



Phenotypic Characterization of Non-Fermentative Gram Negative Bacilli from Clinical Samples

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Abstract:

This is a prospective study conducted in the SRM Medical College Hospital and Research Centre, Tamil Nadu from April 2010 to July 2010 to find out the prevalence of NFGNB in the clinical specimens and to isolate and identify the prevalent NFGNB from different clinical samples received in SRM medical college laboratory. Confirmation of the isolates is done by various biochemical reactions and antimicrobial susceptibility testing with special reference to *Pseudomonas* as per CLSI guidelines. Different samples were collected, processed as per standard test and antimicrobial susceptibility tests were performed. Most of the *Pseudomonas* spp. shows drug resistance. Therefore it is recommended that the above sensitivity pattern should be confirmed by Minimum Inhibitory Concentration. Of the 2129 specimen obtained, 99 isolates were NFGNB and among those *Pseudomonas* (64%) was more prevalent, followed by *Acinetobacter* (19%), NFGNB (10%) and *Moraxella* (6%). Antimicrobial susceptibility test, with reference to *pseudomonas* shows 68% - 70% susceptibility to Aminoglycosides, 34% resistance to Ciprofloxacin. 16% resistance to Imipenem, 72% resistance to Meropenem and cefepime and Aztronam were 100%. Polymixin B (100%) and Colistin (98%) were sensitive. Both Imipenem and Meropenem sensitive were 28%, Imipenem sensitive, Meropenem sensitive 58%, both Imipenem and Meropenem resistant were 14%.

Introduction:

The non-fermentative gram negative bacilli are groups of aerobic, non-sporing bacilli that either do

not use carbohydrates as a source of energy or degrade them through metabolic pathways other than fermentation. Non fermenting gram negative bacteria were considered to be non pathogenic and commensal of little significance. Recently there has been a tremendous interest in these organisms as they are being isolated from clinical specimens with increasing frequency.

Pseudomonas aeruginosa and *Acinetobacter baumannii* are the common non-fermentative gram-negative bacteria found that have minimal nutritional requirements and can survive on a wide variety of surfaces and in aqueous environments. They rarely cause serious infections in otherwise healthy persons and are infrequently identified as normal microbial flora in healthy individuals, but they are of greatest concern in hospitalized patients, particularly those in intensive-care units (ICUs), where these opportunistic pathogens are capable of causing severe invasive infections in critically ill and immunocompromised patients such as bacteremia, pneumonia, urinary tract infection, meningitis, endocarditis, burn wound infection, eye infection, surgical site infection and osteomyelitis. Recent advances in medicine such as the advent of more elaborate surgery and intensive care, the use of immunosuppressive drugs, the availability of the invasive procedures or instrumentation and the increase in number of immuno-compromised patients liable to nosocomial infections with these NFGNBs are on rise. Increase in concern to treat these infections due to NFGNBs as they possesses intrinsic mechanisms of resistance to various groups of

drugs, especially to carbapenems, which are widely used in clinical practice in health care settings.^[1] Various clinically important non-fermentative bacilli are divided according to the motility and the ability to produce cytochrome oxidase. Packaged commercial kit system have been designed for the identification of NFGNBs which can give accurate and reproducible results equal to or better than conventional procedures but the results may not be reliable as always. Bacterial action on the substrates produces turbidity or color changes that interpreted visually. Production of blue-green pigment is indicative of *P. aeruginosa*.^[2] However, some strains of *P. aeruginosa*, particularly the mucoid ones, may not produce pyocyanin, as well as displaying a slow oxidase reaction and may therefore require further tests to confirm identification. Infection with such organisms is relatively uncommon and usually found in immuno-compromised patients is associated with a contaminated medical device.^[3]

Metallo- β -lactamases (Zinc based) are able to hydrolyse penicillins, cephalosporins and carbapenems. An increasing prevalence of carbapenem resistance mediated by acquired metallo- β -lactamases is being reported, particularly for *Pseudomonas aeruginosa* clinical isolates in several countries. The resistance may spread rapidly to various species of gram negative bacilli, as the MBL gene reside in mobile gene cassettes inserted in integrons^[4]. The carbapenems have been the drug of choice for treatment of infections caused by penicillin or cephalosporin-resistant Gram-negative bacilli especially, extended spectrum β -lactamase (ESBL) producing Gram-negative infections.^[5] Polymyxin B is considered as an option to treat infections due to MDR *P. aeruginosa* and to decrease mortality associated with MBL positive strains.

Materials and methods:

It was a Prospective study done from April 2010 to February 2011 at SRM hospital, Tamil Nadu, India. Various clinical specimens are collected. A random environmental surveillance swabs (disinfectants,

bed linen, instruments) are collected from various clinical departments such as ICU, surgical, post operative and ortho wards.

The specimens are cultured on to Mac Conkey agar and Nutrient agar, incubated overnight at 37°C and 42 °C aerobically. Gram staining, motility and Oxidase test are performed from the culture obtained. *Pseudomonas* spp. were identified with pigment production. Non-pigment producing isolates were processed on Pigment enhancing media such as *Pseudomonas* agar. Cetrimide agar was used for selective isolation of *Pseudomonas* spp. exclusively for surveillance swabs collected from hospital environment. *Pseudomonas* isolates recovered from both, clinical and environmental specimens after confirmation were tested for susceptibility to the following antibiotics by Kirby-Bauer disc diffusion method on Mueller Hinton agar as per the Central Laboratory Standard Institute (CLSI) guidelines. Each antibiotic disc is placed on the agar, 20 mm apart. Ciprofloxacin (5 μ g), Ofloxacin (5 μ g), Gentamicin(10 μ g), Amikacin(30 μ g), Tobramycin (10 μ g), Netilmycin (30 μ g), Ceftazidime (30 μ g), Cefepime (30 μ g), Cefoperazone/Sulbactam, Polymyxin B (300 U), Piperacillin/Tazobactam(100/10 μ g), Imipenem (10 μ g), Meropenem (10 μ g), Colistin (Polymyxin E) (10 μ g), Aztreonam (30 μ g) are the drugs chosen for the anti-microbial testing. The spectrum of activity of Imipenem, Meropenem and Polymyxin B drugs against *Pseudomonas* isolates were also assessed by performing Minimum Inhibitory Concentration (MIC) by Agar dilution technique as per CLSI guidelines. With agar dilution, the antimicrobial concentrations and the isolates to be tested are brought together on an agar based medium rather than in broth. Each doubling dilution of an antimicrobial agent is incorporated into a single Mueller Hinton agar plate. The surface of each plate is spot inoculated with 5 μ l (1 x 10⁴ CFU/spot). After overnight incubation at 37°C, plates are examined for growth and compared with one positive growth control plate without the antibiotic agent. One or more bacterial

isolates are tested per plate. MIC is the lowest concentration of an antimicrobial agent in agar that completely inhibits visible growth.

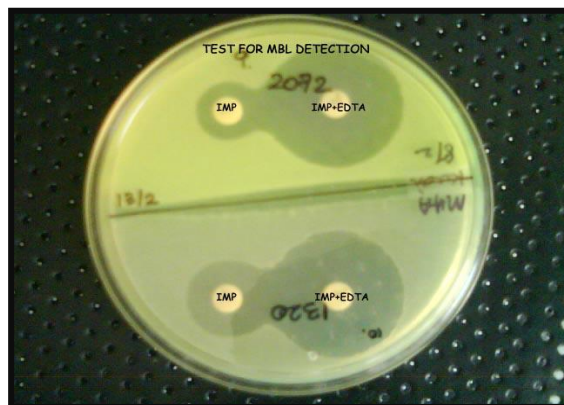
Metallo-β-lactamase (MBL) production is detected by the Imipenem – EDTA double disk synergy test [6]. Imipenem resistant *Pseudomonas* isolates are chosen for the test. The EDTA, a chelating agent, will chelate the Zinc ion present in the MBL enzyme and inhibit the action of this enzyme to the drug. Thus an enhanced zone of inhibition around the disk incorporated with EDTA is considered positive test. Disk potentiation test using an imipenem disk and imipenem with EDTA disk is a simple method to detect MBL producing clinical isolates. Test organisms were inoculated onto plates of MH agar. Two 10 µg imipenem discs were placed on inoculated plates and 10 µl of EDTA solution was added to one of the imipenem disc. The zone of inhibition around imipenem disk alone and imipenem with EDTA was recorded and compared after 16-18 hours incubation at 37°C. An increase in zone size of at least 12 mm around the imipenem-EDTA disc was recorded as positive.

Results and Discussion:

Out of 4763 clinical specimens, subjected to the study, the recovery of NFGNB was 207 (4.35%). *Pseudomonas aeruginosa* was the predominant isolate. Out of 133 *Pseudomonas* isolate recovered, 7 isolates were non-pigmented variety. The number of isolates recovered from blood (5), wound specimens (90), respiratory specimens (20), urine (15) and others (3) respectively. The isolation rate of *Acinetobacter* spp. was 18.35% (n=38) from blood (1), wound specimens (16), respiratory specimens (14), urine (6) and others (1), *Moraxella* was 11 (5.31%) and other non-fermentative gram negative bacilli was (blood -1, wound specimens – 14, respiratory specimens – 1, urine – 8 and others – 1) 25 (12.077%). The 133 isolates of *Pseudomonas* were subjected to antimicrobial susceptibility testing. The resistance pattern detected showed a high level resistance to drugs like Ceftazidime (83%), Cefepime (78%) and Aztreonam (75%). All the isolates were found to

be susceptible to Polymyxin B and 3.7% of isolates were found to be resistant to Colistin (Polymyxin E) 23.3% of isolates were multi-drug resistant (n=31). The percentage of resistance to Carbapenem group of drugs by disc diffusion susceptibility testing to Imipenem and Meropenem were 15.03% (n=20) and 31.57% (n=42) respectively. All the 20 isolates resistant to Imipenem by disc diffusion were susceptible at the MIC breakpoint 8 mg/L. Only 4 out of 42 isolates were resistant to Meropenem at the MIC ≥16 mg/L as per CLSI guidelines. The 20 isolates resistant to Imipenem by disc diffusion tested for Metallo-β-lactamase production by using Imipenem/EDTA (10/750mcg) prepared in-house and Imipenem/EDTA (10/750mcg) yielded 9 Metallo-β-lactamase positive isolates. Out of 108 surveillance swabs taken from various sites of different specialties of the hospital environment, the isolation of *Pseudomonas* was 8 and *Acinetobacter* spp. was 14. The susceptibility profiles of the environmental isolates were found to be almost susceptible to all the drugs. The susceptibility pattern of clinical and environmental isolates was concordant.

Figure 1: MBL detection by Disc Potentiation Method



Pseudomonas aeruginosa infection is a serious cause of nosocomial infections. The present study showed the prevalence or occurrence of 4.35% of NFGNBs in various clinical specimens and 20.37% in environmental specimens. In this study it was

found that the incidence of *Pseudomonas aeruginosa* was predominant and resistance to Fluoroquinolones was found to be 38-39%. This is almost similar to the studies conducted by ^[7] Javia *et al*, which showed 28-37% resistance to Fluoroquinolones. And another study by ^[8] Celia Maria *et al*, showed 23-37% resistance. Hence there is no considerable variation in the resistance to fluoroquinolones. In the present study 23.3% of *Pseudomonas* was considered MDR isolates. *Pseudomonas* isolates resistant to imipenem and meropenem were 15.03% and 31.5% respectively. 23.26% of resistance to carbapenems in this study is more or less similar to the studies conducted by ^[9] Ami Varaiya *et al*, showed 20.8% resistance to imipenem and meropenem and another study by Javia *et al* showed 19.64% resistance to imipenem and meropenem. In our study, all the *Pseudomonas* isolates were susceptible to Polymyxin B whereas a study by Alexandre Prehn, 1.3% resistance to Polymyxin B by *P. aeruginosa* and 11.7% resistance by *Pseudomonas* spp. are observed. This can be considered as a significant finding in this study. With regard to the spectrum of activity of Polymyxin B is identical to Colistin, most of the *P. aeruginosa* isolates are found to be susceptible by a study by Gales AC *et al* but in the present study 3.7% resistance to Colistin (Polymyxin E) by *P. aeruginosa* was observed. In this study, 6.7% (9/133) *Pseudomonas* isolates were found to be MBL producers. In a study conducted by Zavascki *et al* found that 29.9% (86/287) *P. aeruginosa* were MBL producers. Another study by Ami Varaiya *et al*, in ICU patients, 20.8% of *Pseudomonas* isolates were found as MBL producers. When compared to these studies, number of MBL producing *Pseudomonas* isolates was less. In our study, 78% of *Pseudomonas* was resistant to Cefepime and 83% were resistant to Ceftazidime. In a study by Javia *et al* showed remarkable resistance to Ceftazidime, 67.86%. Another study conducted by Celia Maria *et al*, 34% of *Pseudomonas* isolates was susceptible to Cefepime and 57% to Ceftazidime. 40% were resistant to both Cefepime and Ceftazidime and

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37% to Cephalosporins. This shows a considerable variation compared to the above studies. 37% of resistance to Ciprofloxacin showed in a study conducted in the Institute, National de Control, Brazil. In our study 38% were resistant. Piperacillin/Tazobactam found to be the second most active drug by the above study as well as in the present study.

Conclusion:

The study highlights or emphasizes the importance to look for, identify various NFGNBs in the hospital environment as well as clinical specimens recovered in the microbiology laboratory. The identification of isolates can be enhanced by using automated systems. Packaged systems can be recommended, provided one understands their shortcomings and is willing to setup supplemental tests to identify weakly reactive or fastidious strain. The isolation of NFGNBs was more in ICU settings which warrants routine surveillance of ICU to keep in check, these NFGNBs. Patterns of susceptibility and resistance of the isolates recovered in SRM Medical College Hospital & Research Centre, India during the study period was found to be in par with the observations of various studies conducted across national and international institutions. The study suggests the physicians the judicious use of Imipenem and Meropenem and also opt Piperacillin/Tazobactam as the drug of choice therefore of low level of resistance. No resistance was observed to Polymyxin B, because of lack of utility of Polymyxin B for therapy, which can be saved for future use for life threatening mortality associated infections. Identification of MBL producers in these nosocomial isolates is essential, since MBL producing isolates are associated with a high level of mortality especially in immunocompromised patients particularly in ICU setting.

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