Molecular Detection of Extended-Spectrum Beta-Lactamases in Clinical Isolates of *Acinetobacter baumannii*

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Abstract:
Resistance to third-generation oxyimino cephalosporins is emerging, and it is considered a problem in medical field. Extended spectrum β-lactamase (ESBL) producing *Acinetobacter baumannii* have been noticed to be important cause of hospital infections. This study aimed to undertake and determine the occurrence of ESBLs especially SHV, TEM and VEB β-lactamase types. A total of 770 clinical samples were collected from February to June, 2011. The *A. baumannii* isolates were identified according to API 20NE system. Phenotypic detection of ESBL was performed by using the combination disk, disk approximation methods then confirmed by culturing on ESBL CHROM agar medium. The isolates were subjected to polymerase chain reaction (PCR) assays with specific primers for bl*sa*SHV, bl*sa*TEM and bl*sa*VEB. Twelve (1.5%) *A. baumannii* isolates were recovered from clinical infections. All of them were β-lactam resistant (resistant to both ampicillin and amoxicillin). Of the β-lactam resistant isolates, 8/12 (66.7%) were found to be disk combination test positive, and 10 (83.3%) isolates were confirmed as ESBL producers and gave heavy growth on ESBL CHROM agar. In PCR experiments using specific primers for bl*sa*SHV, bl*sa*TEM and bl*sa*VEB genes, out of 12 *A. baumannii* isolates, three (25%) were harbored bl*sa*SHV gene and only one (8.3%) isolate gave positive PCR results for bl*sa*TEM gene. This study demonstrate all isolates were bl*sa*VEB negative. The present study concluded that the emerging of dissemination of ESBL producing *A. baumannii* in Najaf hospitals.

Keywords: key words, *Acinetobacter baumannii*, Extended-Spectrum Beta-Lactamases, Molecular Detection

1. Introduction

*Acinetobacter baumannii* has emerged over the last decade as a significant opportunistic pathogen. Although it is generally associated with benign colonization of hospitalized patients, it is responsible for about 10% of nosocomial infection in intensive care unit (ICU) patients, causing a wide range of infections (Levin et al, 2003; Poirel et al, 1999). They are usually considered to be opportunistic pathogens, and of recent have been reported to cause a number of outbreaks of nosocomial infections in hospitalized patients like septicaemia, pneumonia, wound sepsis, endocarditis, meningitis and urinary tract infection (UTI) (Towner 1997). In *Acinetobacter*-associated nosocomial infection, the major problem encountered by ICU clinicians relates to the readily transferable antimicrobial resistance expressed by this organism (Bergogne-Berezin 2001). In addition to intrinsic resistance, *A. baumannii* has the ability to acquire resistance to many major classes of antibiotics including newer β-lactams (Perilli et al, 1996). The presence of resistance plasmids (R-plasmids) is a significant feature of this organism, and plasmid profiling has been proposed as a method of epidemiological typing for *Acinetobacter* (Joshi 1998). Although *A. baumannii* colonizes hospitalized patients, approximately 30% of isolates are associated with frank infection in ICU patients and, in this setting, tend to demonstrate variable susceptibility profiles (Dy et al, 1999).

Over the last 20 years many new β-lactam antibiotics, specifically designed to resist known β-lactamases, have been developed. However, almost invariably new β-lactamases have emerged to combat each new class of β-lactams. Plasmid-mediate ESBLs emerged in Gram-negative bacilli in Europe in the 1980s (Zeba 2005). ESBL-producing bacteria are typically resistant to penicillins, first-and second-generation cephalosporins as well as the third-generation oxyimino cephalosporins (Jacoby &Medeiros 1991).

Typically, ESBLs are plasmid encoded but also present on chromosomes, often in association with integrons. These enzymes are derivatives, predominantly, of class A and D β-lactamases. Classical ESBLs evolved from class A, TEM (from TEM-1 or TEM-2) and SHV (from SHV-1) enzymes, and these remain the most prevalent types of ESBLs, though class D ESBLs (OXA family) have also been known for some time (Bradford 2001). Hence the fundamental aim of this study is to identify the occurrence of ESBL in *A. baumannii* isolates recovered from Hospital settings in Najaf.

2. Materials and Methods

2.1 Isolation and Identification

A total of 770 clinical samples included (sputum (n= 450), urine (n= 210), and burn wounds (n= 110)) were collected from patients in three separate hospitals (Al-Sader Medical City, Al-Hakeem General Hospital, Al-Furat Teaching Hospital) in Najaf over five months period starting from February to June, 2011. Isolates were
recovered from clinical samples after culturing on MacConkey's agar (Himedia, India) and incubated for overnight at 37°C, non lactose fermenting bacteria (colorless or slightly beige) were subcultured and incubated for additional overnights. Suspected bacterial isolates which their cells are Gram negative cocacobacillary or diplobacillus and negative to oxidase which further identified by the traditional biochemical test according to Holt et al. (1994) and MacFaddin (2000). Isolates were confirmed by API20 NE multi-test systems (BioMerieux ,France).

2.2 Screening Test for β-lactam and Antibiotics Resistance 
Ampicillin and amoxicillin were added separately, from stock solution, to the cooled Muller-Hinton agar at final concentration of 100 and 50 µg/ml, respectively. The medium poured into sterilized Petri dishes, then stored at 4°C. Isolates were cultured on Mueller–Hinton agar and their susceptibilities to different antibiotics were tested by disk diffusion method (NCCLSs, 2003 ). Results were compared with E.coli ATCC 25922 as negative control.

2.3 Phenotypic Detection of ESBL 
2.3.1 Disk Combination Test (Recommended by CLSI 2010)
The phenotypic confirmation of potential ESBL-producing isolates was performed by using disk diffusion method. Cefotaxime alone and in combination with clavulanic acid were tested. Inhibition zone of ≥ 5 mm increase in diameter for antibiotic tested in combination with clavulanic acid versus its zone when tested alone confirms an ESBL producing isolate (Cantarelli et al, 2007).

2.3.2 Disk Approximation Test
All β-lactamase producing isolates tested according to Batchoun et al. (2009). Antibiotic disks of cefotaxime (30µg), cefazidime (30µg), ceftriaxone (30µg), and aztreonam (30µg) were placed 15 mm (edge to edge) around a central disk of amoxi-clav (20µg amoxicillin plus 10 µg clavulanate) on Muller-Hinton agar plates seeded with organism being tested for ESBL production. Plates were incubated aerobically at 37°C for 24 hr . Any augmentation (increase in diameter of inhibition zone) between the central amoxi-clav disk and any of the β-lactam antibiotic disks showing resistance or intermediate susceptibility was recorded, and the organism was thus considered as an ESBL producer.

2.3.3 CHROM agar Technique phenotype detection of ESBLs producing isolates
Extended spectrum β-lactamase CHROM agar plates were streaked in the same day of preparation by overnight growth of A. baumannii. The plates were incubated at 37°C for 24 hr according to manufacturer procedure. Growth of blue colonies indicated to ESBL producer. The reference strain of E. coli ATCC 25922 was inhibited and used as negative control.

2.4 Detection of ESBL bla Genes by Polymerase Chain Reaction
2.4.1 DNA extraction
Extraction of DNA from bacterial cells was performed by salting out method (Pospiech & Neumann, 1995) with some modification to prepare templet DNA.

2.4.2 Polymerase Chain Reaction Protocols
2.4.2.1 PCR Mixture and thermo cycling conditions
The DNA template extracted from A. baumannii isolates were subjected to bla genes by PCR, the protocol was used depending on manufacturer's instruction and the right PCR cycling program parameters conditions were installed as in Table (1).

2.4.2.2 Agarose Gel Electrophoresis
All requirements, technical and preparations of agarose gel electrophoresis for DNA detection and analysis were performed by Bartlett & Stirling (1998). Finally, the gel was photographed using Biometra gel documentation system.

3. Results
Among the 770 clinical samples were collected during study period (Table 2), only 12 (1.5%) isolates had been identified as A. baumannii, all isolates (100%) were resistant to both ampicillin and amoxycillin. Fifty percent of A. baumannii isolates were recovered from urine followed by 4(0.88%) from sputum and 2(1.8%) from burn wound. Production of ESBL was confirmed by three different methods, disk combination, disk approximation tests and ESBL CHROM agar (Table 3). Only 8 (66.7%) isolates demonstrated enhancement of inhibition zone, suggesting production of ESBL by disk combination test, while no remarkable distinct change was noticed when using disk approximation test. In same time most isolates 10 (83.3%) were identified as ESBL producer by CHROM agar. In PCR experiments using specific primers for blaSHV and blaTEM and blaVEB , the results of table (4) show three (25%) isolates were harbored blaSHV gene and only one (8.3%) isolate gave positive PCR results for blaTEM gene (Figure 1 and 2). This study demonstrates all isolates were blaVEB negative. Consequently, table (5) show all isolates that harbored SHV and TEM types gene in their genotype appeared phenotypically as multidrug resistant isolates (resistant for more than three antibiotic classes).
4. Discussion

*A. baumannii* is an important causes of nosocomial infections and has been associated with a wide variety of illness in hospitalized patients, especially patients in the intensive care units (Sinha *et al.*, 2007). During a few decades *A. baumannii* tend to be multidrug resistance (MDR) due to their ability to develop antibiotic resistance (Kusradoze *et al.*, 2011). Also, the extensive use of antimicrobial chemotherapy within hospital has contributed to the emergence and procreation of *A. baumannii* strains which are resistance to a wide range of antibiotic including broad spectrum β-lactams, aminoglycosides and fluoroquinolones (Parisa *et al.*, 2012).

The main mechanisms of resistance to β-lactams in *A. baumannii* is enzymatic degradation by β-lactamase including the extended spectrum β-lactamase (*bla*TEM, *bla*SHV, *bla*VEB and *bla*PER) and metalo-beta-lactamase (*bla*OXA, 51, 23, 24, and 38) (Shahcheraghi *et al.*, 2011). As shown in our results, 83% of isolates showed positive results for CHROM agar media compared with disc disc combination test (66.7%). This may due to the sensitivity of CHROM agar for the detection of enzyme compared with other methods. Also, our results showed no correlation between the existence of *bla* genes and phenotypic resistance against β-lactam antibiotics in *A. baumannii*. This results was in an agreement with other studies that confirmed a specific correlation between genotypic and phenotypic properties of β-lactam resistance among *A. baumannii* (Soroush *et al.*, 2010; Srinivasan *et al.*, 2009; Yan *et al.*, 2009). Several studies reported that resistance to β-lactam antibiotic was largely due to existence of carbapenemase, ESBLs and metalo-beta-lactamase (Taherikalani *et al.*, 2009; Lin *et al.*, 2010; Srinivasan *et al.*, 2009). The vast majority of ESBLs are acquired enzyme encoded by plasmids, this confirmed our results which showed no correlation between genotype and phenotype properties (Parisa *et al.*, 2012; Taherikalani *et al.*, 2008; Papa *et al.*, 2009).

The acquired ESBLs are expressed at various levels and differ significantly in biochemical characteristics such as activity against specific β-lactams (cefotaxim, ceftazidime and aztereonam) (Canton *et al.*, 2012: Kusradoz *et al.*, 2010). On the other hand, a high distribution of multiple antibiotic resistance was found in β-lactamase resistance *A. baumannii* (Table 5), this may due to the co-presence of other resistance mechanisms (other β-lactamase, effluex, altered permeability) (Parisa *et al.*, 2012; Magiorakos *et al.*, 2012; Uma *et al.*, 2009)

References:
and Wilkins, USA.


Figure 1. Ethidium bromide-stained agarose gel of PCR amplified products from extracted DNA of *A. baumannii* isolates and amplified with *bla*<sub>SHV</sub> gene primers. The electrophoresis was performed at 70 volt for 1.5 hr. Lane (L), DNA molecular size marker (10000-bp ladder), Lane (A7, A10, A12) of *A. baumannii* isolates show positive results with (753bp), Lanes (A1-A6 and 8, 9) show negative results.

Figure 2. Ethidium bromide-stained agarose gel of PCR amplified products from extracted DNA of *A. baumannii* isolates and amplified with *bla*<sub>TEM</sub> gene primers. The electrophoresis was performed at 70 volt for 1.5 hr. Lane (L), DNA molecular size marker (10000-bp ladder), Lane (A3) of *A. baumannii* isolate show positive result with (822bp).
Table 1. Programs of PCR thermocycling conditions

<table>
<thead>
<tr>
<th>gene</th>
<th>Initial denaturation</th>
<th>Cycling condition</th>
<th>Final extension</th>
<th>Cycle number</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>denaturation</td>
<td>annealing</td>
<td>extension</td>
</tr>
<tr>
<td></td>
<td></td>
<td>94/30 sec</td>
<td>94/30 sec</td>
<td>60/1 min</td>
</tr>
<tr>
<td></td>
<td></td>
<td>72/10 min</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td>94/30 sec</td>
<td>60/1 min</td>
<td>72/1 min</td>
<td>72/10 min</td>
<td>35</td>
</tr>
<tr>
<td>94/30 sec</td>
<td>45/1 min</td>
<td>72/1 min</td>
<td>72/10 min</td>
<td>35</td>
</tr>
<tr>
<td>93/1 min</td>
<td>55/1 min</td>
<td>72/1 min</td>
<td>72/7</td>
<td>40</td>
</tr>
</tbody>
</table>

Table 2. Number and percentage of β-lactam resistant A. baumannii isolates collected from clinical samples

<table>
<thead>
<tr>
<th>Clinical sample</th>
<th>No. of β-lactam resistant A. baumannii isolate</th>
<th>No. (%) of phenotypic confirmed ESBL producer isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sputum</td>
<td>450</td>
<td>4 (0.88%)</td>
</tr>
<tr>
<td>Urine</td>
<td>210</td>
<td>6 (2.8%)</td>
</tr>
<tr>
<td>Burn swab</td>
<td>110</td>
<td>2 (1.8%)</td>
</tr>
<tr>
<td>Total</td>
<td>770</td>
<td>12 (1.5%)</td>
</tr>
</tbody>
</table>

Table 3. Phenotypic detection of ESBL production in A. baumannii isolates

<table>
<thead>
<tr>
<th>Type of sample</th>
<th>No. of β-lactam resistant A. baumannii isolate</th>
<th>No. (%) of phenotypic confirmed ESBL producer isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Disk Combination Test Disk Approximation Test ESBL* CHROM agar technique</td>
</tr>
<tr>
<td>Sputum</td>
<td>4</td>
<td>2 (50%) 0 (0%) 4 (100%)</td>
</tr>
<tr>
<td>Urine</td>
<td>6</td>
<td>4 (66.7%) 0 (0%) 4(66.7%)</td>
</tr>
<tr>
<td>Burn wound</td>
<td>2</td>
<td>2 (100%) 0 (0%) 2 (100%)</td>
</tr>
<tr>
<td>Total</td>
<td>12</td>
<td>8 (66.7%) 0(0%) 10(83.3%)</td>
</tr>
</tbody>
</table>

L.S.D. (0.05) Samples = 9.143, Methods = 8.94, *ESBL: Extended spectrum β-lactamase
Table 4. Molecular detection of \( \text{bla} \) genes in ESBL producing \( A. \) \textit{baumannii} isolates

<table>
<thead>
<tr>
<th>Type of sample</th>
<th>No. of ( \beta )-lactam resistant ( A. ) \textit{baumannii} isolate</th>
<th>Molecular detection of ESBL ( \text{bla} ) genes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>( \text{bla}_{\text{SHV}} )</td>
</tr>
<tr>
<td>Sputum</td>
<td>4</td>
<td>1 (25%)</td>
</tr>
<tr>
<td>Sputum</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>Burn wound</td>
<td>2</td>
<td>2 (100%)</td>
</tr>
<tr>
<td>Total</td>
<td>12</td>
<td>3 (25%)</td>
</tr>
</tbody>
</table>

Table 5. Antibiotic susceptibility profiles of multi-drug resistant \( A. \) \textit{baumannii} isolates.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Genotype</th>
<th>Antibiotic resistant</th>
</tr>
</thead>
<tbody>
<tr>
<td>AS3</td>
<td>SHV</td>
<td>Ac, CTX, CI, CAZ, ATM, FOX, MEM, FEP, TOB, AK, CN, CIP, GT, LEV, TE, PRL, TIC, PY</td>
</tr>
<tr>
<td>AB4</td>
<td>SHV</td>
<td>Ac, CTX, CI, CAZ, ATM, FOX, MEM, FEP, TOB, AK, CN, CIP, GT, LEV, PRL, TIC, PY</td>
</tr>
<tr>
<td>AB6</td>
<td>TEM</td>
<td>CTX, CAZ, ATM, FOX, FEP, TOB, AK, CN, TEP, TE, PRL, TIC, PY</td>
</tr>
<tr>
<td>AB9</td>
<td>SHV</td>
<td>AC, CTX, CI, CAZ, ATM, FOX, IPM, MEM, FEP, TOB, AK, CN, TEP, CIP, GT, TE, PRL, TIC, PY</td>
</tr>
</tbody>
</table>

PY, Carbenicillin; PRL, Piperacillin; TIC, Ticarcillin; AC, Amoxi-clav; CFX, Cefexime; FOX, Cefoxitin; CAZ, Ceftazidime; CTX, Cefotaxime; CI, Ceftriaxone; FEP, Cefepime; IMP, Imipenem; MEM, Meropenem; ATM, Aztreonam; AK, Amikacin; CN, Gantamycin; TOB, Tobramycin; CIP, Ciprofloxacín; LEV, Levofloxacín; GT, Gatifloxacín; TE, Tetracyclin; TEP, Trimethoprim
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