INTRODUCTION

Acinetobacter spp. is one of the emerging opportunistic and multidrug-resistant (MDR) bacteria among NFGNB (Non Fermentative Gram Negative Bacteria) causing various infections such as pneumonia, urinary tract infection, endocarditis, surgical site infection, meningitis, and septicemia, particularly in immune-compromised patients [1]. Extended-spectrum beta-lactamases (ESBLs) are enzymes produced by gram negative bacteria including Acinetobacter spp. that inactivates extended-spectrum cephalosporins and other beta-lactam antimicrobial agents and are inhibited by clavulanic acid, sulbactam or tazobactam [2]. ESBLs can be plasmid mediated resistance mechanism. The plasmid may spread antibiotic resistance genes to their host bacteria by various mating processes and replicates independently of the host chromosome [3]. Biofilm is the surface-attached (sessile) community of microorganisms growing embedded in a self-produced matrix of extracellular polymeric substances (EPS). Typically biofilm shows an increased antimicrobial resistance as compared to plank tonic bacteria [4]. Frequently, the biofilm production should be found in the clinically relevant infections, and the biofilm producers should be resistance to the commonly used antibiotics and also a variety of disinfectants [5]. This study was designed to know the nature of plasmid as an antibiotic resistance marker in ESBLs and biofilm detection among ESBL producing Acinetobacter species.

MATERIALS AND METHODS

During 8 mo period (May 2013-December 2013), 56 ESBL producing Acinetobacter spp. (isolated from the clinical specimens of routine diagnosis) were obtained in the Department of Microbiology, Sri Venkateswara Institute of Medical Sciences, Tirupati. Kirby Bauer’s method [6] was performed to know the resistance pattern. The CLSI recommended confirmatory phenotypic method [7] was performed to confirm the presence of ESBL production. K. pneumoniae ATCC 700603 (positive control) and E. coli ATCC 25922 (negative control) were used as quality control.

Isolation and detection of molecular weight of plasmid DNA

The plasmid DNA from ESBL producing isolates of Acinetobacter spp. were isolated using alkaline lysis method described by Sambrook et al [8]. The bacterial cultures were grown in LB medium with appropriate antibiotic at 37°C, 150 rpm for 16-18 h. After nucleic acid precipitation, plasmid pellet was dissolved in 50 µl of TE (10 mM Tris, 1 mM EDTA, pH 8) buffer. Agarose gel electrophoresis was performed on 0.8% (w/v) agarose prepared in TBE buffer.

Curing of plasmid DNA

The curing of plasmid DNA was performed in ESBL producing strains of Acinetobacter spp. by the method as described by Deshpande et al [9]. The efficiency of plasmid curing was expressed as a number of colonies with cured phenotype per 100 colonies tested. In cured strains, the antibiotic sensitivity test (disc diffusion method) was repeated to check the presence or loss of resistance character and additionally, agarose gel electrophoresis was performed with the plasmid DNA preparation of respective culture to confirm the physical loss of plasmids.

Biofilm detection by tube method

We screened all isolates for the biofilm formation by tube method (qualitatively) as described by Christensen et al. [10]. Brain-heart infusion (BHI) was inoculated with loopful of test culture from overnight culture plates and incubated for 24 h at 37°C. Then, tubes
were decanted and washed with PBS (pH 7.3) and dried than tubes were stained with crystal violet (0.1%). Excess stain was removed with PBS and tubes were washed with deionized water. Tubes were than dried in inverted position and observed for biofilm formation. Biofilm formation was considered positive when a visible film lined the wall and bottom of the tube. Ring formation at the liquid interface was not indicative of biofilm formation. Tubes were examined and the amount of biofilm formation was recorded as strongly adherent (strongly positive), weakly adherent (weak positive) and non-adherent (negative). Experiments were performed in triplicate and repeated three times.

**Biofilm detection by microtitre method**

Microtitre method (quantitatively) [10] was performed to screen the biofilm formation. A 1:100 dilution of overnight broth culture of the test organism was inoculated in BHI broth with 2% sucrose and transferred to 96 well polystyrene flat-bottomed microtitre plates. Fig. 1: Plasmid profile of ESBL producing Acinetobacter spp M:λ DNA (EcoR1+Hind III digested); 1,2,3,4 and 5: presence of plasmid DNA

After incubation at 37 °C, washed and air dried then stained with crystal violet solution, the glacial acetic acid was added to the wells and optical density was read at 490 nm. The optical density (OD) greater than 0.120 was considered for biofilm formation. The biofilm positive control strain was maintained [11].

All ESBL positive samples were sensitive to tegecyclin (100%) followed by polymixin-B (91.07%) and imipenem (66.66%) and 100% resistance to third generation cephalosporins which includes cefotaxime, ceftazidime, cefepime and monobactam includes aztreonam and resistance pattern for other antibiotics were shown in table 1.

The analysis of plasmid in ESBL producing strains revealed the presence of plasmid DNA with an approximate molecular weight of 21,226 bp in all isolates (fig. 1). Most of the isolates showed thick blue ring at the liquid-air interfaces (fig. 2). In tube method, 11 (19.64%) isolates were strongly positive, 37 (66.66%) were weakly adherent and 08 (14.28%) were non-biofilm producers. Isolates that showed weakly adherent were considered as non-biofilm producers. In the micro-titre plate method, the 09 (16.07%) were showed OD values greater than 0.120 and remaining isolates 47 (82.93%) were considered as negative for biofilm formation. were considere as negative for biofilm formation (fig. 3).

**Table 1: Antibiotic sensitivity pattern of 56 ESBL producing Acinetobacter spp**

<table>
<thead>
<tr>
<th>Serial number</th>
<th>Antibiotic (concentration)</th>
<th>Sensitive (%)</th>
<th>Intermediate (%)</th>
<th>Resistance (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>amikacin (30 μg)</td>
<td>16 (28.57)</td>
<td>03 (5.35)</td>
<td>37 (66.07)</td>
</tr>
<tr>
<td>2.</td>
<td>ampicillin (10μg)</td>
<td>00 (0.00)</td>
<td>03 (5.35)</td>
<td>53 (94.64)</td>
</tr>
<tr>
<td>3.</td>
<td>aztreonam (30μg)</td>
<td>00 (0.00)</td>
<td>00 (0.00)</td>
<td>56 (100)</td>
</tr>
<tr>
<td>4.</td>
<td>cefepime (30μg)</td>
<td>00 (0.00)</td>
<td>00 (0.00)</td>
<td>56 (100)</td>
</tr>
<tr>
<td>5.</td>
<td>cefotaxime (30μg)</td>
<td>00 (0.00)</td>
<td>00 (0.00)</td>
<td>56 (100)</td>
</tr>
<tr>
<td>6.</td>
<td>cefoxitin (30μg)</td>
<td>08 (14.28)</td>
<td>03 (5.35)</td>
<td>45 (80.35)</td>
</tr>
<tr>
<td>7.</td>
<td>ceftazidime (30μg)</td>
<td>00 (0.00)</td>
<td>00 (0.00)</td>
<td>56 (100)</td>
</tr>
<tr>
<td>8.</td>
<td>chloramphenicol (30μg)</td>
<td>08 (14.28)</td>
<td>04 (7.14)</td>
<td>44 (78.57)</td>
</tr>
<tr>
<td>9.</td>
<td>ciprofloxacin (5μg)</td>
<td>09 (16.07)</td>
<td>04 (7.14)</td>
<td>43 (76.78)</td>
</tr>
<tr>
<td>10.</td>
<td>ce-trimoxazole (25μg)</td>
<td>24 (42.65)</td>
<td>05 (8.92)</td>
<td>27 (48.21)</td>
</tr>
<tr>
<td>11.</td>
<td>gentamicin (30μg)</td>
<td>09 (16.07)</td>
<td>04 (7.14)</td>
<td>43 (76.78)</td>
</tr>
<tr>
<td>12.</td>
<td>imipenem (10μg)</td>
<td>37 (66.07)</td>
<td>08 (14.28)</td>
<td>11 (19.64)</td>
</tr>
<tr>
<td>13.</td>
<td>meropenem (10μg)</td>
<td>40 (71.42)</td>
<td>08 (14.28)</td>
<td>08 (14.28)</td>
</tr>
<tr>
<td>14.</td>
<td>nitrofurantoin (30μg)</td>
<td>20 (35.71)</td>
<td>00 (0.00)</td>
<td>36 (64.28)</td>
</tr>
<tr>
<td>15.</td>
<td>polymixin-B (300 units)</td>
<td>51 (91.07)</td>
<td>00 (0.00)</td>
<td>05 (8.92)</td>
</tr>
<tr>
<td>16.</td>
<td>tegecyclin (15 μg)</td>
<td>56 (100)</td>
<td>00 (0.00)</td>
<td>00 (0.00)</td>
</tr>
<tr>
<td>17.</td>
<td>piperacillin/tazobactum (100/10μg)</td>
<td>13 (23.21)</td>
<td>08 (14.28)</td>
<td>35 (62.5)</td>
</tr>
<tr>
<td>18.</td>
<td>cefoperazone sulbactum (75/10μg)</td>
<td>29 (51.78)</td>
<td>07 (12.5)</td>
<td>20 (35.71)</td>
</tr>
<tr>
<td>19.</td>
<td>amoxy clav (20/10μg)</td>
<td>02 (3.57)</td>
<td>14 (25.00)</td>
<td>40 (71.42)</td>
</tr>
</tbody>
</table>

μg: microgram; %: percentage.

**DISCUSSION**

Most commonly, extended spectrum beta lactams and other beta lactam group of antibiotics have been used to treat gram negative bacterial infections including Acinetobacter spp. But the emergence of ESBL producing and another beta-lactamase producing organisms has been posed a serious threat for their continuing use [12, 13]. The nosocomial infections are becoming rising trouble for hospitalized
patients [14]. If the antibiotic resistant bacteria, unlike spread to patients in any transmission mode, subsequently susceptible microbial flora may also be transformed into the resistant strain or susceptible bacteria into resistant, as a result, the risk factors are directly or indirectly increases treatment failure. Acinetobacter baumannii were highly resistant to a β-lactum group of antibiotics which were isolated from the ICU patients [15]. In our study, gentamicin resistance was found to be 76.78% which was high as compared to the previous study reported by Shafaq Aiyaz Hassan et al. [16] (60%) and low (92.6%) when compared with Iroha et al. [17].

The resistance profile of ciprofloxacin among ESBL producers varies with geographic regions 13.7% in Canada, 34.8% in France, 34.2% in Europe to 65.5% in Western pacific [18]. In our study, 76.78% of resistance pattern was documented which was close prevalence to (76.5%) reported in Bangladesh [19].

Treatment options for ESBL producing isolates are available, but they are limited. The risk factor of treatment failure is more in patients infected with ESBL producers than ESBL non-producers [20]. In a previous studies from Pakistan [21] and Bangladesh [19], all ESBL producers were sensitive (100%) to carbapenems. The carbapenems are used to treat the infections caused by ESBL producers, but carbapenem-resistant ESBL isolates have been reported from various parts of the world including United States, Greece, Korea, Israel, and China [22]. In our study, 19.64% and 14.28% of resistance were found against imipenem and meropenem respectively.

In a study from Pune, Acinetobacter spp. isolated from human skin showed a maximum of three plasmids ranging from 1.5 to 40 kb [23] but in our study only single plasmid (approximately 21,226 bp) was observed among all ESBL strains isolated from clinical specimens. The physical loss of plasmid (after curing) is the evidence for the nature of antibiotic resistance marker in the ESBL strains. The cured isolates were found sensitive to third generation cephalosporins.

Microbial biofilms are considered as the virulence factors. In this study, the detection of biofilm production in Acinetobacter spp. was aimed to verify any association between the biofilm formation and ESBLs. The device related nosocomial infection also causes the organisms to resist physical and chemical disinfection, often by forming a biofilm. Therefore, infections due to bacteria that form biofilms are a persistent clinical problem. In a study, both qualitative (tube method) and quantitative (microtitre plate) method showed 30 isolates (60%) as biofilm producers [24]. In the present study, 11 (19.64%) isolates were considered as strong adherence, 08 (14.28%) isolates were weak adherence and 37 (66.07%) were considered as strong adherence, 08 (30) isolates (60%) as biofilm producers [24]. In the present study, 11 (tube method) and quantitative (microtitre plate) method showed a maximum of three plasmids ranging from 1.5 to 40 kb respectively.

Acinetobacter baumannii are highly adherent as biofilm negative and this report was suggested that low adherence alone may not complete the cycle of the process of biofilm formation and there might be many other mechanisms that could explain adherence [25]. So, we categorized isolates that were weakly adherent as biofilm negative and this report was suggested that low adherence [26]. In another study, it was observed that 44% of positive biofilm isolates were isolated from wound infections and 30% of biofilm producers were identified from patients infected from pneumonia. This indicates that in the wound and respiratory infections, the biofilm may contribute associated with antibiotic resistance [25] and in the same study [26] in another study, the qualitative and quantitative method showed 62% of isolates as strong biofilm producers. Fourteen isolates, which were weakly adherent, were additionally picked up by the quantitative microtiter assay asserting its sensitiveness.

We concluded that the nature of plasmid plays a major role in antibiotic resistance with reference to ESBL mediated resistance. In this regard, furthermore, molecular studies to be needed to check whether the prevalence of resistance was due to a specific gene or multiple genes.

CONFLICT OF INTERESTS
Declared none

REFERENCES


