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Antibiotic resistance profile and co-production of extended spectrum beta lactamases and AmpC in *Acinetobacter* spp. in a level 1 trauma center from India

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Abstract:

INTRODUCTION: *Acinetobacter baumannii* has now emerged as a significant nosocomial pathogen in health-care setting ESP in intensive care units. Rapidly growing resistance among clinical isolates suggests a need to detect resistance mechanisms in this organism. The present study was designed to compare the various phenotypic tests available with the gold standard of genotype.

METHODOLOGY: The present study was conducted to include all isolates of *Acinetobacter* spp. isolated over 3 years. Their resistance to various antibiotics was determined and extended spectrum beta-lactamases (ESBL) and AmpC production in the isolates showing resistance to ceftazidime/ceftriaxone/cefotaxime (CAZ/CTR/CTX) was determined. ESBL and AmpC production was confirmed using polymerase chain reaction (PCR).

RESULTS: A total of 154 strains were isolated, and all the strains were tested for ESBL and AmpC detection. Of the strains tested, 15 (9.7%), 17 (11%), 24 (15.6%), 27 (17.5%), 54 (35%), 67 (43.5%), and 72 (46.7%) strains showed ESBL production using CTX/CTX-clavulanate double-disc synergy test (DDST), CTX/CTX-clavulanate E-test, CAZ/CAZ-clavulanate DDST, CAZ/CAZ-clavulanate E-test, Piperacillin/Piperacillin-tazobactam (TZ) DDST, CTR/CTR-Sulbactam DDST, and Piperacillin/Piperacillin-TZ E-test, respectively. 20 (12.9%) and 19 (12.3%) of strains were positive for AmpC production using AmpC disc test and Boronic acid inhibition test, respectively. Genotype analysis using PCR for TEM, SHV, CTXM, PER, and VEB genes was done and 69 (51.5%) strains were positive for TEM gene.

DISCUSSION: ESBL detection in *Acinetobacter* spp. is difficult as standard guidelines for the same are not available unlike in enterobacteriaceae, and there are no zone diameter breakpoints for aztreonam and cefpodoxime. In comparison, piperacillin/piperacillin-TZ E-test had the best sensitivity and specificity for ESBL detection.

CONCLUSION: Standard guidelines for ESBL detection in nil fermenters like *Acinetobacter* spp. must be laid down for ease of detection. Use of piperacillin/piperacillin-tazobactam E-test could be used as one of the standard methods.

Key words:

Acinetobacter spp., AmpC, antibiotic resistance, extended spectrum beta-lactamases

Introduction

Acinetobacter baumannii, named after Paul Baumann, is ubiquitous in soil

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and water and was earlier considered to be a normal commensal of the human flora.^[1] However, it has now emerged as the most significant nosocomial pathogen in the

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health-care setting ESP in intensive care units (ICUs) [2] due to its virulence properties and its ability to acquire resistance to various antibiotics.[3] Reports of community-acquired *Acinetobacter* infections have increased over the past decade.[4] *Acinetobacter* spp. have been associated with a wide range of infections such as pneumonia, bloodstream infection, surgical site infection, and urinary tract infection.[5] The mortality of patients with *A. baumannii* infections in hospitals and in the ICU has ranged from 7.8% to 23% and from 10% to 43%, respectively.[6]

The extent of antimicrobial resistance is more severe in *A. baumannii* isolates from patients in Asian and European ICUs than from patients in American ICUs.[7] A significant increase in antimicrobial resistance has been noted worldwide from 2004 to 2009.[6] *Acinetobacter* spp. have a number of resistance mechanisms such as β lactamase enzyme production, aminoglycoside-modifying enzyme, porin mutation, and efflux pumps which confer resistance to large number of antibiotics. The first MDRAB isolate resistant to almost all available antibiotics in Taiwan was discovered in 1998.[8] A high level of carbapenem-resistant *Acinetobacter* spp. have also been reported from the Indian ICUs with the rate of resistance ranging from 76% to 90%.[9,10] The spread of multidrug-resistance determinants in *A. baumannii* is mostly through plasmid conjugation, transposon acquisition or integron mobilization to gain clusters of genes encoding resistance to several antibiotic families.[11]

Extended spectrum beta-lactamases (ESBLs) in *A. baumannii* are widely reported from many countries, such as India, France, Turkey, Korea, Belgium, Latin America, the United Kingdom, and the United States.[2] *Acinetobacter* inherently produces chromosomally mediated AmpC type cephalosporinases which are also known as *Acinetobacter*-derived cephalosporinases.[12] Co-production of ESBLs and AmpC β -lactamases is a major problem which is responsible for causing therapeutic failures with the use of most of the antibiotics. Rapidly growing resistance among clinical isolates suggests a need to detect resistance mechanisms in this organism. Various methods are available for detection of ESBL and AmpC production. However, none of these tests has been found to be the best in *Acinetobacter* spp.[13] Thus, the present study was designed to compare the various phenotypic tests of ESBL and AmpC production available with the gold standard of genotype.

Methodology

The study was conducted over a period of 3 years (2013–2015) at a 186 bedded level 1 trauma center of

India. All consecutive strains of *Acinetobacter* spp. isolated from the various clinical samples were included in the study. All the strains isolated were identified using the Vitek 2 system, Biomerieux, and France (GNID ref 21341) and their antibiotic sensitivity was done using the disc diffusion method as per the CLSI guidelines. The antibiotics tested were: ceftazidime (CAZ) (30 μ g), cefotaxime (CTX) (30 μ g), ceftriaxone (CTR) (30 μ g), ceftipime (CPM) (30 μ g), CPM/tazobactam (CPM/TZ) (30/10 μ g), cefoperazone/sulbactam (SUL), imipenem (IMI) (10 μ g), meropenem (MERO) (10 μ g), amikacin (30 μ g), netilmicin (30 μ g), levofloxacin (5 μ g), tigecycline (TGC) (15 μ g), and colistin (CL) (10 μ g).

All isolates found resistant to either CTX, CAZ, or CTR were tested for ESBL and AmpC production. ESBL detection was done using a double-disc synergy test (DDST) and E-test for ESBL detection using clavulanic acid or TZ as an inhibitor. AmpC detection was done using AmpC disc test and boronic acid inhibition test.

Extended spectrum beta-lactamases detection

Double-disc synergy test^[13]

The test was performed using ceftazidime/CAZ-clavulanate, CTX/CTX-clavulanate, piperacillin/piperacillin-TZ, and CTR/CTR-SUL discs. A ≥ 5 mm increase in the zone size was considered as positive for ESBL production.

E-test for extended-spectrum beta-lactamases detection^[13]

Minimum inhibitory concentrations (MICs) of CTX and CAZ with and without clavulanic acid; and piperacillin/piperacillin-TZ were tested by the E-test method as per the manufacturer's instructions. An ≥ 8 -fold reduction in cephalosporins/piperacillin MICs in the presence of clavulanate/TZ was taken as confirmatory of ESBL.

AmpC detection

AmpC disc test^[14]

A lawn culture of *Escherichia coli* ATCC 25922 was grown on a Mueller-Hinton Agar plate. Several colonies of test organism were inoculated on sterile discs (6 mm) which were moistened with sterile saline (20 μ l). The inoculated disc was placed beside a cefoxitin disc on agar plate. The plates were incubated overnight at 35°C. A positive test was considered to be either flattening or indentation of the cefoxitin inhibition zone, which indicated enzymatic inhibition of cefoxitin. An undistorted zone was suggestive of a negative test.[14]

Boronic acid disk test method

For this, the 30 μ g cefoxitin disk will be supplemented with 300 μ g of phenylboronic acid. An organism demonstrated a defined increase (≥ 5 mm) in zone diameter around the antibiotic disk with added

inhibitor compound compared to that with the antibiotic-containing disk alone will be considered to be an AmpC producer.^[15]

Genotype analysis

DNA was extracted using the standard method, and PCR for ESBL and AmpC production was performed.

Polymerase chain reaction for extended spectrum beta-lactamases detection^[16]

Suspected/phenotypically confirmed ESBL producing strains were examined for the presence of the *bla*_{TEM'}, *bla*_{SHV}, *bla*_{CTX-M'}, *bla*_{PER'}, and *bla*_{VEB} β-lactamases genes, using the primers listed in Table 1.

Polymerase chain reaction protocol for AmpC production^[16]

Multiplex PCR was performed for the detection of MOX-1, MOX-2, CMY-1, CMY-8 TO CMY-11, LAT-1 TO LAT-4, CMY-2 to CMY-7, BIL-1, DHA-1, DHA-2, ACC, MIR-1T ACT-1, FOX-1 to FOX-5B. The primers used for PCR amplification are listed in Table 2.

Ethical clearance

This study (project code: I-800, grant number: 5/3/3/26/2011-ECD-I) was approved by the Institute Ethics Committee, All India Institute of Medical Sciences, New Delhi.

Statistical analysis

Data were analyzed statistically using the SPSS software version 6.0. (SPSS Inc., Chicago, Illinois, USA).

Results

A total of 154 strains of *Acinetobacter* spp. were isolated and identified. The antibiotic resistance pattern of the strains isolated is shown in Figure 1.

Of the 154 strains tested, only 8 (5.1%), 2 (1.2%), and 3 (1.9%) strains, respectively, were sensitive to CAZ, CTX, and CTR. Thus, all the strains were tested for ESBL and AmpC production. Of the strains tested, 15 (9.7%), 17 (11%), 24 (15.6%), 27 (17.5%), 54 (35%), 67 (43.5%), and 72 (46.7%) strains showed ESBL production using CTX/CTX-clavulanate DDST, CTX/CTX-clavulanate E-test, CAZ/CAZ-clavulanate DDST, CAZ/CAZ-clavulanate E-test, Piperacillin/Piperacillin-TZ DDST, CTR/CTR-Sulbactam DDST, and Piperacillin/Piperacillin-TZ E-test, respectively. 20 (12.9%) and 19 (12.3%) of strains were positive for AmpC production using AmpC disc test and boronic acid inhibition test, respectively.

Genotype analysis using PCR for TEM, SHV, CTXM, PER, and VEB genes showed that 69 (51.5%), 25 (21.1%),

Table 1: Primer and polymerase chain reaction conditions for amplification of β-lactamases

Primer name	Sequence	PCR conditions	Amplicon size (bp)
TEM-F	AGATCAGTTGGGTGCACGAG	1.5 mMg; 55°C annealing	750
TEM-R	TGCTTAATCAGTGAGGCACC		
SHV-F	GGGAAACGGAAGTGAATGAG	1.5 mMg; 55°C annealing	380
SHV-R	TTAGCGTTGCCAGTGCTCG		
CTX-MU-1	ATGTGCAGCACCAAGTAACGT	1.5 mM Mg; 58°C annealing, 1 min	867
CTX-MU-2	TGGGTCAACTAGGTGACCAGA		
PER-F	ATGAATGTCATTATAAAAGC	1.5 mM Mg; 55°C annealing	926
PER-R	AATTTGGGCTTAGGGCAGAA		
VEB-F	GTTAGCGGTAATTTAACCAG	1.5 mM Mg; 55°C annealing	820
VEB-R	TATTCAAATAGTAATCCACG		

PCR=Polymerase chain reaction

Table 2: Primers used for amplification of AmpC genes

Target (s)	Primer	Sequence (5' to 3')	Expected amplicon size (bp)
MOX-1, MOX-2, CMY-1, CMY-8 TO CMY-11	MOXMF	GCT GCT CAA GGA GCA CAG GAT	520
	MOXMAR	CAC ATT GAC ATA GGT GTG GTG C	
LAT-1 TO LAT-4, CMY-2 to CMY-7, BIL-1	CITMF	TGG CCA GAA CTG ACA GGC AAA	462
	CITMR	TTT CTC CTG AAC GTG GCT GGC	
DHA-1, DHA-2	DHAMF	AAC TTT CAC AGG TGT GCT GGG T	405
	DHAMR	CCG TAC GCA TAC TGG CTT TGC	
	ACCMF	AAC AGC CTC AGC AGC CGG TTA	
MIR-1T ACT-1	ACCMR	TTC GCC GCA ATC ATC CCT AGC	302
	EBCMF	TCG GTA AAG CCG ATG TTG CGG	
	EBCMR	CTT CCA CTG CGG CTG CCA GTT	
FOX-1 TO FOX-5B	FOXMF	AAC ATG GGG TAT CAG GGA GAT G	190
	FOXMR	CAA AGC GCG TAA CCG GAT TGG	

40 (30.3%), 50 (44.2%), and 3 (4.3%) strains, respectively, were positive for TEM, SHV, CTXM, PER, and VEB genes. PCR for AmpC genes showed that only 24 (9.4%) strains produced DHAM gene. None of the strains showed production of MOXM, CITM, ACCM, EBCM, and FOXM. Using the SPSS software sensitivity and specificity of the tests was found which is given in Table 3. ESBL and AmpC co-production was seen in 18 (11.7%) strains.

Discussion

MDR *Acinetobacter* spp. have been isolated worldwide. The rate of carbapenem resistance in *Acinetobacter* spp. isolated from Europe has been increasing with the lowest rates being reported from Scandinavia.^[17] According to MYSTIC surveillance study conducted in the year 2002–004 in 48 European hospitals, 26.9%, 30.2%, 67.6%, 66%, and 52.4% of the strains were resistant to MERO, IMI, CAZ, ciprofloxacin, and gentamicin respectively.^[18] The prevalence of carbapenem-resistant *Acinetobacter* spp. from a burns unit in the USA was found to be substantially high, being 87% in the year 2007.^[19] In the Asian subcontinent, the level of resistance to all the available antibiotics including TGC and CL is very high.^[20] The level of CL resistance has been variable but is ever increasing ranging from; 2.7% from Europe,^[20] 2% in the UK,^[21] and only sporadic cases from Slovakia.^[22] Similarly, in our study also very high resistance was seen to all the available antibiotics.

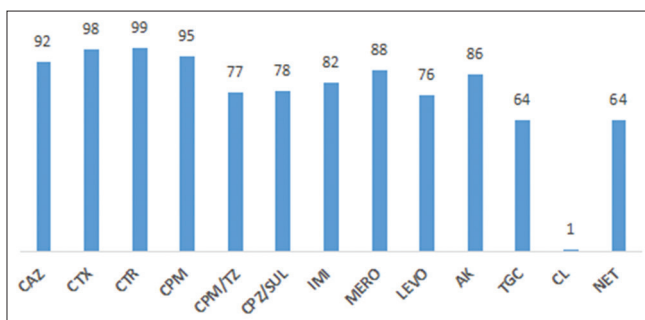


Figure 1: Antibiotic resistance profile (% resistance) of *Acinetobacter* spp.

Table 3: Statistical analysis of the tests

Test	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)
CAZ/Cefta-clav DDST	25.8	62.5	50	36.7
CAZ/Cefta-clav Etest	29.03	65.62	55.1	38.9
CTX/Cefo-clav DDST	16.13	56.25	34.8	31.58
CTX/Cefo-clav Etest	18.28	57.8	38.6	32.7
Piperacillin/Pipera-Tazo DDST	67.1	79.7	83.8	61.5
Piperacillin/Pipera-Tazo Etest	82.42	87.3	90.4	77.5
CTR/Ceftria-Sulbactam DDST	69	79.7	83.8	63
AmpC disc test	83.3	86.1	52.6	96.6
Boronic acid inhibition test	79.2	76.9	38.8	95.2

CAZ=Ceftazidime, CTX=Cefotaxime, CTR=Ceftriaxone, DDST=Double-disc synergy test

ESBL detection in *Acinetobacter* spp. is difficult as standard guidelines for the same are not available unlike in *Enterobacteriaceae*. Unlike *Enterobacteriaceae*, there are no zone diameter breakpoints for aztreonam and cefpodoxime in *Acinetobacter* spp.^[23] Thus, in the present study, isolates showing resistance to CAZ, CTR, and CTX were tested for ESBL detection. ESBL detection was assessed using TZ, sulbactam, and clavulanate as inhibitors and was compared with PCR (taking PCR as the gold standard). In comparison, tests performed using SUL and TZ as inhibitors performed better than tests with clavulanic acid inhibitor. AmpC producing organisms act as hidden reservoirs for ESBLs. Such isolates, when tested using clavulanic acid inhibition test, are induced to produce high levels of AmpC enzymes which may antagonize the synergy arising from inhibition of ESBLs leading to false negative results. SUL and TZ are much less likely to induce AmpC β-lactamases and therefore, are preferable inhibitors for ESBL detection tests.^[12] However, few studies using clavulanic acid as inhibitor for DDST have reported up to 28% ESBL production in *Acinetobacter* spp.^[24]

The prevalence of AmpC β-lactamase producing *Acinetobacter* spp. appears to be increasing and they have been associated with increased nosocomial infections. However, in the present study, only 12% of strains showed AmpC β-lactamase production. Other authors also detected AmpC β-lactamases in 6 (50%) out of 12 isolates of the *Acinetobacter* species using the same method.^[25]

Genotypically, TEM gene was found the most common gene in our hospital isolates. Safari *et al.* from Iran reported SHV gene as the most frequent gene in 58% of the strains.^[26] Pragasam *et al.* from India reported TEM (54%), SHV (16%), and PER (1%) to be the most abundant in strains isolated from South India.^[27]

Most of the ESBL producing strains in our study were resistant to almost all the antibiotics. This could be due to the expression of other resistance mechanisms such as production of carbapenemases, porin mutation, and outer membrane impermeability, The presence of resistance

to beta-lactams is generally associated with resistance to other groups of antibiotics such as aminoglycosides and fluoroquinolones. Resistance mechanisms are generally transferable by way of plasmids.^[2] According to the growing and threatening danger of these bacteria, the rapid identification and detection of this strain have an important role in preventing their spread. Furthermore, these findings illuminate the necessity of review in antimicrobial therapy, use of proper antibiotics, and the utilization of new antimicrobial elements to treat the infections resulting from these bacteria. This is a big lacuna of the current study as it was not clinically correlated with the response of the patient to the antibiotics.

Conclusion

Standard guidelines for ESBL detection in nil fermenters like *Acinetobacter* spp. must be laid down for ease of detection. Use of piperacillin/piperacillin-tazobactam E-test could be used as one of the standard methods.

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Conflicts of interest

There are no conflicts of interest.

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