PREVALENCE OF EXTENDED SPECTRUM β-LACTAM RESISTANCE AMONG BACTERIA CAUSING HOSPITAL INFECTIONS - DETECTION USING PCR

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ABSTRACT

Resistance to contemporary broad spectrum â-lactams mediated by extended spectrum â- lactamases (ESBL) is an increasing problem world wide. The epidemiology of ESBL producing Enterobacteriaceae and the genotypic determinants in an ICU setting in India is largely unknown. The available phenotype tests can sometimes be misleading as more than one type of resistance can be detected in a single isolate or more than one gene is harbored by the same isolate. In the present study, fourteen isolates of E. coli and K. pneumoniae that were antibiotic resistant in a E-strip test were selected. Presence of the ***bla***$_{TEM}$ *and* ***bla***$_{SHV}$ *genotypes among these isolates was screened by PCR. While, four resistant isolates showed no specific amplicons for these two genes, four others carried both the genes. The others either carried the TEM or the SHV type only. This first report from India, clearly highlight the epidemiology of the prevalent genes among the isolates from our ICU.*

Key words : *beta lactamases, polymerase chain reachion*

INTRODUCTION

Hospitals have always acted as a source of infection to patients admitted to them. The concept of asepsis and its application in hospital practice have reduced their incidence but hospital infections still cause considerable morbidity and mortality. It is much higher in crowded hospitals in the developing countries. Even when hospitalization does not lead to obvious infection, it causes a change in the patient's microbial flora. The normal flora is gradually replaced by the drug resistant micro- organisms typical of the hospital environment. In recent decades, the enteric gram negative bacilli E. coli, Klebsiella, Enterobacter, Proteus & Serratia have become the most important group of hospital pathogens in addition to Pseudomonas species and the Gram positive Staphylococci.

Emergence of resistance to antibiotics among the Enterobacteriaceae has serious implications in hospitals and for therapy. Of the many types evident the resistance to β-lactam antibiotics is widespread. Routine methods to detect it include, isolation and culture of these bacteria from patient samples and determining their range of resistance by disc diffusion assays or MIC's. Multiple drug resistance is known in all these species. In most instances this resistance is carried by genes present on plasmids or transposons and on chromososmes [1-4] ([1]et al., 2002; Arlet, et al., [2]1995; [3]1990, [4]2003). Plasmids and transposons being mobile are responsible for the spread of multiple drug resistance among bacteria. The mechanism of drug resistance encoded by these genes includes decreased permeability of drugs, alternative metabolic pathways and production of enzymes that inactivate the drugs.

Of the three mechanisms known, the occurrence of enzymes e.g., β- lactamases (Medeiros, [5]1984) that degrade the lactam ring of the antibiotics is the most common among gram negative bacteria. The first beta-lactamase was identified in an isolate of Escherichia coli in 1940. To date, there are >130 TEM-type and >50 sulfhydryl variable (SHV)-type beta-lactamases, mainly in E. coli, K pneumoniae, and Proteus mirabilis but also in other members of the Enterobacteriaceae family and in some nonenteric organisms, such as Acinetobacter species.[6,7] With emergence of resistance to penicillins, use of broad spectrum antibiotics such as cephalosporins became widespread. But within 5 years of their use in 1983, transferable resistance to these antibiotics in clinical isolates of K. pneumoniae and Serratia marcescens was reported.[8-10] Resistance to these drugs was a result of a new class of β – lactamases, the CTX-type that were similar in sequence to bla$_{TEM}$ but with a few amino acid changes that extended its substrate range for hydrolysis. [6, 11] These β – lactamases differed from TEM types by hydrolysis of cefotaxime or ceftriaxone but were inhibited by clavulanate and cloxacillin. These new class of enzymes called as Expanded spectrum of β – lactamases (ESBLs) are enzymes that mediate resistance to β-lactam antibiotics such as penicillin, extended spectrum (third generation) cephalosporins and monobactams by catalyzing the hydrolysis of -lactam and could inactivate antibiotics with oxyimino groups. Most ESBLs are derivatives of TEM type or SHV type enzymes encoded by ESBL plasmid.

ESBL-producing organisms frequently also possess resistance factors to other classes of antibiotics, such as aminoglycosides and fluoroquinolones, and possibly also piperacillin-tazobactam and cefepime. Different TEM variants were found to coexist within the same cells. Again,

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a patient could harbor two or three different strains that encoded the same enzyme or two indistinguishable isolates that produced distinct TEM, β - lactamases.[3]

The incidence of expanded-spectrum beta-lactamases (ESBLs) varies; depending on which area of the globe the isolates originate from. ESBLs render the oxyimino-cephalosporins ineffective, and hence it is important to know the type of beta lactamase that is prevalent in the clinics. It is, therefore, imperative that microbiology laboratories should routinely test for the presence of antibiotic resistant strains among their isolates and determine the epidemiology of the prevalent types.

In many countries, ESBLs are expressed in 10%-40% of E. coli and K pneumoniae isolates. (ref) In Indian hospitals, several studies on drug resistance among Gram –ve bacteria have been made but most describe their phenotypic response. Recently studied drug resistance among Staphylococcus aureus (MRSA) isolates report the occurrence of both ESBL's and pencillin binding protein genes in these isolates by PCR.[12]

Routine screening for drug resistance is carried out at the Microbiology department of SRMC and RI (DU) by disk diffusion assay, E-tests and MIC. However the molecular type of this resistance is largely unknown and has not been attempted so far except for the brief study by Sireesha (2005).

In this study, gram negative strains were collected from C4 ICU (SRMC&RI) –patient's sample and their ESBL production was determined using double disk method. The minimum inhibitory concentration (MICs) for various antibacterial agents was determined using E test strips. The presence of genes to confirm the production of ESBLs was carried out using gene specific primers. Results and the protocols to detect the TEM type or the SHV type ESBL's are presented here. The prevalence of the specific types of resistance in these isolates and their molecular genotypes are reported here for the first time.

2. MATERIALS AND METHODS

General molecular biology protocols as recommended in Sambrook et al., 2000[15] were followed. All the general chemicals used for molecular techniques were purchased from Sigma or Himedia. DNA polymerase and dNTP's were purchased from Invitrogen, USA or Genie (Bangalore, India).

CULTURES: Bacterial strains used in the present study were collected from C4 – ICU (SRMC & RI) during 2004. First, samples of five different bacteria were chosen to optimize plasmid isolation protocols. They were:

Name of strain	Strain No.	Expected Plasmid
E. coli	SRMC-26	bla_{TEM} Plasmid (>12kb)
K. Pneumonia	SRMC-20	bla_{SHV} Plasmid (>12kb)
E. coli	SRMC-3	bla_{TEM} Plasmid (>12kb)
E. coli	ATCC(25923)	No Plasmid
K. Pneumonia	ATCC(70603)	Plasmid (>12kb)

Isolates obtained from the clinical specimens include the following:

Strain No.	Nature of isolates
Klebsiella	
U9316	Urine sample
E4177	Wound exudates
E4119	Wound exudates
R1738	Respiratory sample
R1756	Respiratory sample
E4146	Wound exudates
E4140	Wound exudates
E. coli	
R1966	Respiratory sample
R1703	Respiratory sample
U8192	Urine sample
R1759	Respiratory sample
U8688	Urine sample
E4159	Wound exudates
E4098	Wound exudates

They were tested for production of β-lactamases using strip E-tests based on the recommendation of the National Committee on Clinical Laboratory Standards (NCCLS[14]; 2003;)[1].

GROWTH AND MAINTENANCE: All isolates were routinely maintained on appropriate antibiotic containing medium. Luria Bertani media (LB; Sambrook; et al. 2000) amended with the antibiotic, ceftazidime (LCA) at a concentration of 5 mg/ml was used routinely to maintain the isolates. Agar at 15 g/L was used to solidify.

ISOLATION OF PLASMID (Alkaline Lysis Method;[15]: Briefly the method involved inoculating liquid cultures with single colony of the isolates in LB broth (50ml) containing ceftazidime (5ppm). They were grown overnight at 37°C in an orbital shaker and the OD was checked at 600nm. Cell lysis and denaturation involved an alkaline lysis buffer. DNA was separated from the solution with potassium acetate. After obtaining the plasmid DNA it was resuspended in 100 ìl of TE buffer. A sample (7ml) was then loaded on agarose gel (0.8%) and electrophoresis was carried out for 45 min at 70V and 50 mA and Tris

Acetic acid and EDTA buffer (pH 8.0). Spectrophotometric quantification of DNA (UV-Visible Spectrophotometer, Techcomp, India) at 260 and 280nm was done.

PCR for bla_{TEM} and bla_{SHV} genes: Plasmids isolated from the antibiotic resistant clinical isolates were used for amplification using TEM and SHV primers. The primers were synthesized from Microsynth, Switzerland. The details of the sequences are:

SHV-OS/F1 (20mer), 5′- TTATCTCCCTGTTAGCCACC-3′ and SHV-OS/R1 (20mer)- 5′-GATTTGCTGATTTCGCTCGG-3′ for the SHV β-lactamase genes (Arlet, 1997).

For the TEM b -lactamase genes (Mabilat et al., 1990) the primers used were:

TEM/F1 (17mer)- 5′- ATAAAATTCTTGAAGAC-3′ and TEM/R1 (17mer)- 5′- TTACCAATGCTTAATCA3′. The PCR reaction mix of 20 uL contained Forward and Reverse primers 5 picomoles each; (1 uL); 2 uL of Buffer (10x; 15mM); dNTPs (2 uL; 2.5mM); plasmid DNA (50 ng); 0.2 uL of Taq Enzyme (1Unit) and the total volume was made up with 12.8 uL of water. The PCR was run using the following conditions in a thermal cycler (MJ Research, USA). Initial denaturation -94^{0}C for 5 minutes was followed by PCR of 35 cycles that included denaturation at 94^{0}C for 1 minute; annealing at 41^{0}C for1 minute; extension at 72^{0}C for 7 minutes; followed by a final extension at 72^{0}C for 10minutes. After the completion of the amplification, the PCR products were checked on 0.8 % agarose gel run in TAE buffer.

RESULTS

Phenotypic characterization of antibiotic resistance of isolates

Seven isolates of E. coli and seven isolates of Klebsiella were tested for growth on antibiotics by disc diffusion and MIC. Their response to antibiotics Ceftazidime (ca), Cephatoxime (ce), Cefoperazone (cfs with sulbactum), Cefoperazone, (cs), Tazobactum with piperacillin (tzp) was done using E strips. Data presented in Table1 clearly confirmed that these isolates were resistant to the tested drugs. Klebsiella isolates showed a MIC as high as >2048 mg/ml when tested for cs. Among the E. coli isolates highest MIC of >2048 mg/ml was exhibited for ce and cs. These results confirm the existence of resistance among these clinical isolates.

Molecular analysis of drug resistant bacteria: Plasmids were obtined from these isolates (Fig. 1) and genotypes, TEM and SHV, were characterized using PCR technique. Yields of plasmid DNA ranged from as low as 1.8 ìg/μL to as high as 24.8 ìg/ μL. All the clinical isolates showed one high molecular weight plasmid and 1-4 forms of the plasmid(s) moving faster on the gel. These plasmid preparations were then amplified by PCR using the primers for bla_{TEM} and $bla_{SHV.}$ The PCR amplified products were resolved on a gel and isolates positive for bla_{TEM} showed an amplicon of ~ 1.2 Kb (Fig. 2; lane 3) while those positive for bla_{SHV} produced an amplicon of ~ 0.9kb (Fig 2; lane 5).

The results from the PCR analysis for all the fourteen isolates are compared with their drug resistance profile in Table1. The seven Klebsiella isolates were tested with both sets of primers. The analysis presented in Table 1 and Fig. 3, 4 shows that out of the 7 isolates, one isolate (R1738) did not show any amplicon with either primer but was drug resistant. Three had both bla_{TEM} and bla_{SHV} (e.g., isolate E4119; Fig. 3a, lane 3- SHV +ve and Fig. 4 a; lane 5- TEM +ve) encoded in their plasmids. Two carried only the bla_{TEM} gene (Fig. 4a; lane 6) and one was positive for bla_{SHV} (Fig. 3b; lane 7). The known test E. coli strain SRMC-26 carrying the bla_{TEM} was positive (Fig. 4b). Out of the seven clinical E. coli strains, three were negative with both sets of primers; two were positive for only bla_{TEM} (Table 1 and Fig. 4a; lanes 3,4). One however, carried both genes bla_{TEM} and bla_{SHV} resistance (Fig. 3a; lane 8) and one was positive for bla_{SHV} (Fig. 3b; lane 4).

DISCUSSION

The prevalence of ESBL among clinical isolates varies from country to country. They can be seen in the community setting as well. Because of their complexity and substrate specificity their detection is a major challenge faced by laboratories.

ESBL producing bacteria are typically associated with multiple drug markers (because genes for other mechanisms of resistance often reside on the same plasmid as the ESBL genes.). Apart from resistance to b – lactams they can have resistance to quinolones, aminoglycosides and Trimethoprim sulphamethoxaczole. Infection with ESBL producing bacteria can result in avoidable treatment factor with resultant increase in the care of patient case and prolonged hospital stay.

Earlier reports on evaluation of resistance to antibiotics also studied the presence of the β-lactamase and the occurrence of the responsible genes bla_{TEM} and bla_{SHV} either in plasmids or the chromosomes. In this study, we screened for the presence of a likely plasmid in these resistant strains as all were antibiotic resistant to a range of antibiotics [Ceftazidime (ca), Cephatoxime (ce), Cefoperazone (cfs with sulbactum), Cefoperazone, (cs), and Tazobactum with piperacillin (tzp)]. Among the 14 isolates tested for the presence of TEM or SHV type b – lactamases, four of them (one Klebsiella and three E. coli)did not show any PCR products but were however resistant to various antibiotics (Table 1). These isolates may have other mechanisms of resistance or it may reside in its chromosome.

A high MIC indicates that the isolate is resistant to the drug but resistance can be mediated by many mechanisms and ESBL is one such. Since it is a transferable, it is important to recognize the type. E-test helps us in knowing the MIC rapidly. Again it has to be

confirmed by agar dilution or broth dilution method which is very cumbersome and best done only in lots or batches.

There is no data on the prevalence of TEM, SHV type β-lactamase in India (except for the preliminary report by Sirashee, 2005). Incidence of ESBL reported from various centres in India is largely based on the phenotypic tests. A knowledge of the genotypic pattern is of importance in the molecular epidemiology of the resistant types and this study has helped us distinguish the occurrence of these genes in the clinical isolates from our ICU.

Acknowledgements:

The authors wish to thank the management of SRMC and RI (DU) for their monetary support, facilities and their encouragement to carry out research. We also wish to thank the Prof. K V Somasundaram, Dean of Faculties for his constant appreciation, motivation and guidance during this effort. T S Lokeswari thanks Ms Anandhi J, Mr Sivanandan and Mr Shyam Sundar, research scholars, for helping us with the techniques and assistance for photography.

Table1: Minimum Inhibitory Concentration (MIC) value of various clinical isolates and the corresponding amplification products with primers for bla_{TEM} and bla_{SHV}

Strain No.	MIC value (μg/ml)					PCR results(this study)
	ca	ce	cfs	cs	tzp	
Klebsiella						
U9316	1024	2048	4	>2048	128	Þ TEM (+)
E4177	128	>1024	32	1024	256	Þ TEM(+)
E4119	512	>1024	12	1024	512	Þ, æ Both +ve
R1738	1024	1024	>256	>2048	1024	Both -ve
R1756	>1024	>1024	16	>2048	512	Þ, æ Both +ve
E4140	>1024	>1024	>256	>2048	512	Þ, æ Both +ve
E4146	>1024	>1024	>256	>2048	512	Æ SHV(+)
E. coli						
R1759	512	1024	2	4	128	Æ SHV(+)
U8688	1024	>1024	8	>2048	256	Both -ve
E4159	1024	>1024	16	>1024	512	Both -ve
E4098	512	>2048	32	>2048	128	Þ TEM(+)
R1966	256	512	4	>2048	64	Þ TEM(+)
R1703	512	>1024	2	1024	128	Both -ve
U8192	128	>1024	2	1024	32	Þ Both +ve

Both - TEM & SHV; +ve / -ve: Presence/ absence of an amplicon; Sizes of amplicon include ~ 1.2kb for bla_{TEM} (Þ) and ~0. 9 kb for bla_{SHV} (Æ)). Note that in four isolates both TEM and SHV could not be amplified. Antibiotics tested are:

ca - Ceftazidime; ce - Cephatoxime; cfs - Cefoperazone with sulbactum; cs - Cefoperazone

tzp - Tazobactum with piperacillin

FIG.1. Presence of a plasmid (s) seen in drug resistant clinical isolates of E. coli (EC) seen in panel (a)- lanes2,6,10: SRMC 26; lanes4,8- SRMC 3; and in panel (b)- lanes 4,5,6 – R1759,U8688 & E4159. Plasmid(s) of Klebsiella (Kl) are shown in panel (a)- lanes 3,7 - SRMC 20; lane 5, 9 - ATCC 70603 (with plasmid); and in panel (b)- lane 2, 3 &7 - R1738; R1756 & E4146. The lane 1 in both panels contained ë DNA / Hin dIII digest. Note the presence of more than one plasmid in some isolates. EC

FIG. 2: A comparison of the sizes of the PCR products on a 1.3% gel from both genes, bla_{SHV} and bla_{TEM} indicating that they are of expected lengths. The lanes were loaded with lane 1- 100bp ladder; lane 2- (TEM +ve; 1.2 kb); lane 3- (SHV +ve; 900 bp); lane 4 – negative control. EC- E. coli; Kl- Klebsiella.

FIG. 3: Detection and identification of $bla_{SHV\ (->)}$ genes by PCR was seen in isolates of E. coli (EC)-lane 8 (panel a) and lane 4 (panel b). Isolates of Klebsieela (Kl) that were positive ca be seen in lane 9 (panel a); lane 3,6,7 (panel b). Lane 1- Lambda DNA/Hind III digest.

FIG: 4 Detection and identification of bla_{TEM} (->) genes by PCR seen in panel (a) of isolates of E. coli (EC)- lane 3,4 and in isolates of Klebsiella (Kl)- lane 5, 6. A positive E. coli sample (SRMC –26) showed a ~1.2 Kb product (panel b). Lane 1: Lambda DNA/Hind III digest (panel a) or a 1.0 Kb ladder (panel b).

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