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## Phenotypic and genotypic characterizations of antimicrobial resistance among gram-negative bacilli of clinical isolates

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### ABSTRACT

One of the biggest threats to human health today is the emergence of resistance among the most significant bacterial diseases. To identify outbreaks and the transmission of clinically important resistance genes and the genetic components associated with them in human infections, there is a need for genomic surveillance of antimicrobial resistance genes. The objective of this study is to evaluate the phenotype and genotype of antimicrobial resistance in clinically isolated gram-negative microorganism strains, as well as their molecular characterization. Microbiological identification was done using the automatic microbiological analyzer and GN ID REF21341 cards. The isolates' susceptibility to antimicrobials was evaluated using conventional disc diffusion. Using particular primers, resistance genes were amplified through PCR (applied biosystems). The identities of all 1470 isolates, including *Escherichia coli* (28%), *Klebsiella pneumoniae* (21%), *Acinetobacter baumannii* (7%), *Serratia marcescens* (6%), *Enterobacter cloacae* (6%), *Proteus mirabilis* (6%), *Pseudomonas aeruginosa* (5%), *Citrobacter freundii* (5%), *Citrobacter braakii*, *Acinetobacter lwoffii*, *Enterobacter aerogenes* and *Proteus vulgaris* (4%). All of these isolates exhibited multidrug resistance (MDR) to several kinds of antimicrobial medicines. Nine Carbapenem-resistant gram-negative bacilli strains were identified to be positive for blaNDM and blaOXA-1. Our study found a concerning association between these diseases' antimicrobial resistance and the routinely prescribed antibiotics. This discovery compromises the medical field's therapeutic options and encourages the use of specialists who have less potent antimicrobial effects.

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### Introduction

The World Health Organization (WHO) claims that antimicrobial resistance (AMR) poses a risk to treating and preventing infectious illnesses. It is a widespread issue primarily brought on by inadequate administration, poor treatment, and indiscriminate, unchecked use of antibiotics (Kumarasamy et al. 2010; WHO 2015; 2016). AMR is becoming more prevalent and severely threatens global public health (Kotwani & Holloway 2011; Zimlichman et

al. 2013). AMR surveillance is the most potent instrument for estimating the burden of AMR and providing the antibiogram data needed to establish local, national, and worldwide treatment programs (Ganguly et al. 2011; Zimlichman et al. 2013). WHO created the Global Antimicrobial Resistance Surveillance System (GLASS) in May 2015 to enable a standardized approach to AMR data collecting, analysis, and sharing on a global scale (WHO 2015). In affluent countries, there are numerous studies on

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AMR surveillance (Leape et al. 1991; Zimlichman et al. 2013; Yokoe et al. 2008). However, studies on AMR surveillance in developing countries, including India, are insufficient. AMR lengthens hospital stays, raises medical costs and increases mortality (Ganguly et al. 2011; Saxena et al. 2019). The emergence and fast spread of germs that are resistant to antibiotics have seriously hampered the ability to treat both common and fatal bacterial diseases. The Global Antibiotic Resistance Partnership (GARP) was created to formulate practical policy recommendations for low- and middle-income nations (Ganguly et al. 2011).

Drug resistance emerged in equine infections between 1992 and 1997, and a German study found a 75% increase in *E. coli* resistance to tetracyclines, by 80% to ampicillin, and by 90% to sulfonamides. In addition, for *Staphylococcus aureus* resistance to penicillins increased by 50% to 60% while gentamicin resistance increased by 400%. The most common species found in swine carcasses is *Campylobacter coli*, which is less frequently linked to illness in people (Ahern & Richardson 2012). Tetracycline, fluoroquinolones, lincosamides, and macrolides are the antibiotics that are most frequently associated with antimicrobial resistance. However, as was already indicated for *C. jejuni*, gentamicin has become resistant in the US as well. When opposed to *C. jejuni*, *C. coli* have historically exhibited higher levels of antibiotic resistance. This phenomenon's origin is a mystery (Ahern & Richardson 2012). Numerous variables, including the location of the hospital, the kind of intensive care units (ICUs) or wards, the patient population, immunity status, and fundamental chronic conditions like hypertension, diabetes, COPD, and chronic kidney disease, affect the incidence of nosocomial infection in hospitalized patients (Moolchandani et al. 2017). However, hospital infection control procedures and, most significantly, hospital management play a vital role (Carattoli 2009; Kumarasamy et al. 2010). The growth in Gram-negative bacteria resistance is primarily attributable to mobile genes on plasmids that can quickly propagate through bacterial populations (Hawkey & Jones 2009). Standardized plasmid typing methods improve our understanding of these elements' host ranges and global distribution. Furthermore, unprecedented human air travel and migration enable bacterial plasmids and clones to move rapidly across countries and continents. Much of this spread goes unnoticed, with resistant clones being carried in the normal human flora and only becoming apparent when they are the source of endogenous diseases (Walsh et al. 2007). In the mid-1990s, the CTX-M-15 extended-spectrum-lactamase (ESBL) encoded by blaCTX-M-15 was first discovered in India. The gene migrated from its original hosts' chromosomes to plasmids, expanding widely, seeking to establish CTX-M-15 as the globally dominant ESBL and the principal origin of attained resistance to next generation (3rd) cephalosporins in Enterobacteriaceae (Livermore et al. 2007).

India has been termed as the "World AMR Capital." While the introduction of emerging MDR organisms poses new diagnostic and therapeutic hurdles, India is still fighting old antagonists like tuberculosis, malaria, and cholera pathogens that are becoming increasingly medication resistant (Chaudhry & Tomar 2017; Taneja & Sharma 2019). Of the 2152 studies on AMR published by Indian institutes, 1,040 (48.3%) were on humans, while only 70 (3.3%) were on animals, 90 (4.2%) were on the environment, and 11 (0.5%) were on One Health ("Scoping Report on Antimicrobial Resistance in India" n.d.). In India, recent studies indicate that 70-90% of Enterobacteriaceae contain ESBLs. While this may be a biased test, it highlights a serious problem requiring the widespread use of restricted antibiotics such as carbapenems (Hawkey & Jones 2009; Kumarasamy et al. 2010). Although cephalosporin resistance rates are low in other countries, the growing number of ESBL manufacturers is sufficient to drive the need for carbapenems. Since there are not many antibiotics in the hoard other than carbapenems, the *Enterobacteriaceae* are under selective pressure for carbapenem resistance, and its rise is a global public health concern (Kumarasamy et al. 2010; Livermore 2009). *Klebsiella pneumoniae* clones harbouring the KPC carbapenemase have already caused concern in the United States, Greece, and Israel. A plasmid encoding the VIM metallo-carbapenemase has also spread to *K. pneumoniae* in Greece.

In 2008, the first example of NDM-1 producer was detected in *K. pneumoniae* isolated from a 59-year-old man who returned to Sweden after being hospitalized in India (Potron et al. 2015). Likewise, an NDM-1 among *E. coli* was found in the patient's stool. Gram-ve bacteria with the NDM-1 resistance flag use at least one zinc molecule at the active site to aid in carbapenem hydrolysis (Yong et al. 2009). The objective of this study is to determine the prevalence of nosocomial infections, identify potential risk factors for these infections and molecular characterization of pathogens.

## Materials & Methods

### Ethical approval

We conducted this prospective study in the Western Diagnostic Laboratory and Maharaj Vinayak Global University, Jaipur between January 2018 to December 2021 according to the Institutional Ethics Committee (Ref. number MVGU/ADM/2020/437(i)).

### Sampling, isolation and identification of bacterial strains

About 1470 isolates of Gram-negative bacteria were found in the patient's samples (blood, urine, faeces, and pus). The samples were collected during the inquiry at the facility and forwarded to the lab. The microbiological characterization was carried out using Gram staining and

morphology on culture media. The enzymatic activity of isolated organisms was examined using biochemical assays such as oxidase tests, citrate utilization tests, and indole tests. The Vitek 2 automatic microbiological analyzer (BioMérieux Inc., USA) and GN ID REF21341 cards were used for microbial identification.

### Antimicrobial susceptibility analysis

The Clinical and Laboratory Standards Institute's (CLSI) Criteria and interpretation guidelines were utilized to detect antibiotic sensitivity phenotypes in bacterial isolates (CLSI 2020). Antibiotic susceptibility assay of the isolate was completed by disc diffusion on MHA media plates for the following antibiotics namely: Aminoglycosides: Amikacin (30µg), Gentamicin (10µg), Fosfomycin (30µg), Tigecycline (30µg), Beta-lactams: Ceftazidime (30µg), Ceftriaxone (30µg), Aztreonam (30µg), Carbapenem: Imipenem (10µg), Meropenem (10µg), Quinolone: Levofloxacin (5µg), Ciproflaxocin (5µg), Norfloxacin (5µg), Polymyxin: Colistin (10µg), and inhibitors CefoperazoneSulbactam and Piperacillin Tazobactam respectively.

### Isolation of bacterial DNA and preparation for PCR

A few bacterial colonies from the culture were combined with 100 µL of sterilized water, incubated at 95 °C for 10 minutes in a water bath, and then centrifuged at 10,000 g for 15 minutes to extract the DNA. Resistant genes were amplified by PCR (Applied Biosystems) using primers NDM forward: GGTGGCGATCTGGTTTC NDM reverse: CGGAATGGCTCATCAGATC for blaNDM and OXA-1 Forward: TCAACTTCAAGATCGCA OXA-1 reverse GTGTGTTTAGAATGGTGA. Gel loading dye (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol) and PCR products (25 µl) were combined. In 1X TAE (40mM Tris-acetate, 1mM EDTA) buffer, 2% agarose gel was created. Ethidium bromide was added at a concentration of 0.5 µg/ml. It was okay to place the gel on the work surface. A 1X TAE running buffer was included after the gel equipment was set up. In wells, 20 µl of sample product and 5 µl of 100 bp DNA ladder were added and photographed by Gel Documentation tool (Bio-Rad Laboratories, USA).

## Results

### Occurrence of antibiotic resistant gram-negative bacteria

Among 1470 isolates, it was observed that *E. coli* (28%) and *K. pneumoniae* (21%) was the most common organism isolated from biological samples. Other common organisms isolated were in decreasing order as depicted in Figure 1. *Acinetobacter* spp. at 7%, *Serratia* spp., *Enterobacter* spp. and *Proteus* spp. at 6%, for *Pseudomonas* spp. and *Citrobacter* spp. (5%), *Citrobacter* spp., *Acinetobacter* spp., *Enterobacter* spp. and *Proteus* spp. were at 4%.

### Antimicrobial susceptibility profile

To assess the antibiotic susceptibility profiles of 1470 clinical isolates of gram-negative bacilli. These isolates were discovered to be extremely resistant to a variety of commonly used antibiotic classes in clinical settings, including Aminoglycosides: Table 1 shows that the following antibiotics were discovered: Gentamicin (43.40%), Amikacin (29.65%), Fosfomycin (21.36%), Tigecycline (8.57%), Beta-lactams: Ceftazidime (31.90%), Ceftriaxone (52.85%), Aztreonam (21.97%), Carbapenem: Imipenem (5.30%), Meropenem (4.89%), Quinolone: Levofloxacin.

### Detection of blaNDM and blaOXA-1 resistant markers

In order to find the blaNDM and blaOXA-1 genes, carbapenem-resistant gram-negative bacterial strains were investigated. According to figures 2 and 3, the strain 09 of 78 carbapenem-resistant gram-negative bacilli was found to be positive for blaNDM and blaOXA-1. Out of 9, 4 isolates produced *E. coli* with blaNDM-1 and blaOXA-1, 2 isolates produced *K. pneumoniae* with blaNDM-4 and blaOXA-1, 1 isolate produced *E. coli* with blaNDM-5 and blaOXA-1, and 1 isolate produced *Enterobacter aerogenes* with blaNDM-1 and blaOXA-1 (Table 2).

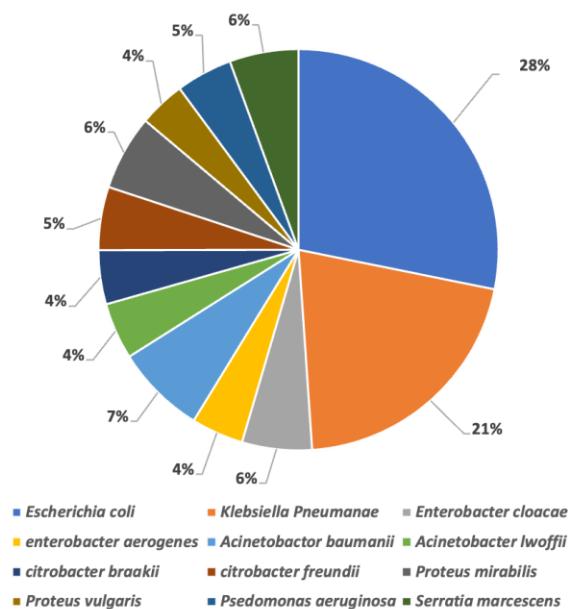


Fig 1. Distribution of isolated bacteria as percentage.

## Discussion

Our present study offers insights into the molecular characterization and antibiotic resistance from blood, urine, faeces, and pus samples, where in *E. coli* were the major cause of infections being identified. The result of this study exhibited the overall hygienic status of treatment, sample collection conditions and outbreak investigations. In the twenty-first century, antimicrobial resistance (AMR) has become one of the most important global public health

**Table 1** Antimicrobial susceptibility of 1470 Gram-negative bacilli clinical isolates

Antibacterial Class	Type of Antibacterial	The number of isolates with indicated susceptibility			The rate of resistant %
		Resistant (R)	Intermediate (I)	Susceptible (S)	
Aminoglycosides	Gentamicin	638	33	799	43.40
	Amikacin	436	57	977	29.65
	Fosfomycin	314	68	1088	21.36
	Tigecycline	126	10	1334	8.57
Beta-lactamas	Ceftazadime	469	03	998	31.90
	Ceftriaxone	777	02	691	52.85
	Aztreonam	323	5	1142	21.97
Carbapenem	Imipenem	78	52	1340	5.30
	Meropenem	72	8	1390	4.89
Quinolone	Levofloxacin	737	09	724	50.13
	Ciprofloxacin	717	59	694	48.77
	Norfloxacin	791	52	627	53.80
Polymyxin	Colistin	45	01	1424	3.06
Inhibitor	Cefoperazone	72	17	1381	4.89
	Sulbactam				
	Piperacillin	92	34	1344	6.25
	Tazobactam				

**Table 2** Genotypic characteristics of blaNDM-5 and blaOXA-1 producing Gram-negative bacilli

S. No.	Species	NDM type	OXA type
1	<i>E. coli</i>	NDM-1	OXA-1
2	<i>E. coli</i>	NDM-1	OXA-1
3	<i>E. coli</i>	NDM-1	OXA-1
4	<i>E. coli</i>	NDM-1	OXA-1
5	<i>K. pneumoniae</i>	NDM-4	OXA-1
6	<i>K. pneumoniae</i>	NDM-1	OXA-1
7	<i>K. pneumoniae</i>	NDM-1	OXA-1
8	<i>E. aerogenes</i>	NDM-5	OXA-1
9	<i>E. aerogenes</i>	NDM-1	OXA-1

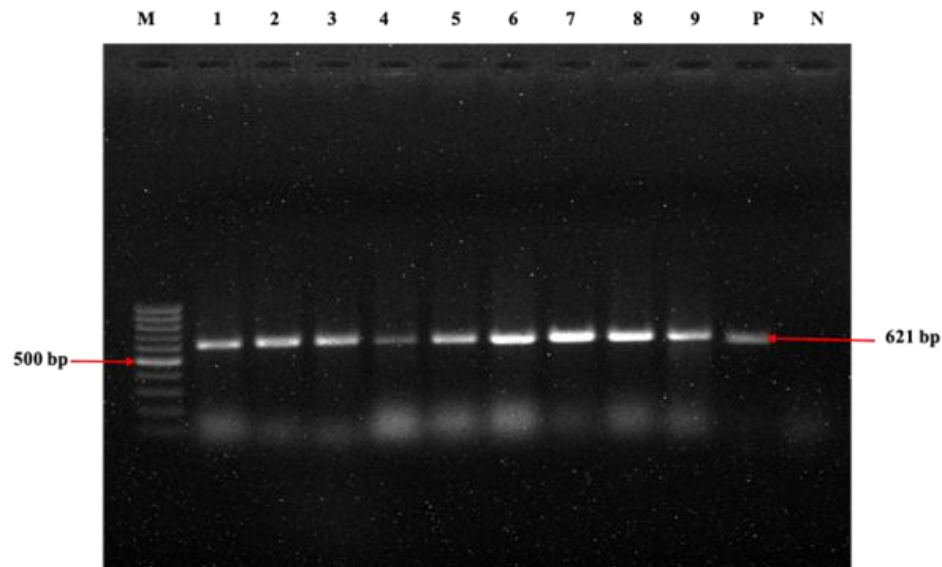
challenges (WHO 2015; Arora et al. 2017). AMR arises when bacteria mutate and reduce the efficiency of medicines used to treat diseases, is one of the most critical challenges affecting public health in the twenty-first century. Human-animal-environment interactions are critical for AMR containment, according to the One Health concept (Dahal et. al 2017; Taneja & Sharma 2019). Antibiotic use in medical, veterinary care, agriculture, and poultry has contributed significantly to the evolution of bacterial resistance. The same is true for India, where AMR rates have risen across all three industries (Taneja & Sharma 2019).

AMR occurs commonly in bacteria due to protein overexpression, which permits a subgroup of resistant bacteria to develop naturally (Munita & Arias 2016). Understanding how AMR arises in bacteria and developing novel medicines to combat the threat of widespread AMR rely on identifying these proteins. Other reasons of antibiotic resistance include genetic changes, antibiotic

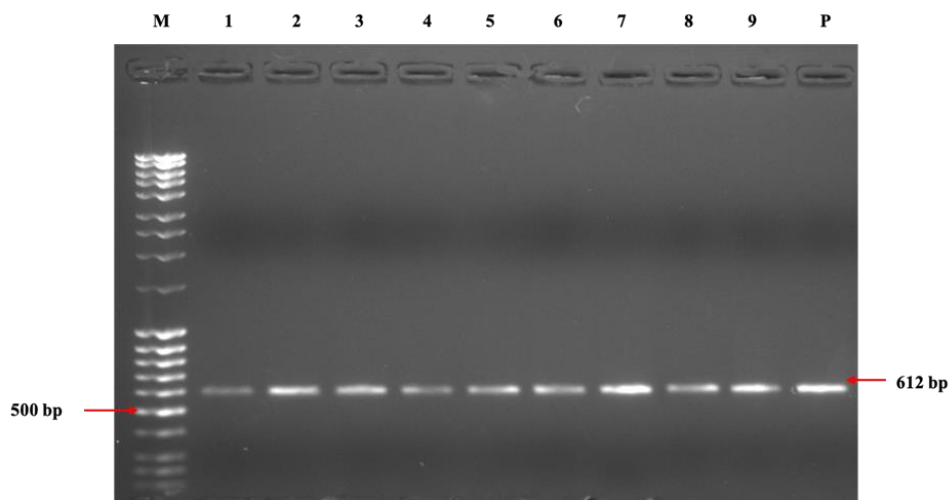
abuse and/or overuse in humans and animals, inappropriate broad-spectrum antibiotic prescribing, antibiotic trafficking, and antimicrobial manufacturing contaminations (Ganguly et al. 2011).

Enterobacteriaceae are a large family of Gram-negative microorganisms that include many well-known pathogens such as *Enterobacter* spp., *Serratia* spp., *Klebsiella* spp., *Escherichia* and others (Levy & Marshall 2004). However, the resistance patterns of the bacterial populations varied between nations, which may reflect changes in antimicrobial treatments (Kumari et al. 2007; Saxena et al. 2019). The resistance phenotypes of bacteria isolated from human samples have been documented from numerous countries (Sands et al. 2021). Increased antimicrobial resistance is making treatment options difficult, particularly with high carbapenemase-producing Gram-negative bacilli microorganisms, as verified and described in most countries (Kumari et al. 2007). The clinical influence of carbapenemase producers has emerged

globally. The propagation of carbapenem-resistant is resistance threat in Gram-ve bacteria (Jin et al. 2020). currently the utmost persuasive



**Fig 2.** PCR amplification of blaNDM. Lane M: 100 bp DNA Ladder, Lane 1-9: blaNDM, Lane P: Positive control, Lane N: Negative control.



**Fig 3.** PCR amplification of blaOXA-1. Lane M: DNA Ladder, Lane 1-9: blaOXA-1, Lane P: Positive control

Medically valuable carbapenem-resistant microbiota are a threat due to their resistance profile, accelerated emergence, and rapid transmission from bacteria to bacteria. Additionally, it can spread to other genera of Gram-ve organisms through infectious genetic factors (e.g., plasmids, insertion sequences, and transposons) (Jin et al. 2020). Additionally, XDR or MDR features caused by additional antibiotic resistance markers found in communicable genetic components severely restrict the range of available therapeutic alternatives. In this study, the results demonstrated that a total of 1470 Gram-negative bacilli were identified, wherein the 12 Gram-negative bacterial

species were identified to cause infections throughout the study period (Tamma & Simner 2018; Workneh et al. 2019). Generally, *E. coli* (28%), and *K. pneumoniae* (21%) are the main pathogens isolated in this study. In Nigeria, Osifo and Aghahowa reported that *K. pneumoniae* and *E. coli* were the most frequently isolated pathogens (Osifo & Aghahowa 2011).

Table 3 (supplementary materials) demonstrates that *Ceftriaxone* is the most resistant antibiotic among the isolates of *E. coli* (55.7%), *K. pneumoniae* (60.9), *C. freundii* (46.6%), *E. aerogenes* (45.1%) and *S. marcescens* (45.8%). Furthermore, Levofloxacin is the second most resistant

antibiotic against *A. baumannii* (59.8%), *A. lwoffii* (59.7%), *E. cloacae* (51.8), *P. vulgaris* (52.7%), *C. braakii* (57.8%), and *P. aeruginosa* (56.7%), with Ciproflaxocin showing the highest resistance against *P. mirabili* (52.8%). Four isolates showed co-existence with blaNDM-1 and blaOXA-1 producing *E. coli* in the molecular characterization; two isolates were associated with blaNDM-1 and blaOXA-1, and one was blaNDM-4 co-existing with blaOXA-1 among *K. pneumoniae*; one isolate was associated with blaNDM-5 and blaOXA-1, and one was blaNDM-1 co-existing with blaOXA-1. The previously discovered blaNDM and blaOXA-1.15NDM-5, which were first discovered in *E. coli*, are also found in Bangladesh (Manyahi et al. 2022). These alterations were responsible for the high resistance of NDM-5 bacteria compared to NDM-1 bacteria (Hornsey, Phee, & Wareham 2011). Resistance to commonly used antibiotics is common in *E.aerogenes* isolates from patients; in particular, the use of carbapenems meropenem and imipenem as first-line antimicrobial agents for considering emergence infections has resulted in a significant increase in the predominance of carbapenemase-producing *E. aerogenes* (Chen et al. 2015)

The current study examined carbapenemase-encoding genes to see if they were associated with antibiotic resistance. The blaNDM and blaOXA-1 genes were found in the majority of the isolates, showing that blaNDM and blaOXA-1 are the primary mechanisms underlying carbapenem resistance in the screened samples. The high association for resistance characteristics in our current study could be attributable to the fact that the NDM and OXA-1 gene is a frequent variant with benefits in obtaining resistance determinants and surviving in the nosocomial environment. The discovery of an antibiotic resistance connection is critical in developing antimicrobial resistance control and treatment strategies for each serotype. Although virulence genes are distributed evenly across serotypes, some of these genes are associated with certain serotypes.

## Conclusion

In This study sheds light on the numerous illnesses produced by NDM and OXA-1 in Gram-negative bacilli. In this investigation, a large number of MDR Gram-negative bacteria from cases at the Western Diagnostic Laboratory in Meerut were found. We were also able to identify these isolates as well as distinct categories of antibiotic resistance genes. As a result, periodic monitoring of the antibiotic susceptibilities of pathogenic bacteria isolated from human samples, along with molecular identification of this resistance, would be a useful metric in detecting resistance emergence and transmission. The Indian Health Ministry has already prioritized the expansion of Enterobacteriaceae in India and its spread to other nations to implement strategies to tackle the threat of NDM and OXA-1 producers. Current and earlier research indicates that it is critical to restrict the spread of carbapenem-resistant Gram-negative bacilli in both the hospital and the community.

This control will be required to develop rapid, low-cost, and accessible diagnostic procedures for identifying carbapenemase production. Furthermore, given the presence of blaNDM and its association with blaOXA-1 in India, caution should be maintained. Urgent need for more studies to assess the prevalence of multidrug-resistant microorganisms and develop strategies to combat their spread in India and worldwide.

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## Conflict of interest

The authors declare that there is no conflict of interest.

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