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Molecular characterization of genes encoding AmpC beta-lactamases in clinical isolates of *Pseudomonas* and *Acinetobacter* species

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ABSTRACT

Emergence of AmpC beta-lactamases in isolates of *Pseudomonas* and *Acinetobacter* species, is a threatening condition as they mediate resistance to a wide variety of β-lactam drugs, including α-methoxy-β-lactams, such as cefoxitin, narrow-, expanded- and broad-spectrum cephalosporins, aztreonam and are poorly inhibited by β-lactam inhibitor combinations. The present study was conducted to determine the occurrence of *bla*$_{ampC}$ genes in these pathogenic non-fermenters for their rapid and accurate detection. Monoplex PCR was done to detect *bla*$_{ampC}$ genes in 40 non-duplicate clinical *Pseudomonas* and *Acinetobacter* isolates, that were found resistant to any of the third-generation cephalosporin and cefoxitin. Multiplex PCR assay was carried out to identify family-specific AmpC beta-lactamase genes within *Pseudomonas* and *Acinetobacter* spp. PCR detected *bla*$_{ampC}$ in 43.24% of *Pseudomonas* and 33.33% of *Acinetobacter* isolates. Overall 42.50% of the total isolates were found to harbour *bla*$_{ampC}$ genes by PCR. By multiplex PCR, total eight (20%) isolates yielded a positive amplicon with AmpC-specific primers. High prevalence of *bla*$_{ampC}$ genes in cefoxitin-resistant isolates of *Pseudomonas* and *Acinetobacter* isolates emphasizes that molecular detection methods should be carried out to know the exact prevalence of beta-lactamases.

INTRODUCTION

Antibiotic resistance is now a linked global problem. It increases the morbidity, mortality and costs of treating infectious diseases. The threat from resistance (particularly multiple resistance in bacterial strains that have disseminated widely) has never been so great; however there are recent concerns about the increasing frequency of antibiotic resistance. The key factors driving this threat are increased antibiotic usage (in both human and animal medicine), greater movement of people and increased industrialization. The major forces behind the development of resistance in bacteria are exposure to antibiotics followed by selection and dissemination of resistant strains. The health care setting provides a favorable environment in this context. Resistant strains evolve and disseminate by direct and indirect contacts, where large numbers of people are congregated under one roof and where the usage of antibiotics is high. Therapeutic options for infections caused by nosocomial isolates of *Pseudomonas aeruginosa* and *Acinetobacter* spp., expressing plasmid-mediated AmpC beta-lactamases are limited as these organisms are usually resistant to all beta-lactams except cefepime, cefpirome and carbapenams. Therefore, infection by these nosocomial isolates adversely affects clinical outcomes and patient treatment costs (Harris *et al*. 1999; Aloush *et al*. 2006). Since the information on the molecular documentation of AmpC β-lactamases among *Pseudomonas* and *Acinetobacter* spp. are fragmentary in India; this study was carried to determine occurrence of *bla*$_{ampC}$ genes in these pathogenic non-fermenters for their accurate detection.

MATERIALS AND METHODS

The present prospective study was conducted in the department of Microbiology, J. N. Medical College, Aligarh Muslim University, Aligarh, during a period of one year (October 2010 to September 2011). Various clinical specimens received for routine culture and susceptibility testing in the clinical microbiology lab was studied. Clinical samples received in the laboratory were inoculated on the blood agar plate (BA) and teepol lactose agar (TLA) and then incubated overnight at 37 °C.

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Pseudomonas and *Acinetobacter* spp were identified by different microbiologcal methods. Antibiotic susceptibility testing was done. 30µg cefoxitin disc was used for initial screening of AmpC beta-lactamase production. A total of forty isolates (23 *Pseudomonas* and 17 *Acinetobacter* spp.), that were found resistant to any of the third-generation cephalosporin and cefoxitin were randomly selected for molecular characterization.

Detection of bla_{ampC} genes by monoplex PCR

The study isolates were subjected to PCR as described by Feria *et al*., (2002) with some modifications to detect bla_{ampC} genes. AmpC-F-5'-CCCCGCTTATAGAGCAACAA-3' (634bp) and AmpC-R-5'-TCAATGGTCGACTTCACACC -3' primers were used. The reaction mixture was placed in MJ-mini Bio-Rad thermal cycler (Bio-Rad, USA). The PCR amplification cycle was performed with cycling conditions consisting of an initial denaturation step at 95˚C for 15 min, followed by 35 cycles of 94˚C for 60 sec., 58˚C for 2 min., 72˚C for 3 min. and the process was completed with a final elongation step at 72˚C for 10 min. Amplified PCR products were analysed by gel electrophoresis with 2% agarose (Bangalore Genei, India) gel containing ethidium bromide. After electrophoresis DNA fragments were visualized by Bio-Rad Gel documentation system (Bio-Rad, USA). Previously characterized isolates provided by Prof. Daniel Jonas, Germany, were used as positive controls.

Detection of plasmid-mediated AmpC β-lactamase by multiplex PCR

Multiplex PCR was carried out to detect family-specific plasmid-mediated AmpC β-lactamase genes by implementing protocol as described by Perez-Perez and Hanson (2002). The total volume of reaction mixture was 25 µl (Table 1). Primers were synthesised by Operon Biotechnologies, Cologne, Germany.

RESULTS AND DISCUSSION

As it is very crucial to know the exact prevalence of AmpC beta-lactamase harboring isolates of *Pseudomonas* and *Acinetobacter* spp., these isolates were subjected to genotypic detection by PCR. Table 2 shows that monoplex PCR detected - bla_{ampC} in 43.24 % of *Pseudomonas* and 33.33% of *Acinetobacter* isolates. Overall 42.50% of the total isolates were found to harbour bla_{ampC} genes by monoplex PCR (Figure 1). Multiplex PCR assay was carried out to identify family-specific AmpC beta-lactamase genes within *Pseudomonas* and *Acinetobacter* spp. As shown in table 2, total eight (20%) isolates yielded a positive amplicon with AmpC-specific primers.

Five isolates, out of total eight positive isolates corresponded to the MOX-family, while only one isolate belonged to CIT-family. Rest 2 isolates were confirmed to be of FOX-family. Two distinct bands were noticed in only one isolate, which belonged to the genus *Pseudomonas*. In the isolate showing two bands in multiplex PCR, the upper band corresponded to the CIT family and the lower band was obtained at 200bp, which corresponds to the expected position of the FOX family (Figure 2). To identify the exact family to which lower band belongs, monoplex PCR was done for all the families of AmpC β-lactamases and an amplified product corresponding to EBC family was obtained.

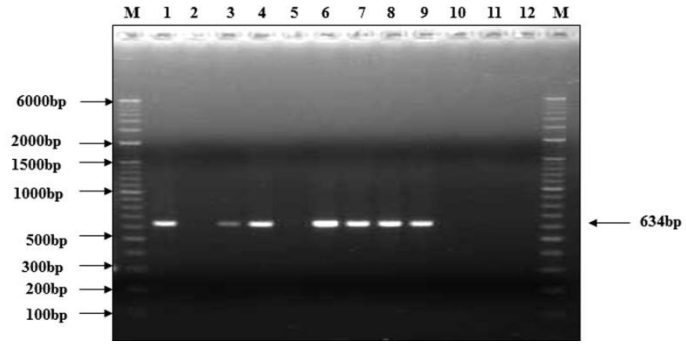

Fig. 1: 2% agarose gel is showing amplification pattern of bla_{ampC} genes. Lanes M are showing Fermentas high range DNA ruler. Lane 1 shows positive control strain for $bla_{ampC,}$ while lane 2 shows negative control. Lanes 3, 4, 6-9 shows clinical isolates harbouring bla_{ampC} genes, while lanes 10-12 shows negative isolates.

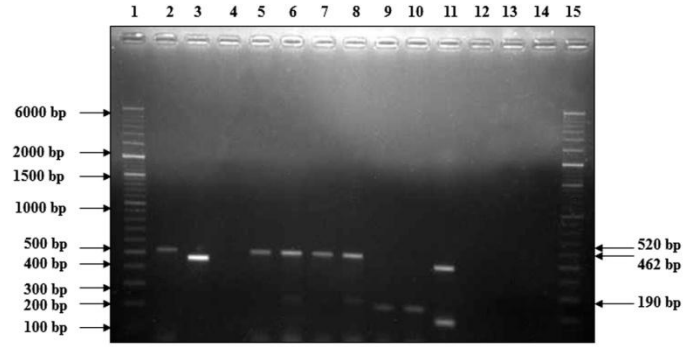

Fig. 2: Electrophoretogram (on 2% agarose) showing results of multiplex PCR for the detection of blaampC alleles. Lanes 1 and 15 show ladders (Fermentas high range DNA ruler). Lane 3 shows positive control of CIT family, whereas lane 4 shows negative control. Lanes 5-8 shows amplicons of MOX family in test strains. Lanes 9 and 10 shows FOX-family in test strains. Lane 11 shows test isolate possessing both CIT and EBC family. Lanes 12-14 shows negative isolates.

Apart from fragmentary reports regarding the prevalence of AmpC beta-lactamases in *Pseudomonas* and *Acinetobacter* spp, the actual prevalence is still unknown, as most of these studies are based on phenotypic detection methods only. One important fact is that, there is currently no clear consensus regarding guidelines for phenotypic screening or confirmatory tests for AmpC β-lactamase-producing organisms (Shahid *et al*., 2004). Our study reveals high prevalence of bla_{ampC} (42.50%) among clinically important *Pseudomonas* and *Acinetobacter* isolates from our centre. In contrary to our results, from North India, 20 % of *P. aeruginosa* (Delhi) and 20.7 % of Gram-negative organisms (Aligarh) and 47.8% *E. coli*, 17.3% *P. aeruginosa*, *13% K. pneumoniae* (Kolkata) were reported as AmpC β-lactamase producers (Manchanda and Singh, 2003; Shahid *et al.*, 2003; Suranjana and Manjusri, 2005). 37.50% isolates have been reported as AmpC-producers from Chennai (Subha *et al*., 2003).

Table 1: Preparation of reaction mixture for detection of plasmid-mediated AmpC β-lactamases.

Reagents	Quantity (µl), for 25 µl of reaction mixture
Primers	
FOXMR-5'- CAA AGC GCG TAA CCG GAT TGG-3' (190 bp)	0.16
FOXMF-5'- AAC ATG GGG TAT CAG GGA GAT G-3'	0.16
EBCMR-5'- CTT CCA CTG CGG CTG CCA GTT-3' (302 bp)	0.20
EBCMF-5'- TCG GTA AAG CCG ATG TTG CGG-3'	0.20
ACCMR-5'- TTC GCC GCA ATC ATC CCT AGC-3' (346 bp)	0.20
ACCMF-5'- AAC AGC CTC AGC AGC CGG TTA-3'	0.20
DHAMR-5'- CCG TAC GCA TAC TGG CTT TGC-3' (405 bp)	0.24
DHAMF-5'- AAC TTT CAC AGG TGT GCT GGG T-3'	0.24
CITMRb-5'-TTT CTC CTG AAC GTG GCT GGC-3' (462 bp)	0.24
CITMF-5'-TGG CCA GAA CTG ACA GGC AAA-3'	0.24
MOXMR-5'- CAC ATT GAC ATA GGT GTG GTG C-3' (520 bp)	0.24
MOXMF-5'-GCT GCT CAA GGA GCA CAG GAT-3'	0.24
PCR master mixture	12.5
Template DNA solution	2.5
Water, nuclease free	7.44

F denotes forward primer, R denotes reverse primer

Table 2: bla_{ampC} Detection by monoplex and multiplex PCR.

Organism	Monoplex PCR		Multiplex PCR	
	Positive	**Negative**	**Positive**	**Negative**
***Pseudomonas* spp. (n=37)**	16 (43.24%)	21 (56.76%)	8 (21.67%)	29 (78.38%)
***Acinetobacter* spp. (n=3)**	1 (33.33%)	2 (66.67%)	0	3 (100%)
Total (n=40)	17 (42.50%)	23 (57.50%)	8 (20%)	32 (80%)

In another study performed by Singhal *et al*. (2005), prevalence of AmpC β-lactamases was reported as 8%, which is much lower than our finding in *Pseudomonas* and *Acinetobacter* spp. In 2007, Hemlatha *et al*., from Chennai reported 47.3% AmpC-producers in *Escherichia coli* and *Klebsiella* isolates. In 2010, Upadhyay *et al*., from Varanasi, reported 59.4% isolates of *P. aeruginosa* as AmpC-producers. In the same year, Mohamudha *et al*. (2010), reported 93.6% Gram-negative clinical isolates as AmpC-producers, based on three-dimensional extract method. They reported 66.6% and 55.5% plasmid-mediated AmpC-producers in *Acinetobacter* and *Pseudomonas* respectively. 33 (32.7%) isolates of *Pseudomonas aeruginosa* were confirmed to be positive for AmpC beta-lactamase, in study conducted by kumar *et al*. in 2012.

Similar results were reported by Sreeshma *et al*. in 2013 where 48% of isolates were inducible AmpC producers and only 2% produced plasmid mediated AmpC beta-lactamase. Oteo *et al*., (2010) from Spain have reported the occurrence of plasmid-mediated bla_{ampC} in 38% isolates. Tan *et al*. (2009) detected bla_{ampC} in 47% isolates. In a study carried out by Shahid *et al.* (2009) in Aligarh between 2003 and 2005, 39.1% of isolates belonging to family *Enterobacteriaceae*, were found to carry bla_{ampC} genes. In a recent study conducted in Iran 60.8% of tha *Pseudomonas aeruginosa* isolates harbored bla_{ampC} (Roya *et al*., 2014).

CONCLUSION

The detection of bla_{ampC} reflects the emergence of beta-lactamases among antimicrobial resistant *Pseudomonas and Acinetobacter* isolates. The present study also highlights that molecular methods should be carried out in clinical laboratories to know the accurate prevalence of beta-lactamases, so that effective steps can be taken to prevent the further spread of these resistant strains.

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