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ORIGINAL ARTICLE

The Plasmid Profile of Multidrug Resistant Nonfermenters Isolated From Different Clinical Specimens Of Beed And Akola District Of Maharashtra, India

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ABSTRACT
Nonfermenters are defined as aerobic non-spore forming Gram-negative bacilli which cannot catabolize carbohydrates as a source of energy or degrade them via oxidative rather than fermentative metabolic pathways. In human infection nonfermenters play a critical role due to their multi-drug resistant property against most commonly used antibiotics. Multidrug resistance in nonferentative Gram-negative bacilli now represents a major challenge to treat infections and become a major issue for the management of antimicrobial therapy. In nonfermenters rate of resistant's against cephalosporins, quinolone and carbapenemase are increasing rapidly because of sequentional chromosomal mutations , this is because of either plasmid encoding or chromosomally encoded resistant genes which may lead to the overproduction of intrinsic beta-lactamases, hyper-expression of efflux pumps, target modifications and permeability alterations. Nonfermenters like Pseudomonas aeruginosa and Acinetobacte rbaumannii also have the ability to acquire mobile genetic elements encoding resistance genes. In this study 94 nonfermenters were identified and 5 were found multidrug resistant and identified as Pseudomonas aeruginosa, Acinetobacter baumannii, Acinetobacter calcoaceticus, Alcaligenes faecalis and Pseudomonas sp which were further subjected for plasmid profiling. 25KB Plasmid DNA was found common in all isolated multi drug resistant nonfermenters which were found positive to carry resistant genes against selective antibiotics and capable for cross species trans- mission.
Keywords: Plasmid Profile, Multidrug Resistant Nonfermenters , *Gram-Negative Bacilli*

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INTRODUCTION

Plasmid are extra chromosomal element of finite size DNA usually stably inherited within a bacterium cell line and are capable of transfer between strains, species or genera [1]. Plasmids were first discovered in enteric pathogen and from late 1950S on wards were increasingly associated with antibiotics resistant's genes which are responsible for the increase pathogenicity of most bacteria [2].

Nonfermenters are defined as aerobic non-spore forming Gram-negative bacilli which cannot catabolize carbohydrates as a source of energy or degrade them via oxidative rather than fermentative metabolic pathways [3]. Organisms of this group are generally resistant to quaternary ammonium compounds and chlorhexidine [4, 5]. In human infection Nonfermenters play a critical role due to their multi-drug resistant property against all commonly used antibiotics [5]. In this group *Pseudomonas aeruginosa* is eminent pathogen followed by *Acinetobacter baumanni* [6].

Pseudomonas aeruginosa becomes one of the important pathogens from this group because of its intrinsic multi-drug resistance capabilities. In its large genome 6.3 million base pairs (bp) and 8 virulence genes are identified. Large genome size of *Pseudomonas aeruginosa* increases the probability of possible mutation sites and which gives reasons for its virulence versatility [7]. In non-fermenter rate of resistant gene spread has been suspected as the major cause of increased antibiotic resistant. Plasmid carry genes

that could be spread by transduction or by conjugation while the genome based resistant genes are also spread by replication. The current study, aimed to determining the antimicrobial resistance patterns and plasmid profiles of Nonfermenters isolated from different clinical specimens of Akola and Beed District of Maharashtra to understand the extent of drug resistance, genetic diversity and possibility of cross species trans- mission of the organisms.

MATERIAL AND METHODS

Isolation, identification and antibiotic susceptibility testing: A total 310 numbers of different clinical samples were collected from different diagnostic centers, clinic and govt. hospital of Beed and Akola district of Maharashtra, India from 2014 to 2016 and identification was carried out according to Koneman's Color atlas and Textbook of Diagnostic Microbiology (Sixth edition). Antibiotic susceptibility test were performed by disc-diffusion method. Antibiotics disc of Cefexime-5µg, Cefepime-30µg, Cefotaxime-30µg, Ceftazidime-30µg, Ceftizoxime-30µg, Ciprofloxacin-5µg, Levofloxacin-5µg, Norfloxacin-10µg, Gentamicin 10µg, Polymyxin-B, Meropenem-10µg and Colistin-10 µg were used [8].

Screening of Extended spectrum β lactamases producers: Ceftazidime (ACZ) 30µg and Cefotaxime (CTX) 30µg antibiotics discs were used as screening materials for extended spectrum β lactamases producers according to CLSI guidelines. Isolates that were resistant against both of these drugs were further subjected for phenotypic conformation of Extended spectrum β lactamases production by using ESBL's detection kit Cefotaxime/Clavulanic acid 30/10 (Hi-Media, India).

Phenotypic identification of carbapenemase production: Isolated Nonfermenters were subjected for Imipenem-EDTA and Imepenem disc test; a zone diameter difference of ≥8 mm between Imipenem discs &Imipenem + EDTA discs should be interpreted as phenotypically Metallo-β-Lactamse positive [9].

Determination of minimum inhibitory concentration (MIC): Minimum inhibitory concentration was determined by agar dilution method where ciprofloxacin, cefotaxime and meropenem were used as selective antimicrobial agents. Different concentrations of 50µg to 256 µg/ml were prepared with incorporation of 10% volume of total media.

Plasmid DNA isolation and molecular weight estimation: Isolation and purification of Plasmid DNA was performed by using Quagen plasmid DNA isolation and purification test kit. Estimation of molecular weight of plasmid DNA was done by using 25 KB high range ladder running on 1% agarose gel [10] along with purified samples.

Transformation: Competent cells were prepared by calcium chloride procedure and transformation was done according to [11]. Purified plasmid DNA isolated from multi-drug resistant Nonfermenterswas transferred to *E.coli* JM 107 as the recipient and it was sensitive to all tested drugs. The strain *E.coli* JM107 were collected from Department of Microbiology Asam University, Shilchor India

Resistance test of transformants: Transformed strains of *E.coli*JM107 were grown at 37^0C for 24 hrs in Nutrient broth and were spread on Müller-Hinton agar plate. The antibiotic disc of Cefepime-30 µg, Cefotaxime-30 µg, Ceftazidime-30 µg, Ceftizoxime-30 µg, Ciprofloxacin-5 µg, Levofloxacin-5 µg, and Norfloxacin-10µg, were placed on plate and incubated at 37^0C for 24 hrs, respectively. Presence of clear zone around the disk was the index of sensitivity and absence of which may indicate resistant. Transformation of resistance genes were further confirmed by spreading transformed culture on Mueller-Hinton agar containing 100µg/ml of different antibiotic solutions. Absence of growth indicates strains were sensitive to selective concentration where presence of growth indicates resistant to selective concentration of antibiotics.

RESULT AND DISCUSSIONS

Total 94 Nonfermenters were isolated from 310 numbers of different clinical specimens. The most frequently isolated non-fermentative gram negative bacilli were *Pseudomonas aeruginosa* followed by *Aceinetobacter baumannii*. From antimicrobial susceptibility test 5 isolates were found active against all most all tested antibiotics, which were considered as multidrug resistant non-fermentative bacterial pathogens and they were further identified as *Pseudomonas aeruginosa, Acinetobacter baumannii, Acinetobacter calcoaceticus, Alcaligenes faecalis* and *Pseudomonas sp.,* results of which are recorded at table I. Phenotypic determination of extended spectrum β lactamases and carbapenemase production were carried out to confirm the presence of multidrug resistant genes and it was found positive for all 5 isolates results of which are recorded at table II & III. To estimate minimum inhibitory concentration, 5 isolates were further expose to *quinolones*, cephalosporin and *carbapenems* classes of antibiotics (Ciprofloxacin, Cefotaxime and Meropenem) as this three antibiotics are commonly used to treat infections. Minimum inhibitory concentration of all 5 isolates were found MIC ≥256 µg/ml against tested antibiotics, according to CLSI guidelines MIC above 256 µg/ml will be considered as resistant pathogens

against expose antibiotics results are recorded at table IV(a-c). After identification of multidrug resistant Nonfermenters they were finally subjected for plasmid profiling andit was found that, *Pseudomonas aeruginosa, Acinetobacter baumannii, Acinetobacter calcoaceticus, Alcaligenes faecalis* and *Pseudomonas sp*. were having 3, 2, 1, 1, and 1nuber of plasmids DNA respectively. It was further observed that *Pseudomonas aeruginosa* was having three plasmids with molecular weight 2.5KB, 10 KB and 25KB, *Acinetobacter baumannii* was having two plasmids with molecular weight 1 KB and 25 KB, *Acinetobacter calcoaceticus, Alcaligenes faecalis* and *Pseudomonas sp.* was having single plasmid of 25 KB results are recorded at table V. Purified plasmids were transformed to *E.coli*JM107 recipient bacterial cell and transformation of resistance genes were further confirmed by spreading transformed culture on Mueller-Hinton agar containing 100µg/ml of different antibiotic solutions. From transformation studies it was confirm that horizontal transability of resistant genes were found positive for all multidrug resistant non-fermenters.

Table 1.Antibacterial resistance pattern exhibited by multidrug resistant non-fermenters.

Sl. No.	Name of Antibiotics	*Pseudomonas aeruginosa* N=1	*Acinetobacterbaumanni* N=1	*Acinetobacter calcoaceticus* N=1	*Alcaligenes faecalis* N=1	*Pseudomonas sp.* N=1
1.	Cefexime-5µg	R	R	R	R	R
2.	Cefepime-30 5µg	R	R	R	R	R
3.	Cefotaxime-30 µg	R	R	R	R	R
4.	Ceftazidime-30 µg	R	R	R	R	R
5.	Ceftizoxime-30µg	R	R	R	R	R
6.	Ciprofloxacin-5µg	R	R	R	R	R
7.	Levofloxacin-5 µg	R	R	R	R	R
8.	Norfloxacin-10 µg	R	R	R	R	R
9.	Gentamicin 10 µg	R	R	S	R	S
10.	Polymyxin-B	S	S	S	S	S
11.	Meropenem-10 µg	R	R	R	R	R
12.	Colistin-10 µg	R	R	S	R	R

Table 2.Phenotypic determination of extended spectrum β-lactamases (ESBL) by combined disk diffusion method.

Organism N=1	Ceftazidime 30µg	Cefotaxime 30 µg	Cefotaxime/Clavulanic acid 30/10
Pseudomonas aeruginosa	R	R	+
Acinetobacterbaumannii	R	R	+
Alcaligenesfaecalis	R	R	+
Acinetobactercalcoaceticus	R	R	+
Pseudomonas sp.	R	R	+

Table 3.Phenotypic determination of metallo β-lactamases (MBL) by Imipenem-EDTA 10-750 µg disk diffusion method.

Organism N=1	Zone Diameter difference	Interpitation of results
Pseudomonas aeruginosa	>8 mm	MBL Positive
Acinetobacterbaumannii	>8mm	MBL Positive
Alcaligenesfaecalis	>8mm	MBL Positive
Acinetobactercalcoaceticus	>8mm	MBL Positive
Pseudomonas sp.	>8mm	MBL Positive

Table 4(a):Minimum inhibitory concentration of multidrug resistant Nonfermenters against Ciprofloxacin

Name of organism N=1	Antibiotic concentration			
	50 µg/ml	100 µg/ml	200 µg/ml	256 µg/ml
Pseudomonas aeruginosa	R	R	R	R
Acinetobacterbaumannii	R	R	R	R
Alcaligenesfaecalis	R	R	R	R
Acinetobactercalcoaceticus	R	R	R	R
Pseudomonas sp.	R	R	R	R

Table 4(b):Minimum inhibitory concentration of multidrug resistant Nonfermenters against Cefotaxime.

Name of organism	Antibiotic concentration			
	50 µg/ml	100 µg/ml	200 µg/ml	256 µg/ml
Pseudomonas aeruginosa	R	R	R	R
Acinetobacterbaumannii	R	R	R	R
Alcaligenesfaecalis	R	R	R	R
Acinetobactercalcoaceticus	R	R	R	R
Pseudomonas sp.	R	R	R	R

Table 4(c):Minimum inhibitory concentration of multidrug resistant Nonfermenters against Meropenem.

Name of organism N=1	Antibiotic concentration			
	50 µg/ml	100 µg/ml	200 µg/ml	256 µg/ml
Pseudomonas aeruginosa	R	R	R	R
Acinetobacterbaumannii	R	R	R	R
Alcaligenesfaecalis	R	R	R	R
Acinetobactercalcoaceticus	R	R	R	R
Pseudomonas sp.	R	R	R	R

Table 5.Estimation of molecular weight of plasmid DNA isolated from multi drug resistant non-fermenters.

Sl. No	Name of organism	Number of band observed	Size of each band
1.	*Pseudomonas aeruginosa*	**3**	**2.5 Kb, 10 Kb, 25 Kb**
2.	*Acinetobacterbaumanni*	**2**	**1 Kb, 25 Kb**
3.	*Acinetobactercalcoaceticus*	**1**	**25 Kb**
4.	*Alcaligenesfaecalis*	**1**	**25 Kb**
5.	*Pseudomonas sp.*	**1**	**25 Kb**

Table 6.Horizontal gene transfer ability of multi drug resistant non-fermenters to *Escherica coli JM107* competent cell.

Sl. No	Source of plasmid	Recipient bacteria	Drug used to confirm the presence of horizontal gene transfer		
			Ciprofloxacin	Cefotaxime	Meropenem
1.	*Pseudomonas aeruginosa*	*E.coliJM107*	Growth	Growth	No-Growth
2.	*Acinetobacterbaumannii*	*E.coliJM107*	No-Growth	Growth	No-Growth
3.	*Acinetobactercalcoaceticus*	*E.coliJM107*	No-Growth	No-Growth	Growth
4.	*Alcaligenesfaecalis*	*E.coliJM107*	Growth	No-Growth	Growth
5.	*Pseudomonas sp.*	*E.coliJM107*	Growth	No-Growth	No-Growth

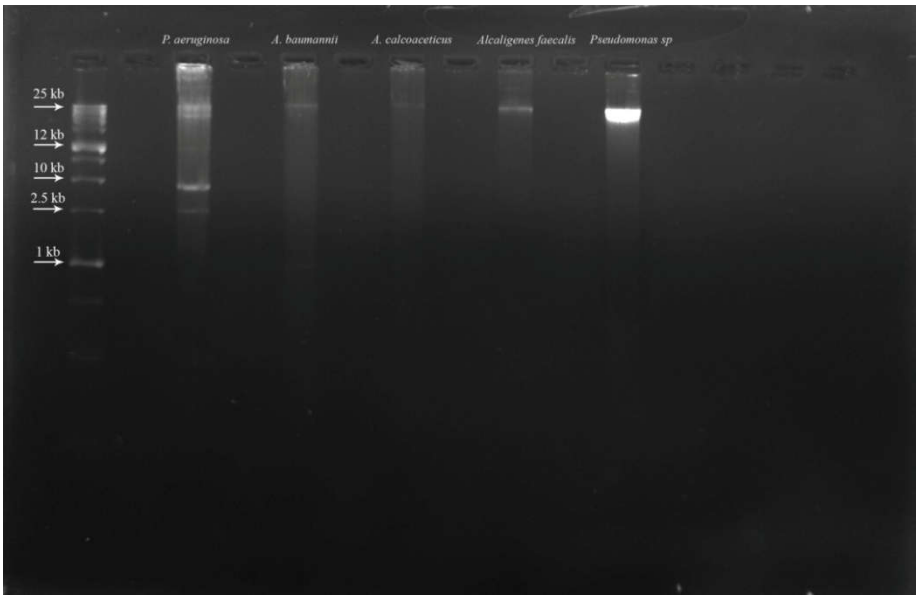

Fig. 1. Profiling of plasmid DNA isolated from multi drug resistant non-fermenters

CONCLUSIONS

Clinical isolates of nonfermenters are constantly exposed to hospital environment where they have gained resistance against most commonly used antibiotics by various mechanisms. Antibiotic resistance pattern of these isolated nonfermenters were ranged from 1 to 9 antibiotics against commonly prescribed items. From plasmid profile it was observed that all multidrug resistant Nonfermenters were having common plasmid DNA of 25KB. *Pseudomonas aeruginosa* and *Acinetobacter baumanni* were having multiple copy of plasmid DNA and show plasmid mediated resistance against commonly prescribed drugs. This study confirms the important role of plasmid numbers and plasmid size that controls the resistance characteristics in non-fermenters.

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