Original Article

Detection of Metallo-Beta-lactamase Production in Rare Carbapenem-Resistant Non-fermentative Gram-Negative Bacilli Isolated in a Tertiary Care Hospital, Visakhapatnam, India

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Introduction: Non-fermentative Gram-negative bacilli (NFGNB) are occasionally involved in infectious diseases pathology, but have shown resistance to multiple antibiotics and the capability to gain new resistance factors in the hospital environment. The present study was aimed to investigate the antibiotic susceptibility pattern of rare NFGNB isolated from different clinical samples and the prevalence of Metallo-beta-lactamase (MBL) producing non-fermenters among the carbapenem-resistant isolates. **Methods:** A total of 250 clinical samples from the patients suffering from various infections were analyzed by using different standard microbiological techniques like microscopy, culture methods, biochemical reactions and antibiotic susceptibility using Kirby-Bauer method. MBL detection was performed by imipenem-EDTA combined disc test and imipenem-EDTA double disc synergy test (DDST). **Results:** The non-fermenters bacteria rate isolated from different clinical samples was 4.8%. The highest rate of non-fermentative isolates was observed in patients with hospital-acquired infections (91.6%). The various species of NFGNB included *Pseudomonas putida* (33.3%), *Pseudomonas stutzeri* (25%), *Burkholderia cepacia* (16.6%), *Achromobacter xylosoxidans* (16.6%) and *Ochrobactrum anthropic* (8.33%). The isolates showed high resistance to carbapenems, and the incidence of MBL producing non-fermenters among the carbapenem-resistant organisms was found to be 100%. **Conclusion:** NFGNB are now emerging as organisms of nosocomial infections. The use of broad spectrum antibiotics should be avoided, and quick detection and efficient infection control measures are essential to prevent further spread of MBLs to other Gram-negative bacilli. Detection of MBL production and rationale antibiotic usage are the most important factors which control the gradually increasing NFGNB related infections. *J Med Microbiol Infec Dis, 2016, 4 (1-2): 31-36*.

Keywords: Non-fermenting Gram-negative bacilli, Carbapenems, Metallo-beta-lactamase.

INTRODUCTION

Non-fermenting Gram-negative bacilli (NFGNB) are a group of aerobic, non-sporing organisms that either does not use carbohydrates as a source of energy or consume them through metabolic pathways other than fermentation [1]. These organisms are abundant in nature like soil, water, plants, decaying vegetation, food-stuffs and normal flora of humans. In hospitals, nebulizers, disinfectants, dialysate fluids, saline and catheter devices are the common sources of infection by these opportunistic bacteria which can cause severe infections in immune-compromised hosts [2]. Most of the NFGNB have emerged as important nosocomial pathogens in hospitalized patients with severe burns, urinary tract infections, wound infections, septicemia, pneumonia, osteomyelitis, peritonitis, meningitis and cirrhosis of the liver. These bacilli have been isolated from pus, sputum, urine, ascites, catheters, tracheal aspirates and blood cultures of patients admitted to high-risk units like oncology, nephrology, burns, Neonatal Intensive Care Unit (NICU) and Cardiology [3]. They account for around 15% of all bacteria species isolated from clinical samples [4]. The Antibiotic resistance pattern showed that many isolates were multi-drug resistant [5]. *Burkholderia cepacia* due to its intrinsic resistance to multiple antibiotics can survive in the hospital environment. It is commonly non-pathogenic to healthy individuals, but occasionally colonizes and causes life-threating infections and also non-fatal infections of the urinary tract and respiratory tract. The primary concern regarding *B. cepacia* is its colonization in the lungs of cystic fibrosis patients, which in some cases is associated with rapidly fatal fulminating pneumonia and septicemia [6]. Human infection with *B. cepacia* is usually through direct contact with contaminated foods or devices such as respiratory equipment or medical solutions like disinfectants. Person-to-person transmission also has been documented [7].

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Infection with *Ochrobactrum anthropi* is commonly associated with implantation of intravenous catheters or other medical devices in patients with a debilitating illness. Acquisition of *O .anthropi* via contaminated pharmaceuticals, puncture wound, and contaminated medical material has frequently been reported in immunocompromised patients, and rarely, if ever occurs in healthy people, suggests that these bacteria have relatively low virulence [8]. The ability of an organism to adhere to the silicone material of catheters may contribute to this organism propensity to cause catheter-related infections [8]. *Achromobacter xylosoxidans* is found in the moist areas of the hospital environment, and its transmission usually involves exposure to contaminated fluids like intravenous, hemodialysis and irrigation fluids as well as soaps and disinfectants. It causes infections in the urinary tract, biliary tract, wounds and may result in peritonitis, pneumonia, and bacteremia [9]. *Pseudomonas putida* belongs to a fluorescent group of the pseudomonads isolated from the hospital environment and causes infections like pneumonia, catheter-associated infections, bacteremia, cholecystitis, thrombophlebitis, tonsillitis, and skin and soft tissue infections [10]. *Pseudomonas stutzeri* is a non-fluorescent denitrifying bacterium distributed widely in the environment and colonizes the hospitalized patients and causes nosocomial infections. The most common clinical samples from which this bacterium was isolated were surgical wounds, blood, respiratory tract, and urine [11]. The non-fermenting organisms have shown multidrug resistance, and a nosocomial outbreak of carbapenem-resistant non-fermenters is attributed to Metallo-beta-lactamase (MBL) enzyme production. So this enzyme is responsible for multidrug resistance and possesses high hydrolytic activity that leads to degradation of higher generation cephalosporins. Plasmid-mediated MBL genes spread rapidly to other species of Gram-negative bacilli. Therefore rapid detection of MBL production is necessary to modify therapy and to initiate effective infection control to prevent their dissemination [6].

In the present study, we attempted to identify some rare NFGNB isolated from different clinical materials and examined their various biochemical characters. We also explored the production of MBL by these bacteria.

MATERIAL AND METHODS

Different clinical samples were collected from 2015 to 2016 (one year). The samples included pus, urine, endotracheal aspirates, sputum, blood, catheter tips, ascetic fluid, pleural fluid and cerebrospinal fluid (CSF) from 250 patients. The cases included individuals of both sexes and all age groups. Gram staining was initially performed to study the morphological characteristics of the clinical isolates.

Culture and Biochemical reactions. All the clinical specimens were primarily processed to identify the non-fermenters from the other Gram-negative bacilli. Clinical samples including pus, respiratory secretions, and other specimens were inoculated onto blood agar, MacConkey agar, and Nutrient agar, and urine samples were inoculated into the cystine lactose electrolyte deficient (CLED) agar and incubated aerobically at 37°C for 24 h. The wound swabs were inoculated into the nutrient broth and incubated for 3-4 h at 37°C. After the appearance of turbidity due to bacteria growth, they were subcultured into MacConkey and Blood agar and incubated for 12-18 h at 37°C. Later, the motility of organisms was observed through Hanging drop method. Colonial morphology provisionally identified non-fermenting organisms and pigment production; the isolated bacteria were subjected to various biochemical and biological tests for confirmation [12]. The tests included carbohydrate fermentation with glucose, lactose, xylose, and mannitol. The organisms were also tested for indole production, citrate utilization, nitrate reduction, gelatin hydrolysis, amino acid decarboxylation, and the ability of growth on nutrient agar containing 6% NaCl, and growth at 42°C. Also, they were subjected to Methyl Red (MR), Voges-Proskauer (VP), urease, oxidase, catalase, Triple sugar iron (TSI), Hughs-Leifsons (O/F) tests. *Pseudomonas cepacia* agar or oxidative-fermentative base polymyxin B-bacitracin lactose (OFPBL) was specifically used to isolate *B. cepacia* or *P. cepacia* from respiratory secretions. The medium contains crystal violet, bile salts, polymyxin-B and ticarcillin to inhibit Gram-positive and rapidly growing Gram-negative organisms. It also contains pyruvate so that the organism breaks pyruvate creating an alkaline pH and resulting in a color change of the pH indicator.

Antibiotic susceptibility testing of isolated non-fermenters. Overnight broth culture of the isolated bacteria was used as inoculums. Antibiotic susceptibility testing was carried out by disc diffusion method according to The Clinical and Laboratory Standards Institute (CLSI) guidelines on Muller-Hinton (MH) agar. The antibiotic discs used in this study were piperacillin (100µg), amikacin (30µg), gentamicin (10µg), cefoperazone (75µg), ceftazidime (30µg), ceftriaxone (30µg), cipro-floxacin (5µg), imipenem (10µg), piperacillin/tazobactam (100/10µg), polymyxin B (300U) and meropenem (10µg). The inhibition zone was measured according to CLSI guidelines.

Detection of MBL Production. Screening for MBL production was done in multidrug resistant isolates of the non-fermenters. Production of MBL was performed by imipenem-EDTA combined disc test and imipenem-EDTA double disc synergy test (DDST). In imipenem-EDTA combined disc test, the tested organisms were inoculated onto plates with Muller-Hinton agar. Two 10µg imipenem discs were placed on the plate, an amount of 10µl of EDTA solution was added to one of discs to obtain the desired concentration (750µg). The inhibition zones of the imipenem and imipenem-EDTA discs were compared after 16-18 h incubation at 37°C. In the combined disc test, if the increase in inhibition zone with the imipenem-EDTA was >7mm comparing to the imipenem disc alone, it was considered as MBL positive [13]. In imipenem-EDTA DDST, 10µg imipenem discs and an imipenem plus EDTA 750µg were placed on MH agar. Another disc containing only 750µg EDTA was also placed as a control. After overnight incubation, the established zone diameter difference of >7mm between imipenem discs and imipenem plus EDTA was interpreted as EDTA synergy positive.

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They were compared with ATCC 17588 strains of *Pseudomonas* as a negative control [12].

RESULTS

Among the 250 clinical samples, 12 harbored uncommon non-fermenters isolates, equivalent to 4.8% of the total cases. These 12 isolates were used for further processing. Nearly 150 clinical samples showed the growth of other *Enterobacteriaceae* members, and Gram-positive cocci and 48 were negative (Fig. 1). Different types of infections were noticed in the age group 60-69 (n=58, 23.2%) and a lower number of cases in the age group 70-79 (n=10, 4%). The number of males (n=180, 72%) attending the hospital with various infections was higher than the number of females (n=70, 28%). About 91.6% of non-fermenters were isolated from the hospitalized patients and 8.33% from individuals in Out Patient Department (OPD). Table 1 depicts the various microorganisms isolated from different clinical samples in the present study.

The non-fermenter isolates were differentiated by using various cultural and biochemical tests. *B. cepacia* was identified by its grey-white color colonies surrounded by red color zone on the *P. cepacia* (PC) agar medium that might be due to a change in the pH of the medium to alkaline. The overall antibiotic sensitivity pattern of uncommon NFGNB isolated from various clinical samples is shown in Table 2. Among the five isolated bacteria *P. putida*, *P. stutzeri* and *B. cepacia* showed a high resistance to carbapenems and sensitivity to polymyxin-B (300U). *P. stutzeri* was more susceptible to aminoglycosides and the second-generation quinolone compounds whereas *B. cepacia* showed maximum sensitivity to the third generation cephalosporins. Among carbapenem-resistant isolates of non-fermenters, 100% showed MBL production (Table 3 and Fig. 2-3). The other two cultures *A. xylosoxidans* and *O. anthropi* were susceptible to carbapenems.

Table 1. Clinical samples with culture positive and negative cases of various NFGNB

Clinical isolates	Number of culture positive cases	Percentage (%)	Clinical samples with positive growth	Clinical samples with negative growth
P. putida	04	33.3	tracheal aspirates -02 catheter tips -01 pus sample -01	Blood, urine, sputum, ascetic fluid, CSF
P. stutzeri	03	25	tracheal aspirates-02 sputum -01	pus exudates, catheter tips, blood, urine, ascetic fluid, CSF
B. cepacia	02	16.6	sputum-01 pus sample-01	tracheal aspirates, catheter tips, blood, urine, ascetic fluid, CSF
A. xylosoxidans	02	16.6	urine-01 sputum-01	pus exudates, catheter tips, blood, tracheal aspirates ascetic fluid, CSF
O. anthropic	01	8.33	catheter tips-01	pus exudates, sputum, urine, blood, tracheal aspirates ascetic fluid, CSF

Table 2. Antibiotic susceptibility pattern of the isolates

S. No	Antibiotics	*P. putida* (n=4)	*P. stutzeri* (n=3)	*B. cepacia* (n=2)	*A. xylosoxidans* (n=2)	*O. anthropi* (n=1)	Percentage (%) of Resistance
1.	Piperacillin	R (100%)	R (100%)	R (100%)	S (100%)	R (100%)	83.33%
2.	Amikacin	R (100%)	S (100%)	R (100%)	R (100%)	S (100%)	66.6%
3.	Gentamicin	R (100%)	S (100%)	R (100%)	R (100%)	R (100%)	75%
4.	Ciprofloxacin	R (100%)	S (100%)	R (100%)	R (100%)	S (100%)	66.6%
5.	Cefoperazone	R (100%)	R (100%)	S (100%)	R (100%)	R (100%)	83.33%
6.	Ceftazidime	R (100%)	R (100%)	S (100%)	R (100%)	R (100%)	83.33%
7.	Ceftrixone	R (100%)	R (100%)	S (100%)	R (100%)	R (100%)	83.33%
8.	Piperacillin/ Tazobactum	R (100%)	R (100%)	S (100%)	S (100%)	R (100%)	66.6%
9.	Imipenem	R (100%)	R (100%)	R (100%)	S (100%)	S (100%)	75%
10.	Polymyxin-B	S (100%)	S (100%)	S (100%)	S (100%)	S (100%)	0%
11.	Meropenem	R (100%)	R (100%)	R (100%)	S (100%)	S (100%)	75%

Table 3. Susceptibility of isolates to carbapenem in relation to MBL production

S. No	Type of organisms	Number of isolates	Resistant to carbapenems	Percentage	MBL production	Percentage
1.	*P. putida*	4	4	100%	4	100
2.	*P. stutzeri*	3	3	100%	3	100
3.	*B. cepacia*	2	2	100%	2	100
4.	*A. xylosoxidans*	2	0	0	0	0
5.	*O. anthropi*	1	0	0	0	0
	Total	12	9	75%	9	75%

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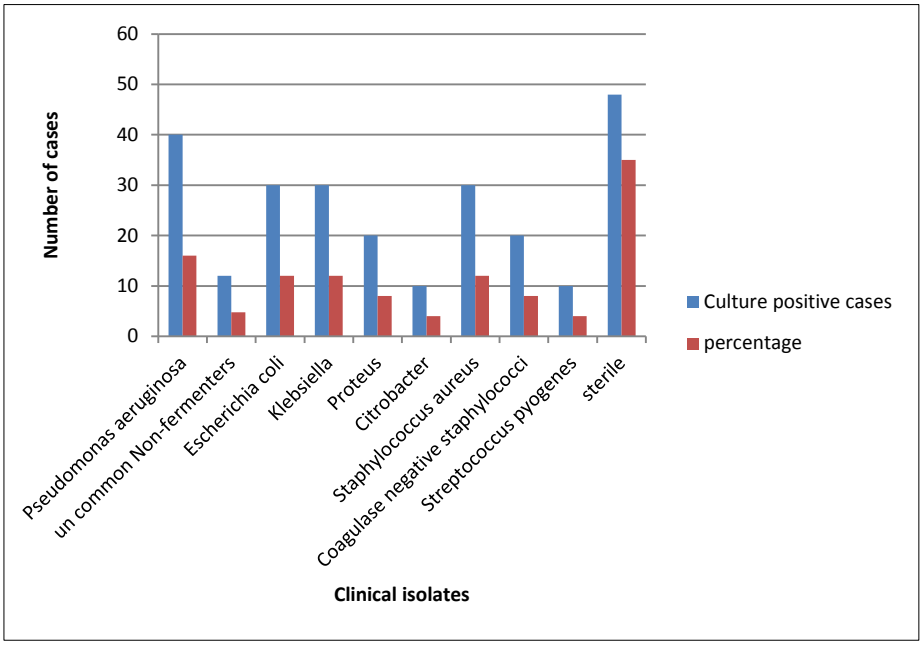


Fig. 1. Different types of bacterial isolates in 250 clinical samples

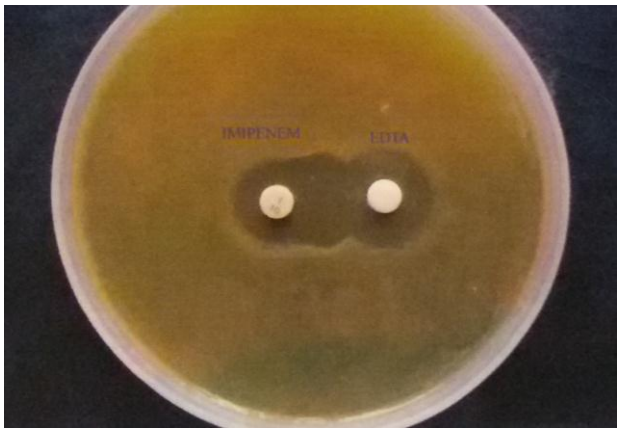


Fig. 2. Imipenem-EDTA Double synergy test of *P. putida*



Fig. 3. Combined disc test showing MBL production by *P. stutzeri*

DISCUSSION

The present study was undertaken to evaluate the role of uncommon NFGNB in infections especially in hospitalized patients and to know the prevalence of MBL among these bacteria. While NFGNB are considered as commensals or contaminants, their potential pathogenicity has been well documented by their recurrent isolation from clinical samples and their association with clinical disease [14]. The outbreaks of nosocomial infections, emerging antimicrobial resistance, and epidemiology complexity have made NFGNB remarkable organisms [15]. In the recent years, they have emerged as important nosocomial pathogens, and their resistance to antimicrobials has increased over the years. The NFGNB are innately resistant to many antibiotics and are known to produce extended spectrums of beta-lactamase and MBL enzymes [16]. Out of 250 different samples collected and processed in the present study, the rate of recovery of uncommon non-fermenters was 4.8% which is comparable with the study of Jayanthi and Jeya (9.1%) [17]. In contrast, Omar *et al.* [18] reported a higher rate of only *B. cepacia* isolates (85.7%) from various clinical samples. Gales *et al.* [19] showed that 62.7% of positive cases were due to *B. cepacia*, and Bisharat *et al.* [20] reported *P. stutzeri* as the causative agent in more than 90% infections. Lee *et al.* [21] reported that 75% positive cultures belonged to *P. putida* and *P. stutzeri*, whereas Matros *et al.* [22] reported a lower isolation rate (1.6%) for *A. xylosoxidans* and *B. cepacia*. The 33.3% isolation of *P. putida* was in agreement with results reported by Matros *et al.* [22] that isolated 4 strains of *P. putida* from 357 clinical specimens. Lee *et al.* [21] and Guzel *et al.* [23] reported a lower rate of isolates (1%). *P. stutzeri* was the second most common non-fermenter (n=3, 25%), in our study which is almost similar to of Lee *et al.* [21] work reporting isolation of *P. stutzeri* from 4 clinical samples. The higher incidence of the isolate was reported by Bisharat (90%) [20]. The

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incident rate of *P. stutzeri* infection (25%) was greater than that reported by Jayanthi and Jeya (6.1%) [17]. About 16.66% of isolates in the present study were *B. cepacia* and *A. xylosoxidans* whereas in the studies by Matros *et al.* [22] and Santosh *et al.* [24] only 0.3% and 2.69% of the isolates belonged to these species. Also, identification of one isolate (8.33%) as *O. anthropi* in the present work was similar to the results reported by Menezes *et al.* [25]. Most of the isolates in our study were from tracheal aspirates which are in agreement with studies by Matros *et al.* [22]. Regarding the antibiotic susceptibility pattern, the highest sensitivity rate was observed with polymyxin-B (100%) followed by piperacillin, and the third generation cephalosporins (83.33%). The least sensitivity was observed to amikacin, PIP/TZB, and ciprofloxacin (66.66%). In our study, *P. putida* showed the maximum resistance (100%) to all antibiotics including β-lactum antibiotics, quinolone antibiotics and aminoglycosides and no resistance (0%) to polymyxin-B. Similar results were reported by Guzel *et al.* [23], but the isolated organism showed sensitivity to ceftazidime (0% resistance). In the present work, *P. stutzeri* isolates showed the highest resistance to penicillin, the third generation cephalosporins, and carbapenem antibiotics, but were susceptible to quinolone compound ciprofloxacin (100%) and aminoglycosides, which is in agreement with the findings by Bisharat *et al.* [20]. *B. cepacia* isolates showed maximum resistance (100%) to piperacillin, aminoglycosides, quinolones, and carbapenems, but were 100% sensitive to third generation cephalosporins like ceftazidime (100%). The reports of Matros *et al.* [22] and Omar *et al.* [18] indicated 100% and 60% of the sensitivity of *B. cepacia* isolates to ceftazidime, respectively. The isolated strains were 100% carbapenem-resistant, similar to the findings reported by Matros *et al.* [22] and Gautam *et al.* [26]. About 36% of *A. xylosoxidans* isolates showed the highest sensitivity to carbapenems, polymyxin-B and PIP/TZB (100%) similar to findings reported by Matros *et al.* [22]. The species *O. anthropi* was more susceptible to antibiotics like amikacin, ciprofloxacin, and carbapenems (100%) which is in agreement with previous findings by Menezes *et al.* [25] (100%) and Thoma *et al.* [27] (97.1%) reports. All the pathogenic cultures isolated in the current investigation showed 100% sensitivity to polymyxin-B. Antibiotic options are limited in multiple-resistant NFGNB infections; polymyxins, which have become available again, are an option in the treatment of resistant strains and are widely used in clinical practices for NFGNB infections. Our results showed that 75% of *Pseudomonas* spp. strains were resistance to the carbapenems, and all (100%) of the carbapenem-resistant strains were MBL producers, which corroborated the previous reports of Santosh *et al.* [24], Guzel *et al.* [23] and Lee *et al.* [21].

NFGNB are emerging as nosocomial pathogens with developed resistance mechanisms capable of hydrolyzing all beta-lactam antibiotics including carbapenems through producing MBL. The excess use of carbapenems and other broad spectrum antibiotics should be avoided and early detection, and prompt infection control measures are essential to prevent further spread of MBLs to other Gram-negative bacilli. Education of health care staff, hospital infection control, antibiotic management, implementation of an active surveillance system, limit of non-human use of antimicrobials and distribution of data about resistant strains at the national and international level are suggested to control the population of resistant bacteria.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest associated with this manuscript.

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