Journal of Chemical Health Risks

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JCHR (2023) 13(6), 73-80 | ISSN:2251-6727

Exploring Carbapenem-Resistant NFGNB in Northern Kerala: Phenotypic Detection and Characterization of MBL Producers in Clinical Samples

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(Received: 07 October 2023 Revised: 12 November Accepted: 06 December)

KEYWORDS
Non-Fermenting Gram-Negative Bacilli; Carbapenem-Resistance;Acinetobacter baumannii; Pseudomonas aeruginosa; MBL Producers.

ABSTRACT:

Background: The Non-Fermenting Gram-negative bacilli (NFGNB) are considered an emerging threat owing to their multi-drug resistance and ability to cause nosocomial infections. In the recent decade, carbapenem resistance in NFGNB has increased due to several drug resistance mechanisms, the most common beingthe production of carbapenemases. Fewer data are available on the species diversity of carbapenem-resistant NFGNB in the clinical samples from Northern Kerala, India.

Materials and methods: All clinical isolates are carbapenem-resistantNFGNB and were collected from a tertiary care hospital and screened for Carbapenem resistance or MBL. Metallo β – lactamases (MBL) enzyme production was detected by using a combined disc diffusion (CDD) test and E-test for the screening of MBL strain

Result: Out of 100 carbapenem resistant NFGNB isolates, 51 were carbapenem-resistant *Acinetobacter baumannii*and 29 were *Pseudomonas aeruginosa.* Among the carbapenem-resistant *Acinetobacter baumannii,* 41 strains were found to be MBL producers from the E-test and 37 were positive for the CDD test. Among the 29 carbapenem-resistant *Pseudomonas aeruginosa*, 23 strains were found to be MBL producers from the E-test and 23 were positive to CDD test.

Conclusion: Our study highlights the high prevalence of carbapenem resistance NFGNB among the clinical isolates in the regions of North Kerala, India.

Introduction

Non-fermentative- Gram-negative bacteria are a class of aerobic, gram-negative bacilli that do not utilize carbohydrates as a source of energy or break them down via an oxidative pathway as opposed to a fermentation process [1]. Gram-negative non-fermentative bacteria are present everywhere in nature and can be found in large quantities in sewage, water, plants, soil, and even on the mucous membranes of people and animals. Additionally, they thrive in water baths, sinks, faucets, aerators, and respirators. They are known to contaminate prescription drugs or sterile liquids meant for intravenous therapy [2]. These bacteria can infect immunocompromised patients, colonize them, and then spread disease, or enter normally sterile body sites through trauma. The Non-Fermenting Gram-Negative Bacilli (NFGNB) is comprised of 15 families [3]. Due to their intrinsic resistance and ability to produce infections in immunosuppressed individuals, NFGNBs pose a challenge to microbiologists and clinicians. In hospital settings, multi-drug-resistant NFGNB is a major issue since it can lead to numerous infections. Many gram-negative bacteria have the enzymes carbapenemases and metallocene-lactamases, which enable the bacteria to preferentially hydrolyse carbapenems and so develop drug resistance. Because the organisms that produce carbapenemase are also resistant to other classes of medications, nosocomial acquired carbapenemase-

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JCHR (2023) 13(6), 73-80 | ISSN:2251-6727

producing NFGNB infections present a significant challenge to the treating physician.

In 2017, the World Health Organisation released the first-ever global priority pathogen list for investigating and creating innovative therapeutics [4]. The list was drawn up to guide and promote research and development (R&D) of new antibiotics, as part of WHO's efforts to address growing global resistance to antimicrobial medicines. Since there are now only a small number of Antibiotics available and none have been found in the last ten years, antimicrobial resistance is a serious danger. One of the infections that causes the most trouble in healthcare institutions is *Acinetobacter baumannii*, which has spread around the world and evolved into a drug-resistant bacterium. [5-6].Resistance to antimicrobial agents is a major public health problem all over the world,especially in India [7]. The high morbidity and mortality associated with nosocomial infections are due to the expansion and spread of multidrug resistance of most gram-negative bacteria(GNB) [8]. One of the infections that causes the most trouble in healthcare institutions is *Acinetobacter baumannii*, which has spread around the world and evolved into a drug-resistant bacterium. Most of the *Acinetobacter.* spp are members of the *Acinetobactercalcoaceticus*– baumannii complex (Acb complex),the most resistant bacteria encountered in clinical practice are *Acinetobacter. spp* [9-10].There are many different species of *Acinetobacter spp*. in the soil, water, and food supply. They have been linked to ventilators, humidifiers, catheters, and other medical equipment in the hospital setting. Reports say that about 25% of adults and 7% of children have the organisms in their pharynx. Hospitalized people are easily colonized if they do not already have *Acinetobacter* spp [11]. *A. baumannii* is associated with UTIs, pneumonia, ventilator-associated pneumonia, tracheobronchitis, or both; endocarditis,septicaemia; meningitis, frequently as a side effect of intrathecal chemotherapy for cancer; and cellulitis, typically brought on by contaminated indwelling catheters, trauma, burns, or the introduction of a foreign body [12].

The opportunistic pathogen *Pseudomonas*is a frequent causeof acquired infection [13-16] and is known for its cultural characteristic which produces the blue-green coloration ingrowth. *Pseudomonas aeruginosa* is the primary cause of nosocomial infections, including pneumonia and bacteraemia [17]. It has been linked to a wide range of clinical illnesses, including bacteraemia, which frequently manifests as ecthyma gangrenosum of the skin, wound infections, pulmonary disease, particularly in people with cystic fibrosis (CF), nosocomial urinary tract infections (UTIs), endocarditis, infections after burns or trauma, and, in rare instances, infections of the central nervous system, including meningitis [18]. Infection is to be suspected when *P. aeruginosa* is isolated from a sterile bodily location, such as blood, pleural fluid, joint fluids or tissues, or cerebrospinal fluid (CSF). The oropharynx is one mucosal surface that P. aeruginosa can colonize. Hospitalized patients are at risk for contracting *Pseudomonas aeruginosa*, *Burkholderia cepacia*, and *Stenotrophomonas maltophilia*, among other bacteria. Patients in the intensive care unit (ICU) subjected to mechanical ventilation may quickly become colonized with *P. aeruginosa*[19-20]. Once the infection has started, the organism is usually always difficult to get rid of. Further genetic research is required to understand the multidrug resistance of the NFGNB isolates.

There are two main categories of carbapenemases or carbapenem resistance is such as serine beta-lactamase and metallo beta-lactamases. Little is known regarding the incidence of carbapenem-resistant Gram-negative bacteria in India, particularly in the southern state of Kerala. In the current study, we have focussed on Metallo beta-lactamase carbapenemases due to their high prevalence in India.Clinical catastrophes can be avoided by using phenotypic approaches to stop the spread of MBL genes by early detection, and timely treatment of resistant strains. To determine the frequency of carbapenem resistance and to define carbapenemase enzymes, we tested MDR Gram-negative bacterial isolates from clinically relevant cases for carbapenem resistance in the current investigation.

Materials and Methods

Sample Collection:

Carbapenem-resistant NFGNB (resistant to imipenem and meropenem) are collectedfrom clinical specimens (blood, urine, exudative specimens, which included pus, wound swabs, drain fluids, cerebrospinal fluid (CSF), and lower respiratory secretions) from patients at Aster Mims Hospital Calicut, India. The study protocol was approved by the Institutional Ethics Committee

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JCHR (2023) 13(6), 73-80 | ISSN:2251-6727

[CAU/SHS/2400/IEC/2014]. The age-wise distribution and types of sample sources have been graphically represented in figure 1 and figure 2 respectively.

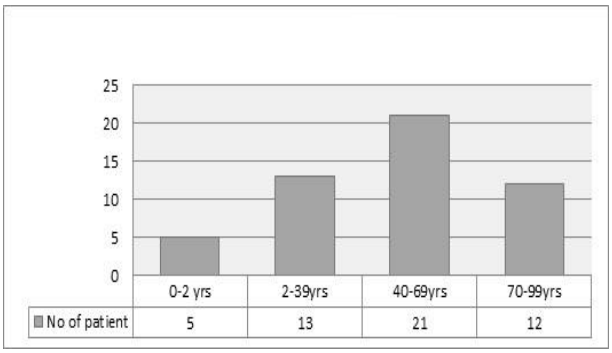

Fig 1: Age Interval of Patients

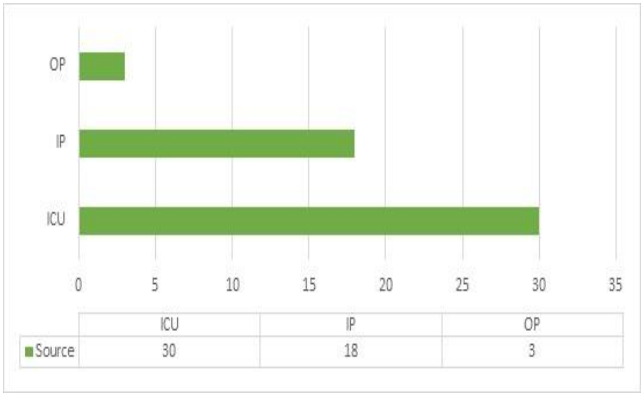

Fig 2: Source of Specimen (ICU-intensive care unit; IP- in patient clinic; OP- outpatient clinic)

Identification of NFGNB:

All isolates were collected and processed according to standard laboratory protocols. The collected strains were classified based on their colony morphology, staining properties, and various biochemical reactions, as well as through the use of the Vitek 2 compact automated system.

Antimicrobial susceptibility testing:

Isolated bacterial strains were subjected to antibiotic susceptibility testing for Meropenem and Imipenem by disc diffusion assay according to the 2019 CLSI guideline for selecting carbapenem-resistant organisms [21]. Since the study was profoundly focused on the Metallo β – lactamases nonfermenting gram-negative bacteria for antimicrobial resistance,the obtained carbapenem-resistant NFGNBwere subjected to the Combined Disc Diffusion (CDD) test and E test for the screening of MBL strain:

Screening and confirmation of MBLs:

Combined Disc Diffusion (CDD) test:

The carbapenem-resistant strains were screened for MBL by CDD test using IMP (10µg) + 5µl of 0.5 M EDTA(930µg) and MER (10 µg) + 5µl of 0.5 M EDTA(930µg). The zone diameter was compared with the presence of EDTA and those with IMP or MER alone were tested and the presence of an MBL was considered a positive test. Further confirmation of IMP + IMP-EDTA E-test strips (Himedia) for MBL production was done (Figure 3).

Fig 3: CDD test and E test for *Acinetobacter boumanni*

E-test method:

The test organism was lawn cultured on the culture plate, a single strip impregnated with a concentration gradient of imipenem (4 µg/ mLto 56 µg/ mL) was kept on one end and that of imipenem (1 µg/ mLto 64 µg/ mL) fused with a stable concentration of EDTA on the other end was placed. The 8-fold increase in the ellipse of imipenem and EDTA in the culture plate indicated the presence of MBL (Figure 4).

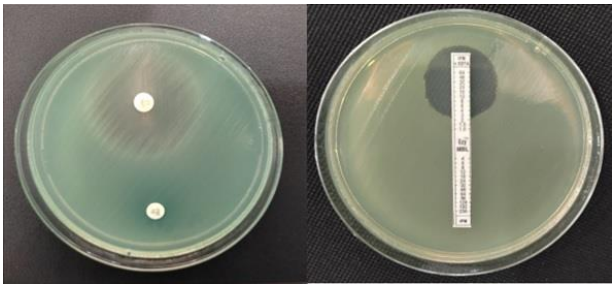

Fig 4: CDD test and E test for *Pseudomonas aeruginosa*

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JCHR (2023) 13(6), 73-80 | ISSN:2251-6727

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 8 software using One-way ANOVA. The sensitivity, specificity, positive predictive value, and negative predictive value of the test were evaluated by using online MedCalc Statistical Software (MedCalc Software bvba, Belgium; version 18.9; 2018).

Results

Identification of NFGNB:

Vitek 2 compact automated system was used to identify the clinical isolates. All the 100 samples are Carbapenem-resistantnon-fermentinggram-negative bacteria (CRNFGNB), the most predominant organisms being *Acinetobacter baumannii, Acinetobacter junii, Acinetobacter lwoffii, Burkholderia cepacia, Chryseobacteriumindologenes,Elizabethkingia meningoseptica, Pseudomonasaeruginosa, Pseudomonas fluorescens, and Stenotrophomonas maltophilia.* The distribution of organisms identified has been graphically represented in Figure 5 and Table 1.

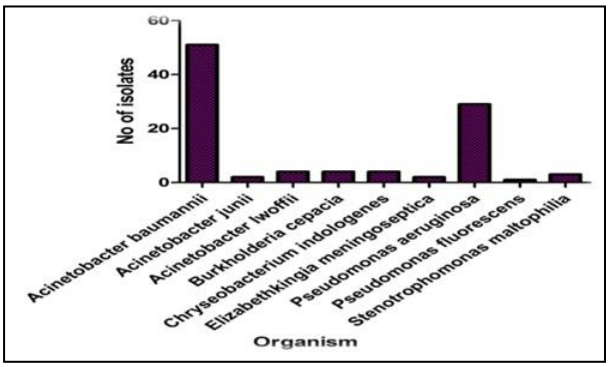

Fig 5: Distribution of Different Non-Fermenting Gram-Negative Bacteria obtained from the clinical samples

Table 1: Isolates obtained from various clinical samples.

***These value were statistically significant (p<0.0001)**

Organism	BAL	Blood	Bronchial wash	ET Tip	Pus	Sputum	Suction Tip	Tissue	Urine	Grand Total
Acinetobacter baumannii	0	1	3	3	15	21	2	2	4	51*
Acinetobacter junii	0	0	0	0	0	0	0	0	2	2
Acinetobacter lwoffii	0	1	1	1	0	1	0	0	0	4
Burkholderia cepacia	0	3	0	0	0	0	0	0	1	4
Chryseobacteriumindologenes	0	0	1	0	0	0	1	0	2	4
Elizabethkingiameningoseptica	0	2	0	0	0	0	0	0	0	2
Pseudomonas aeruginosa	2	1	0	0	6	10	0	1	9	29*
Pseudomonas fluorescens	0	0	0	0	0	0	0	0	1	1
Stenotrophomonas maltophilia	0	0	2	1	0	0	0	0	0	3
Grand Total	**2**	**8**	**7**	**5**	**21**	**32**	**3**	**3**	**19**	**100**

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Antibiotic susceptibility testing:

Isolated bacterial strains were subjected to antibiotic susceptibility testing for Meropenem (Table 2) and Imipenem by disc diffusion assay (Table 3) according to the 2019 CLSI guideline for selecting carbapenem-resistant organisms. Among the 100 carbapenem-resistantnonfermentinggram-negative bacilli obtained, 64 organisms are carbapenemase-producing (MBL) and 36 are non-carbapenemase-producingOrganisms (Table 4). The statistical analysis revealed the sensitivity and specificity of the tests performed to be 100 % (Table 5). The percentage of prevalence of drug resistance was found to be 66.13%. The P value <0.001 was statistically significant. The Positive Predictive Value and Negative Predictive Value for the test were 100%.

Table 2: Result of the combined disc diffusion method for Meropenem resistance (combined disc test).

Combined disc diffusion method (combined disc test)	
Positive	62
Negative	38

Table 3: Result of the MBL IP/IPI E Test, showing Imipenem resistance

MBL IP/IPI E Test	
Positive	64
Negative	36

Table 4: No of carbapenem-resistant /MBL resistant organisms obtained among the clinical isolates

Organisms	**Total**	**Positive**	**Negative**
Acinetobacter baumannii	51	41	10
Acinetobacter junii	2	0	2
Acinetobacter lwoffii	4	0	4
Pseudomonas aeruginosa	29	23	6
Pseudomonas fluorescens	1	0	1
Stenotrophomonas maltophilia	3	0	3
Burkholderia cepacia	4	0	4
Chryseobacteriumindologenes	4	0	4
Elizabethkingiameningoseptica	2	0	2

Table 5: Relationship between various parameters and the confidence interval

Statistic	**Value**	**95% CI**
Sensitivity	100.00%	91.40 % to 100.00%
Specificity	100.00%	83.89 % to 100.00%
Disease Prevalence (*)	100.00%	83.89 % to 100.00%
Positive Predictive Value (*)	66.13%	52.99 % to 77.67 %
Negative Predictive Value (*)	100.00%	
Accuracy (*)	100.00%	94.22 % to 100.00%

Discussion

Multi-drug resistant Non-Lactose Fermenting Gram Negative Bacteria is a major problem in healthcare settings as it causes numerous types of infections [22]. Many gram-negative bacteria have the enzymes carbapenemases and metallo-b-lactamases, which enable the bacteria to preferentially hydrolyse carbapenems and so develop drug resistance. Since the organisms that cause nosocomial acquired carbapenemase-producing NFGNB infections are resistant to carbapenems as well as other drug classes, such as fluoroquinolones and aminoglycosides, they present a significant risk of morbidity and mortality for the patients.

In our study, among the NFGNB isolates, Acinetobacter was found to be the predominant organism. This is in agreement with thestudiesof Esther et al., 2017, were reported that of the the1302 bacterial isolates, 598 were NFGNB, including *Pseudomonas species, Acinetobacter, Stenotrophomonas maltophilia, and Burkholderia cepacia*. Although the types of organisms

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discovered were comparable to the panel used in Ester's investigation, Acinetobacter accounted for the majority of cases [23].

In the study by BenachinmardiKirtilaxmi K et al., [24] with 100 samples, *Pseudomonas aeruginosa* (57.7%) and *Acinetobacter baumannii (42.3%)* were the two NFGNB species found to be resistant amongst all the NFGNB. This is concordant with our study in terms of the identified organisms but the percentage is varied as about 51 % of the samples were *Acinetobacter baumannii* and 29 % were *Pseudomonas aeruginosa*. The majority of the cases were detected from pus samples (40.4%) but this is in disagreement to our study as about 32% of the isolates. This is also concordant with the study by BenachinmardiKirtilaxmi K et al., [24]. The majority of the carbapenem-resistant isolates were from those above 60 years of age which was in agreement with our study. Elderly patients are considered highly prone to drug-resistant infections due to their waning immunity and comorbid conditions [25].Sonika Agarwal et al., 2017 revealed that 99 NFGNB were isolated, and the most common sample was ET aspirate sample 256 (64.5%) which is in disagreement to our study. However, in their study, *Acinetobacter baumannii* was the most common NFGNB isolated 63 (63.63%) followed by *Pseudomonas aeruginosa* 25 (25.25%), *Elizabethkingiameningoseptica*and *Strenotro phomonasmaltophilia*. This is concordant with our study [26]. Rit et al., 2013 in their study reported in their study with 1,650 clinical samples that non-fermenters isolated were *Pseudomonas aeruginosa* (50.24%), *Acinetobacter baumannii* (24.87%) which is in disagreement with our study as the majority of the samples were *Acinetobacter baumannii* followed by *Pseudomonas aeruginosa*[27]. In another study by Ahmed NH et al.,2015, the majority of the isolates resistant to carbapenems were *Acinetobacter baumannii* followed by *Pseudomonas aeruginosa* which is in agreement with our study [28].

In order to prevent the spread of infection, and therapeutic failure and to decrease the mortality rate, the detection of MBL is important [4,29-30]. It is a crucial step toward large-scale monitoring of the emerging resistant strains that produce MBL. Many simple screening tests have been designed for the detection of CRNFGNB [29]. Our study was capable of analyzing the prevalence of the circulating Carbapenemase resistant species.

Conclusion

Surveillance projects conducted by various institutions have analyzed significant data on disease incidence and antibiotic resistance across several geographic regions. In order to create effective treatment plans and infection control strategies, it is crucial to identify and keep track of carbapenem-resistant *Acinetobacter baumannii* and *Pseudomonas aeruginosa*. It is also crucial to screen for the prevalence of metallo beta-lactamases in the clinical laboratory.Our study highlights the high prevalence of carbapenem resistance NFGNB among the clinical isolates in the regions of North Kerala, India. Among the carbapenemresistance NFGNB , the dominant organism was found to be *Acinetobacter* species followed by *Pseudomonas* species. The routine screening for the identification of the MBL-producing organisms that are resistant to carbapenem may help in limiting the spread of infection with appropriate infection control practices and treatment plans.

Conflict of Interest

The authors declare no conflict of interest.

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