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Non-fermenting Gram-negative bacilli: Phenotypic identification and a correlation between biofilm formation and antibiotic susceptibility testing

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ABSTRACT

Non-fermenting Gram-negative bacilli (NFGNB) are the primary cause of nosocomial infections worldwide. They produce biofilm, which is life-threatening if not monitored by proper surveillance and treatment. Thus, this study was conducted to identify the non-fermenters, to analyse their antimicrobial susceptibility and biofilm formation. The cross-sectional study was done in the department of microbiology at Saveetha Medical College and Hospital. Identification was made based on P.C. Schrekenberger matrix. Biofilm formation was done by microtitre plate method and the antimicrobial susceptibility testing was done by Kirby Bauer disc diffusion testing. In this study, 3500 samples were cultured, out of which 240 yielded NFGNB. Predominant (102) were *Pseudomonas aeruginosa*, followed by other non-fermenters. 99 multidrug resistant and 19 pan drug-resistant strains were isolated. 19 were weak, 19 were moderate and 97 were strong biofilm producers. Biofilm pro- ducers were highly sensitive to Amikacin. In this study, 13 species of non- fermenters were isolated. After performing different biochemical tests, the study could derive a simple flowchart for identification with reference to P.C. Schreckenberger matrix. Moreover, there is no significant correlation be- tween the antibiotic susceptibility and biofilm formation. Hence, it can be stated that all biofilm producers will not show resistance to antibiotics. How- ever, after the biofilm has been produced, because of certain factors like less penetration power etc., the antibiotic susceptibility will vary. Thus, the com- prehensive surveillance and monitoring of these organisms are mandatory to control the hospital-acquired infections.

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INTRODUCTION

Non-fermenters (also non-fermenting bacteria) are a taxonomically heterogeneous group of bacteria of the division Proteobacteria that cannot catabolize glucose and are thus unable to ferment. This does not necessarily exclude that species can catabolize other sugars or have anaerobiosis like fermenting bacteria. Non-fermenting Gram-negative bacilli (NFGNB) are ubiquitous and causes opportunistic infections in immunocompromised patients. They were also the major cause of nosocomial infections because of their ability to survive in various (moist and dry) environments and exhibiting resistance to disinfectants and antibiotics. This increases the morbidity and mortality rate in the society. Among NFGNB, *Pseudomonas aeruginosa* is the main source of nosocomial infection followed by other organisms.

Moreover, they show different drug susceptibility depending on endemicity. Rapid and accurate identification of these organisms is mandatory in a clinical microbiology laboratory. These organisms

are capable of producing biofilms. So it becomes important for proper surveillance and management of these organisms. Otherwise, it will be life-threatening in hospitalised patients who were under specific medical interventions like catheterization, ventilation etc. Many laboratories have automated machines for accurate identification of NFGNB. 16s rRNA detection is the one who can give a 100 % identification report. However, this can be done only for research purpose and cannot be followed in all the laboratories for diagnostic purpose. Hence in this study, phenotypic detection of NFGNB will be done using the reference of P.C. Schrekenberger matrix and their antibiotic susceptibility will be correlated with the biofilm formation. This will show whether all biofilm producers exhibit resistance to antibiotics or not.

MATERIALS AND METHODS

The cross-sectional study on identification, biofilm formation and antibiotic susceptibility pattern of NFGNB was done in the clinical microbiology laboratory of Saveetha Medical College and Hospital, Thandalam, Chennai, Tamilnadu, India, during the period of April- October 2017, after getting approval from the institution human ethical committee and scientific review board (010/08/2015/IEC/SU). Identification was done using P.C. Schrekenberger matrix (Winn W *et al.,* 2006), the biochemical tests done were: oxidase, oxidative fermentative-dextrose, phenyl pyruvic acid test, lysine decarboxylation, polymixin-B sensitivity, acetamide utilization, arginine dihydrolase test, oxidative fermentative-maltose, and motility test was also done. Microtitre plate method (Hoiby N *et al.,* 2010) was used for biofilm formation identification and antibiotic susceptibility testing was done using Kirby Bauer disc diffusion technique and compared with the central laboratory standard institute 2016 guidelines (CLSI, 2016). Biofilm analysis was made to determine the cut-off value of negative control optical density and determined as Non-producers, weak, moderate and strong biofilm producers (Di Bonaventura G *et al.,* 2004; Reid G, 1999; Costerton JW *et al.,* 1999). Here the antibiotics used were, amikacin (AK), gentamicin (G), Cefotaxime(CTX) ceftazidime (CAZ), cefepime (CPM), ciprofloxacin (CIP), ofloxacin (OF), Piperacillin-tazobactam (PIT), imipenem (IMP) and meropenem (MR). *Escherichia coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were used as the control strains (Geller DE *et al.,* 2011; Waters V *et al.,* 2006; Nieshimura S *et al.,* 2006; Presterl E *et al.,* 2007).

RESULTS

In this study 3500 samples were cultured, out of which 240 (7%) yielded NFGNB. 102 were *Pseudomonas aeruginosa*, 19 strains were *Acinetobacter baumannii*, 26 strains were *Acinetobacter lwoffii*, 9 strains were *Burkholderia cepacia,* 12 were *Stenotrophomonas maltophila*, 5 were *Achromobacter xylosoxidans*, 12 were *Ochrobactrum anthropi*, 6 were *psychrobacter immobilis*e, 6 were *Burkholderia gladioli*, 7 were *Oligella urealytica*, 11 were *Pseudomonas stutzeri*, 24 were *Pseudomonas putida* and 1 was *Moraxella atlantae*. The identification flowchart was derived after multiple biochemical tests were done with reference from P.C. Steenbergen matrix (Winn W *et al.,* 2006). Antibiotic susceptibility of 13 species was shown in table 1. 99 multi-drug resistant and 19 pan-drug resistant strains were isolated. 19 were weak, 19 were moderate and 97 were strong biofilm producers. Distribution of biofilm-forming isolates was tabulated in table 2. Biofilm producers were highly sensitive to Amikacin. The comparison between biofilm production and antibiotic susceptibility was shown in table 3**.**

Statistical analysis: Wilcoxon rank sum test (Lihua Qi *et al.,* 2016) was used for comparison of biofilm formation between isolates susceptible/non-susceptible to each antimicrobial category. Data analysis was performed using XLSTAT 1995-2017. $P < 0.05$ was considered statistically significant for all tests. All the results were described in figure 1-5. There is no significant correlation between the antimicrobial susceptibility and biofilm production in non-fermenters.

DISCUSSION

In this study, the prevalence rate of non-fermenter is 7% where could isolate 13 different species of non-fermenters. Performing different biochemical tests, the study could derive the flowchart for 13 species identification of non-fermenters. 42% were *Pseudomonas aeruginosa*. This is well correlated with the study done by Gokhale *et al.,* 2012 in which out of 130 NFGNB, Pseudomonas aeruginosa was the commonest non-fermenter accounting for 82.3% followed by *Acinetobacter baumanii* (15.4%). Other significant NFGNB isolated were: Acinetobacter lwofii (0.76%), *Burkholderia pseudomallei* (0.76%) and *Moraxella species* (0.76%). *Pseudomonas aeruginosa* showed good sensitivity to meropenem (96.2%), ciprofloxacin (50%) and amikacin (49.5%). *Acinetobacter baumanii* showed 96.2% sensitivity to meropenem and 45% sensitivity to ciprofloxacin. In this study, 19 were weak, 19 were moderate, and 97 were strong biofilm producers. In a study done by Shingai *et al.,* 2013, 80% of Gram negative bacilli were moderate to strong biofilm producers (Al-Thahab *et al.,* 2018; Lateef et al., 2018). In this study, only 48% were biofilm producers. This may be due to they have included all fermenting and non-fermenting Gram negative bacilli in their study. In this

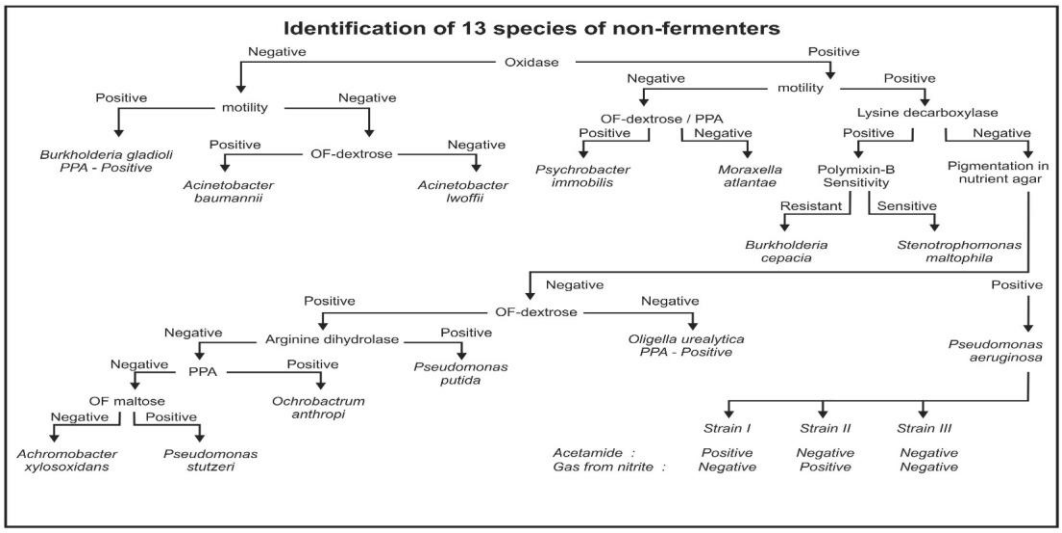

Figure 1: Flowchart of the identification of non-fermenters based on P.C.Schrecken berger matrix

Table 1: Antibiotic susceptibility of 13 species

S.No.	Name of the Organism	Total No. of isolates	Antibiotic Sensitivity-Resistant (R%)*									
			AK	G	CTX	CAZ	CPM	CIP	OF	PIT	IMP	MR
1.	*Pseudomonas aeruginosa*	102	12	26	ND*	42	40	33	31	19	10	18
2.	*Acinetobacter baumannii*	19	16	26	68%	ND*	63	58	58	37	26	32
3.	*Acinetobacter lwoffii*	26	19	35	65%	ND*	62	58	46	46	30	38
4.	*Burkholderia cepacia*	9	22	44	ND*	66	66	22	22	11	Nil	Nil
5.	*Stenotrophomonas maltophilia*	12	25	25	ND*	33	33	25	25	25	17	17
6.	*Achromobacter xylosoxidans*	5	20	60	ND*	60	80	40	40	40	40	40
7.	*Ochrobactrum anthropic*	12	Nil	8	ND*	42	42	17	17	8	8	8
8.	*Psychrobacter immobilise*	6	Nil	33	ND*	67	67	17	17	33	33	33
9.	*Burkholderia gladioli*	6	33	33	ND*	33	33	33	33	33	17	17
10.	*Oligella ureolytica*	7	Nil	Nil	ND*	43	43	Nil	Nil	Nil	Nil	Nil
11.	*Pseudomonas stutzeri*	11	54	64	ND*	73	73	82	73	73	18	18
12.	*Pseudomonas putida*	24	17	21	ND*	58	58	33	33	21	17	17
13.	*Moraxella atlantae*	1	Nil	Nil	ND*	100	100	100	100	100	100	100

**ND- Not done, *R%- Resistant percentage*

study, biofilm non-producers and strong producers have shown similar susceptibility to all nine drugs. However, in the study done by Lihua Qi *et al.,* 2016 stated that non-producers had shown increased resistance when compared to strong biofilm producers. In contrary, the study done by Jubair (Jubair H H, 2015), said that the strong biofilm producers had shown increased resistance to antibiotics (Azhar Omaran, 2017). Microbial biofilm is more resistant to antimicrobial agents and therefore more difficult to control, remain largely unexplored (Limsong *et al.,* 2004). Their inherent resistance to antimicrobial agents is at the root of many persistent and chronic bacterial infections (Weiss K *et al.,* 2006). Biofilms have been reported to be less susceptible to antimicrobial agents and

Table 2: Distribution of biofilm producing NFGNB

Name of the organism	Status of Biofilm formation				Total
	Non-producers	Weak	Moderate	Strong	
Pseudomonas aeruginosa	69	9	10	14	102
Acinetobacter baumannii	10	4	2	3	19
Acinetobacter lwoffii	13	3	5	5	26
Burkholderia cepacia	2	0	0	7	9
Stenotrophomonas maltophilia	3	0	1	8	12
Achromobacter xylosoxidans	2	0	0	3	5
Ochrobactrum anthropi	1	1	0	10	12
Psychrobacter immobilis	2	0	1	3	6
Burkholderia gladioli	0	0	0	6	6
Oligella ureolytica	0	1	0	6	7
Pseudomonas stutzeri	2	0	0	9	11
Pseudomonas putida	1	1	0	22	24
Moraxella atlantae	0	0	0	1	1
Total	105(44%)	19(8%)	19(8%)	97(40%)	240

Table 3: Comparison of biofilm production and antibiotic susceptibility

Antibiotics	Biofilm production			
	Non-producers	Weak	Moderate	Strong
Aminoglycosides	15	4	3	17
Quinolones	39	6	4	35
Cephalosporin	52	10	7	51
Beta-lactam inhibitors	29	5	4	19
Carbapenem	26	5	3	15

have reduced sensitivity to inhibitors. The resistance shown by biofilm to various antibiotics is a matter of concern (Thomas *et al.,* 2011).

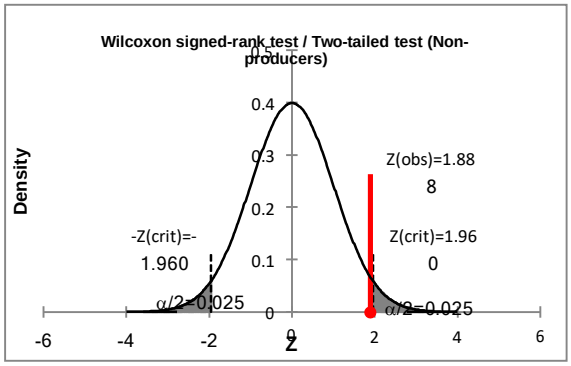

Figure 2: Antibiotic susceptibility and non-biofilm producers

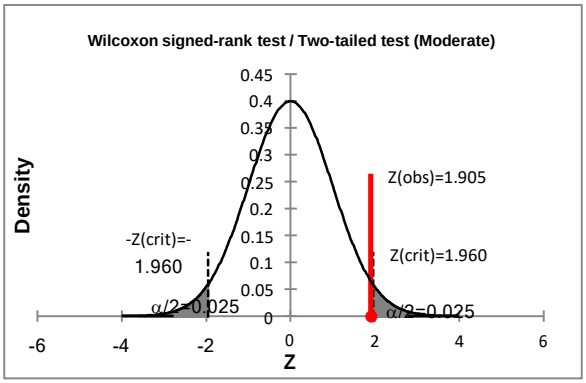

Figure 3: Antibiotic susceptibility and moderate biofilm producers

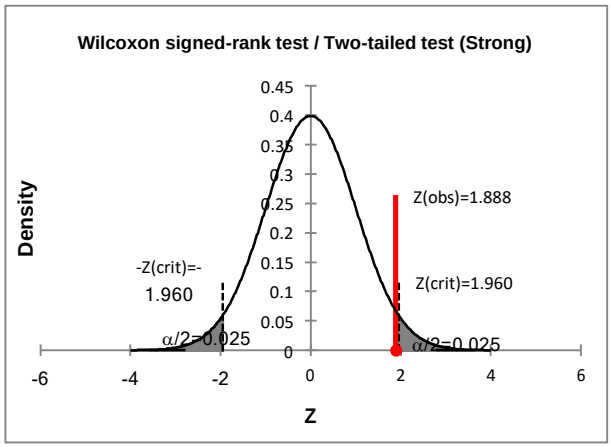

Figure 4: Antibiotic susceptibility and strong biofilm producers

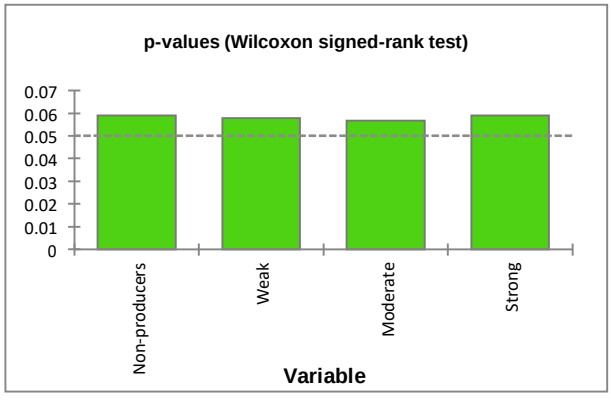

Figure 5: Antibiotic susceptibility and biofilm production

CONCLUSION

NFGNB were a strong source of nosocomial infections, because of their ability to survive in disinfectants and exhibiting resistance to antibiotics. They are, of course, strong biofilm producers when compared to other Gram-negative bacilli. However, if an NFGNB produces a biofilm, it is not that, they are resistant to antibiotics also. The results clearly stated that the majority of resistant organisms were non-biofilm producers. Hence, if we exactly could monitor these organisms without forming biofilms in the devices, we can reduce the nosocomial infections of multi-drug and pan-drug resistant organisms. Because once the biofilm is produced in the surfaces or the devices, the disinfectants or the antibiotics will have less penetration power to kill the microbes. Thus, complete surveillance of these organisms should be done periodically in hospitals.

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Conflict of interest: Nil

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