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RESEARCH ARTICLE

POLY-MICROBIAL AEROBIC GROWTH IN SINGLE CULTURES: CLINICAL AND MICROBIOLOGICAL PROFILE OF A COHORT OF HOSPITALIZED PATIENTS – A PILOT STUDY

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ABSTRACT

Co-colonization of anatomical niches by microorganisms is well documented. Growth of more than one type of Gram negative bacilli (GNB) from single cultures is commonly observed in Microbiology laboratories. The study aimed to determine whether any of the patient's demographic and/or clinical factors influence poly-microbial growth from clinical samples.Various clinical samples that grew two types of GNB from a single sample were selected for the study. In the cohort of 82 patients, the most common samples were pus (52.4%). 62.2% of the samples grew a combination of glucose Non Fermenting Gram Negative Bacilli (NFGNB) and an Enterobacteriaceae species. 40.2% samples grew a combination of a susceptible and a resistant GNB. In 69.3% of the samples there was an association between the Gram stain report (presence of inflammatory cells with or without bacteria) and clinical condition suggestive of infection, but this was not statistically significant (p = 0.322). Duration of hospital stay of more than 5 days was significantly associated with the antibiotic resistance pattern of the isolates, both by univariate (p=0.002) and multivariate (p=0.003) analysis. Similar results were seen with the pattern of organisms isolated and their antibiotic resistance pattern.

INTRODUCTION

Co-colonization of anatomical niches by microorganisms is well documented. Although co-infections are increasingly described, their impact is not well understood (O'Fallon E *et al*, 2009; Weintrob AC *et al*, 2010; Lautenbach E *et al*, 2009; Trick WE *et al*, 2001).

Many risk factors are known to influence the presence, type and relative numbers of bacteria in these niches. Appropriate sampling techniques have been described to help reduce the relative contamination by the background flora and aid in laboratory diagnosis. Gram stain of the samples has been used to aid in reporting. Quantitative culture techniques have been described to help in the enumeration of the different species to reflect the relative numbers in samples like - tracheal secretions and wound biopsies; the microbiologist then decides on which species to process further for identification and sensitivity testing.

Inspite of these protocols, microbiologists face a constant dilemma when presented with mixed growth of various kinds of aerobic bacteria in cultures.

The clinicians can use the report as a guide to prescribe appropriate antibiotics.

Gram positive pathogens e.g. *Staphylococcus aureus*, *Streptococcus pneumoniae and Beta haemolytic Streptococci* are reported inspite of mixed growth with Gram negative bacilli(GNB). Issues arise when there is mixed growth of more than one type of GNB and with variable susceptibility patterns. The dilemma for the microbiologist is- what to report and for the clinician- how to treat (Wiener-Well Y *et al*, 2013).

When more than one type of GNB is isolated on direct plating it is difficult to conclude which of them is the pathogen or the colonizing flora, especially when isolated from respiratory samples (bronchioalveolar lavage, endotracheal tip/secretions, tracheal trap), urine and deep wounds communicating to the outside.

To add to this dilemma, is the simultaneous isolation of bacteria with different antimicrobial susceptibility pattern from the same clinical sample, which is further, complicated when one of them is multidrug resistant. In this study we have focussed on the simultaneous growth of two GNB in aerobic culture. Simultaneous growth of Gram positive and GNB was not included in the study.

The primary objectives of the present study were to –

a) Analyze the microbiological and clinical profile of patients growing two types of GNB in a single sample sent for microbiological culture;
b) Determine the presence of demographic or clinical factors which could influence the simultaneous isolation of different GNB.

The secondary objective was to determine whether factors like Gram stain, clinical parameters of the patient (total leukocyte counts, condition of the wound, rise in body temperature, X-ray findings) at the time of isolation of poly-microbial growth

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could help determine if the growth was possibly contributing to infection or colonization.

METHODS AND MATERIAL

This study was conducted at a tertiary care centre, between May 2012 and August 2012. It was a cross sectional study. Subjects (inpatients only) were recruited as and when a sample from them, in single culture, grew two types of GNB either both sensitive or both resistant to cephalosporins and carbapenems, in significant numbers (>10^5cfu/ml from samples like tracheal secretions and urine) or in heavy growth (from samples like wound and sputum). All samples were collected for routine microbiological diagnosis.

Data collection

Data was collected from the in-patient charts according to a proforma which included patient's demographic details like- age, sex and clinical details like- type of sample, type of ward, length of stay in the hospital and treatment details before the receipt of sample, presence of any co-morbid conditions or devices, presence of fever, total leukocyte counts, condition of the wound and x-ray findings depending on the type of case.

Microbiological methods

All samples were processed as per standard guidelines (Henry. E. Isenberg 1992).

Tips (e.g., drain tips, central line tips) received for cultures were directly rolled over the culture plates without Gram stain being done.

Significant growth of organisms was documented, identified and reported as per standard protocols [e.g., Kass criteria for urine, semi-quantitative culture method for bronchioalveolar lavage(BAL)] (Henry. E. Isenberg 1992).

Antibiotic susceptibility test was performed using Kirby Bauer's disc diffusion method & interpreted according to the CLSI guidelines (2012).

Descriptions used to summarise data

All clinical samples with growth of two types of GNB on primary plates within 48 hours, with their susceptibility pattern were included in the study.For comparison, patients were categorised as ward and ICU patients. At the time of, or any time prior to the sample collection if the patient was in the ICU he was considered as ICU patient otherwise as ward patient.

Treatment details prior to the time of sampling, whether the patient was on a single antimicrobial, or a combination of Treatment details prior to the time of sampling, whether the patient was on a single antimicrobial, or a combination of different antimicrobials with or without steroids, or was not on any antimicrobials were collected. different antimicrobials with or without steroids, or was not on any antimicrobials were collected.

Gram stain of the sample and clinical parameters of the patient (total leukocyte counts, condition of the wound, rise in body temperature, X-ray findings) were used to associate the poly-microbial growth as possible cause of infection.

Since, a variable susceptibility pattern was noticed among the isolates, a working definition as 'multi-drug resistant (MDR) isolate' was given to those isolates which were resistant to both, cephalosporins and carbapenems (http://www.cdc.gov/hicpac/pdf/MDRO/pages5_6MDROGuideline2006.pdf.). If the isolate was susceptible to atleast one of them it was not considered as a 'multi-drug resistant isolate'. Considering the susceptibility pattern of both the isolates from the same clinical sample, the patients were divided into 3 categories as follows: category A- both isolates MDR, category B- both isolates sensitive, category C- Combination of sensitive and MDR isolates.

The choice of antimicrobials will be limited when there is isolation of MDRGNB as poly-microbial growth hence we have combined all patients who grew atleast one MDR GNB into a single category (category A plus category C) to check if such resistance pattern has any association with the other variables.

Statistical Analysis

All the statistical analysis was performed using SPSS version 18.0. Descriptive statistics such as median [(Interquartile range (IQR)] for age and N (%) for nonparametric variables were used to describe the sample characteristics. For categorical variables, a Pearson Chi-squared test was used to determine the statistical significance of the association between the variable and sensitivity pattern. A univariate logistic regression predicting the sensitivity pattern was fit to determine the statistical significance of the associated factors.

RESULTS

During the 4-month study, a cohort of 82 patients were included, 61 (74.4%) were male and 21 (25.6%) were female, with median age of 51 years (IQR 34 - 60). Majority of them were in the age group 41- 60 years (42.7%).

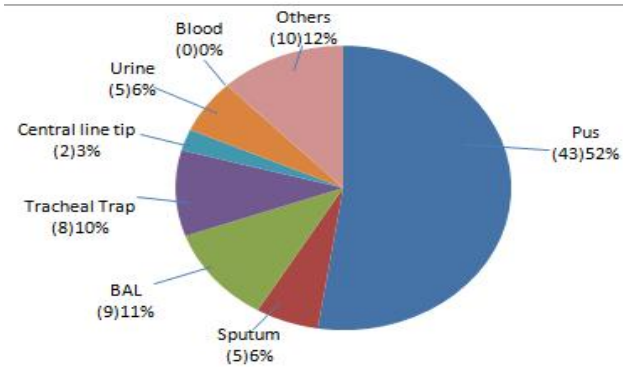

Figure 1 Sample Distribution (numbers in parenthesis)

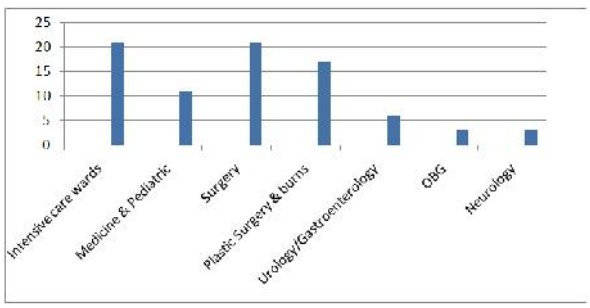

Figure 2 Ward wise distribution of samples (n=82)

Two types of GNB were isolated commonly from pus samples. (Fig 1)
25.6% of the cohort was from intensive care and general surgery wards each. (Fig 2)

Table 1 Sensitivity pattern of the isolates and associated factors

Variables (n)	Sensitivity pattern of the isolates			χ^2 value	p value
	Category A:Both isolates MDR (%)	Category B: Both isolates sensitive (%)	Category C: Combination of sensitive and MDR isolates (%)		
Age in years					
≤ 20 (9)	2 (22.2)	2 (22.2)	5 (55.6)		
21-40 (19)	3 (15.8)	6 (31.6)	10 (52.6)	5.064	0.281
≥ 41 (54)	7 (13.0)	29 (53.7)	18 (33.3)		
Sex					
Male (61)	11(18.0)	26(42.6)	24(39.3)	2.258	0.323
Female (21)	1(4.8)	11(52.4)	9(42.9)		
Type of stay					
Ward (44)	5 (11.4)	23 (52.3)	16 (36.4)	2.125	0.346
ICU (38)	7 (18.4)	14 (36.8)	17 (44.7)		
Co-morbid conditions*					
Nil (25)	4 (16.0)	8 (32.0)	13(52.0)	2.653	0.265
≥ 1 (57)	8 (14.0)	29 (50.9)	20 (35.1)		
Devices†					
Nil (42)	5 (11.9)	22 (52.4)	15 (35.7)	1.833	0.390
≥ 1 (40)	7 (17.5)	15 (37.5)	18 (45.0)		
Hospital stay					
<5 days (27)	1 (3.7)	19 (70.4)	7 (25.9)	11.024	0.004
≥ 5 days (55)	11 (20.0)	18 (32.7)	26 (47.3)		
Treatment					
One antibiotic (21)	3 (14.3)	12 (57.1)	6 (28.6)		
Combination (56)	9 (16.1)	23 (41.1)	24 (42.9)	3.009	0.556
No drugs (5)	0 (0.0)	2 (40.0)	3 (60.0)		
Gram stain correlation with clinical condition					
Yes (52)	7 (13.5)	22 (42.3)	23 (44.2)	1.269	0.530
No (23)	4 (17.4)	12 (52.2)	7 (30.4)		
Organisms isolated					
Both Enterobacteriaceae (22)	0 (0.0)	17 (77.3)	5 (22.7)		
Both NFGNB (9)	4 (44.4)	1 (11.1)	4 (44.4)	19.075	0.001
Combination (51)	8 (15.7)	19 (37.3)	24 (47.1)		

*Diabetes mellitus, hypertension, use of steroids or antibiotics, transplant recipients, bedridden patients, malignant conditions, frequent hospitalization, alcoholism, smoking, cardiac problems, etc. †Central line, urinary catheter, ventilator, anydrain, etc.

Table 2 Univariate analysis of sensitivity pattern with the associated factors.

Variables	Category A plus C (atleast one of the isolate being MDR)	Category B (both isolates being sensitive)	Unadjusted OR (95% CI)	p value
Hospital stay				
≥ 5days	37 (67.3)	18 (32.7)	4.89 (1.79, 13.27)	0.002
< 5days	8 (29.6)	19 (70.4)	1	
Age in years				
0 – 20	7 (77.8)	2 (22.2)	0.25 (0.5, 1.30)	0.098
21 – 40	13 (68.4)	6 (31.6)	0.40(0.13, 1.20)	0.102
> 40	25 (46.3)	29 (53.7)	1	
Type of stay				
ICU	24 (63.2)	14 (36.8)	1.87 (0.77, 4.55)	0.163
Ward	21 (47.7)	23 (52.3)	1	
Devices				
Yes (≥ 1)	25 (62.5)	15 (37.5)	1.83 (0.76, 4.43)	0.178
No	20 (47.6)	22 (52.4)	1	
Co-morbidities				
Yes (≥ 1)	28 (49.1)	29 (50.9)	2.20(0.82, 5.91)	0.118
No	17 (68.0)	8 (32.0)	1	
Treatment				
One antibiotic	9 (42.9)	12 (57.1)	0.50 (0.07, 3.65)	0.963
Combination	33 (58.9)	23 (41.1)	0.96 (0.15, 6.19)	0.494
No drugs	3 (60.0)	2 (40.0)	1	
Gram stain correlation with clinical condition				
No	11 (47.8)	12 (52.2)	1.50 (0.555, 3.987)	0.43
Yes	30 (57.7)	22 (42.3)	1	
Organisms isolated				
Both NFGNB	8 (88.9)	1 (11.1)	27.20 (2.71, 272.83)	0.005
Combination	32 (62.7)	19 (37.3)	5.73 (1.82, 18.04)	0.003
Both Enterobacteriaceae	5 (22.7)	17 (77.3)	1	

51 (62.2%) samples grew a combination of NFGNB and an Enterobacteriaceae species (Table 1). 33 (40.2%) samples grew a combination of a susceptible and a resistant GNB; 37 (45.1%) samples grew a combination of both susceptible GNB.

Of the 164 isolates from this cohort, 69 were NFGNB and 95 were Enterobacteriaceae. Most common were *Pseudomonas aeruginosa* 45 (27.4%) among the NFGNB, Klebsiella species 42 (25.6%) and *Escherichia coli* 26(15.85%) among Enterobacteriaceae

Table 3 Multivariate logistic regression analysis.

Variable	Adjusted OR(95% CI)	p value
Hospital stay		
≥ 5days	5.27 (1.74, 15.96)	0.003*
< 5days	1.00	
Organisms isolated		
Both NFGNB	6.16 (1.82, 20.87)	0.003*
Combination	0.21 (0.02, 1.99)	0.173
Both Enterobacteriaceae	1.00	

*The variable 'hospital stay' and 'both NFGNB' only were found to be statistically significant in the multivariate analysis adjusting for all other factors of age, type of hospital stay, presence of devices and co-morbidities.

The difference in sensitivity pattern of the poly-microbial growth among patients with different periods of hospital stay is statistically significant(p=0.004), since majority of those who have stayed for <5 days show a grown pattern of 'both isolates being sensitive' (70.4%) as compared to those who have stayed for ≥ 5 days (32.7%)(Table1).

When the isolated organisms were a combination of both being Enterobacteriaceae, the probability that both of them could be drug sensitive was high, as compared to the other combinations, which had higher chances of MDRGNB; which was statistically significant (p=0.001) (Table 1).

Those who had hospital stay of more than 5 days were nearly 5 times more likely to grow MDR bacteria, than those who had less than 5 days which is statistically significant, OR (95%CI) = 4.89 (1.79, 13.27), p=0.002 (Table 2).

Compared to the group of patients who grew both organisms as Enterobacteriaceae, those who grew 'both NFGNB' were nearly 27 times more likely to grow MDR bacteria, which is also statistically significant, OR (95%CI) = 27.20 (2.71, 272.83), p=0.005 and those who grew a combination of Enterobacteriaceae and NFGNB were 5.73 times more likely to grow MDR bacteria, which is also statistically significant, OR (95%CI) = 5.73 (1.82, 18.04), p=0.003 (Table 2).

All variables with p-values <0.25 in univariate analysis were included in the multivariate analysis. The results are shown in table 3.

In 69.3% of the samples (52 out of 75 samples, the remaining samples were device tips) there was an association between the Gram stain report and clinical condition of the patient but was not statistically significant. Table 4

Table 4 Distribution of Gram stain findings with respect to clinical condition.

	Clinical condition suggestive of infection (%)	Clinical condition not suggestive of infection (%)
Bacteria seen in Gram stain (n=56)	47 (83.9)	9 (16.1)
No bacteria in Gram stain (n=19)	14(73.7)	5(26.3)

χ^2= 0.98, p = 0.322

The association between Gram stain and clinical condition is not statistically significant.

DISCUSSION

During the study period, all blood cultures grew solitary bacterial isolates; pus samples ranked the highest in growing more than one bacterial isolate. This probably reflects on the method of sample collection and method of processing; blood samples being collected with greater sterile precautions. Moreover patients with open wounds are at an increased risk of getting colonized/ infected with hospital environmental strains and have higher chances of showing growth of multiple bacterial isolates which tend to complicate microbiological reporting.

Length of hospitalization and the type of organisms grown have had a significant effect on the multiple isolation as evident from both univariate and multivariate analysis. Increased length of hospital stay increases the chance of colonization/infection with hospital environmental strains. Here, we have seen that hospital stay of ≥ 5 days is significantly associated with isolation of MDRGNB. This could probably reflect on the hospital infection control practices and would demand greater attention towards cleaning and disinfection activities. The choice of antibiotics being limited for infections with MDR organisms, the above finding could also have an impact on the hospital antibiotic policy.

Studies have begun to elucidate the characteristics of MDRGNB colonization by describing prolonged duration of colonization for up to five months (O'Fallon E *et al*, 2009; Weintrob AC *et al*, 2010), polymicrobial growth with different strains of the same Gram-negative bacterial species (Lautenbach E *et al*, 2009), and with methicillin resistant *Staphylococcus aureus*(MRSA) and vancomycin-resistant Enterococci (VRE) (Trick WE *et al*, 2001). Mechanisms of acquisition of MDRGNB include patient-to-patient spread and transfer of resistance genes among the GNB in the host's gut. The potential to acquire MDRGNB both exogenously and endogenously suggests that patients may have a substantial risk of becoming colonized with more than one different MDRGNB species. Understanding the epidemiology of MDRGNB co-colonization would have important implications for preventive efforts aimed at limiting their spread.

We have also observed that in patients who have been hospitalized for more than 5 days, the chances of isolating more than one MDR organism is high, either both being NFGNB or a combination of Enterobacteriaceae and NFGNB. Carbapenem-resistant Enterobacteriaceae (CRE) and glucose non fermenting gram-negative bacilli, such as *Acinetobacter baumannii* and *Pseudomonas aeruginosa*, are among the most challenging pathogens to treat and contain in the hospital. Because of lack of effective therapeutic agents, infections due to these MDRGNB are associated with devastating outcomes (Nordmann P *et al*, 2009; Schwaber MJ *et al*, 2008; Abbo A *et al*, 2007; Boucher HW *et al*, 2009; . Livermore DM, 2009; Paterson DL, 2008). Data on the prevalence and epidemiology of patients demonstrating polymicrobial growth with both CRE and carbapenem resistant *Acinetobacter baumannii* and or carbapenem resistant *Pseudomonas aeruginosa* are limited, and the significance of this epidemiologic feature has not been thoroughly evaluated (Maragakis LL *et al*, 2008; Snyder GM *et al*, 2011).

Study by Marchaim *et al*, has demonstrated that, of 86 patients included in the study, 34 patients (40%) were "co-colonized", and 26 (79%) had MDR *Acinetobacter baumannii* or *Pseudomonas aeruginosa* isolated on the same day as the CRE(Marchaim D *et al*, 2012).They also found that recent stay at a long-term acute-care facility and previous therapy with antimicrobials with activity only against Gram-positive microorganisms also were associated with co-colonization, but did not remain significant independent predictors.

Wiener-Well *et al*l evaluated the clinical significance of urine cultures from patients with an indwelling urinary catheter from which 2 different pathogens were isolated and they concluded that laboratory work-up of 2 pathogens from patients with an indwelling catheter may be discarded (Wiener-Well Y *et al*, 2013)

Gram stain findings (presence of bacteria and inflammatory cells) and patients' clinical condition are very useful tools in assessing significance of the culture reports and determining treatment options. We have seen that the association between Gram stain findings and the patients' clinical condition does not significantly correlate with poly-microbial growth. This means to say that Gram stain alone, cannot be relied upon to associate poly-microbial growth with patients' clinical condition; because, even with the presence of bacteria on the Gram smear, patients did not have any contributing signs of infection, and vice-versa,.

With the findings of our study, it can be concluded that, length of hospitalization and type of organisms grown has some influence on the polymicrobial growth and their antibiotic resistance pattern. It would help us plan for more appropriately designed studies in future overcoming the study limitations and narrowing down the objectives to the ones which will contribute in providing better patient care.

CONCLUSION

Isolation of more than one GNB of different species and varying antibiogram confound microbiologists while reporting. In this study, based on the analysis of patient demographics, length of hospital stay, presence of invasive devices, co-morbid conditions, admission to ICU and source of sample, hospital stay of more than 5 days was found to be significantly associated with isolation of a more than one type of MDRGNB. This preliminary information may help influence laboratory and clinical decisions. It also helps to plan for appropriately designed studies to analyse these findings further.

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