectrum β lactamases. Indian J Med Microbiol, 28(4): 332-6, (2010).
7. Jain A, Mondal R. TEM and SHV genes in extended spectrum beta-lactamase producing Klebsiella species and their antimicrobial resistance pattern. Indian J Med Res, 128: 759-64, (2008).
8. Koneman EW. Allen SD, Janda WM, Schreckenberger PC, Win WC, Editors. The Enterobacteriaceae. In: Color atlas and textbook of Diagnostic Microbiology, 5th edition, Lippincott Publications: 171-230, (1997).
9. National Committee for Clinical Laboratory Standards. Performance Standards for Antimicrobial Susceptibility Testing: Nineteenth informational supplement: CLSI document M100-S19.Wayne PA: Clinical and Laboratory Standards Institute; 2009.
10. Jarlier Y, Nicolas MH, Fourier G, Phillippon A. Extended broad spectrum beta lactamases conferring transferable resistance to newer beta lactam agents in Enterobacteriaceae, hospital prevalence and susceptibility patterns. Rev Infect Dis, 10: 867-78, (1988).
11. Thomson KS, Sander CC. Detection of extended spectrum beta lactamases in members of the family Enterobacteriaceae: Comparison of double disc and three dimensional tests. Antimicrob Agents & Chemother, 16(9): 1877- 1882, (1992).
12. Subhash Chandra Parija. Isolation of plasmids. In: Textbook of Practical Microbiology. 1st Edition. Ahuja Publishing House, New Delhi: Lipee Scan Pvt Ltd: 145-146, (2007).
13. SA Jemima, S Verghese. Molecular characterization of nosocomial CTX-M type beta lactamase producing Enterobacteriaceae from a tertiary care hospital in South India. Ind J Med Microbiol, 26(4): 356 – 358, (2008).
14. Ju-Hsin Chia, Chishih Chu, Lin-Hui Su, Cheng-Hsun Chiu, An-Jing Kuo, Chien-Feng Sun1 and Tsu-Lan Wu . Development of a multiplex PCR and SHV melting curve for detection of some SHV and CTX-M beta lactamases of Escherichia coli, Klebsiella pneumonia and Enterobacter cloacae in Taiwan. J Clin Microbiol, 43(9): 44896 – 4491, (2005).
15. S Baby Padmini, B Appala Raju, KR Mani. Detection of *Enterobacteriaceae* producing CTX-M extended spectrum β-lactamases from a tertiary care hospital in south India. Indian J Med Microbiol, 26(2): 163-6, (2010).
16. Drik M. Leinberger, Verena Grimm et al. Integrated detection of extended spectrum beta-lactam resistance bt DNA microarray based genotyping of TEM, SHV and CTX-M genes. J Clin Microbiol, 48(2): 460 – 471, (2010).
17. Pattarachai Kiratisin, Anucha Apisarnthanarak, Chaitat Laesripa, and Piyawan Saifon. Molecular characterization and epidemiology of extended spectrum lactamase producing Escherichia coli and Klebsiella pneumonia isolates causing health care associated infection in Thailand, where the CTX-M family is endemic. J Antimicrob Agents Chemother, 52(8): 2818 – 2824, (2008).
18. James S. Lewis II, Monica Herrera, Brian Wickes, Jan E. Patterson and James H. Jorgensen. First report of the emergence of CTX-M type extended spectrum beta-lactamases (ESBL) as the predominant ESBL isolate in a US health care system. Antimicrob Agents Chemother, 51(11): 4015 – 4021, (2007).
19. Elif Burcu Bali, Leyla Acik and Nedim Sultan. Phenotypic and molecular characterization of SHV, TEM, CTX-M and extended spectrum betalactamase produced by Escherichia coli, Acinetobacter baumannii and Klebsiella isolates in a Turkish hospital. Afr J Microbiol Res, 4(8): 650 – 654, (2010).