Genome-Wide Identification of Carbapenem-Resistant Gram- Negative Bacterial (CR-GNB) Isolates Retrieved From Hospitalized Patients in Bihar, India

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Abstract

Carbapenemase-producing clinical isolates are becoming more common over the world, posing a severe public health danger, particularly in developing nations like India. Carbapenem-resistant Gram-negative bacterial (CR-GNB) infection has become a fast-expanding global threat with limited antibiotic choice and significant mortality. The aim of this study was to highlight the carbapenem-resistance among clinical isolates of hospital admitted patients in Bihar, India. A cross-sectional study was conducted with 101 clinical isolates of *E. coli*, *K. pneumoniae*, *A. baumannii*, and *P. aeruginosa*. All GNB isolates were tested for their antimicrobial susceptibility using double disc synergy test / modified hodge test (DDST/MHT) and subsequently confirmed carbapenemase-producing isolates were evaluated for carbapenem-resistance genes using whole-genome sequencing (genotypically) method. The overall percentage of carbapenem-resistance among GNB was (17/101) 16.83%. The AMR analysis demonstrates a significantly high prevalence of bla_{CTX-M} followed by bla_{SHV}, bla_{TEM}, bla_{OXA} and bla_{NDM} β -lactams carbapenem-resistance genes among clinical isolates of GNB. Co-occurrence of carbapenemase-encoding genes with bla_{NDM} was found in 70.6% of carbapenemase-producing isolates. Our study highlights the mechanism of carbapenem-resistance to curb the overwhelming threat posed by emergence of drug-resistance in India.

Introduction

Antimicrobial-resistance is an public health issues worldwide due to the rough use of antibiotics (Katiyar et al., 2020). It has been investigated as a foremost medical and community health issues since the inadequate treatment option to cure contagions triggered by antimicrobial-resistant bacteria. The growing microbial resistance rates to most available antibiotics, including penicillin, cephalosporins and carbapenem made a severe risk (Nordmann & Poirel, 2019). The evaluation of antibiotic resistance throughout the world increases and has become very hard to control due to the growth rates of multidrug-resistance and the lack of consistent observation methods (Manandhar et al., 2020). The WHO recently enumerated β -lactams carbapenemase-producing Gram-negative bacteria (GNB) of serious importance (Wyres et al., 2020). Gram-negative bacteria (GNB), especially *Escherichia coli*, and *Klebsiella pneumoniae* have proven resistance to a wide-ranging variety of antimicrobials accountable for noteworthy mortality all over the world (Zavascki et al., 2013). The development of carbapenem-resistance in GNB is a foremost medical problem, predominantly for immunocompromised patients with serious infections (Nair et al., 2021).

Carbapenem are the most effective drug of choice against pathogenic bacteria offering a wide range of antibacterial activity (Zagui et al., 2020). This antimicrobials are painstaking as one of the last option antibiotics against drug-resistant GNB (Elbadawi et al., 2021). The pathogen which are resistant to carbapenem habitually display high intensities of resistance to commonly used antibiotics. This is not only major cause of high death rates, but also creates difficult situations for the patients who spend prolonged time in the hospital and having high medical expenditures gather, employing an sensitive, monetary liability on families, particularly in inadequate sources countries. Hence, precise identification of AMR in GNB is an indispensable for the appropriate administration of apposite antimicrobials. To find AMR in GNB, *in vitro* cultures were used to monitor the development of bacteria for various concentrations of drugs and may need at least 72 hours to acquire precise antibiotic susceptibility results. Advancement in whole-genome sequencing (WGS) reinforced the evaluation of the complete DNA sequence of bacteria. WGS delivers vital description for genotype of an individual organism. WGS data can give mechanistic insight of the antibiotic-resistance for drugs not being tested routinely (Sawa et al., 2020).

India is a prime location for AMR pathogens because of overuse of antibiotics. However, less data on carbapenemase genes from Bihar region are available to correlate genotypes with the phenotypes. Hence, the main aim of this study was to determine the resistance patterns in carbapenemase-producing clinical isolates from in-patients at I.G.I.M.S., Patna, India.

Methods

Study design

A cross sectional investigation was conducted on CR-GNB strains isolated from routine clinical samples of hospital admitted patients coming to microbiology laboratory of Indira Gandhi Institute of Medical Sciences, Patna, Bihar during a period of 10 months from March 2019 to December 2019. All of the methods followed the guidelines set out by the Clinical and Laboratory Standards Institute (CLSI). The lab work and data analysis were completed at Indira Gandhi Institute of Medical Sciences (I.G.I.M.S.), Patna and All India Institute of Medical Science (A.I.I.M.S.), New Delhi, respectively. Written informed consent was taken by the participants and the study was reviewed and approved by the ethical committee of IGIMS, Patna, India (451/IEC/2018/IGIMS).

Bacterial isolates

Gram-negative bacteria (GNB) including *E. coli*, *K. pneumoniae*, *A. baumannii*, and *P. aeruginosa* were isolated and identified by standard manual conventional method from culture of the routine clinical samples like blood, vascular catheter tip, urine, bile, ascitic fluid, pus, sputum, endotracheal tube-aspirate and broncho-alveolar lavage fluid (BAL).

Antimicrobial susceptibility testing of GNB isolates

Antimicrobial susceptibility testing of isolates was performed using the standard Kirby-Bauer Disc Diffusion Method. For quality control, suitable ATCC control strains were used. The following antibiotics (Hi-Media disc in mcg) were tested: ampicillin (10mcg), amoxicillin clavulanic acid (20/10mcg), cefotaxime (30mcg), ceftriaxone (30mcg), ceftazidime (30mcg), piperacillin-tazobactam (100/10mcg), sulfamethoxazole-trimethoprim (25mcg) nitrofurantoin (100mcg), aztreonam (30mcg), ciprofloxacin (5mcg), gentamicin (10mcg), amikacin (30mcg), minocycline (30mcg), meropenem (10mcg) and imipenem (10mcg). The Clinical and Laboratory Standards Institute (CLSI) 2018 standards were used to quantify and interpret zone diameter.

Detection of carbapenemase production

Carbapenem-resistance screening of GNB isolates was done using meropenem (10ug) disc. The Modified Hodge Test (MHT) and meropenem Double Disc Synergy Test (DDST, employing EDTA disc) were used to validate phenotypic identification of carbapenemase production in these resistant isolates. In MHT, a susceptible lawn of *E. coli* was spread across the plate with a meropenem disk. The expected zone of inhibition was streaked with positive controls, negative controls, and test strains. Diffusible carbapenemase, which appears as an indentation in the zone of inhibition, protects otherwise vulnerable *E. coli* from being killed. In DDST, enhancement of the inhibition zone (by > 5 mm in the area between meropenem disc and the EDTA disc in comparison with the zone of inhibition on the far side of the drugs) was considered to be a positive result. All of the isolates were maintained at 80°C in nutritional broth containing 7.5 percent (v/v) glycerol. These stocked carbapenem-resistant isolates were sub-cultured on blood agar prior to undergo whole-genome sequencing.

Whole-genome sequencing

Genomic DNA of pathogens were isolated from a total of 17 freshly sub-cultured colonies of carbapenem-resistant bacteria using DNeasy Blood and Tissue Kit (Qiagen, Catalogue # 69505), in accordance with the manufacturer's recommendations. DNA concentration of the purified DNA was calculated on Qubit (Thermo Fischer Scientific), using Qubit™ 1X dsDNA HS Assay Kit (Invitrogen, Catalogue # Q33231). The genomic DNA was stored at -20°C. The Illumina MiSeq (Illumina, San Diego, CA, 107 USA) technology was used to sequence these DNA isolates. Using the Nextera DNA Flex Library Prep Kit, a MiSeq short-read sequencing library was created with 1ng pure DNA (Illumina, Cat. No. 20018704). Bead-linked transposomes (BLTs) were employed in this kit to facilitate simultaneous DNA fragmentation and Illumina sequencing primer tagging (Tagmentation). These sequencing ready DNA fragments were amplified by PCR and indexes (for sample identification) and adapters were further added to them (using Nextera DNA CD Indexes, Cat. No.20018707). For each library, the normalization was performed to bring them to a standard concentration of 4nM. Sequencing ready fragments were further washed and pooled. The average size of the libraries was found to be around 550bp that was calculated with the help of Agilent Bioanalyzer 2100 using High Sensitivity DNA Assay Kit (5067-4626). The libraries were next loaded on to the sequencer Illumina MiSeq 500 platform for sequencing by synthesis/bridge amplification. Casava (v.1.8.2) was used to de-multiplex the output data files and convert them to FASTQ files (Illumina, Inc, USA).

Preprocessing and *de-novo* assembly

FastQC-0.11.9 (<https://www.bioinformatics.babraham.ac.uk>) was used to assess the read quality. Trimmomatic-0.39 was used to trim adapters and low-quality sequences (Bolger et al., 2014). Velvet was used to build contigs using clean reads (Zerbino and Birney, 2008). QUAST was used to evaluate the assembled genome's quality (Gurevich et al., 2013). Prokka (v1.12) was used to annotate the assembled bacterial genomes (Seemann, 2014). Under the accession number **PRJNA744890**, the sequencing SRA data were submitted to the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>).

Detection of resistance genes

The RGI-CARD (Comprehensive Antibiotic Resistance Database) and Pathogenwatch were used to predict the resistance genes in the assembled Gram-negative bacteria genomes (Center for Genomic Pathogen Surveillance databases). We utilized 50 percent sequence identity and 70 percent query coverage as cut-off criteria. The acquired antimicrobial resistance genes and genes associated with chromosomal point mutations were identified using the ResFinder webserver 3.0 (<https://cge.cbs.dtu.dk/services/ResFinder/>).

Result

Out of 101 Gram-negative bacteria (GNB) isolated from different clinical samples, 17(16.8%) were carbapenemase producer.

Demographic distribution

The isolates found were from different age group patients ranging from 6 years to 76 year old. Isolates from males 76.5% (13/17) were more in number as compared to isolates from female 23.5% (4/17) inpatients. Maximum numbers of these carbapenemase producing isolates were found from Medicine ICU 47% (8/17) followed by surgery ward 35.3% (6/17) admitted patients. Isolates generating carbapenemase were found in the following clinical specimens: blood 11.8% (2/17), vascular catheter tip 5.9% (1/17), urine 47.1% (8/17), bile 5.9% (1/17), pus 11.8% (2/17), sputum 5.9% (1/17), endotracheal tube aspirate 5.9% (1/17) and BAL 5.9% (1/17). Urine samples had the highest number of carbapenem resistant isolates. Sample-wise distribution and phenotypic carbapenem-resistance rate of isolated GNB is given in Table 1.

Antimicrobial susceptibility and phenotypic screening

Table 2 shows the phenotypic antimicrobial resistance pattern of isolated GNB. For 101 GNB isolates tested, the highest percentage of resistance was recorded in ampicillin (95.8%) followed by ciprofloxacin (94.4%), amoxicillin-clavulanic acid (89.3%), cefotaxime (88.8%), ceftriaxone (84%), piperacillin-tazobactam (78.9%), ceftazidime (76.2%), tobramycin (66.7%), sulfamethoxazole-trimethoprim (66.2%), gentamicin (60.7%) and amikacin (30.3%). Meropenem and imipenem both had a 22.1 percent resistance rate. The percentage of bacterial resistant to carbapenems was highest in *K. species*. Amikacin showed good sensitivity in GNB among aminoglycosides.

Prevalence and distribution of β -lactams carbapenemase genes

Gram-negative bacterial isolates (n=17) were screened for β -lactams carbapenemase and ESBLs-resistant genes using antimicrobial repositories (such as CARD, Pathogenwatch and ResFinder), yielding 87 types of carbapenem gene. Among them, 37.93% types of genes were bla_{CTX-M} followed by bla_{SHV} (28.74%), bla_{TEM} (16.09%) and bla_{OXA} (6.90%) (Figure 1; Supplemental Table S1). Analysis revealed that bla_{CTX-M-15}, bla_{NDM-5}, bla_{TEM-1B}, and bla_{OXA-10} were the most frequent subtype in their respective groups of GNB isolates (Supplemental Figure S1). β -lactams carbapenemase including bla_{NDM-1} (Sands et al., 2021),

(Annavaiah et al., 2019) *bla*_{NDM-5} (Annavaiah et al., 2019), *bla*_{NDM-16} (Taggar et al., 2020), and *bla*_{NDM-20} (Liu et al., 2018) were observed which makes bacteria resistant to a broad range of beta-lactam antibiotics. Others carbapenemase includes *bla*_{OXA-1} (Livermore et al., 2019), *bla*_{OXA-10} (Kotsakis et al., 2018), *bla*_{OXA-50} (Girlich et al., 2004; Petrova et al., 2019), *bla*_{OXA-181} (Anaïs et al., 2011; Sands et al., 2021), *bla*_{OXA-23} (Yousefi Nojookambari et al., 2021), *bla*_{OXA-488} (Hao et al., 2021) and *bla*_{DIM} (Manohar et al., 2018). In addition, colistin-resistance genes (MgxB/PmrB) were also observed in *E. coli*, *K. pneumoniae* and *P. aeruginosa* strains. The strain-wise prevalence of β -lactams carbapenem-resistance gene was *bla*_{CTX-M} (13/17), followed by *bla*_{NDM} (12/17), *bla*_{OXA} (10/17), *bla*_{SHV} (9/17), and *bla*_{TEM} (9/17). Table 3 and Supplementary Table S2 provide a genotypic description of the antimicrobial-resistance genes of all 17 isolates. The gene occurrence of *bla*_{NDM} and *bla*_{OXA} was dominantly observed in *K. pneumoniae*, followed by *E. coli* whereas *bla*_{CTX-M} was mainly found in *K. pneumoniae*, followed by *E. coli* (Figure 2 and Supplemental Table S3).

Co-resistance genes

It has been observed that the majority of GNB isolates had more than one β -lactams carbapenem-resistance gene, where co-resistance genes were mostly found in *E. coli* and *K. pneumoniae* isolates. Among, co-resistance of two genes namely "*bla*_{CTX-M} + *bla*_{NDM}" (1/17), and "*bla*_{CTX-M} + *bla*_{SHV}" (1/17), was observed in *K. pneumoniae* and *E. coli*, respectively, whereas co-resistance of three genes "*bla*_{CTX-M} + *bla*_{NDM} + *bla*_{OXA}" was commonly observed in both *K. pneumoniae* and *E. coli*. Likewise, "*bla*_{CTX-M} + *bla*_{TEM} + *bla*_{OXA}" and "*bla*_{CTX-M} + *bla*_{NDM} + *bla*_{SHV}" pattern of three genes was found in *K. pneumoniae*, whereas "*bla*_{CTX-M} + *bla*_{NDM} + *bla*_{TEM}" co-resistance genes pattern was found exclusively in *E. coli*. Co-resistance of four genes namely "*bla*_{CTX-M} + *bla*_{NDM} + *bla*_{SHV} + *bla*_{TEM}" was detected in 3 species (*K. pneumoniae*, *E. coli* and *A. baumannii*), whereas "*bla*_{CTX-M} + *bla*_{NDM} + *bla*_{OXA} + *bla*_{TEM}" was observed in *E. coli* only. Interestingly, co-resistance of five genes "*bla*_{CTX-M} + *bla*_{NDM} + *bla*_{SHV} + *bla*_{OXA} + *bla*_{TEM}" was found in 3 isolates of *E. coli*. A high co-resistance rate in GNB may provide further insight into the epidemiology of resistance acquisition. Table 4 shows the distribution of co-resistance genes among different Gram-negative bacteria. Analysis revealed β -lactams co-resistance genes with *bla*_{NDM} in 12 (70.6%) of carbapenemase-producing isolates.

Genotype and phenotype correlations

The findings of the genotypic method (WGS) were compared with phenotypic method (MHT/DDST) and observed that the concordance between genotypic and phenotypic was 100% for β -lactams carbapenem-resistance genes (Table 5). We also observed resistance for ceftazidime, piperacillin-tazobactam, meropenem and imipenem antimicrobials genotypically and phenotypically for *E. coli*, *K. pneumoniae*, *A. baumannii* and *P. aeruginosa* strains. These findings concluded a strong correlation between genotypic and phenotypic correlations among GNB isolates.

Discussion

Carbapenemase-producing Gram-negative bacterial (GNB) infection is increasing worldwide including India where it is a cause for major concern (Meletis, 2015). Carbapenems are currently the medicine of choice for treating serious hospital-acquired infections, however carbapenem-resistance has been reported very high in India and the Indian subcontinent in recent decades. The accurate identification of carbapenemase-producing microbes is interesting, and it necessitates phenotypic and genotypic studies to identify all genes linked to carbapenemase-production.

This study was carried out in 1060 bedded super-specialty tertiary care hospital in Bihar, India. The majority of patients were referred after using antimicrobials. In addition, 47% of the isolates in the study were from the intensive care unit, where patients are more prone to undergo invasive procedures. These factors and prolonged hospital stay may have contributed for the high prevalence of carbapenem-resistant in admitted patients. Carbapenemase activity has been known in β -lactamases classes namely A, B, and D (Walsh, 2010; Fonseca et al., 2012). The prevalence of CR-GNB in the current study was 16.8% as compared to the 31.77% in Western Maharashtra (Pawar et al., 2018). Similarly, Wattal et al found a prevalence rate of 13 to 51 percent in a tertiary care hospital in Delhi (Wattal et al., 2010). Nair et al found it to be around 12.26 percent in a study in Mumbai (Nair et al., 2013), while Gupta et al found it to be between 17 and 22 percent in a study in northern India (Gupta et al., 2016). This is consistent to our data.

The most frequently detected β -lactams carbapenem-resistance types of gene was *bla*_{CTX-M} (37.93%) followed by *bla*_{SHV} (28.74%), *bla*_{TEM} (16.09%), *bla*_{OXA} (6.90%) and *bla*_{NDM} (4.65%) in GNB isolates in this study. This is consistent with finding from other parts of India. For example, study conducted on 130 clinical samples in *E. coli* and *K. pneumoniae* taken from Aligarh, Varanasi (Uttar Pradesh; North India) and Hubli (Karnataka; South India) have shown the prevalence of *bla*_{CTX-M-15} gene (Nair et al., 2021). In another study, 300 isolates of *E. coli* tested and found that *bla*_{CTX-M-15} was the most dominant gene (Rohit et al., 2019). Likewise, the study conducted on carbapenem-resistance genes in urinary isolates of *K. pneumoniae* (from Southern India) showed high prevalence of *bla*_{CTX-M-15} gene (Muzaheed et al., 2008), where's analysis of 1275 strains from *E. coli* and *K. pneumoniae* showed the increasing prevalence of *bla*_{CTX-M-15} gene in the patients from the rural community of North India (Devi et al., 2020).

Similar trends for *bla*_{CTX-M-15} genes were observed in neighbor countries including Nepal (Manandhar et al., 2020), Bangladesh (Khan et al., 2018), Brazil (Rocha et al., 2015), China (Xia et al., 2014), Pakistan (Abrar et al., 2019), Ethiopia (Zeynudin et al., 2018), Switzerland (Yuki et al., 2021,) (Marie-Frédérique et al., 2007), Argentina (M. et al., 2003), Netherlands (N. et al., 2006), Japan (Zhao & Hu, 2013) and United States in GNB isolates. For example, the study conducted in Ethiopia showed the prevalence of *bla*_{CTX-M-15} type extended-spectrum β -lactamases in *E. coli* (92.3%) and *K. pneumoniae* (96.7%) among clinical isolates of GNB (Zeynudin et al., 2018). In another study, ESBL-producing *E. coli* contained higher prevalence of *bla*_{CTX-M-15} (58.4%) gene in patients admitted at hospital, Kathmandu, Nepal (Pokhrel et al., 2014). The abundance of *bla*_{CTX-M-15} gene was also observed in *E. coli* clinical isolates from community and hospital-based infection in China (Xia et al., 2014). High prevalence of ESBL-encoding *bla*_{CTX-M-15} gene was observed in 2372 clinical samples of GNB including *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *Enterobacter spp.* and *A. baumannii* obtained from the hospitals and diagnostic research center of Lahore, Pakistan (Nair et al., 2021). The carbapenemase activity for *bla*_{CTX-M-15} has been reported earlier (Laurent et al., 2021) (Walsh, 2010).

The abundance of bla_{CTX-M} genes in different species suggests horizontal gene transfer is occurring now or in the past. For example, *E. coli* from healthy food animals can be key repositories of bla genes and may contribute to the spread and transmission of these β -lactamase genes, and lateral transfer of resistance genes between animals and humans. In contrast, bla_{NDM} and bla_{OXA} was observed to be highly prevalent in GNB isolates in Tamil nadu (Nachimuthu et al., 2016) as well as Mumbai (Kazi et al., 2015). Likewise, bla_{VIM} (Okochi et al., 2015), and bla_{IMP} (Mushi et al., 2014) was observed to be the most common gene in CR-GNB isolates. We found 3 types of carbapenemase gene namely bla_{NDM} (4.65%), bla_{OXA} (6.90%) and bla_{DIM} (1.15%) in our study. Though these carbapenemase gene are not common, yet it is concerning because it can be resistant to even more number of antibiotics. Carbapenem-resistant isolates may exhibit multidrug-resistance as they possessed bla_{NDM-1} or bla_{NDM-5}, along with other antimicrobial-resistance factors. Among, subclass B1 metallo-beta-lactamase (bla_{NDM}), higher prevalence of bla_{NDM-5} was detected in GNB isolates which may confers higher resistance against carbapenems than bla_{NDM-1} as reported earlier (Hornsey et al., 2011). Varying geographic locations, different levels of healthcare institutions engaged, different levels of exposure to healthcare environments, antibiotic use, and antibiotic stewardship procedures may all contribute to these disparities.

In our research, multiple co-existence genes within the same isolate were observed, where β -lactams co-resistance genes with bla_{NDM} was found in 70.6% of carbapenemase-producing isolates. Carbapenem co-resistance retain genes that make them resistant to other antibiotics, making them multi-drug resistant and it threatens global antibiotic chemotherapy, patients' recovery, and the economy (Kopotsa et al., 2020); (Mmatli et al., 2020). Resistance to carbapenem can be caused by the presence of bla_{NDM}, bla_{CTX-M}, bla_{TEM}, bla_{SHV}, and bla_{OXA} gene family as well as impermeability (Rawat & Nair, 2010). This is particularly problematic in India, where β -lactams carbapenemase prevalence is quite high. This suggests that the detection of carbapenemase-encoding genes is an important index for phenotype in CR-GNB isolates. In our study, more than half of the isolates tested positive for multidrug resistance (MDR) to the most commonly used antibiotics. The acquisition and horizontal transfer of resistant genes from a variety of sources, including pathogenic bacteria, the environment, and animals, could be the main causes of resistance's uncontrolled expansion (Fair & Tor, 2014). Poor infection management in the country might be another reason for the high incidence of MDR and the acquisition of resistance genotypes, necessitating immediate action to combat the burgeoning AMR.

Conclusions

Carbapenemase-producing bacteria were detected in abundance in the Bihar region. The present study highlights the overwhelming threat of the β -lactam group to explore the mechanism of carbapenem-resistance in GNB. A high co-resistance rate in multidrug resistant GNB was observed which may provide further insight into the epidemiology of resistance acquisition. The prevalence of carbapenemase-encoding genes (bla_{NDM}, bla_{OXA} and bla_{DIM}) found from this study is a rising threat in India which requires immediate attention from the healthcare perspective. Therefore, strict antibiotic policy to prevent the misuse of antibiotics should be imposed to control the drug-resistance in India.

Abbreviations

CR-GNB: carbapenem-resistant Gram-negative bacterial; **CLSI:** Clinical and Laboratory Standards Institute; **MHT:** Modified Hodge Test; **DDST:** Double Disc Synergy Test; **CARD:** Comprehensive Antibiotic Resistance Database; **AMR:** antimicrobial resistance; **CTX-M:** cefotaxime-hydrolyzing β -lactamase–Munich; **NDM:** New Delhi metallo- β -lactamase; **OXA:** oxacillin carbapenemase/oxacillinase; **SHV:** sulfhydryl variant of the TEM enzyme; **TEM:** temoneira class A extended-spectrum β -lactamase; **VIM:** verona integron-encoded metallo- β -lactamase, metallo- β lactamases (MBLs)

Declarations

Data availability

Whole genome sequences have been deposited in the NCBI under the accession number PRJNA744890.

Acknowledgement

All the work and data analysis were completed at Indira Gandhi Institute of Medical Sciences (I.G.I.M.S), Patna and All India Institute of Medical Science (A.I.I.M.S.), New Delhi, respectively. IGIMS ethical committee approval (45/IEC/ 2018/IGIMS) was taken.

Author contribution

NK-contributed to the experimental design, **MK and AK**-contributed to whole-genome sequencing and AMR data analysis, interpreted the results and drafted the manuscript, **ABK, PP and BK**- contributed to phenotypic data analysis, **NK, NRB and PK**-reviewed and revised the manuscript. All authors have read and approved the manuscript for publication.

Ethics statement: Written informed consent was taken by the participants and the study was reviewed and approved by the ethical committee of IGIMS, Patna, India (451/IEC/2018/IGIMS).

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Tables

Table 1
Distribution and phenotypic carbapenem-resistance rate of isolated Gram-negative bacteria

SI No.	Specimen	GNB isolates	Carbapenem-resistance rate (%)
1	Blood and vascular catheter tip	6	<i>K. pneumoniae</i> – 1/06 (16.7%)
			<i>A. baumannii</i> – 1/06 (16.7%)
			<i>E. coli</i> – 1/06 (16.7%)
2	Pus and body fluids	36	<i>E. coli</i> –2/36 (5.6%)
			<i>K. pneumoniae</i> – 1/36 (2.8%)
3	Urine	49	<i>K. pneumoniae</i> – 2/49 (4.1%)
			<i>E. coli</i> – 5/49 (10.2%)
			<i>P. aeruginosa</i> – 1/49 (2.0%)
4	Lower respiratory samples (Endotracheal aspirate, Broncho-alveolar Lavage, Sputum)	10	<i>K. pneumoniae</i> – 1/10 (10%)
			<i>E. coli</i> – 1/10 (10%)
			<i>P. aeruginosa</i> – 1/10 (10%)
		Total=101	Total=17, confirmed by MHT / DDST

Table 2
Antimicrobial-resistance (Kirby-Bauer Disc Diffusion) rates (%) of isolated Gram-negative bacteria

Antimicrobials (n=101)	<i>E. coli</i> (n=71)	<i>K. pneumoniae</i> (n=12)	<i>A. baumannii</i> (n=7)	<i>P. aeruginosa</i> (n=11)
Ampicillin	68 (95.8%)	11 (91.7%)	7 (100%)	-
Amoxycillin-clavulanic acid	60 (84.5%)	10 (83.3%)	7 (100%)	-
Cefotaxime	59 (83.1%)	10 (83.3%)	7 (100%)	-
Ceftriaxone	59 (83.1%)	10 (83.3%)	6 (85.7%)	-
Ceftazidime	57 (80.3%)	9 (75%)	6 (85.7%)	7 (63.6%)
Piperacillin-tazobactam	59 (83.1%)	10 (83.3%)	6 (85.7%)	7 (63.6%)
Sulfamethoxazole-trimethoprim	43 (60.6%)	9 (66.7%)	5 (71.4%)	-
Nitrofurantoin	-	-	-	-
Aztreonam	-	-	-	9 (81.8%)
Ciprofloxacin	65 (91.5%)	11 (91.7%)	7 (100%)	-
Ofloxacin	-	-	-	8 (72.7%)
Gentamicin	35 (49.3%)	7 (58.3%)	5 (71.4%)	7 (63.6%)
Amikacin	19 (26.8%)	4 (33.3%)	3 (42.8%)	2 (18.2%)
Tobramycin	49 (69.01%)	8 (66.7%)	6 (85.7%)	5 (45.4%)
Minocycline	-	-	1 (14.3%)	-
Meropenem	10 (14.1%)	5 (41.7%)	1 (14.3%)	2 (18.2%)
Imipenem	10 (14.1%)	5 (41.7%)	1 (14.3%)	2 (18.2%)

Table 3
Genotype profiling of 17 carbapenem-resistant Gram-negative bacteria (CR-GNB)

*Strain	Beta-lactams/ESBLs				Carbapenem												Colistin
	AmpC1	PDC-10	SHV-11	SHV-148	TEM-104	TEM-1D	CTX-M15	DIM-1	NDM-1	NDM-5	OXA-1	OXA-10	OXA-23	OXA-50	OXA-181	OXA-488	MgrB
AB01	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
EC02	1	0	0	0	0	0	1	0	1	0	0	1	0	0	1	0	0
EC03	0	0	0	0	0	1	1	0	0	1	0	0	0	0	0	0	0
EC04	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
EC05	0	1	1	0	0	1	1	1	0	1	0	1	0	0	0	1	0
EC06	0	0	0	1	0	1	1	0	0	1	0	0	0	0	1	0	0
EC07	0	0	1	0	0	1	1	0	0	1	0	1	0	0	0	0	0
EC08	1	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0
EC09	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
EC10	0	0	1	0	0	1	0	0	0	1	0	1	0	0	0	0	0
KP11	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
KP12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
KP13	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0
KP14	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
KP15	1	0	0	0	0	0	1	0	1	0	0	1	0	0	1	0	0
PA16	1	0	0	0	1	0	0	0	0	1	1	0	1	0	0	0	0
PA17	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
*AB: Acinetobacter baumannii; EC: Escherichia coli; PA: Pseudomonas aeruginosa; KP: Klebsiella pneumonia; 1: present; 0: absent																	

Table 4
Co-existence genes conferring resistance to β -lactams carbapenem drugs in Gram-negative bacteria

β -lactamases genes	E.coli (n=8/9)	K. Pneumonia (n=5/5)	P. Aeruginosa (n=0/2)	A. baumannii (n=1/1)
CTX-M + NDM	0	1	0	0
CTX-M + SHV	1	0	0	0
CTX-M + NDM + OXA	1	1	0	0
CTX-M + TEM + OXA	0	1	0	0
CTX-M + NDM + SHV	0	1	0	0
CTX-M + NDM + TEM	1	0	0	0
CTX-M + NDM + OXA + TEM	1	0	0	0
CTX-M + NDM + SHV + TEM	1	1	0	1
CTX-M + NDM + SHV + OXA + TEM	3	0	0	0

Table 5
Genotypic-Phenotypic correlation of carbapenemase-producing antimicrobial-resistance genes in Gram-negative bacteria

Antimicrobials (n=17)	<i>E. Coli</i> (n=9)		<i>K. Pneumoniae</i> (n=5)		<i>A. Baumannii</i> (n=1)		<i>P. Aeruginosa</i> (n=2)	
	Genotype (%)	Phenotype (%)	Genotype (%)	Phenotype (%)	Genotype (%)	Phenotype (%)	Genotype (%)	Phenotype (%)
Ampicillin	R (77)	R (100)	R (100)	R (100)	R (100)	R (100)	R (100)	-
Amoxycillin-clavulanic acid	R (67)	R (88.9)	R (100)	R (100)	R (100)	R (100)	R (50)	-
Cefotaxime	R (67)	R (88.9)	R (100)	R (100)	R (100)	R (100)	R (100)	-
Ceftriaxone	R (89)	R (88.9)	R (100)	R (100)	R (100)	R (100)	R (100)	-
Ceftazidime	R (77)	R (88.9)	R (100)	R (100)	R (100)	R (100)	R (100)	R (100)
Piperacillin-tazobactam	R (67)	R (88.9)	R (100)	R (100)	R (100)	R (100)	R (100)	R (100)
Sulfamethoxazole-trimethoprim	-	R (77.8)	-	R (80)	-	R (100)	-	-
Nitrofurantoin	-	-	-	-	-	-	-	-
Aztreonam	R (89)	-	R (100)	-	R (100)	-	R (100)	R (100)
Ciprofloxacin	R (22)	R (100)	R (100)	R (100)	-	R (100)	-	R (100)
Ofloxacin	-	-	-	-	-	-	-	R (100)
Gentamicin	R (22)	R (55.6)	-	R (60)	-	R (100)	-	R (100)
Amikacin	R (22)	R (33.3)	-	R (40)	-	R (100)	-	R (50)
Tobramycin	-	R (88.9)	-	R (80)	-	R (100)	-	R (100)
Minocycline	-	-	-	-	-	R (100)	-	-
Meropenem	R (89)	R (100)	R (83)	R (100)	R (100)	R (100)	R (50)	R (100)
Imipenem	R (77)	R (100)	R (83)	R (100)	R (100)	R (100)	R (50)	R (100)
*R-resistance								

Figures

Figure 1

Types of carbapenem-resistance gene in Gram-negative bacteria.

Figure 2

Isolates-wise prevalence and distribution of carbapenemase genes in Gram-negative bacteria

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