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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 undertaken 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 bla_{SHV}, bla_{TEM} and bla_{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 bla_{SHV}, bla_{TEM} and bla_{VEB} genes, out of 12 *A. baumannii* isolates, three (25%) were harbored bla_{SHV} gene and only one (8.3%) isolate gave positive PCR results for bla_{TEM} gene. This study demonstrate all isolates were bla_{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 _- 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 cephaloporins 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 coccobacillary 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 (NCCLs, 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), ceftazidime (30µg), ceftriaxione (30µg), and azetreonam (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 recoverd 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 isolate 10 (83.3%) were identified as ESBL producer by CHROM agar.In PCR experiments using specific primers for bla_{SHV} and bla_{TEM} and bla_{VEB} , the results of table (4) show three (25%) isolates were harbored bla_{SHV} gene and only one (8.3%) isolate gave positive PCR results for bla_{TEM} gene (Figure 1 and 2). This study demonstrates all isolates were bla_{VEB} negative.Consequently, table (5) show all isolates that harbored SHV and TEM types gene in their genotype appeared phenotypicaly 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 flouroquinolones(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 ($bla_{OXA51,23,24,and\ 58}$) (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 a*l , 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)

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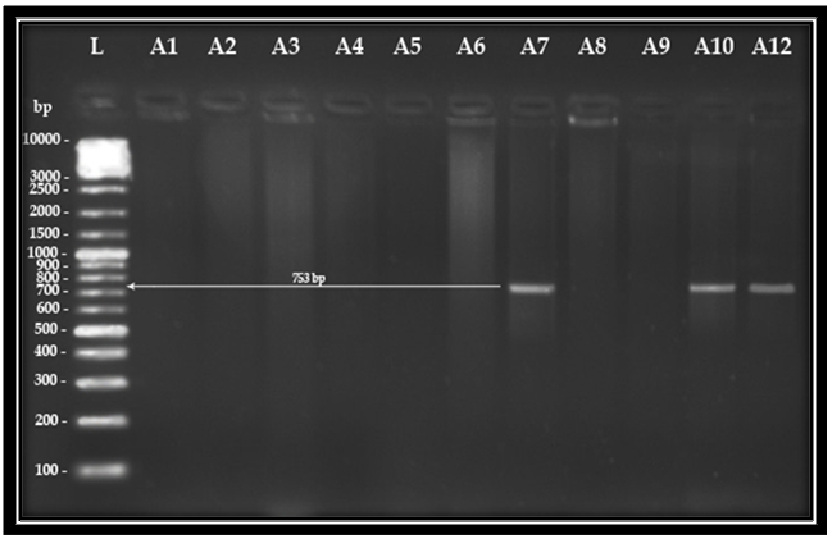

Figure 1. Ethidium bromide-stained agarose gel of PCR amplified products from extracted DNA of *A. baumannii* isolates and amplified with bla_{SHV} gene primers. The electrophoresis was performed at 70 volt for 1.5 hr. Lane (L), DNA molecular size marker (l0000-bp ladder), Lane (A7, A10, A12) of *A. baumannii* isolates show positive results with (753bp), Lanes (A1-A6 and 8, 9) show negative results.

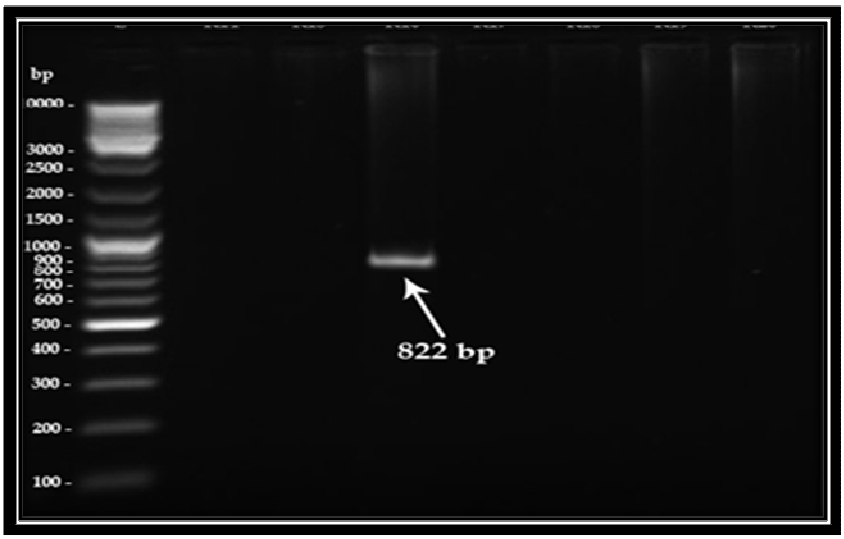

Figure 2 . Ethidium bromide-stained agarose gel of PCR amplified products from extracted DNA of *A. baumannii* isolates and amplified with bla_{TEM} gene primers. The electrophoresis was performed at 70 volt for 1.5 hr. Lane (L), DNA molecular size marker (l0000-bp ladder), Lane (A3) of *A. baumannii* isolate show positive result with (822bp).

Table 1. Programs of PCR thermocycling conditions

gene	Temperature(°C) / Time					Cycle number
	Initial denaturation	Cycling condition			Final extension	
		denaturation	annealing	extension		
bla$_{SHV}$	94/30 sec	94/30 sec	60/1 min	72/1 min	72/10 min	35
bla$_{TEM}$	94/30 sec	94/30 sec	45/1 min	72/1 min	72/10 min	35
bla$_{VEB}$	93/3 min	93/1 min	55/1 min	72/1 min	72/7	40

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

Clinical sample	No.	No. (%) of β-lactam resistant *A. baumannii* isolate
Sputum	450	4 (0.88%)
Urine	210	6 (2.8%)
Burn swab	110	2 (1.8%)
Total	770	12 (1.5%)

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

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

L.S.D. (0.05) Samples = 9.143, Methods = 8.94, *ESBL: Extended spectrum β-lactamase

Table 4 . Molecular detection of *bla* genes in ESBL producing *A. baumannii* isolates

Type of sample	No. of β-lactam resistant *A. baumannii* isolate	Molecular detection of ESBL *bla* genes		
		bla_{SHV}	bla_{SHV}	bla_{SHV}
Sputum	4	1 (25%)	0	0
Sputum	6	0	0	0
Burn wound	2	2 (100%)	1 (33.3%)	0
Total	12	3 (25%)	1 (8.3%)	0

Table 5 . Antibiotic susceptibility profiles of multi-drug resistant *A. baumannii* isolates.

Isolate	Genotype	Antibiotic resistant
AS3	SHV	Ac, CTX, CI, CAZ, ATM, FOX, MEM, FEP, TOB, AK, CN, CIP, GT, LEV , TE, PRL, TIC, PY
AB4	SHV	Ac, CTX, CI, CAZ, ATM, FOX, MEM, FEP, TOB, AK, CN, CIP, GT, LEV , PRL, TIC, PY
AB6	TEM	CTX, CAZ, ATM, FOX, FEP, TOB, AK, CN, TEP, TE, PRL, TIC, PY
AB9	SHV	AC, CTX, CI, CAZ, ATM, FOX, IPM, MEM, FEP, TOB, AK, CN, TEP, CIP, GT, TE, PRL, TIC, PY

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, Ciprofloxacin; LEV, Levofloxacin; GT, Gatifloxacin; TE, Tetracyclin; TEP, Trimethoprime