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Blood Stream Infections caused by Non-Fermenting Gram Negative Bacilli, Clinical Correlation, MIC for Colistin, Gene Detection

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Abstract

To study the risk factors and outcome of blood stream infection caused by non-fermenting gram negative bacilli (NFGNB) and their pattern of antibiotic susceptibility and genes. We included sepsis cases with blood culture positive for NFGNB. MIC for colistin was determined by broth microdilution method. Multiplex PCR was used to detect Bla_{IMP}, Bla_{VIM}, Bla_{KPC}, Bla_{NDM-1} genes in cephalosporin and carbapenems resistant Acinetobacter spp. isolates. Out of 4,664 cases of sepsis, 50 (1.07%) were positive for NFGNB. *Acinetobacter* spp. 29 (58%) was the predominant isolate, of which 16 (55.17%) isolates were resistant to cephalosporins and carbapenems. We detected Bla_{KPC} and Bla_{NDM-1} genes in two of these isolates. We did not detect Bla_{IMP}, Bla_{VIM}, Bla_{KPC} and Bla_{NDM-1} genes in any other NFGNB isolates. Majority of the strains of *Pseudomonas* spp. showed sensitivity to all the antibiotics tested. NFGNB sepsis patients with respiratory illness correlated well with fatal outcome (p <0.05; OR 21). More numbers of *Acinetobacter* spp. sepsis cases had fatal outcome (p <0.05; OR 12.83). NFGNB sepsis patients with respiratory illness and those which yielded *Acinetobacter* spp. correlated positively with fatal outcome. We detected Bla_{KPC} and Bla_{NDM-1} genes in two strains of drug resistant *Acinetobacter* spp.

Keywords: Bacteremia, colistin, Drug resistance, Gram negative bacilli, Risk factors

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INTRODUCTION

Gram negative bacilli which are non-fermenters (NFGNB) are a heterogeneous category of microorganisms which do not have the ability to ferment sugars. NFGNB, which were considered as contaminants in the past, have emerged as important major pathogenic organisms responsible for causing a variety of infections in recent years[1]. NFGNB cause opportunistic healthcare-associated infections. They are commonly found on skin of healthcare-workers, instruments such as ventilator machines, humidifiers and mattresses[2-4]. Among NFGNB *Burkholderia cepacia, Pseudomonas aeruginosa*, *Acinetobacter baumanni* complex, *Alcaligenes faecalis*, *Sphingomonas paucimobilis* and *Stenotrophomonas maltophilia* are some pathogens known for causing health care associated bloodstream infections[5].

Multidrug resistance (MDR) property of NFGNB is the reason that facilitated the way for the reconsideration of previously used antibiotic colistin into clinical use. For the treatment of infections caused by multi-drug resistant gram-negative bacilli (MDRGNB), colistin may have crucial and reliable role as potent antibiotic.[6,7] The clinical use of colistin was restricted because of reports of serious nephrotoxicity and discovery of less toxic antibiotics[6,7]. So we intend to study the antibiotic sensitivity pattern of NFGNB from sepsis cases and correlate with the clinical condition of the patient. We also determine MIC of Colistin and also detect Bla_{IMP}, Bla_{VIM}, Bla_{KPC}, Bla_{NDM-1} genes in cephalosporin and carbapenems resistant *Acinetobacter* spp. isolates.

MATERIALS AND METHODS

It is a prospective time bound study conducted from (1st November 2018 to 30th April 2019) at the Microbiology laboratory of a tertiary care center. Sepsis cases with blood culture positive for NFGNB were included in the study. Blood culture samples which did not grow NFGNB and samples received on days other than the duration specified were excluded. Non random sampling strategy was used. Clinical data was taken from the records section of the hospital. Institutional ethics committee clearance has been taken for the project.

Bac T/Alert bottles with patient's blood received in microbiology laboratory, for blood culture were processed as per protocol, in Bac T/Alert 3D system. From the positive bottles, the subcultures were done on MacConkey agar, Blood agar and Chocolate agar. The identification of NFGNB up to the species level was done by VITEK ®2 Compact(C) system using the Gram-negative Identification (GN-ID) 21341 card, and antibiotic sensitivity testing (AST) was done with AST 281 card. Both the Bac T/Alert 3D system and VITEK®2 Compact (C) system were from bioMerieux company (North Carolina, USA),

MIC for colistin by broth micro dilution method

MIC for colistin by broth microdilution method was carried out as per CLSI guidelines.[8] A 100 mL stock solution of colistin sulfate (Sigma Aldrich, USA) of concentration 256 µg/mL was prepared according to the directions provided by the drug manufacturer. Two fold dilutions of the antibiotic were made to get a final dilution of 128, 64, 32, 16, 8, 4, 2, 1, 0.5 & 0.25 µg per ml of colistin respectively in wells 1 to 10 of microtitre plate.

Inoculum was prepared from 4 to 5 isolated colonies of similar colony morphology grown over night (18- 24 hours) on Mac Conkey's agar. The colonies were inoculated into cation adjusted Mueller Hinton broth (CAMHB), kept at 37°C for 4 to 6 h to get log phase growth and adjusted to 0.5 McFarland (1 x 10^8 CFU/ml). This broth was repeatedly diluted to get the final concentration of bacteria as approximately 5 x 10^4 CFU/ ml. Now 10µl of inoculum was pipetted into wells 1 to 11. Well 11 acted as the growth control and 12th well as broth sterility control with sterile CAMHB. The microtitre plates were incubated for 12-18 h at 37°C. *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were run as controls. The lowest concentration of antibiotic at which there is no visible growth of organism was taken as MIC endpoint. According to CLSI, ≤ 2 µg/ml and ≥ 4 µg/ml were considered as the susceptible and resistant break points respectively.

Multiplex PCR[9,10]

The DNA was extracted according to CDC protocol using boiling method. The primer sets were purchased from sigma Aldrich Pvt. Ltd (Table 1). The master mix was prepared by adding ready mix Taq PCR reaction mix with $MgCl_2$ (California, USA), in which the respective primer sets i.e, Forward (F') as well as Reverse (R') and the testing DNA were added and this master mix

was subjected to amplification. Amplification was done with following cycling conditions;10 min denaturation at 95°C, then 35 cycles of (45s denaturation at 94°C, 45 s primer annealing at 54°C, and 50 s primer extension at 72°C). The last cycle, an additional 7 min extension step at 72°C in Rotor gene –Q (Qiagen, germany) and the products were kept at 4°C. The amplified product was detected by using 2% agarose gel electrophoresis. *K.pneumoniae* ATCC BAA 1705 and *K.pnemoniae* BAA 1706 were used as positive and negative control. Clinical isolates known to be positive for Bla_{IMP}, Bla_{VIM} Bla_{NDM-1} genes were also used as controls.

Statistical analysis

Statistical analysis was performed using SPSS version 16.0. Multiple logistic regression analysis was done for correlation of risk factor and fatal outcome. Chi Square test was also used to correlate other variables.

RESULTS

Out of 4,664 cases of sepsis 50(1.07%) patient's blood samples yielded non fermenting gram negative bacilli. Of these 3(6%) were *Acinetobacter lwoffi*; 23(46%) *A. baumannii*; 1(2%) *A. juni*; 2(4%) *Acinetobacter* spp; 14(28%) *Pseudomonas aeruginosa*; 2(4%) *P. putida*; 3(6%)

Table 1. The primer sets were in PCR reaction

Primers for detection of genes	Primer sequence	Product size (bp)
bla $_{IMP}$	5'GGAATAGAGTGGCTTAAYTCTC 3' 5' GGTTTAAYAAAACAACCACC 3'	232
bla $_{VIM}$	5' GATGGTGTTTGGTCGCATA 3' 5' CGAATGCGCAGCACCAG 3 '	390
bla $_{NDM-1}$	5' CACCTCATGTTTGAATTCGCC 3' 5' CTCTGTCACATC GAAATCGC 3'	561
bla $_{KPC}$	5'-CATTCAAGGGCTTTCTTGCTGC-3' 5'-ACGACGGCATAGTCATTTGC-3'	538

Table 2. The antibiotic sensitivity pattern of *Acinetobacter* spp. isolated from blood of patients with symptoms of sepsis.

Antibiotics	Sensitive (%)	Intermediate (%)	Resistant (%)
Amikacin	26 (89.7)	0	3(10.34)
Ciprofloxacin	15(51.72)	0	14(48.28
Ceftazidime	17(58.62)	0	10(34.48)
Cefepime	11(37.93)	0	18(62.07)
Cefaperazone_Sulbactam	16(55.17)	5(17.24)	8(27.59)
Ceftriaxone	10(34.48)	2(6.7)	17(58.62)
Gentamicin	13(44.83)	0	16(55.17)
Imipenem	12(41.38)	0	17(58.62)
Meropenem	13(44.83)	0	16(55.17)
Netillin	16(55.17)	0	13(44.82)
Ofloxacin	18(62.07)	0	10(34.48)
Piperacillin	17(58.62)	1(3.45)	11(37.93)
Piperacillin_Tazobactam	11(37.93)	1(3.45)	17(58.62)
Ticarcillin_Clavulinic acid	15(51.72)	1(3.45)	13(44.83)
Trimethoprim _Sulphamethoxazole	16(55.17)	0	13(44.83)
Tigecycline	25(86.21)	3(10.34)	1(3.45)

were *P.stutzeri*; 1(2%) *Burkholderia cepacia*; 1(2%) *Brevundomonas vescularis*. Of the 50 cases of NFGNB, 28(56%) were male and 22(44%) were females; 22(44%) were more than 50 years of age, 16(32%) were 31 to 50 years of age, 7(14%) were 11-30 years of age and 5(10%) were less than 10 years of age.

Of the 29 isolates of *Acinetobacter* spp. most of them were sensitive to amikacin 26(89.7%) followed by tigecycline 25(86.21%) and 16(55.17%) isolates were resistant to carbapenems and cephalosporins (Table 2). All the isolates were sensitive to colistin with MIC values from 0.25 to 2 µg/ml. Majority of *Pseudomonas* spp. strains showed sensitivity to all the antibiotics tested (Table 3). Only one of these isolates was resistant to cephalosporins and carbapenems. MIC values for colistin among these isolates were between 0.25 and 8 µg/ml of which two isolates were resistant. The single isolate of *Brevundomonas vescularis* was sensitive to cephalosporin, carbapenems and resistant to aminoglycosides, fluoroquinolones, Trimethoprim/sulfamethoxazole. The single isolate of *Burkholderia cepacia* was resistant to most of the antibiotics tested, but sensitive to carbapenems, fluoroquinolones and tigecycline.

Of the 16 isolates of the *Acinetobacter* spp. resistant to cephalosporins and carbapenems

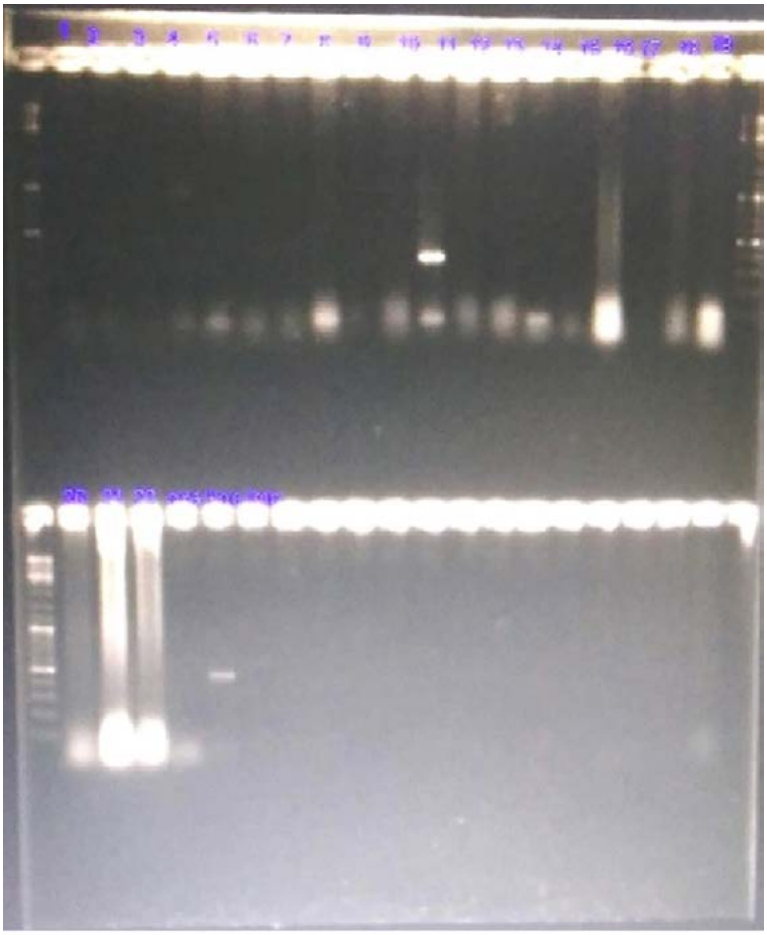
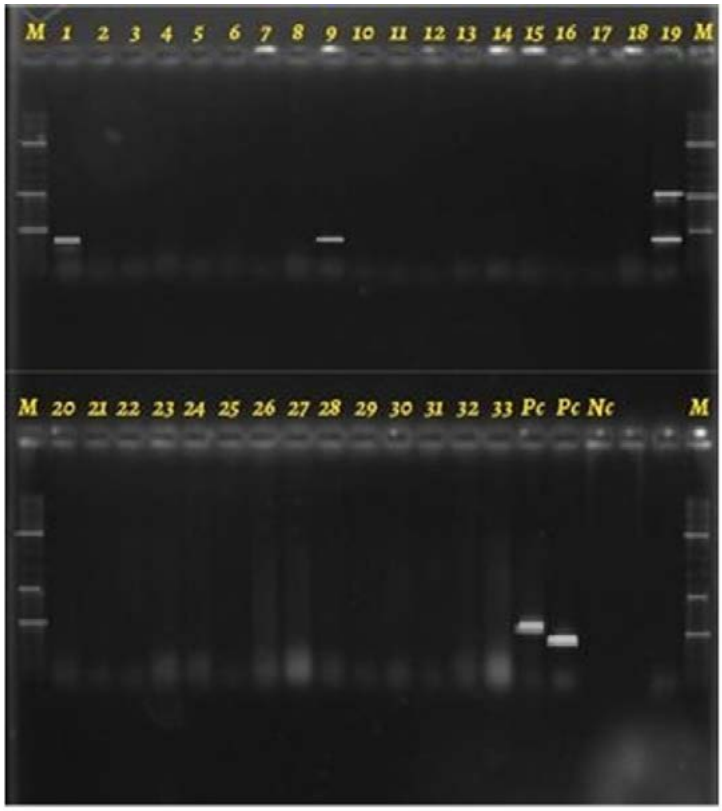


Fig. 1. Detection of Bla_{IMP}, Bla_{VIM}, Bla_{KPC} and Bla_{NDM-1} genes by multiplex PCR in the drug resistant *Acinetobacter* species isolated from cases of blood stream infection

Bla$_{KPC}$ and *Bla*$_{NDM-1}$ genes were detected in two of these isolates. We did not detect *Bla*$_{IMP}$, *Bla*$_{VIM,}$ *Bla*$_{KPC}$ and *Bla*$_{NDM-1}$ genes in any other NFGNB isolates. (Fig. 1 and 2)

Of the 50 cases of NFGNB we got detailed clinical history for 23 cases. Of these 23 cases, 14(60.9%) expired due to multiple underlying conditions. Most of the fatal outcome was due to a combination of chronic obstructive pulmonary disease (COPD) respiratory failure, cardiac arrest, renal failure or in other words multi-organ failure. Most of the cases of NFGNB, the patients had multiple underlying clinical conditions like diabetes mellitus 9(39.13%), cardiac anomalies 8(34.8%), malignancy 6(26%), respiratory illness 14(60.9%), malaria 4(17.4%), HIV 3(13%), GIT anomalies 3(13%), renal anomalies 4(17.4%), hepatic anomalies 7(30.4%), CNS infection 3(13%), wound infection 4(17.4%). Cardiac anomalies included cases of cardiac arrest, cardiac valve dysfunction, tachycardia, atrial fibrillation; malignancy included lymphoma, leukemia, nasopharyngeal carcinoma, multiple myeloma, osteocarcinoma; respiratory illness included COPD, ventilator associated pneumonia (VAP), lower lobe pneumonia, aspiration pneumonia, bronchial asthma, respiratory failure; GIT anomalies included esophageal candidiasis, progressive abdominal fistulation, GIT bleeding; renal anomalies included acute renal failure, decreased urine output, renal



Lane 1 and 9: *Bla*KPC gene
Lane 19: *Bla*KPC and *Bla*NDM-1 genes
Lane 34: *bla* IMP gene
Lane 35: *bla* VIM gene

Fig. 2. Multiplex PCR conducted on Klebsiella clinical isolates run as positive and negative controls

insufficiency; hepatic anomalies included portal hypertension, hepatomegaly, jaundice; CNS anomalies included myasthenia gravis, PCOM aneurysm; wound infection included cellulitis, surgical wound infection.

When underlying clinical conditions were correlated with fatal outcome, patients with respiratory illness (p <0.05) correlated well with fatal outcome (OR 21, Cl = 2.397-183.99) (Table 4). When the organisms were correlated with fatal outcome, more numbers of patients infected with *Acinetobacter* spp. had fatal outcome (p <0.05; OR 12.83, Cl = 1.695-97.193) (Table 4).

DISCUSSION

Among NFGNB *Burkholderia cepacia, Pseudomonas aeruginosa*, *Acinetobacter baumannii* complex, *Alcaligenes faecalis* and *Sphingomonas paucimobilis* are some pathogens known for causing health care associated bloodstream infections[5-7]. In the present study, *Acinetobacter* spp. 29(58%), *Pseudomonas* spp. 19(38%), followed by one isolate each of *Burkholderia cepacia* and *Brevundomonas vescularis* were isolated.

In a study on resistance of NFGNB isolated from blood cultures from an emergency hospital in Brazil found that from 87(100%) isolated and analyzed strains, 11(13%) were NFGNB. *Acinetobacter* spp. was most often occurring organism (55%) succeeded by *Pseudomonas* spp. (18%), *Stenotrophomonas* spp. (18%) and *Burkholderia* spp. (9%). *Acinetobacter* spp. were resistant to gentamycin, meropenem, imipenem, amikacin, ciprofloxacin, ceftazidime and ceftriaxone. *Pseudomonas* spp. was sensitive to gentamycin, meropenem, imipenem, amikacin, levofloxacin, norfloxacin, ceftazidime and cefepime. All *Stenotrophomonas* spp. were resistant to levofloxacin and chloramphenicol. *Burholderia* spp. were sensitive to the antibiotics tested[11]. A study showed *A.baumannii* isolated from sepsis cases was highly resistant to aminoglycosides in comparison to other *Acinetobacter* spp. *P. aeruginosa* strains were sensitive to all the aminoglycosides tested.[12] We have similar results.

Rate of isolation of NFGNB from blood vary in studies conducted from various places from 0.7 to 20.8[13,14]. In a study by Grewal US et al isolation rate of NFGNB was 11.6%. Commonest NFGNB isolated in the study was *P. aeruginosa* (87.96%), followed by *A. baumannii* (7.87%). 24.2 % of *P. aeruginosa* strains were identified as multi drug resistant (MDRPA). About 100% of the MDRPA

Table 3. The antibiotic sensitivity pattern of *Pseudomonas* spp. isolated from blood of patients with symptoms of sepsis

Antibiotics	Sensitive (%)	Intermediate (%)	Resistant (%)
Amikacin	19(100)	0	0
Ciprofloxacin	19(100)	0	0
Ceftazidime	18(94.74)	1(5.26)	0
Cefepime	19(100)	0	0
Cefaperazone_Sulbactam	18(94.74)	0	1(5.26)
Ceftriaxone	17(89.47)	1(5.26)	1(5.26)
Gentamicin	18(94.74)	1(5.26)	0
Imipenem	19(100)	0	0
Meropenem	17(89.47)	1(5.26)	1(5.26)
Netillin	19(100)	0	0
Ofloxacin	19(100)	0	0
Piperacillin	18(94.74)	1(5.26)	0
Piperacillin_Tazobactam	17(89.47)	1(5.26)	1(5.26)
Ticarcillin_Clavulinic acid	13(68.42)	5(26.32)	1(5.26)
Trimethoprim _Sulphamethoxazole	17(89.47)	0	2(10.53)
Tigecycline	18(94.74)	0	1(5.26)

isolates were found to be susceptible to polymyxin B. However, 60.9% of the MDRPA isolates in their study showed resistance to imipenem, which is usually the first therapeutic choice for treating the infections caused by them. Isolates of *A. baumannii* in their study showed maximum susceptibility to imipenem (88.2%), followed by cefoperazone + sulbactam (76.4%). Maximum resistance was shown to aztreonam (11.7%). 11 isolates of *A. baumannii* showed multidrug resistance (MDRAB) in the study. Isolation of multi drug resistant *P. aeruginosa* and multi drug resistant *A. baumannii* in the study raised the concern of emerging antibiotic resistance in this group[14].

In an another study on predominance of NFGNB and sensitivity pattern in Tamil Nadu, found that out of 5052 clinical samples, 517 (10.2%) samples were NFGNB. *Acinetobacter and Pseudomonas* spp. were the most frequently found organisms. Colistin and imipenem showed maximum sensitivity for all clinical samples. Highest resistance was seen with gentamycin ceftazidime and ciprofloxacin[15].

An antibiotic trend analysis over a period of seven years in patients suffering from nosocomial infections showed elevated levels of resistance to frequently used antibiotics and hence a significant rise in colistin use especially in patients who were critical[16].

In a retrospective study factors deciding the existence of patients with *Acinetobacter baumannii* bacteremia were evaluated. Relevant details of hospitalized patients who developed *Acinetobacter baumannii* bacteremia were collected in Beirut between 2010 and 2015. Presence of diabetes mellitus, large amount of steroids, ventilator use, early treatment with tigecycline and colistin, critical care unit and septic shock correlated with worse result by univariate analysis. Multivariate analysis showed significant correlation with large doses of steroids and septic

Table 4. Correlation of underlying clinical conditions with fatal outcome

Variables	Non-fatal (n=9)	Fatal (n=14) (n=14)	p value	Odds ratio
Age group (11-30)	2(22.2%)	1(7.1%)	0.295	0.26
31-50	5(55.5%)	6(42.9%)	0.552	0.6
More than 50	2(22.2%)	7(50%)	0.183	3.5
Male	5(55.5%)	11(78.6%)	0.242	2.933
Female	4(44.4%)	3(21.43%)	0.242	0.341
Under lying disease conditions				
Diabetes Mellitus	2(22.2%)	7(50%)	0.311	2.625
Malignancy	3(33.3%)	3(21.4%)	0.526	0.545
Cardiac anomalies	2(22.2%)	6(42.9%)	0.311	2.625
Respiratory illness*	2(22.2%)	12(85.71%)	0.002*	21*
Hepatic anomalies	2(22.2%)	5(35.7%)	0.493	1.944
Renal anomalies	1(11.1%)	3(21.4%)	0.524	2.182
GIT anomalies	1(11.1%)	2(14.3%)	0.825	1.33
HIV	0	3(21.4%)	0.136	1.273
Malaria	1(11.1%)	3(21.4%)	0.524	2.182
Wound infection	11(84.6)	3(21.4%)	0.524	2.182
CNS complaints	2(22.2%)	1(7.1%)	0.295	0.269
Organisms				
Pseudomonas spp.	6(66.7%)	2(14.3%)	0.01	0.083
Acinetobacter spp.*	2(22.2%)	11(78.6)	0.008*	12.83*
Burkholderia cepacia	1(11.1%)	0	-	-
Brevundomonas vesiculosis	0	1(7.1%)	-	-

* Statistically significant

shock. Approximate fatality was 63.5%. Bacteremia showed 70.3% of the deaths. An extended hospital admission is usually correlated with failure of performance, prior use of steroid and early exposure of antibiotic[17].

In our study there were no positive correlation among death and diabetes mellitus. But as in previous studies we got positive correlation between death and underlying respiratory illness like, VAP, COPD, bronchial asthma and pneumonia. We also found that mortality was associated with sepsis in *Acinetobacter* spp.

In a study on carbapenem resistant strains of coliforms isolated from cases of blood stream infection bla_{NDM-1} & bla_{KPC} genes were detected. They found that 16 isolates harbored bla_{NDM-1}, and bla_{KPC} gene was not detected in any of the isolates[9]. In a study period of 2008 and 2012, 166 uropathogens isolated from cases with complicated UTI, 34(43.6%) isolates had bla_{VIM} gene, 5(6.4%) had bla_{IMP}, bla_{NDM-1} and bla_{KPC} genes were absent. Among the isolates from 2012, 47(53.4%) had bla_{NDM-1} gene 19(24.4%) had bla_{VIM}, one (1.1%) had bla_{IMP} and bla_{KPC} gene was absent[10]. In the present study one each of Bla_{KPC} and Bla_{NDM-1} genes were detected in two strains of drug resistant *Acinetobacter* spp. Further studies with a larger sample size may give more conclusive results.

CONCLUSION

Rate of isolation of NFGNB from blood from sepsis cases was 1.07%. Most commonly isolated bacteria were *A. baumannii* and *P.aeruginosa*. NFGNB sepsis patients with respiratory illness and those which yielded *Acinetobacter* spp. correlated positively with fatal outcome. Most isolates of *Acinetobacter* spp. were sensitive to amikacin and tigecycline. Sixteen isolates of *Acinetobacter* spp. were resistant to cephalosporin and carbapenems. Most strains of Pseudomonas spp. were sensitive to all antibiotics tested. Only one isolate was resistant to carbapenems and cephalosporin. All strains of *Acinetobacter* spp. were sensitive to colistin and Two isolates of Pseudomonas species were resistant to colistin. One each of Bla_{KPC} and Bla_{NDM-1} genes were detected in two strains of drug resistant *Acinetobacter* spp. Further studies with a larger sample size may give more conclusive results.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

Ms. AV: Review of literature, conduct of the research and data collection, result analysis, writing the project report, Dr. JU: Planning of the topic, review of literature, research protocol, guiding the first author in conduct of research project, result analysis, writing project report, Dr. PR: Guidance regarding the gene detection, result analysis and writing of the project report, Dr. ES: Guidance regarding the topic selection, result analysis and writing of the project report. All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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ETHICS STATEMENT

Institutional ethics committee clearance has been taken for the project. IEC KMC MLR 10-18/355.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

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