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Microbiology Section

Co-production of ESBL and AmpC β-Lactamases in Clinical Isolates of *A. baumannii* and *A. lwoffii* in a Tertiary Care Hospital From Northern India

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ABSTRACT

Background: *Acinetobacter baumannii* is an important cause of health care associated infections which are difficult to control and treat, because of widespread antimicrobial resistance which is possessed by this organism.

Aim: The aim of the present study was to know the prevalence of ESBLs and AmpC β-lactamases in clinical isolates of *Acinetobacter spp*. which were cultured from various clinical specimens by using different phenotypic methods.

Settings and Design: Study was conducted over a period of one year at the Microbiology Department of a tertiary care teaching hospital. A total of 100 consecutive, non-duplicate strains of *Acinetobacter* species which were isolated from various clinical samples were included.

Materials and Methods: All the isolates were identified by standard microbiological procedures and antimicrobial susceptibility testing was done by Kirby-Bauer disc diffusion technique. Isolates which showed reduced susceptibilities to third generation cephalosporins were tested for ESBL production by CLSI double disc synergy method and also by using sulbactam as an inhibitory agent. Isolates which showed reduced susceptibilities to cefoxitin were tested for AmpC detection by doing AmpC disc test.

Statistical Analysis: SPSS, version 17 was used to calculate p-value. If the p-value was <0.05, it was considered to be significant.

Results: Out of 100 isolates, 82 were *Acinetobacter baumannii* and 18 were *Acinetobacter lwoffii*. ESBL were mentioned in 4% of the Acinetobacter isolates and in 77% of the isolates by using clavulanic acid and sulbactam as inhibitory agents respectively. AmpC β-lactamase production was detected in 60% isolates of *Acinetobacter spp*. Co-production of both ESBL and AmpC enzymes were seen in 29% of the *Acinetobacter* strains.

Conclusion: Failure in detecting β-lactamases contributes to their uncontrolled spread and therapeutic failures. Hence, these β-lactamases should be detected routinely and they should be reported to clinicians in time, so that inappropriate use of antibiotics can be stopped in time.

Keywords: *Acinetobacter*, Extended spectrum β-lactamases, AmpC β-lactamases, Multi-drug resistance, Cephalosporins

INTRODUCTION

Members of the genus *Acinetobacter*, in particular, *Acinetobacter baumannii*, are being reported increasingly as the causative agents of numerous hospital outbreaks which occur in several countries. They are responsible for causing a number of nosocomial infections like septicaemia, pneumonia, wound sepsis, endocarditis, meningitis and urinary tract infections (UTIs), especially in intensive care settings [1]. The infections caused by these organisms are often extremely difficult for clinicians to treat, because of the widespread resistance of these bacteria to the major groups of antibiotics like aminoglycosides, fluoroquinolones, ureidopenicillins and third generation cephalosporins. Moreover, the ability of resistant strains of *A. baumannii* to survive for prolonged periods in the hospital environment, contributes significantly to antimicrobial resistance, thereby posing a difficult challenge for those who are involved in infection control services[1,2].

Resistance to β-lactams appears to be primarily caused by production of β-lactamases which include extended spectrum β-lactamases (ESBLs), metallo-β-lactamases, and most commonly, oxacillinases [3]. *Acinetobacter* inherently produces chromosomally mediated AmpC type cephalosporinases which are also known as *Acinetobacter* derived cephalosporinases (ADCs), which mediate resistance to cephalothin, cefazolin, cefoxitin, most of the penicillins and β-lactamase inhibitor β-lactam combinations. More than 25 varieties of AmpC β-lactamases that share ≥ 94% protein sequences have been described for *Acinetobacter spp* [4].

PER-1 was the first ESBL to be reported in *A. baumannii* and strains harbouring PER-1 demonstrate a high level resistance to penicillins and extended spectrum cephalosporins, but it fortunately does not confer resistance to carbapenems. Routine detection of strains harbouring ESBLs may be difficult, because the synergy existent between third-generation cephalosporins and clavulanic acid, which is typically observed with ESBL-producing Enterobacteriaceae, which tends to be minimal with *Acinetobacter* spp. Therefore, it is uncertain as to what extent class A ESBLs are distributed in *A. Baumannii* [5,6].Co-production of ESBLs and

AmpC β-lactamases is a major problem which is responsible for causing therapeutic failures with use of most of the antibiotics. It has been observed by many workers that coproduction of ESBLs and AmpC β-lactamases is fairly a common phenomenon which is seen in many gram-negative isolates. However, no detailed study on *Acinetobacter* spp. has been done. Hence, in view of the increasing significance of coexistence of β-lactamases, the present study was undertaken to know the prevalence of coexistence of ESBLs and AmpC β-lactamases in clinical isolates of *Acinetobacter* spp. by using different phenotypic methods.

MATERIALS AND METHODS

The present prospective study was conducted in the Department of Microbiology, Pt. B. D. Sharma PGIMS, Rohtak, during a period of one year (May 2010 to April 2011). A total of 100 strains of *Acinetobacter* species which were isolated from various clinical samples like blood, lower respiratory tract (LRT) samples (endotracheal aspirates, bronchoalveolar lavage, sputum), urine, pus, throat swabs, high vaginal swabs (HVS), CSF and other body fluids were included in the present study. All the isolates were identified by using standard microbiological procedures and antimicrobial susceptibility testing was done by Kirby-Bauer disc diffusion technique as per Clinical and Laboratory Standard Institute (CLSI) criteria [7]. Antibiotic discs used in the study were procured from Hi-media Laboratories, Mumbai, India and from BD Diagnostics, USA. American Type Culture Collection (ATCC) strain viz. *E. coli.* ATCC 25922 was employed as a control strain. Discs of the following antimicrobial agents, with their disc concentration, in brackets, were put up: ceftazidime (30μg), cefepime (30μg), ceftriaxone (30μg), cefotaxime (30μg), amoxycillin/clavulanic acid (20μg/10μg), imipenem (10μg), meropenem (10μg), piperacillin/tazobactam (100μg/10μg), ticarcillin/clavulanic acid (75μg/10μg), gentamicin (10μg), amikacin (30μg), netilmicin (30μg), ciprofloxacin (5μg), doxycycline (30μg), cotrimoxazole (25μg), aztreonam (30μg), polymyxin B (300 units), colistin (10μg) and cefoxitin (30μg).

ESBL Detection

Isolates showing reduced susceptibility to third generation cephalosporins were tested for ESBL production by CLSI double disc synergy method [7] and also by using sulbactam as an inhibitory agent [8].

Method in which sulbactam was used as an inhibitory agent: The test organism was inoculated on Mueller-Hinton agar (MHA) plate as per CLSI guidelines. One 30μg disc of ceftazidime, ceftriaxone and cefepime each were placed on surface of MHA plate and another 30/15μg disc of ceftazidime/sulbactam, ceftriaxone/sulbactam and cefepime/sulbactam each were placed on the same agar plate at a distance of approximately 15mm from the ceftazidime, cefotaxime and cefepime discs respectively. A > 5mm increase in zone diameter produced by antimicrobial agents which were tested in combination with sulbactam versus its zone when it was tested alone was considered as positive for ESBL production.

AmpC Detection

Isolates showing reduced susceptibility to cefoxitin were tested for AmpC detection by using AmpC disc test. A lawn culture of E. coli ATCC 25922 was grown on an MHA plate. Several colonies of test organism was inoculated on sterile discs (6mm) which were moistened with sterile saline (20μL). 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 [9].

STATISTICAL ANALYSIS

Two or more sets of variables were compared by using SPSS, version 17. If the p-value was <0.05, it was considered to be significant.

RESULTS

Out of 100 clinical isolates of *Acinetobacter*, 82 were *Acinetobacter baumannii* and 18 were *Acinetobacter lwoffii*. Among isolates of *A. baumannii*, resistance against cefotaxime, ceftriaxone, cefepime and ceftazidime was observed in 97.56%, 96.34%, 95.12% and 93.9% strains respectively and among isolates of *A. lwoffii*, resistance to ceftriaxone was observed in 83.33% of isolates, 72.22% isolates showed resistance to ceftazidime and cefepime and resistance to cefotaxime was observed in 44.44% of isolates. ESBLs were detected in 4% of the *Acinetobacter* isolates and in 77% of the isolates by using clavulanic acid and sulbactam as inhibitory agents. Comparison of two methods with respect to ESBL detection has been shown in [Table/Fig-1]. AmpC β-lactamase production was detected in 64.63% isolates of *A. baumannii* and in 38.88% isolates of *A. lwoffii* [Table/Fig-2]. Coproduction of both ESBL and AmpC enzymes were seen in 29% of the Acinetobacter strains, out of which 23 were *A. baumannii* and six were *A. lwoffii*.

DISCUSSION

ESBL producing *Acinetobacter* isolates continue to be a major problem in clinical setups worldwide. So, knowledge on their prevalence is essential, to guide clinicians towards providing appropriate antibiotic therapies. In the present study, by using clavulanic acid as inhibitory agent, ESBLs were detected in 4% of the *Acinetobacter* isolates. Various studies done in past by using clavulanic acid as inhibitory agent had reported ESBL production in 2.08%, 21.4%, 28% and 44% isolates of *Acinetobacter* species [10-13].

Acinetobacter species	Number of ESBL producing isolates by CLSI method n(%)	Number of ESBL producing isolates by sulbactam n(%)	p-value
A. baumannii (82)	4 (4.8)	65 (79.2)	<0.001
A. lwoffii (18)	0	12 (66.6)	<0.001
Total (100)	4 (4)	77 (77)	<0.001

[Table/Fig-1]: Comparison of the two methods for ESBL detection in 100 isolates of *Acinetobacter* species

Acinetobacter species	Number of cefoxitin resistant isolates	Number of isolates showing flattening		Number of isolates showing indentation		Total number of AmpC producing isolates	
		n	%	n	%	n	%
A. baumannii(82)	82	27	50.94	26	49.05	53	64.63
A. lwoffii(18)	18	5	71.42	2	28.57	7	38.88
Total	100	32	53.33	28	46.66	60	60.0

[Table/Fig-2]: Distribution of AmpC β-lactamase producing *Acinetobacter* isolates

As is evident from the results of present study and data of other authors, the prevalence of ESBL producing *Acinetobacter* species varies greatly in different geographical areas and also from hospital to hospital. By using sulbactam as an inhibitory agent, we detected ESBLs in 77% of the *Acinetobacter* isolates. Our results were in accordance with those of other authors, who reported presence of ESBLs in 75% of the *Acinetobacter* isolates with use of sulbactam [14].

On comparing the two methods of ESBL detection, a highly significant difference (p value <0.01) in the rate of ESBL production was observed in our study. This may be due to the reason that *Acinetobacter* species also contain additional resistance mechanisms

to β-lactam antibiotics, which can mask the presence of ESBL activity. This organism inherently possess chromosomally encoded inducible AmpC cephalosporinases which can hydrolyze all β-lactam antibiotics. AmpC producing organisms act as hidden reservoirs for ESBLs. Such isolates, when they are tested by clavulanic acid inhibition test, are induced to produce high levels of AmpC enzymes which may antagonize the synergy arising from inhibition of ESBLs, which produces a false negative result. Sulbactam and tazobactam are much less likely to induce AmpC β-lactamases and therefore, are preferable inhibitors for ESBL detection tests [15].

As very few studies have been carried out, which have reported ESBL production in *Acinetobacter* species on using phenotypic methods, only limited data is available for comparison. Various past studies which used PCR analysis and isoelectric focusing, have reported PER-1 type of ESBLs in 54.6% and 46% of isolates of *Acinetobacter* species [16,17]. However, Naas et al reported VEB-1 type of ESBLs in 95% of *Acinetobacter* isolates, which was higher than that seen in the present study [18]. An outbreak occurred in France, in which VEB-1 type of ESBLs was detected in all the 12 isolates of A. *baumannii* which were studied[19]. These authors detected genes which were responsible for production of ESBLs, but many a times, there might be presence of silent genes which are not expressed phenotypically and this could be the reason for lower rate of ESBL production in our isolates.

In the current study, on comparing the antimicrobial resistance patterns of ESBL producing and ESBL non producing *Acinetobacter* isolates, a highly significant difference (p value <0.001) was observed for resistance to aminoglycosides and fluoroquinolones. Resistance to ciprofloxacin, netilmicin, gentamicin, amikacin and meropenem was observed in 83.11%, 74.02%, 84.41%, 77.92% and 70.12% of ESBL producing isolates of *Acinetobacter* species respectively, whereas resistance to same drugs was observed in 34.78%, 13.04%, 60.86%, 21.73% and 4.34% non ESBL producing isolates of *Acinetobacter* species respectively [Table/Fig-3]. Similar findings have been reported by other authors [14,17,19].This may be due to the reason that genes coding for ESBLs reside on plasmids and that these plasmids carrying ESBL genes also carry resistant genes for other antibiotics. The most frequent co-resistance found in ESBL producing organisms was that for aminoglycosides, fluoroquinolones, tetracyclines, chloramphenicol and trimethoprim-sulfamethoxazole [20].

Antimicrobial drugs	ESBL producers (n=77)		ESBL non-producers (n=23)		p-value
	n	%	n	%	
Ciprofloxacin	64	83.11	8	34.78	<0.001
Netilmicin	57	74.02	3	13.04	<0.001
Gentamicin	65	84.41	14	60.86	<0.05
Amikacin	60	77.92	5	21.73	<0.001
Meropenem	54	70.12	1	4.34	<0.001
Imipenem	55	71.42	15	65.21	>0.05
Cotrimoxazole	64	83.11	15	65.21	>0.05
Doxycycline	51	66.23	15	65.21	>0.05

[Table/Fig-3]: Comparison of the antimicrobial resistance pattern of ESBL producing and ESBL non-producing *Acinetobacter* isolate to antibiotics other than β-lactams

AmpC β-lactamase enzymes confer resistance to a wide variety of β-lactam antibiotics, except carbapenems. The prevalence of AmpC β-lactamase producing *Acinetobacter spp*. appears to be increasing and they have been associated with increased nosocomial infections. In the present study, AmpC β-lactamases were detected in 60% of the *Acinetobacter* isolates by AmpC disc method. Other authors also detected AmpC β-lactamases in 6 (50%) out of 12 isolates of the *Acinetobacter* species by using same method [21].The results of our study were in accordance with those of these studies. However, some studies have described a lower rate of AmpC β-lactamase production in *Acinetobacter* spp., which ranged from 20% to 45% [9,22,23]. This could be explained by the fact that the number of isolates which were included in these studies was much lower than that which was included in the current study. Different selection criteria used for *Acinetobacter* isolates could be another reason for obtaining different rates of AmpC β-lactamase production in current study than those seen in the above mentioned studies. In the present study, we performed AmpC disc test only for those isolates which were resistant to cefoxitin, whereas in other studies, tests for detection of AmpC β-lactamase were done for all the isolates which were included in study, irrespective of cefoxitin resistance. As no CLSI recommendations exist, regarding the method which has to be used for detection of AmpC β-lactamases, different methods have been used by different authors. This could be another reason for obtaining a different and higher rate of AmpC production in our study.

The current study showed that no AmpC β-lactamase enzymes were detected in 40.4% of cefoxitin resistant *Acinetobacter* isolates. One previous study had reported no AmpC β-lactamase activity in 55.55% of the cefoxitin resistant isolates of *Acinetobacter* species which were studied[9]. Other authors have also reported that 8 (80%) out of 10 cefoxitin resistant isolates of *Acinetobacter* species did not show any production of AmpC β-lactamase enzymes [23].This could be due to the reason that cefoxitin resistance could occur in AmpC non-producing isolates because of mechanisms other than AmpC production, such as lack of permeation of porins or there could have been a low level expression of ampC genes, which had not been detected by the present method.

In the present study, coproduction of ESBL and AmpC enzymes was detected in 29% of the *Acinetobacter* isolates. During literature search, it was observed that only limited studies had been conducted on coproduction of β-lactamases in *Acinetobacter* spp. Rajini et al reported coproduction of these enzymes in 2 (20%) out of 10 isolates of *Acinetobacter* species which were studied [15]. Nagano et al studied three *Acinetobacter* isolates for the production of β-lactamases and they reported coproduction of both ESBL and AmpC enzymes in all three (100%) isolates [24].One study showed coproduction of metallo- β-lactamases and AmpC β-lactamases in 54% of the *Acinetobacter* isolates which were studied [25].Another study reported ESBL production in 17.95% isolates and production of AmpC β-lactamases in 56.67% of *Acinetobacter* isolates which had been studied, but this study did not highlight the prevalence of *Acinetobacter* isolates which had shown coproduction of β-lactamases [26].The major lacunae in past studies which showed coproduction, was the lesser number of *Acinetobacter* strains which were studied. Hence, these could not be considered as statistically significant. It has been suggested that such studies should be conducted on more isolates obtained from geographically diverse areas, with molecular confirmation of these enzymes, so that suitable conclusions can be made regarding this aspect.

CONCLUSION

β-lactams are the most widely used antimicrobials worldwide, which are favoured because of their efficacies, broad spectra and low toxicities. *Acinetobacter* is a pathogen which is well known for its high antimicrobial resistance and it most commonly shows resistance to β-lactams, as it produces β-lactamases. Rapid phenotypic detection of combined mechanisms of antimicrobial resistance, such as ESBL and AmpC expressions, is crucial for epidemiological purposes and for implementing appropriate antimicrobial therapies and infection control measures.

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