Epidemiology of bacterial colonization at intensive care unit admission with emphasis on extended-spectrum β -lactamase- and metallo- β -lactamase-producing Gram-negative bacteria – an Indian experience

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An important risk factor for nosocomial infection in an intensive care unit (ICU) is prior colonization. This study was undertaken to determine the spectrum of bacterial colonization and predisposing risk factors in patients being admitted to an ICU in India, with special emphasis on extended-spectrum β -lactamase (ESBL)- and metallo- β -lactamase (MBL)-producing Gram-negative bacteria. Nasal, oral and rectal swab samples were collected and processed for isolation of ESBL-producing Gram-negative bacteria and MBL-producing *Pseudomonas aeruginosa* and *Acinetobacter* species. Bacterial colonization (of one or more sites) on admission was detected in 51 out of 96 patients included in the study. Non-fermenters, i.e. *P. aeruginosa* and *Acinetobacter baumannii*, were the most common colonizers, present in 37 patients, with simultaneous colonization in 12 patients. A total of 16 patients were colonized with MBL-producing members of the family *Enterobacteriaceae*, out of which 11 isolates (from 5 patients) were also carrying ESBL-encoding genes. As for MBLs, most of our patients have shown colonization with ESBL-producing bacteria. On admission, 47 of 51 patients (92%) have been colonized by ESBL-producing members of the family *Enterobacteriaceae*, at one or more of the three anatomical sites. The most common MBL subtype was *bla*_{IMP} (51.56%), whereas *bla*_{CTX} was the most common gene (84.9%) identified among ESBL producers. Risk factors for colonization on admission to the ICU were hospitalization for more than 48 h, use of ≥ 3 groups of antibiotics, co-morbidities and mechanical ventilation for more than 48 h prior to ICU admission. There is an increasing incidence of MBLs and ESBLs in the Indian population. The identified risk factors can be used as a guide for empiric antibiotic therapy targeted to these resistant bacteria.

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INTRODUCTION

The incidence of nosocomial infections in critically ill patients is much higher than in general ward patients despite the immense advancement in therapeutic technologies (Bryan-Brown, 1992; Brawley *et al.*, 1989). Severe nosocomial infections contribute to prolonged intensive care unit (ICU) stays, increased morbidity and mortality, and of course increased resource utilization (Heyland *et al.*, 1999; Inan *et al.*, 2005). An important risk factor for

nosocomial infection is prior colonization (Bonten & Weinstein, 1996). The Indian literature lacks evidence about the incidence and patterns of colonization of multidrug-resistant bacteria [especially the metallo- β -lactamase (MBL)- or extended-spectrum β -lactamase (ESBL)-producing bacteria] amongst critically ill patients.

This study was undertaken to determine the spectrum of bacterial colonization in patients admitted to an ICU and to assess the predisposing risk factors for colonization. Emphasis was laid on ESBL- and MBL-producing Gram-negative bacteria.

Abbreviations: ESBL, extended-spectrum β -lactamase; ICU, intensive care unit; MBL, metallo- β -lactamase.

METHODS

Settings. The study was conducted in a 700 bed tertiary care hospital, in North India, with a 12 bed ICU that receives patients from within the hospital as well as ones referred from outside. It manages approximately 250–300 critically ill patients annually.

Study population. After ethical clearance by the institute (Sanjay Gandhi Postgraduate Institute of Medical Sciences) and after taking informed consent from the next of kin, all patients admitted between March 2008 and July 2009 were prospectively included in the study. Patients were excluded if they were neutropenic, had pneumonia on admission or had received previous selective digestive decontamination.

Collection of data. Demographic data, clinical characteristics and details of previous hospitalizations were analysed. Nasal, oral and rectal swab samples were collected at the ICU upon admission.

Microbiological studies. All the swabs were processed for isolation of ESBL-producing Gram-negative bacteria and other Gram-negative bacilli, in addition to MBL-producing *Pseudomonas aeruginosa* and *Acinetobacter* species. Gram-negative bacteria were isolated on Chrome agar (supplied by Himedia). Isolates were identified to the species level by standard biochemical tests. The antibiotic susceptibility pattern for the following antibiotics were obtained by the Kirby–Bauer disc diffusion method on Muller–Hinton agar (Himedia) according to Clinical and Laboratory Standards Institute guidelines (CLSI, 2006): amoxicillin, ceftazidime, ceftriaxone, cefotaxime, aztreonam, cefpodoxime, ciprofloxacin, cefoxitin, levofloxacin, gentamicin, amikacin, imipenem, meropenem.

The Gram-negative isolates (including members of the family *Enterobacteriaceae*, *Pseudomonas* spp. and *Acinetobacter* spp.) that were non-susceptible to imipenem (10 µg disc) and/or meropenem (10 µg disc) were tested phenotypically for MBL production using a modified Hodge and imipenem-EDTA combined disc test (Lee *et al.*, 2001; Franklin *et al.*, 2006) while the members of the family *Enterobacteriaceae* showing resistance (or decreased zone diameter according to the ESBL screening method of the Clinical and Laboratory Standards Institute) to third generation cephalosporins (i.e. ceftazidime, ceftriaxone, cefotaxime, aztreonam, cefpodoxime) were tested for ESBL production using disc potentiation and disc approximation methods (NCCLS, 2000; Jarlier *et al.*, 1988).

Further, genotypic confirmation was carried out for the presence of these enzymes. All infections were divided into specific infection sites using standard criteria established by the Centers for Disease Control and Prevention (Garner *et al.*, 1988).

Genotyping

Genotypic detection of MBL genes. A multiplex PCR assay was performed to detect and differentiate five families of acquired MBL-encoding genes (VIM, IMP, SPM, GIM and SIM families) in a single reaction. Conserved regions of all available *bla*_{IMP}, *bla*_{VIM}, *bla*_{SPM-1}, *bla*_{GIM-1}, *bla*_{SIM-1} alleles were identified in CLUSTAL multiple alignments (Ellington *et al.*, 2007). Five primer pairs, specific for each family of acquired MBLs, were designed to amplify products of 188 bp (IMP), 390 bp (VIM), 271 bp (SPM-1), 477 bp (GIM-1), 570 bp (SIM-1) (Ellington *et al.*, 2007) (Table 1).

DNA template was prepared by emulsifying five colonies in 100 µl PCR-grade water and adding 2 µl to the PCR mixture prior to thermal cycling. The cycling conditions were: initial DNA release and denaturation at 94 °C for 5 min; followed by 40 cycles of 94 °C for 30 s, 52 °C for 40 s and 72 °C for 50 s; followed by a single, final, elongation step at 72 °C for 5 min. The amplified PCR product was analysed on 2% agarose containing 1 µl (0.5 µg ml⁻¹) ethidium bromide ml⁻¹. The amplified product was examined for the presence of multiple bands under UV transillumination (UVS system).

Genotypic detection (PCR) of ESBL-encoding genes. A PCR assay was performed to detect and differentiate *bla*_{TEM}, *bla*_{SHV} and *bla*_{CTX-M} genes. DNA template was prepared by extracting plasmid DNA by the alkali lysis method (Sambrook *et al.*, 1989). Extracted DNA of all the phenotypically confirmed ESBL-producing strains was subjected to amplification by PCR using *bla*_{TEM}/*bla*_{SHV}/*bla*_{CTX-M}-specific primers. The primers to amplify the targeted genes were chosen from earlier published studies and were able to recognize all the known TEM, SHV and CTX-M variants (Edelstein *et al.*, 2003; Lee *et al.*, 2000).

The primers were procured from OPERON Biotechnologies. A final reaction volume of 25 µl was prepared with H₂O (Milli-Q grade), 20 pmol both primers, 1 mM each dNTP, 1 unit *Taq* polymerase, 2.5 µl 10× PCR buffer, 1.5 mM MgCl₂, 100 ng DNA template. Amplification reactions were carried out in a thermocycler (PTC-100; MJ Research) under the following conditions: initial denaturation at 96 °C for 3 min, followed by 35 cycles of denaturation at 96 °C for 30 s, annealing as specified and elongation at 72 °C for 30 s. The final elongation step was extended to 3 min at 72 °C.

Statistical analysis. All the statistical calculations were performed using Epi Info (version 3.5.1) statistical software. Statistical analyses of individual risk factors were done by Fischer's exact test and χ^2 test. Yates correction was applied where required. A *P* value <0.05 was considered significant.

Table 1. Primers used in PCR of MBL-encoding genes

Primer (product size)	Orientation	Sequence
IMP (188 bp)	Forward	5'-GGAATAGAGTGGCTTAAYTCTC-3'
	Reverse	5'-CCAAACYACTASGTTATCT-3'
VIM (390 bp)	Forward	5'-GATGGTGTGTTGGTCGCATA-3'
	Reverse	5'-CGAATGCGCAGCACCAG-3'
SPM-1 (271 bp)	Forward	5'-AAAATCTGGGTACGCAAACG-3'
	Reverse	5'-ACATTATCCGCTGGAACAGG-3'
GIM-1 (477 bp)	Forward	5'-TCGACACACCTTGGTCTGAA-3'
	Reverse	5'-AACTTCCAACCTTGCCATGC-3'
SIM-1 (570 bp)	Forward	5'-TACAAGGGATTCCGGCATCG-3'
	Reverse	5'-TAATGGCCTGTCCCATGTG-3'

RESULTS

Demographic data of patients

A total of 96 patients, 78 male and 18 female (age 19–76 years, mean $\pm$ SD 54.3 $\pm$ 14) met the inclusion criteria. Two-thirds of the patients had been admitted to hospital $\geq$ 48 h prior to ICU admission, and 80 of 96 (83 %) had been treated with $\geq$ 3 groups of antibiotics. Among pretreated patients, 23 of 96 (24 %) were surgical patients and received a perioperative infection prophylaxis, the rest of the patients were medical patients of which the majority had received $>$ 3 groups of antibiotics. The demographic characteristics of all patients are summarized in Table 2.

Bacterial pattern of colonization on admission

All 3 sites of the 96 patients were sampled to assess colonization. Bacterial colonization (of 1 or more sites) on admission was detected in 51 patients. Non-fermenters, i.e. *P. aeruginosa* and *Acinetobacter baumannii*, were the most common colonizers, present in 37 patients, with simultaneous colonization in 12 patients. Sixteen patients were colonized with MBL-producing members of the family *Enterobacteriaceae*, out of which 11 isolates (from 5 patients) were also carrying ESBL-encoding genes. The spectrum of bacterial colonization on admission is given in Table 3.

Table 2. Patient characteristics on admission to the ICU ($n=96$)

Descriptive data	No. of patients (%)
Co-morbid conditions	
Respiratory disease (COPD)	60 (63)
Diabetes mellitus	39 (41)
Cardiac disease	54 (56)
Neurological disease	25 (26)
Malignancy	8 (8)
Chronic renal failure	35 (36)
Chronic liver disease	31 (32)
Chronic alcoholic	24 (25)
Chronic smoker	54 (56)
Immunosuppressed	4 (4)
On steroids	41 (43)
Before ICU admission	
Hospitalization for more than 48 h	67 (70)
History of antimicrobial treatment	90 (94)
History of treatment with $\geq$ 3 groups of antibiotics	80 (83)
History of mechanical ventilation for more than 48 h	25 (26)
Mode of ICU admission	
Medical	73 (76)
Surgical	23 (24)

COPD, Chronic obstructive pulmonary disease.

Table 3. Spectrum of bacterial colonization on admission in ICU patients ($n=51$)

Colonizing micro-organism	No. of patients colonized (%)
Non-fermenters (MBL)	
<i>P. aeruginosa</i>	29 (57)
<i>A. baumannii</i>	20 (39)
Enterobacteriaceae (MBL)	
<i>E. coli</i>	8 (16)
<i>K. pneumoniae</i>	5 (10)
<i>Enterobacter</i> species	3 (6)
Enterobacteriaceae (ESBL)	
<i>E. coli</i>	32 (63)
<i>K. pneumoniae</i>	30 (59)
<i>Enterobacter</i> species	3 (6)
<i>Citrobacter</i> species	4 (8)

Risk factors for colonization

Risk factors for colonization identified at ICU admission were mechanical ventilation $\geq$ 48 h before admission ($P \leq 0.0001$), use of $\geq$ 3 groups of antibiotic ($P \leq 0.0009$), co-morbidities ($P \leq 0.0013$) and a hospital stay for $\geq$ 48 h prior to ICU admission ($P \leq 0.0021$). Factors not associated with risk of colonization were sex, age, smoking and alcohol abuse (Table 4).

Genotypic detection of MBL-encoding genes in the isolates

The most common MBL subtype was *bla*_{IMP} (51.56 %) in all the isolates except *Klebsiella pneumoniae* in which *bla*_{VIM} was the most common subtype (Tables 5 and 6).

Genotypic detection of ESBL-encoding genes in the isolates

On admission, 47 out of 51 patients (92 %) had been colonized by ESBL-producing members of the family *Enterobacteriaceae*, at one or more of the three anatomical sites. In our isolates, *bla*_{CTX} was the most common gene (83.3 %) identified among ESBL producers (Table 7).

DISCUSSION

The socioeconomic impact of this colonization leading to nosocomial infections can adversely affect the patients', as well as the nation's, economic burden (Jarvis, 1996). In this study, we have analysed the incidence and pattern of bacterial colonization including multidrug-resistant Gram-negative bacteria in patients at ICU admission.

The majority of our patients showed colonization with MBL-producing bacteria. On admission, amongst the 51 colonized patients, 37 patients (73 %) had been colonized by MBL-producing *P. aeruginosa* or *Acinetobacter* species, at one or more of the three anatomical sites.

Table 4. Univariate analyses of individual risk factors for colonization at admission ($n=51$)

Risk factor	Colonization on admission		
	Present (n)	Absent (n)	P value
Age ≥ 65 years ($n=42$)	23	19	0.9384
Age <65 years ($n=54$)	28	26	
APACHE II score ≥ 20 ($n=63$)	36	27	0.3817
APACHE II score <20 ($n=33$)	15	18	
Smoker ($n=50$)	26	24	0.9796
Non-smoker ($n=46$)	25	21	
Alcoholic ($n=32$)	17	15	1.00
Non-alcoholic ($n=64$)	34	30	
Female ($n=18$)	10	8	0.8187
Male ($n=78$)	41	37	0.9739
Mechanical ventilation for ≥ 48 h before admission ($n=25$)	25	0	$<0.0001^*$
No mechanical ventilation or ventilation for <48 h before admission ($n=71$)	26	45	
Hospital stay prior to ICU ≥ 48 h ($n=67$)	43	24	0.0021*
Hospital stay prior to ICU <48 h ($n=29$)	8	21	
No. of co-morbidities ≥ 2 ($n=73$)	46	27	0.0013*
No. of co-morbidities <2 ($n=23$)	5	18	
Use of antibiotics ≥ 3 groups ($n=80$)	49	31	0.0009*
Use of antibiotics <3 groups ($n=16$)	2	14	
Immunosuppressed ($n=4$)	3	1	0.62
Immunocompetent ($n=92$)	48	44	

* $P \leq 0.05$ is considered as being significant.

Amongst ESBL producers, *bla*_{CTX} was the most common gene (83.3 %) identified. To the best of our knowledge, this may be the one of the few studies from India where a high prevalence of the *bla*_{CTX} gene in ESBL strains from critically ill patients is being detected (Muzahed *et al.*, 2008; Ensor *et al.*, 2006). Another interesting finding is that from different colonization sites we have isolated several strains of *P. aeruginosa*, *A. baumannii* and *Enterobacteriaceae* having multiple MBL-encoding genes.

The reason for the high prevalence of *bla*_{CTX} may be the clonal spread of the plasmid containing the *bla*_{CTX-M} gene amongst the various members of the family *Enterobacteriaceae* in different hospitals including ours (Sanjay Gandhi Postgraduate Institute of Medical Sciences). Our findings

support the hypothesis that CTX-M enzymes are the dominant ESBLs worldwide. Pournaras *et al.* (2004) reported an 87 % prevalence of CTX-M enzyme amongst ESBL producers. In a multicentre study from Russia, Edelstein *et al.* (2003) observed positive amplification for the *bla*_{CTX-M} gene in 28 (35.9 %) *Escherichia coli* and 87 (34.9 %) *K. pneumoniae* isolates expressing the ESBL phenotype. CTX-M β -lactamases were detected in 21 (75 %) surveyed hospitals from the cities representing geographically distant areas of Russia, and in five (17.9 %) hospitals this was the predominant ESBL. Brenwald *et al.* (2003) reported an outbreak of CTX-M harbouring ESBL in the UK. In a nationwide survey in Italy, Luzzaro *et al.* (2006) found CTX-M-producing isolates in 10 of the 11 participating centres distributed across the Italian national territory, although at remarkably variable

Table 5. Results of multiplex PCR for MBL

MBL subtype	<i>A. baumannii</i> (38)	<i>P. aeruginosa</i> (58)	<i>E. coli</i> (16)	<i>K. pneumoniae</i> (10)	<i>Enterobacter</i> spp. (6)
<i>bla</i> _{IMP}	18	24	6	1	1
<i>bla</i> _{VIM}	12	14	3	4	1
<i>bla</i> _{SIM-1}	2	4	2	1	1
<i>bla</i> _{SPM-1}	1	3	0	0	0
<i>bla</i> _{GIM-1}	0	2	1	0	0
<i>bla</i> _{IMP} + <i>bla</i> _{VIM}	2	4	2	0	2
<i>bla</i> _{IMP} + <i>bla</i> _{SIM}	1	3	0	2	0
<i>bla</i> _{VIM} + <i>bla</i> _{SIM}	2	4	2	2	1

Table 6. Overall MBL types detected by multiplex PCR for MBL

Overall MBL types	No. of isolates (%) (n=128)
<i>bla</i> _{IMP}	66 (51.6)
<i>bla</i> _{VIM}	55 (42.3)
<i>bla</i> _{SIM}	27 (21.1)
<i>bla</i> _{SPM}	4 (3)
<i>bla</i> _{GIM}	3 (2.3)

rates in different centres (1.2 to 49.5% of the ESBL producers). In our study, a total of 83 (56.8%) isolates contained multiple β -lactamase genes; the *bla*_{CTX-M} gene was associated with TEM in 64 isolates and with SHV in 27 isolates. Thirteen isolates showed all the three ESBL types.

Major risk factors associated with colonization at admission were prior hospitalization for >48 h, use of ≥ 3 groups of antibiotics, associated co-morbidities and mechanical ventilation for ≥ 48 h prior to ICU admission, which corroborates the findings of some other related studies (Drakulovic *et al.*, 2001; Bonten *et al.*, 1996; Harris *et al.*, 2007). The limitation of this study is that we could not study any specific antibiotic groups as we didn't have complete patient information on admission, maybe because of the poor referral system still prevailing in our country.

There is an increasing trend of MBLs and ESBLs in the Indian population. Approximately 50% of our patients were colonized by MBL- and ESBL-producing *P. aeruginosa*, *Acinetobacter* species or *Enterobacteriaceae*, at one or more of three anatomical sites. The risk factors identified in our study are potentially important because they can help determine which patients may need empiric antimicrobial drug therapy targeted to the ESBL-producing and MBL-producing bacteria. In addition, the colonization pattern and the risk factors identified may be of use to hospital antimicrobial drug stewardship and public health programmes.

Table 7. Results of PCR for ESBL

ESBL subtype	No. of isolates (%) (n=138)
<i>bla</i> _{TEM} + <i>bla</i> _{SHV} + <i>bla</i> _{CTX}	21 (15.2)
<i>bla</i> _{TEM} + <i>bla</i> _{CTX}	38 (27.5)
<i>bla</i> _{TEM} + <i>bla</i> _{SHV}	4 (2.9)
<i>bla</i> _{SHV} + <i>bla</i> _{CTX}	15 (10.9)
<i>bla</i> _{TEM} only	12 (8.7)
<i>bla</i> _{SHV} only	5 (3.6)
<i>bla</i> _{CTX} only	41 (29.7)
Overall ESBL subtype	
<i>bla</i> _{TEM}	75 (54.3)
<i>bla</i> _{SHV}	45 (32.6)
<i>bla</i> _{CTX}	115 (83.3)

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