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Presence of metallo-beta-lactamases (MBL), extended-spectrum beta-lactamase (ESBL) & AmpC positive non-fermenting Gram-negative bacilli among Intensive Care Unit patients with special reference to molecular detection of bla_{CTX-M} & bla_{AmpC} genes

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***Background & objectives*: Non-fermenting Gram-negative bacilli (NFGNB) including *Pseudomonas aeruginosa* and *Acinetobacter baumannii* have been implicated in a variety of infections, particularly in the Intensive Care Units (ICUs). This study was aimed to overview the burden of multidrug-resistant NFGNB causing infections in ICU and also to assess the occurrence of extended-spectrum beta-lactamases (ESBLs), AmpC and metallo-beta-lactamases (MBLs) among these isolates.**

***Methods*: Bacterial culture, identification and antibiotic susceptibility were carried out. ESBLs and AmpC were detected both phenotypically and genotypically. MBL was detected by modified Hodge and imipenem-ethylenediaminetetraacetic acid double-disc synergy test.**

***Results*: NFGNB represented 45 (37%) of total 121 Gram negative isolates. Multidrug resistance was observed in 66.9 per cent and 72.5 per cent isolates of *P. aeruginosa* and *A. baumannii*, respectively. Detection by phenotypic methods showed presence of ESBL, AmpC and MBL in 21.4, 51.1 and 21.4 per cent isolates, respectively. When detected genotypically by polymerase chain reaction, ESBL and AmpC were detected in 21.4 and 41.4 per cent of NFGNB isolates, respectively. Bla_{CTX-M} (21.4%) was the most prevalent gene responsible for ESBL production.**

***Interpretation & conclusions*: Most of the NFGNB isolated from ICU patients were multidrug-resistant and producers of ESBL, AmpC and MBL. A regular surveillance is required to detect ESBL, AmpC and MBL producers, especially in ICU patients.**

Key words *Acinetobacter baumannii* - Intensive Care Unit - multidrug-resistance - *Pseudomonas aeruginosa*

Non-fermenting Gram-negative bacilli (NFGNB) including *Pseudomonas aeruginosa* and *Acinetobacter baumannii* have been implicated in a variety of infections, including bacteraemia, urinary tract and surgical site infections among patients admitted in Intensive Care Unit (ICU)[1,2]. These

may be intrinsically resistant or may have acquired resistance to antibiotics due to impermeability of the cell surface, multidrug efflux pumps and production of β-lactamases [AmpC β-lactamase, extended-spectrum β-lactamases (ESBLs) and metallo-beta-lactamases (MBLs)][3]. Multiple beta-lactamase-producing *P. aeruginosa* can cause major therapeutic failure and poses a significant clinical challenge. Reports on carbapenemase-producing NFGNB are on the rise globally due to the increased carbapenem usage and selection of resistant bacteria under antibiotic pressure[4]. Therefore, early identification and detection of isolates that produce these enzymes are essential to avoid therapeutic failures and nosocomial outbreaks.

This study was designed to assess the burden of multidrug-resistant *P. aeruginosa* and *A. baumannii* in ICU patients. The occurrence of ESBL, AmpC and MBL among these isolates was also assessed.

Material & Methods

The present study was carried out in the department of Microbiology on patients admitted to the ICU of J. N. Medical College, Aligarh Muslim University, Aligarh, India from February 2012 to October 2013. Totally, 125 patients admitted to the ICU were included in the study. A complete history was taken from each patient. Informed written consent was obtained before the study from all the patients, and the study was performed after getting approval from the Institutional Ethics Committee.

The patients were chosen consecutively, and clinical samples were obtained from each patient (endotracheal aspirate, blood, pus, urine). All specimens were collected aseptically and were promptly sent to the microbiology laboratory. All samples were collected within 48 h of the patient admission in the ICU and those collected after 48 h of admission were not included in the study. Wounds (surgical site infections) have been classified according to the Southampton grading[5]. The majority of the cases belonged to Grade IV (purulent discharge along the wound) and Grade V (wound dehiscence). Standard methods for isolation and identification of NFGNB[6] were used.

Susceptibility testing of bacterial isolates was performed using the disc diffusion method as described by the Clinical and Laboratory Standards Institute[7]. Antimicrobial discs used were imipenem (10 µg), cefpodoxime (10 µg), cefotaxime (30 µg), cefepime (30 µg), cefixime (5 µg), cefoperazone (75 µg), cefoperazone/sulbactam (75/10 µg), ticarcillin (75 µg), piperacillin (100 µg), piperacillin/tazobactam (100/10 µg), ceftazidime (30 µg), ceftazidime/clavulanic acid (30/10 µg), cefotaxime/clavulanic acid (30/10 µg), ceftriaxone (30 µg), amikacin (30 µg), gentamicin (10 µg), tobramycin (10 µg), ofloxacin (5 µg), levofloxacin (5 µg), polymixin B (300 units) and colistin (10 µg). All discs were obtained from Hi-Media Labs, Mumbai, India.

Phenotypic methods for ESBL detection: NFGNB isolates were first screened for the production of ESBL by the disc diffusion method (screening test) using cefotaxime, ceftriaxone, cefepime and ceftazidime[7] and later on confirmed by the cephalosporin/clavulanate combination disc (disc potentiation test)[8] and double-disc synergy test[8,9]. *Escherichia coli* ATCC 25922 (non-ESBL producer) was used as a control strain.

Phenotypic methods for AmpC detection: Cefoxitin discs were used to screen AmpC producers, by disc diffusion method[10]. Isolates resistant to cefoxitin were considered as potential AmpC producers.

Phenotypic methods for MBL detection: The isolates were tested for sensitivity to imipenem (10 µg) using Kirby-Bauer method as recommended by the CLSI[7]. All the isolates with a zone of inhibition ≤16 mm or which demonstrated heaping, or if the zone was >16 but ≤20 mm, were tested for MBL production; however, there is no CLSI guideline for MBL detection available for *P. aeruginosa*. These isolates were confirmed by modified Hodge test and imipenem-ethylenediaminetetraacetic acid (EDTA) double-disc synergy test[11,12].

Genotypic methods for the detection of ESBL and AmpC production: Template DNA was prepared from freshly cultured bacterial isolates by suspending bacterial colonies in 50 µl of molecular grade water and then heating at 95°C for five minutes and immediately chilling at 4°C. Molecular detection of bla_{CTX-M}, bla_{TEM}, bla_{SHV} and bla_{AmpC} was performed using polymerase chain reaction (PCR) according to methods described previously with minor modifications (thermal profile of bla_{CTX-M}[12], primer profiles of bla_{CTX-M}, bla_{TEM}, bla_{SHV}, bla_{AmpC}[13]). The primers and cycling conditions for detection of bla_{AmpC} genes were the same as those described by Shahid *et al*[12] and Féria *et al*[13]. The quality-control strain, *Klebsiella pneumoniae* ATCC 700603 (ESBL producer) was used.

Results & Discussion

Among the 125 patients admitted to the ICU, 160 isolates were identified. Of these, Gram-negative bacilli, 121 (75.6%) predominated, followed by 22 (13.8%) Gram-positive cocci and 10.6 per cent (n=17) fungal isolates. NFGNB represented 45 (37%) of the Gram-negative isolates (n=121) of which *P. aeruginosa* (n=35, 29%) was the incriminatory pathogen in majority, followed by *A. baumannii* (n=10, 8%).

Antimicrobial resistance was observed to be higher in *A. baumannii* than in *P. aeruginosa*. Antibiotic resistance pattern of *P. aeruginosa* and *A. baumannii* is shown in Table I. Table II depicts the organisms isolated from different samples of patients in ICU. The positivity for ESBL, AmpC and MBL by phenotypic methods is shown in Table III.

$Bla_{CTX\text{-}M}$, bla_{SHV}, bla_{TEM} and bla_{AmpC} genes were detected in phenotypic ESBL and AmpC producers. $Bla_{CTX\text{-}M}$ was the predominant gene among ESBL producers as it was observed in four (11.4%) isolates of *P. aeruginosa* and one (10%) *A. baumannii*. Bla_{AmpC} was detected in 15 (42.8%) *P. aeruginosa* and four (40%) *A. baumannii* isolates. Bla_{SHV} and bla_{TEM} were not detected in any of the isolates.

Table I. Antibiotic resistance pattern of 45 non-fermenting Gram-negative bacilli detected by disc diffusion method

Antimicrobial agents	No. of isolates (%) showing antibiotic resistance	
	Pseudomonas aeruginosa (n=35)	*Acinetobacter baumanii* (n=10)
Levofloxacin	22 (62.8)	8 (80)
Ofloxacin	28 (80)	7 (70)
Amikacin	22 (62.8)	7 (70)
Gentamycin	25 (71.4)	8 (80)
Tobramycin	20 (57.1)	8 (80)
Cefotaxime	21 (60)	7 (70)
Cefepime	26 (74.2)	7 (70)
Cefixime	24 (68.5)	6 (60)
Ceftazidime	24 (68.5)	7 (70)
Cefpodoxime	23 (65.7)	6 (60)
Cefotaxime-clavulanic acid	12 (34.2)	7 (70)
Ceftazidime-clavulanic acid	10 (28.7)	8 (80)
Piperacillin	26 (74.2)	8 (80)
Piperacillin-tazobactam	22 (62.8)	7 (70)
Imipenem	5 (14.28)	1 (10)

Table II. Distribution of pathogens isolated from endotracheal aspirate and urinary tract infection in Intensive Care Unit patients

Sample	Pathogen	No. of isolates (per cent)
Endotracheal aspirate (n=55)	*Pseudomonas aeruginosa*:	22 (40)
	Klebsiella pneumonia	10 (18.2)
	Escherichia coli	8 (14.5)
	Citrobacter koseri	7 (12.7)
	Aspergillus fumigatus	5 (9.1)
	Acinetobacter baumannii	4 (7.3)
Urine (n=19)	*E. coli*	6 (31.5)
	K. oxytoca	5 (26.3)
	P. aeruginosa	5 (26.3)
	Candida albicans	2 (10.5)
	A. baumannii	1 (5.2)
Pus (n=41)	*E. coli*	15 (36.5)
	Staphylococcus aureus	12 (29.2)
	C. koseri	11 (26.8)
	K. oxytoca	10 (24.3)
	C. freundii	10 (24.3)
	P. aeruginosa	8 (19.5)
	Serratia species	7 (17.1)
	A. baumannii	3 (7.3)
Blood (n=10)	*K. pneumonia*	3 (30)
	S. aureus	3 (30)
	A. baumannii	2 (20)
	C. albicans	1 (10)
	Coagulase negative *Staphylococcus* species	1 (10)

NFGNB including *P. aeruginosa* and *A. baumannii* have been implicated in a variety of ICU infections. In this study, *P. aeruginosa* represented 29 per cent of isolates; similar results were reported by Hadadi *et al*[14]. In other studies, *P. aeruginosa* represented 15.6 per cent of the total isolates[15,16]. *A. baumannii* represented eight per cent of Gram-negative ICU infections in this study. However, other investigators found a higher incidence of *A. baumannii* (20.5 and 24.1%, respectively)[14,15].

In this study, lower respiratory tract infections (LRTIs) were the most common infection in ICU patients (34.3%). Al-Ghamdi *et al*[17] reported 8.9 per cent LRTIs among ICU cases. In our study, *P. aeruginosa* and *A. baumannii* were frequently isolated from LRTIs (40 and 7.3%, respectively), as also shown by Abd El-Fattah[18].

Table III. Number and percentage of extended-spectrum β-lactamase (ESBL)-producing *Pseudomonas aeruginosa* and *Acinetobacter baumannii* by phenotypic methods

NFGNB	Phenotypic methods for ESBL production			Phenotypic methods for AmpC production		Phenotypic methods for MBL production		
	Disc diffusion test (%)	Double-disc synergy test (%)	Disc combination test (%)	β-lactam inhibitors (%)	Cefoxitin disc (%)	Disc diffusion test (%)	Modified Hodge test (%)	Imipenem-EDTA double-disc synergy test (%)
Pseudomonas spp. (n=35)	5 (14.3)	3 (8.5)	4 (11.4)	18 (51.4)	18 (51.4)	5 (14.3)	3 (8.5)	4 (11.4)
Acinetobacter spp. (n=10)	1 (10)	1 (10)	1 (10)	5 (50)	5 (50)	1 (10)	1 (10)	1 (10)

MBL, metallo-beta-lactamase; NFGNB, non-fermenting Gram-negative Bacilli; EDTA, ethylenediaminetetraacetic acid

In our study 76.1 and 70.2 per cent of NFGNB were resistant to fluoroquinolones and aminoglycosides, respectively. Antibiotic resistance is a serious problem in developing countries, especially due to the easy availability of antibiotics over the counter[19,20]. The resistance of *P. aeruginosa* and *A. baumannii* to ceftazidime (68.5%, 70%), cefotaxime (57.1%, 70%) and cefpodoxime (65.7%, 70%) was comparable to results reported by others[14,21].

Screening by disc diffusion method in this study revealed that 24.3 per cent of NFGNB were ESBL producers. These results were comparable to that of Aggarwal *et al*[22], however, lower than results reported by Jiang *et al*[23]. Confirmation with double-disc synergy test (DDST) and disc combination tests revealed that 18.5 per cent of NF Gram-negative isolates were ESBL producers as also reported by Jiang *et al*[23]. For AmpC production, both disc diffusion test and screening by cefoxitin disc revealed same results that 51.4 per cent of *P. aeruginosa* and 50 per cent of *A. baumannii* isolates were AmpC producers. Similar results were reported by Bhattacharjee *et al*[24]. The most prevalent gene for ESBL production was $bla_{\text{CTX-M}}$ which was detected in 11.4 per cent of *Pseudomonas* isolates, while bla_{TEM} and bla_{SHV} genes were not detected in any of the isolates. These results were in agreement with Picão and Gales[25]. PCR detected bla_{AmpC} gene in 42.8 per cent of *P. aeruginosa* and 40 per cent of *A. baumannii* isolates. These results were comparable to that of Khanal *et al*[26].

Comparison between modified Hodge test and DDST in our study revealed that DDST was more sensitive for detecting MBL. The same observation was reported by Jesudason *et al*[27]. In this study, amikacin, tobramycin, imipenem, polymyxin B and colistin demonstrated maximum sensitivity against NFGNB. Therefore, use of these antibiotics should be restricted to severe infections, especially in critically ill ICU patients, to avoid rapid emergence of resistant strains.

In conclusion, our results showed isolation of a high percentage of NFGNB in ICU patients' samples, which were multidrug resistant and producers of ESBL, AmpC and MBL. A regular surveillance of antimicrobial susceptibility status of such isolates is necessary to curb the infection.

Conflicts of Interest: None.

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