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Phenotypic ESBL Detection in *Acinetobacter baumannii*: A Real Challenge

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Abstract: Clinical Laboratory Standards Institute (CLSI) recommends two-step approach for extended-spectrum-β-lactamase (ESBL) detection which includes the screening of recommended agents and the phenotypic confirmation of ESBL using a combination of screening agent and β-lactamase inhibitor. To investigate this approach, we screened 145 β-lactamase-producing *Acinetobacter baumannii* isolates for ESBL from tracheal secretions using double disk synergy test (DDST) and phenotypic confirmatory disc diffusion test (PCDDT), the determination of minimum inhibitory concentration (MIC) for ceftazidime and cefotaxime (with/without clavulanic acid), and the unique disc placement scheme. Eighteen of 145 (12.4%) isolates showed ESBL production. All 18 isolates showed positive PCDDT. MIC of these isolates were extremely high (>512 µg/ml), and eight fold decrease in MIC was shown by only one isolate. The unique disc placement scheme detected 13 (72.2%) and 3 (16.7%) of ESBL producers and ampC producers, respectively. High level resistance to cefoxitin and cefotaxime among these isolates is suggestive of the derepressed mutants. The PCDDT was most effective ESBL detection method while the unique disc placement scheme showed advantage of detection of ampC β-lactamase, derepressed mutants and multiple β-lactam resistance mechanism in these isolates. This is a rare report comparing different tests for phenotypic ESBL detection in clinical isolates of *A. baumannii*.

Keywords: *Acinetobacter baumannii*, β-lactamase Inhibitor, Extended-Spectrum-β-Lactamase, Double Disk Synergy Test, Phenotypic Confirmatory Disc Diffusion Test, Unique Disc Placement

Introduction

In *Acinetobacter baumannii*, the β-Lactamases represents the most prevalent mechanism of β-lactam resistance in clinical isolates which greatly impacts the clinical outcome of infected patients and challenges antimicrobial chemotherapy (Jacoby and Munoz-Price, 2005; Thomson and Bonomo, 2005). The organism is known to produce naturally occurring chromosomal β-lactamases. New β-lactamase arises with the introduction of newer β-lactam antibiotic in therapeutics. A selective pressure and superfluous use of this antimicrobial agent in clinical practice is the major cause for emergence of new variant of β-lactamase (Bradford, 2001). As on today, there are more than 200 different types of β-lactamases described worldwide.

The ESBLs mediate resistance to penicillins, cephalosporins (particularly oxyiminocephalosporins) and monobactams, but spare cephamycin and carbapenems from their action. Most ESBLs have their specific set of β-lactams that they hydrolyze preferentially. A most peculiar feature of ESBLs is that these are inhibited by β-lactamase inhibitor such as clavulanic acid. This property is utilized while designing ESBL detection tests (Bush, 2001). However, the presence of inhibitor-resistant β-lactamase masks the inhibition action and gives a false negative finding of ESBL detection test (Thomson, 2010).

Acinetobacter baumannii is known to be "Intrinsically insensitive" to most β-lactams, particularly cephalosporins. The commonest mechanism of resistance to β-lactam agents is inactivation of these

compounds by β-lactamases encoded either by the chromosome or by plasmids (Bergogne-Berezin and Towner, 1996; Joshi *et al.*, 2006; Joshi and Litake, 2013). Cephalosporinases seems to be the predominant β-lactamases in *A. baumannii* isolates (Vila *et al.*, 1993; Bergogne-Berezin and Towner, 1996). ESBLs in *A. baumannii* are widely reported from many countries, such as India, France, Turkey, Korea, Belgium and Latin America, the United Kingdom, and the United States (Poirel *et al.*, 1999; Vahaboglu *et al.*, 2001; Yong *et al.*, 2003; Capoor *et al.*, 2005; Naas *et al.*, 2006; Pasteran *et al.*, 2006; Litake *et al.*, 2009; Joshi and Litake, 2013). Rapidly growing resistance among clinical isolates suggests a need to detect resistance mechanisms in this organism.

The β-lactamases that can act on a broad range of β-lactam agents challenge the selection of antimicrobial therapy. Also, it is known that the ESBL production in pathogen may co-exist with resistance to other classes of antimicrobials such as aminoglycosides, flouroquinolones, tetracyclines, chloramphenicol and co-trimoxazole (Chaudhary and Aggarwal, 2004; Joshi *et al.*, 2006). From clinicians' perspective of treating bacterial infection, it is more important to know whether the organism is susceptible or resistant to particular antimicrobial therapeutic agent (Bush, 2001; Litake, 2008).

There are several phenotypic methods available to detect the presence of ESBL such as double disc synergy test, phenotypic confirmatory disc diffusion test, three dimensional test, inhibitor potentiated disc diffusion test, disc approximation test, MIC reduction test (Chaudhary and Aggarwal, 2004). Many commercial ESBL detection methods are available such as VITEK ESBL test, the E-test ESBL detection, Phoenix ESBL test and MicroScan ESBL plus confirmation panel, with advantages and disadvantages (Sturenburg *et al.*, 2004; Spanu *et al.*, 2006). None of the method is perfect for accurate detection of ESBLs from clinical isolates of Gram-negative bacteria (Nasim *et al.*, 2004) and ESBL detection relies on molecular techniques. In rural and small town hospitals molecular diagnostic set up is not available and hence the clinicians mainly rely on clinical laboratory findings of traditional phenotypic tests. Therefore, the present work compares the phenotypic methods of ESBL detection from clinical isolates of *A. baumannii* from tracheal secretions and evaluate of the findings to demonstrate which one more suitable.

Materials and Methods

Total 145 β-lactamase positive isolates of *A. baumannii* from tracheal secretions identified by VITEK-2 system, were screened for the presence of β-lactamases using Cefinase disc test (Difco Laboratories, USA) (Montgomery *et al.*, 1979) and as per report (Litake *et al.*, 2009).

Phenotypic Detection of Extended Spectrum β-Lactamases

All β-lactamase positive isolates were screened for the presence of Extended Spectrum β-Lactamase (ESBL) using Double Disk Synergy Test (DDST) and Phenotypic Confirmatory Disc Diffusion Test (PCDDT). The tests were performed by standard disc diffusion assay on the Muller Hinton agar (NCCLS, 1999). To confirm the reproducibility of results each test was performed in duplicates. The commercially available antibiotic discs (Hi-media Laboratories, India and Difco Laboratories, USA) were used for ESBL detection.

Double Disc Synergy Test

A modified double disc synergy test was used to screen the isolates. The antibiotic discs of ceftazidime (30 µg), cefotaxime (30 µg), ceftriaxone (30µg), cefepime (30 µg), aztreonam (30 µg) and amoxyclav (Augmentin) (20/10 µg) were used as described (Sanguinetti *et al.*, 2003; Capoor *et al.*, 2005). Augmentin disc was placed in the center and rest of the discs around it. The distance between two discs was maintained to 15 mm (Babypadmini and Appalaraju, 2004; Shukla *et al.*, 2004). As all the isolates were multi-drug resistant (pre-determined by VITEK-2), the minimum distance was kept rather than the 25-30 mm distance as recommended in original test (Jarlier *et al.*, 1988). Along with this, a cefoxitin (30µg) disc was also placed on the same plate to determine the susceptibility to cephamycin. The ESBL production test was considered positive, if a clear extension of the edge of the zone of inhibition of cephalosporin toward Augmentin observed or if neither discs were inhibitory alone but the bacterial growth inhibition observed where the two antibiotics diffuse together (Shukla *et al.*, 2004).

Phenotypic Confirmatory Disc Diffusion Test

The second test performed to detect ESBL production in isolates was the phenotypic confirmatory disc diffusion test (PCDDT) as recommended by CLSI guidelines for antimicrobial disc susceptibility tests (NCCLS, 2003b). The test involved use of ceftazidime (30µg) and cefotaxime (30µg), alone and in combination with clavulanic acid (10µg) (the β-lactamase inhibitor) (Figure 1). The test was considered positive when an increase in the diameter of zone of inhibition resulted in ≥5 mm around ceftazidime/clavulanic acid against ceftazidime alone, and ≥3 mm around the cefotaxime/clavulanic acid against cefotaxime (Shukla *et al.*, 2004). The isolates which were found ESBL producer with either of these tests were further investigated with two more tests, viz. determination of the minimum inhibitory concentration (MIC) for ceftazidime (GlaxoSmithKline Pharmaceuticals Ltd., India) and cefotaxime (Alkem Laboratories Ltd., India) alone and in

combination with clavulanic acid and the unique disc placement suggested (Rodrigues *et al.*, 2004).

Determination of Minimum Inhibitory Concentration

The MIC was determined by agar dilution method as per CLSI guidelines for dilution antimicrobial susceptibility tests (NCCLS, 2003a). Ceftazidime and cefotaxime (1µg/ml through 512µg/ml) alone and in combination with clavulanic acid (4µg/ml) were used in MIC determination. *E. coli* (NCIM 2931) was used as an internal control. The higher concentrations of antibiotics were selected owing to the pre-determined resistance through earlier tests. The test was considered positive when the isolate showed three twofold (total eight fold) reduction in the MIC of antibiotic in combination with clavulanic acid over the antimicrobial agent alone (Chaudhary and Aggarwal, 2004).

Unique Disc Placement Test

A novel disc placement was performed (as shown in Figure 2) for ESBL detection from *Ab* isolates as described (Rodrigues *et al.*, 2004). The test is also useful for the detection of an ampC type β-lactamase at the same time. Seven antibiotic discs were used in this test, viz. imipenem (10µg), cefotaxime (30µg), cefoxitin (30µg), ceftazidime (30µg), ceftazidime/clavulanic acid (30/10µg), aztreonam (30µg) and ceftriaxone (30µg). Imipenem, the inducer of β-lactamase, was placed in the center, and the remaining discs were placed as per instructions of this test, and the test was interpreted as either the isolate is ESBL producer, inducible ampC producer or a derepressed mutant or have multiple mechanisms of resistance as suggested (Rodrigues *et al.*, 2004)

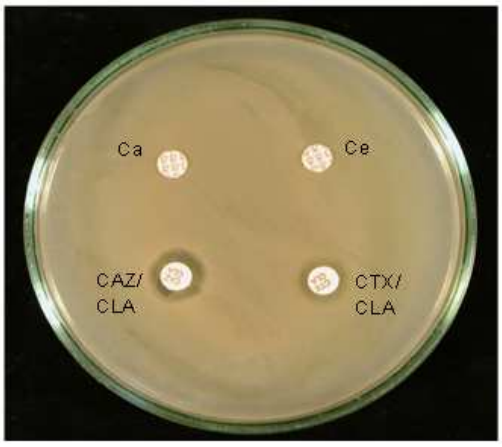

Fig. 1. Depiction of ESBL detection in *Acinetobacter baumannii* by PCDDT. Ca, Ceftazidime; Ce, Cefotaxime; CAZ-CLA, Ceftazidime/Clavulanic acid; CTX-CLA, Cefotaxime /Clavulanic acid

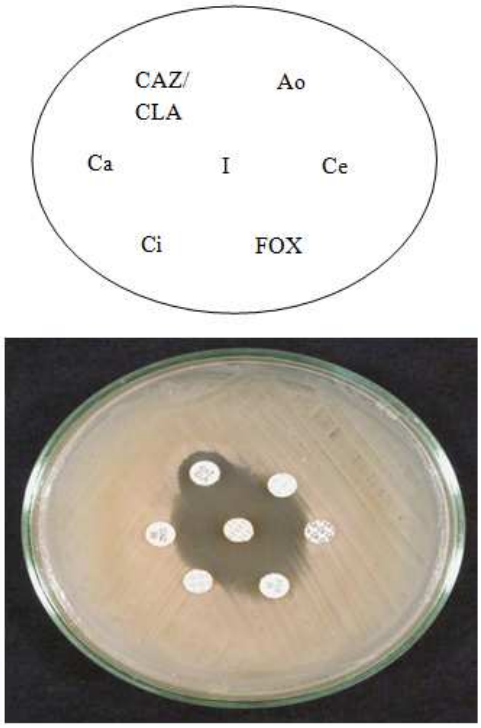

Fig. 2. Representation of ESBL detection in *A. baumannii* by the unique disc placement scheme (above, schematic; below inoculated plate). I, imipenem; Ce, cefotaxime; FOX, cefoxitin; Ca, ceftazidime; CAZ/CLA, ceftazidime/clavulanic acid; Ao, aztreonam; Ci, ceftriaxone

Results

Out of 145 β-lactamase-positive isolates 18 (12.41%) found ESBL producers. None of the isolates showed a clear-cut positive double disc synergy test. Only two isolates showed little indication of positive DDST, and the findings of DDST were in doubts. Compared to DDST, the results of PCDDT were convincing. All 18 isolates showed positive PCDDT. Figure 1 is a representative test plate of one of the isolate.

The findings of MIC of these 18 *A. baumannii* isolates are shown in Table 1. Most of the isolates showed extremely high MIC values (≥ 512 µg/ml) for ceftazidime and cefotaxime alone and in combination with clavulanic acid, and therefore the criteria of eight fold decrease in MIC values, was fulfilled by only one isolate (positive test). Additional one isolate showed only two fold reduction in MIC for ceftazidime and ceftazidime/clavulanic acid (categorized in negative test). As the test included the highest concentration of drugs (512µg/ml), the MIC values beyond this point couldn't be measured for majority of the isolates. The concentrations above 512µg/ml are not reported and not recommended for this test, and the test was inconclusive as the criterion of eight fold reduction in MIC was not fulfilled.

Table 1. Comparison of ESBL detection methods in *A. baumannii* (n = 18)

ESBL detection methods	No. of positive isolates	No. of negative isolates	No. of indeterminate isolates
DDST	2	16	--
PDDCT	18	0	--
MIC	1	1	16
Unique disc placement	13	5	--

Unique disc placement scheme for detection of ESBL and multiple mechanisms of β-lactam resistance is shown in **Figure 2**. The unique disc placement scheme detected 13 of 18 (72.2%) isolates as ESBL producers, and the rest 5 (27.8%) isolates showed no ESBL detection by this test. Among all the eighteen isolates tested three (16.7%) isolates showed positive indication of the ampC production. High resistance to cefoxitin and cefotaxime among these isolates is suggestive of the derepressed mutants (Jacoby, 2009). Thus all these isolates indicated multiple mechanisms of β-lactam resistance. The unique disc placement scheme has an added advantage for the detection of ampC β-lactamase, presence of derepressed mutants and multiple β-lactam resistance mechanism in these isolates. A comparison of four methods viz. DDST, PCDDT, MIC determination and unique disc placement scheme for ESBL detection suggests that PCDDT is a superior method for ESBL detection (Table 1).

Discussion

Bacteria are known to produce β-lactamases, but the improvident clinical use of β-lactams mounts a selective risk factor for enormous production of broad-spectrum β-lactamases by pathogenic bacteria. The type and rate of β-lactamase production differs with geographic variation (Livermore, 1995; Jacoby and Munoz-Price, 2005; Turner, 2005). The major threat to global healthcare is due to variety of ESBL-producing opportunistic pathogens. Due to difficulty in the detection, many ESBL producers can remain unnoticed. As there are no clear CLSI guidelines for detection and reporting of ESBLs from non-fermentative bacteria such as Acinetobacter, one has to follow the guidelines for E. coli and Klebsiella sp. for ESBL detection in this pathogen (Thomson, 2001). According to the CLSI guidelines the organism that has MIC ≥ 2µg/ml for ceftazidime, cefotaxime, ceftriaxone, cefodoxime or aztreonam was considered as a potential ESBL producer. (NCCLS, 1999; Chaudhary and Aggarwal, 2004). In present work, the isolates showed variable findings on level of resistance.

The incidence of ESBL in present study isolates is almost double than earlier report from India (Capoor *et al.*, 2005) and explains the increasing ESBL-producing isolates in hospital set-up. This situation suggests a need to detect ESBLs in *A. baumannii* isolates routinely before starting antimicrobial therapy. One hundred percent resistance to cefoxitin in these isolates indicates the selection of porin-deficient mutants or production of ampC β-lactamases (Livermore, 1995; Jacoby and Munoz-Price, 2005). The escape of the ESBL producers could be a reason for the spread of ESBL-producing *A. baumannii*. The present study confirms that PCDDT is efficient in detection of ESBL (Babypadmini and Appalaraju, 2004) as compared to DDST (Lim *et al.*, 2007), and detected 100 ESBL-producers as opposed to ~15% by DDST. The PCDDT is reported as efficient method among all tested ESBL detection methods which identified majority of the ESBL producing isolates from elsewhere too (Giriyapur *et al.*, 2011; Ndiba, 2013). The DDST is not a standardized procedure and the choice of the drug to be tested and the optimum distance between the discs varies in different studies (Datta *et al.*, 2004).

Determination of MIC to ceftazidime and cefotaxime alone and with clavulanic acid was in vain in most of the study isolates due to extremely high resistance to these antibiotics and concurrent presence of ampC might have masked their detection. The unique disc placement scheme in this regard, had provided the advantage of detection of ampC producers, derepressed mutants and multiple resistance mechanisms along with ESBL production. The co-production of ampC in ESBL-positive isolates that hides the ESBL detection is also documented by other researchers (Rajni *et al.*, 2008). Only disadvantage of this test is a lack of cefotaxime/clavulanic acid disc, as some of the test isolates showed positive ESBL detection only with cefotaxime/clavulanic acid disc, indicates that this method needs to be revised.

Although phenotypic responses are variable, it is recommended to detect ESBLs (even if they are responsible for low level resistance) (Bush, 2001). The accurate and rapid methods in routine clinical diagnostics are still unmet (Vercauteren *et al.*, 1997; Thomson, 2001). As per the CLSI recommendations, the organism producing an ESBL should be reported as resistant to penicillins, cephalosporins and monobactams regardless of the actual antimicrobial profile (NCCLS, 2003b). There are no β-lactam agents under development that can evade the β-lactamases and successfully treat the life threatening *A. baumannii* infection (Jacoby and Munoz-Price, 2005). Also many ESBL producing isolates harbor co-resistance to other classes of antibiotics, especially aminoglycosides and fluoroquinolons (Turner, 2005) and suggests a need for customary ESBL detection in diagnostic procedures.

Conclusion

The presence of multiple mechanisms of β-lactam resistance and lack of accurate test for ESBL detection in *A. baumannii* are challenging and possess limitations for appropriate antibiotic therapy. Our studies suggest that PCDDT is more reliable among all tested methods of phenotypic ESBL detection in clinical isolates of *A. baumannii*.

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Author's Contributions

The study was discussed and experiments designed by all the authors and experiments carried out by Geetanjali M. Litake. Manuscript developed by Geetanjali M. Litake and Suresh G. Joshi and edited by all authors.

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The authors declare no conflict of interest.

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