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Research Article

PREVALENCE OF CEPHALOSPORIN-RESISTANT GRAM-NEGATIVE BACILLI FROM CLINICAL SAMPLES

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ABSTRACT

Objective: Beta-lactams are the group of antibiotics that contain a ring called as "beta-lactam ring," which is responsible for the antibacterial activity. The presence of resistance among Gram-negative organisms is due to the production of beta-lactamases enzymes that hydrolysis the beta-lactam ring thereby conferring resistance to the organism. This study is undertaken to determine the prevalence of extended-spectrum beta-lactamase (ESBL) producing Gram-negative organism from clinical samples.

Methods: A total of 112 clinical samples were taken for this study. The combined disc synergistic test (CDST) was used for the phenotypic detection of ESBL producers from the clinical samples. The genotypic identification of ESBL producers was carried out by alkaline lysis method by isolation of plasmid DNA.

Result: A total of 87 bacterial isolates were isolated and identified. Among them, *Klebsiella* (41%) was the predominant organism followed by *Escherichia coli* (33%), *Proteus* (10%), *Pseudomonas* (10%), and *Serratia* (6%). Among the various bacterial isolates, *Klebsiella* showed a higher percentage of resistance. The CDST showed that 8 isolates of *Klebsiella,* 3 isolates of *E. coli,* and 1 isolate of *Pseudomonas* were found to be ESBL producers. The genotypic confirmation showed that the two bacterial isolates, namely, *Klebsiella* and *E. coli* were found to possess temoniera (TEM) gene which was the 400-500 bp conferring resistance to the antibiotics.

Conclusion: The results of this study suggest that early detection of ESBL producing Gram-negative organism is a very important step in planning the therapy of patient in Hospitals. CDST continues to be a good indicator in the detection of ESBL producers.

Keywords: Beta-lactamases, Gram-negative bacilli, Extended-spectrum beta-lactamase, Resistance, Combined disc synergistic test.

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INTRODUCTION

Antibiotic resistance poses an important medical problem to public health as it poses a great challenge to microbiologist and pharmacologist in the treatment of infectious diseases caused by resistant organisms. Beta-lactam antibiotics are mostly used for the treatment of these infections in the hospitals and health-care centers due to its broad spectrum of activity [1,2]. Frequent and improper usage of beta-lactam antibiotics leads to the development of resistance in the Gram-negative organism [3]. These resistant bacteria produce beta-lactamases enzymes which hydrolyze the amide bond of the beta-lactam ring. Beta-lactamases have been categorized into four groups based on their functions, namely, (a) Penicillinase, (b) extended-spectrum of beta-lactamases (ESBLs), and (c) AmpC and carbapenems [4].

Extended-spectrum beta-lactamase (ESBL) is a plasmid-mediated beta-lactamase which can resist and hydrolyze the first generation penicillins, second- and third-generation cephalosporins, and aztreonam. Nowadays, these enzymes hydrolyze even the fourth-generation cephalosporins [5]. However, clavulanic acid (CAC) inhibits the activity of ESBLs because they are the beta-lactamase inhibitors [6]. ESBL is generally produced by *Klebsiella* spp., *Escherichia coli,* and other Gram-negative bacilli. ESBL-Eb (Enterobacteriaceae) is not only reported to be isolated from hospitals but they are also present in our environments in the farm animals and derived foods, domestic and even in wild animals, healthy humans, wastewater, vegetables, etc. [7].

Unsuitable treatment of infections caused by ESBL producers has been increased mortality and hospital stay. Obligatory, we should confirmation of bacterial resistance pattern before starting treatment in health care and hospitals [8]. As these ESBLs producers are showing resistance to major antibiotics which are used for the treatment of pathogens, microbiologist uses higher antibiotics such as colistin and polymyxin B to treat these ESBLs producers. These drugs are not routinely used as they cause various side effects such as kidney and nerve damage. Most ESBLs are derivatives of the more common beta-lactamases known as temoniera (TEM) or sulfhydryl variable (SHV) types [9]. CTX M1, 2, 8, 9, and 25 then OXA are the genes responsible for the production of ESBLs [10]. Hence, identification of these ESBLs producers has become a very important problem in a clinical setting to avoid the prolonged stay of the patients in the hospitals and restrict the appropriate usage of antibiotics in the treatment. Hence, this present study was undertaken to find the prevalence of ESBLs producing Gram-negative organism from various clinical specimen collected from the local hospital and determine the genes conferring resistance to these organisms.

METHODS

Sample collection

A total of 112 clinical samples were collected from the local Medical Hospitals, Salem, Tamil Nadu, in sterile containers and transported immediately to the laboratory for future work.

Isolation and identification

All the samples were inoculated onto Mac Conkey's agar for the isolation of Gram-negative bacilli. The isolated organisms were identified and confirmed by Bergey's manual of determinative bacteriology.

Antibiotic susceptibility test

The antibiotics susceptibility testing of Gram-negative organisms was done following the CLSI guidelines using ceftazidime (CAZ), ceftriaxone, cefotaxime, amikacin, ampicillin, ciprofloxacin, co-trimoxazole, gentamicin, and imipenem.

Phenotypic detection of ESBL producers

The Gram-negative isolates showing resistance to CAZ were screened for ESBL producers by combined disc synergistic test (CDST). CAZ-30 µg and CAZ+CAC-30/10 µg) were used for this test. 0.5 McFarland of the test culture was swabbed onto Mueller–Hinton agar plates. CAZ-30 µg and CAZ+CAC (CAC-30/10 µg) were placed at a distance of 20 mm (center to center) on Mueller–Hinton agar plates. The plates were incubated for 24 hrs at 37°C. After the incubation period expanded zone of inhibition up to 5 mm around CAZ+CAC disc than CAZ disc were confirmed as ESBL producers based on the CLSI guidelines.

Genotypic detection of ESBL producers

The genotypic identification of ESBL producers was carried out by alkaline lysis method by isolation of plasmid DNA following the method of Sadasivam and Manickam, 2008 with minor modifications. About 1 ml of an overnight culture was transferred into an Eppendorf tube. The cells were sediment by centrifuging briefly (5000 rpm) in the microfuge, and the supernatant was drained off. The pellet was resuspended by adding 100 µL of solution A, and the contents were mixed by vortex. Then, 100 µl solution B was added and mixed well by invert the content (4-5 times). To the above viscous content, 100 µL of solution C was added and the content was inverted 4-5 times to get mixed, the bulk of genomic DNA and other cell debris will precipitate into a viscous clump [23]. It was centrifuged at 12000 rpm in micro centrifuge, and the clump was removed. The clear lysate (supernatant) was transferred to another Eppendorf tube. 150 µl of 100% isopropanol was added and mixed well and centrifuged the content at 12000 rpm for 30 minutes. The supernatant was drained off, and 150 µL of absolute alcohol was added and centrifuged the content at 10000 rpm for 20 minutes. The supernatant was drained off and dissolved the DNA pellets with 20 µL TE buffer. The extracted plasmid DNA was confirmed by running of agarose gel electrophoresis.

Isolates were screened for the resistance genes SHV, TEM, CTX-M, and OXA by a multiplex polymerase chain reaction (PCR) assay by following the protocol of Hong Fang *et al*., (2008) (Table 1). PCR amplification reactions were performed in a volume of 25 µl containing 12.5 µl of ×2 Promega PCR Master Mix (USA), 0.2 µM concentrations of each primer, and 2 µl of DNA template. The cycling parameters were as follows: An initial denaturation at 95°C for 15 minutes followed by 30 cycles of 94°C for 30 seconds, 62°C for 90 seconds, and 72°C for 60 seconds and with a final extension at 72°C for 10 minutes. The amplified PCR products were subjected to electrophoresis at a 1.5% agarose gel in ×1 TBE buffer. A 100 bp ladder molecular weight marker (Helini, India) was used to measure the molecular weights of amplified products.

Table 1: Forward and reverse primer sequence of SHV, TEM, CTX m, and OXA genes with amplification size

Gene name	Primer sequence	Amplified size (bp)
SHV	CTT TAT CGG CCC TCA CTC AA AGG TGC TCA TCA TGG GAA AG	237
TEM	CGC CGC ATA CAC TAT TCT CAG AAT GA ACG CTC ACC GGC TCC AGA TTT AT	445
CTXm	ATG TGC AGY ACC AGT AAR GTK ATG GC TGG GTR AAR TAR GTS ACC AGA AYC AGC GG	593
OXA	ACA CAA TAC ATA TCA ACT TCG C AGT GTG TTT AGA ATG GTG ATC	813

SHV: Sulfhydryl variable, TEM: Temoniera

RESULTS

A total of 112 clinical samples which included 39 urine samples, 30 Swab, 27 Pus samples, and 16 Sputum were taken for this study. A total of 87 bacterial isolates were identified. Among them, *Klebsiella* (41%) was the predominant organism followed by *E. coli* (33%), *Proteus* (10%), *Pseudomonas* (10%), and *Serratia* (6%).

Among the various bacterial isolates, *Klebsiella* showed a higher percentage of resistance compared to other bacterial isolates (Table 2). For the detection of ESBL producers using CDST 29 isolates were taken of which 8 isolates of *Klebsiella,* 3 isolates of *E. coli, and* 1 isolate of *Pseudomonas* were found to be ESBL producers (Fig. 1).

Genotypic detection of ESBL producers

The results of the multiplex PCR showed that the two bacterial isolates were found to possess TEM gene which was 400-500 bp as confirmed by running the PCR amplified product on the agarose gel electrophoresis. This gene is said to be responsible for conferring resistance by the production of ESBL enzyme against the third generation of cephalosporin antibiotics (Fig. 2).

The extracted plasmid DNA of *Klebsiella* spp. was marked as Lane 1; the extracted plasmid DNA *E. coli* was marked as Lane 2.

DISCUSSION

In our study, 8 isolates of *Klebsiella,* 3 isolates of *E. coli,* and 1 isolate of *Pseudomonas* were found to be ESBL producers out of 87 isolates. The multiplex PCR showed that the presence of TEM gene which is responsible for the resistance. The results of this study were similar to the one observed by Mojtaba *et al*., in which presence of TEM and SHV genes were found in 65.8% and 15% of isolates, respectively. The presence of both TEM and SHV was seen in 14 isolates (19.2%) [11].

In this study, *Klebsiella*, *E. coli,* and *Pseudomonas* were found to be ESBL producers which is similar to one reported by Chong *et al*. in various clinical samples. In their study, from 2003 to 2011, the main ESBL-

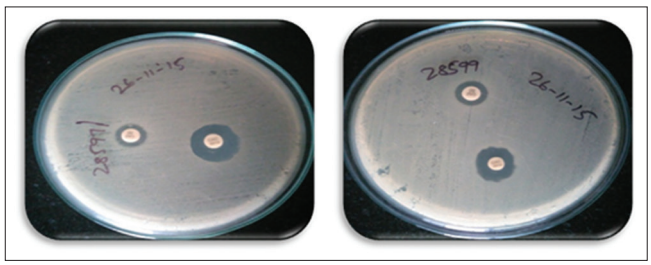

Fig. 1: Phenotypic detection of extended-spectrum beta-lactamase producers in this study. Figure is showing the enhancement zone of inhibition; left - *Klebsiella*; right - *Escherichia coli*

Table 2: Resistant pattern of the Gram-negative organisms isolated from clinical specimens in this study

Name of the organisms	Total Number of isolates	Antibiotics (number of isolates and % of resistance)						
		PIP	CIP	GEN	AK	ATM	CAZ	IPM
Klebsiella	28	8 (12)	16 (24)	8 (12)	1 (1)	20 (29)	15 (22)	1 (1)
E. coli	22	3 (6)	17 (33)	4 (8)	-	18 (35)	9 (18)	-
Proteus	7	2 (8)	3 (13)	5 (22)	3 (13)	5 (22)	3 (13)	2 (9)
Pseudomonas	7	-	2 (14)	3 (22)	3 (22)	3 (21)	2 (14)	1 (7)
Serratia	4	-	1 (25)	2 (50)	-	1 (1)	-	-

PIP: Piperacillin tazobactam, CIP: Ciprofloxacin, GEN: Gentamycin, AK: Amikacin, ATM: Azithromycin, CAZ: Ceftazidime, IMP: Imipenem, *E. coli: Escherichia coli*

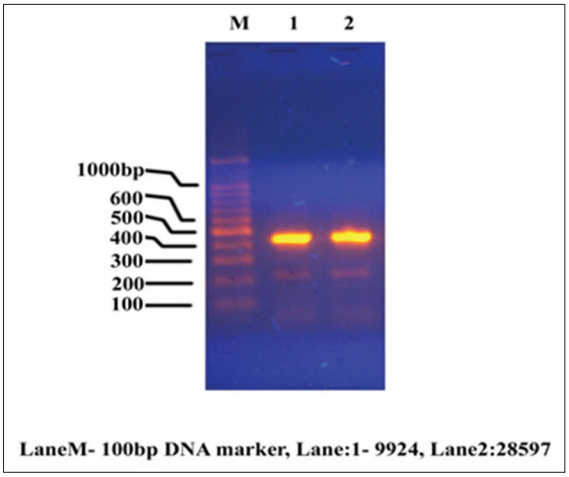

Fig. 2: Genotypic detection of extended-spectrum beta-lactamase producers in this study

producing bacteria isolated from outpatients were *E. coli, Klebsiella pneumoniae,* and *Proteus mirabilis* and 321 (6.3%) of 5137 isolates were identified as ESBL producers. The number of each ESBL-producing bacterium detected was compared between out and inpatients in our hospital. ESBL producing *E. coli* were isolated in the year 2003. These strains steadily increased in number and reached ~20% in 2011. During the same period, the out and inpatients also carried ESBLs in other bacteria, including *K. pneumoniae* and *P. mirabilis*. Interestingly, the frequent detection of ESBLs was first observed in the *E. coli* strains and was followed by the *K. pneumoniae* and *P. mirabilis* strains, irrespective of whether the patients were out-or inpatients. The detection rates of ESBL-producing *K. pneumoniae* and *P. mirabilis* strains increased during recent years, similar to that of the *E. coli* strains, and reached ~10 and ~30%, respectively [12,13].

The current study has proved that most of the isolates were susceptible to imipenem and amikacin. In some previous study, many isolates were showed 100% sensitivity to imipenem and tigecycline. By the phenotypic confirmatory test, the prevalence of ESBL among *E. coli* isolates was found to be 36% [14]. The change in the antibiotic phenotype pattern in different ESBLs clearly differentiated the resistance mechanism of beta-lactamase producing isolates which was resolved by PCR, which accurately distinguished between different types of ESBL genes.

CONCLUSION

From the results of this study, it can be concluded that the early detection of ESBL producing Gram-negative organism is a very important step in not only reducing the mortality but also reducing the prolonged stay of patients in the Hospitals. CDST continues to be a good indicator in the detection of ESBL producers. However, a proper Coordination is very essential among clinicians, pharmacologists, and the microbiologist in the emergence and prevention of drug resistance strains.

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