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 healthcare 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) Ampicillin and carbapenemases [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 (CA) 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 increased mortality and hospital stay. Obligatory, we should confirm the 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 ESBL 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 ESBL-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), ceftiraxone, 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 Sadashivam 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 of solution B was added and mixed well by inverting 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 TE buffer. The extracted plasmid DNA was confirmed by running of agarose gel electrophoresis 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 TE buffer. The extracted plasmid DNA was confirmed by running of agarose gel electrophoresis.

The multiplex PCR showed that the presence of TEM gene which is 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.

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

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Primer sequence</th>
<th>Amplified size (bp)</th>
</tr>
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<tbody>
<tr>
<td>SHV</td>
<td>CTT TAT CGG CCC TCA CTC AA</td>
<td>237</td>
</tr>
<tr>
<td></td>
<td>AGG TGC TCA TCA TGG GAA AG</td>
<td></td>
</tr>
<tr>
<td>TEM</td>
<td>CGG CGC ATA CAC TAT TCT CAG AAT GA</td>
<td>445</td>
</tr>
<tr>
<td></td>
<td>AGG CTC ACC GCC TCC AGA TTT AT</td>
<td></td>
</tr>
<tr>
<td>CTXm</td>
<td>ATG TGC AGY ACC AGT AAR GTK ATG GC</td>
<td>593</td>
</tr>
<tr>
<td></td>
<td>TGG GTR AAR TAR GTS ACC AGA AY AGC GG</td>
<td></td>
</tr>
<tr>
<td>OXA</td>
<td>AGA CAA TAC ATA TCA ACT TCG C</td>
<td>813</td>
</tr>
<tr>
<td></td>
<td>AGT GTG TTT AGA ATG GTG ATC</td>
<td></td>
</tr>
</tbody>
</table>

SHV: Sulphhydril variable, TEM: Temsoniera

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

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

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

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

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).

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-
in a tertiary care hospital in
and
Extended-spectrum beta-lactamase-producing gram-negative
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et al
isolates from pus samples in a
3. Samaha-Kfoury JN, Araj GF. Recent developments in beta-lactamases
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essential among clinicians, pharmacologists, and the microbiologist in
patients in the Hospitals. CDST continues to be a good indicator in the
not only reducing the mortality but also reducing the prolonged stay of
From the results of this study, it can be concluded that the early detection
CONCLUSION
accurately distinguished between different types of ESBL genes.

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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