<u>Original Research Article</u>

A Study on Analysis of the Sputum Gram Staining and Culture in Patients with Lower Respiratory Tract Infections

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Abstract:

Background: One of the persistent problems we are facing now is the lower respiratory tract infections. For diagnosis of Lower respiratory tract infections (LRTIs), expectorated sputum is the most commonly received sample in the laboratory. But usually is contaminated with normal resident bacteria of the oropharynx. The value of sputum microscopy and culture in the diagnosis, management and outcome of LRTIs is a matter of controversy. The following study was conducted to evaluate the correlation of Gram's stain grading using Barletts grading criteria and culture in sputum samples for the diagnosis of lower respiratory tract infections.

Materials & Methods: The present study was during a one year period from Feb 2022 to Feb 2023. A total of 1058 sputum samples were received. All the samples were processed through Gram's stain and culture. The Gram's smear was examined for the presence of polymorphs, epithelial cells and bacterial forms by Bartlett's grading system. All the sputum samples were inoculated onto Blood agar, Chocolate agar and Mac Conkey agar and were incubated overnight at 37degree celsius. After 24 hrs inoculated plates were observed for the presence of growth. By using standard protocols bacterial isolates were identified from the growth. Kirby Bauer disc diffusion method on Mueller Hinton agar was performed for antibiotic susceptibility testing.

Results And Conclusions: Out of 1058 sputum samples processed, 465 (44%) were acceptable and rest 593 (56%) were not meeting the standard criteria of Bartlett. All the sputum samples were processed for culture and 316 samples showed growth (30%).The predominant growth observed was gram negative bacilli. Among the gram negative bacilli Klebsiella pneumoniae in 156 samples (49%) were the highest followed by Pseudomonas species 80 samples. (25%).Among the gram positive cocci Staphylococcus aureus was highest with sixteen samples (5%) In this present study, we recommend initial screening of sputum samples for clinically relevant results, and reject the non-acceptable samples, with help of grading system and repeat test for the diagnosis of true pathogen, thereby providing appropriate antibiotics and avoiding antibiotic resistance and a better treatment outcome for the patient.

Keywords: Sputum, Gram's stain, Bartlett's criteria, sputum culture.

Introduction

One of the persistent and pervasive public health problems that we are facing nowadays are acute lower respiratory tract infections. It is causing a greater burden of disease worldwide than human immunodeficiency virus, malaria, cancer, or heart attacks. [1,2]

Hence identification of the bacterial etiology of lower respiratory tract infections (LRTI) is very crucial to ensure a narrow-spectrum, targeted antibiotic treatment. But Gram stain and culture results are often difficult to interpret as they depend strongly on sputum sample quality. [3] In clinical practice, microbiological analysis of sputum by Gram stain and culture can be of great help in the diagnosing the etiological agent of LRTI and thereby enabling targeted antibiotic treatment.

[4,5,6] The sputum is usually contaminated with normal flora of the oropharynx or saliva (upper respiratory tract secretions). So, a large number of different species overgrow in the culture thereby preventing the growth of the true pathogen. [7,8] As Community-acquired pneumonia (CAP) is a frequent infection with significant morbidity and mortality, especially in extreme ages of life and in patients with other comorbidities and is one of the reason for high economic burden, longer hospital stay and thereby leading to long term effects in quality of life and prognosis. [9,10]

This study was conducted to evaluate the correlation of Gram's stain grading using Barletts grading criteria and culture in sputum samples for the diagnosis of lower respiratory tract infections.

Materials & Methods

The samples received for a period of one year was taken for the study. Gram stained sputum smears were observed under microscope for presence of organisms, pus cells and epithelial cells.

Quality of expectorated sputum samples were assessed by using Bartlett's grading system (Table 1).

All the sputum samples were inoculated onto Blood agar, Chocolate agar and Mac Conkey agar and were incubated overnight at 37degree celsius. After 24 hrs inoculated plates were observed for the presence of growth. By using standard protocols bacterial isolates were identified from the growth. Kirby Bauer disc diffusion method on Mueller Hinton agar was performed for antibiotic susceptibility testing. The isolation of significant pathogenic organisms from a specimen indicates culture positive and isolation of scanty or insignificant growth from a specimen considered as culture negative. When mixed growths of significant organisms were isolated, they were counted according to the predominant growth.

Staphylococcus aureus ATCC 25923, Pseudomonas aeruginosa ATCC 27853, Escherichia coli ATCC 25922, Klebsiella pneumonia ATCC 700603 were included as quality control strains. The antibiotic susceptibility pattern will be assessed from the positive culture samples for gram positive and gram negative bacteria. The data was analyzed by using Microsoft Excel sheet and expressed in percentage using descriptive statistics.

Table 1: Bartlett's Criteria

Number of Neutrophils/10XLPF	Grade
<10	0
10-25	+1
>25	+2
Presence of mucus	+1
Number of Epithelial cells/10XLPF	
10-25	-1
>25	-2
Total Score	

Results: We performed study for a period of one year from February 2022 to February 2023.Totally we received 1058 samples, Male-652, female-406. According to age groups the highest received in the age group of 51 -70 age group and lowest received in the age group less than twelve. Table no: 2

Table 2: Sample Received According Different Age Groups

Age	Total Sample Received
<12	12
13-30	187
31-50	274
51-70	449
<70	136

According to Barletts grading system the final score value of less than or equal to zero indicates a salivary contamination of sputum sample (non- acceptable sputum sample). The final score of 1 and above to be considered as accepted samples. We received a total of 465 samples with score of ≥ 1 and rest of the samples scored less than zero which makes them salivary contaminated samples (Table no: 3)

Table 3: Total sample according to Barletts scoring

Gram Stain	Total Sample Received
-2	144
-1	214
0	235
1	299
2	166

Table 4: Score wise distribution according to age groups

Age	Gram Stain									
	-2	%	-1	%	0	%	1	%	2	%
<12	1	0.5	4	2	4	2	2	1	1	1
13-30	46	32	50	24	32	14	40	13	19	11
31-50	40	28	54	25	62	26	74	25	44	26
51-70	43	30	82	38	107	45	138	46	79	48
<70	14	10	24	11	30	13	45	15	23	14

In the age group 51-70 salivary contamination rate was higher (52%) compared to other age groups. (Table no 4)

After the scoring the samples were inoculated onto Blood agar, Chocolate agar and Mac Conkey agar and were incubated overnight at 37degree celsius. After 24 hrs inoculated plates were observed for the presence of growth.

By using standard protocols bacterial isolates were identified from the growth. Kirby Bauer disc diffusion method on Mueller Hinton agar was performed for antibiotic susceptibility testing. The isolation of significant pathogenic organisms from a specimen indicates culture positive and isolation of scanty or insignificant growth from a specimen considered as culture negative. When mixed growths of significant organisms were isolated, they were counted according to the predominant growth. There was no growth in 742 samples and growth was observed in 316 samples (Chart 1).In 316 samples growth was observed in forty eight salivary contaminated samples/unacceptable samples (15%) (Chart 2)
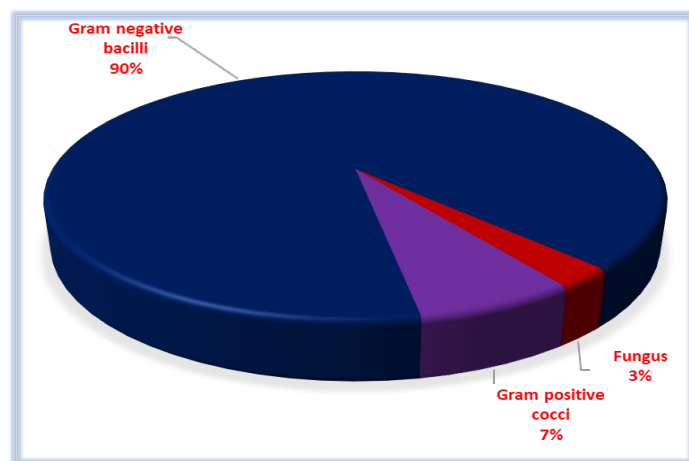
Chart 1: Organism type
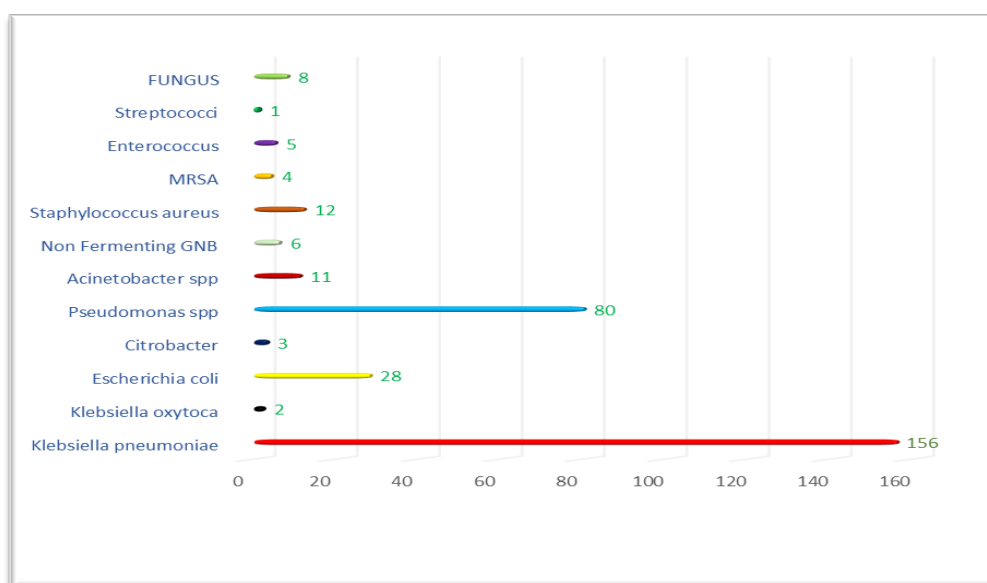
Chart 2: Isolate wise distribution

Higher number was isolated from gram negative bacilli .Among them highest isolated was Klebsiella species followed by Pseudomonas spp. Out of 316 samples eight isolates of fungus also included. A mong the gram positive organism four isolates of MRSA was also isolated. (Chart 2)

Antibiotic susceptibility was performed with Kirby Baeur disc diffusion method. Panel of drugs were tested for gram positive and gram negative organisms. Even though Enterococcus rarely reported we have also taken in to account the growth of Enterococcus. For Citrobacter and Acinetobacter sps 50% resistance was observed for Imipenem. .For Acinetobacter sps only 25 % sensitivity was observed for third generation cephalosporins. For Klebsiella pneumonuae only

35% sensitivity observed for cefepime.50 % sensitivity was observed for Vancomycin, Teicoplanin and 60% sensitivity for Linezolid in case of Staphylococus aureus. Increasing resistance trend of third generation cephalosporins was observed for Klebsiella pneumoniae and E.coli. Out of the twelve staphylococcus aureus isolated 4 of them were cefoxitin resistant, thereby methicillin resistant Staph aureus.

Monotherapy with aminoglycosides are not recommended in the CLSI 2024, because of treatment failure. For streptococcus sps drugs tested were all sensitive making them 100% sensitive. Few of the drugs were not tested because of limited availability in our institution.

Table 5: Antibiotic susceptibility sensitivity pattern in percentage

Organism	Antibiotic panel																
	AMC	AMP	CIP	COT	GEN	AK	CTR	CTX	CX	PIT	CAZ	AT	IPM	CPM	CL	PB	TG
Klebsiella pneumoniae	59	NT	65	69	59	65	56	54	NT	72	NT	37	70	35	55	80	NT
Klebsiella oxytoca	50	NT	100	75	100	75	50	100	100	100	100	100	100	75	100	100	NT
E.coli	31	13	62	62	62	81	50	50	50	88	56	44	75	75	NT	NT	NT
Citrobacter sps	50	50	100	100	50	50	NT	NT	NT	50	75	50	50	NT	NT	NT	NT
Pseudomonas aeruginosa	NT	NT	93	NT	NT	50	NT	NT	NT	82	81	67	80	53	NT	NT	NT
Acenitobacter sps	50	NT	25	50	50	NT	25	25	0	75	NT	0	50	NT	100	100	100
Non fermenting gram negative bacilli	25	NT	25	25	100	75	25	25		50	50	50	75	NT	NT	NT	NT

	P	RIF	E	LE	DO	TR	CX	CIP	CTR	COT	AMC	CD	VA	TEI	AK	HLG	LZ
Staphylococcus aureus	100	40	40	10	30	0	34	NT	NT	30	NT	50	50	50	NT	NT	60
Enterococcus sps	100	50	75	100	100	100	NT	75	NT	NT	NT	100	100	75	100	100	NT
Streptococcus sps	0	NT	0	100	NT	NT	NT	NT	NT	NT	NT	0	100	100	NT	NT	100

Abbreviations: AMC- Amoxicillin Clavulanic Acid, AMP- Ampicillin, CIP- ciprofloxacin, COT- Co-trimoxazole, GEN-, AK- amikacin, CTR- ceftriaxone, CTX- cefixime, CX- cefoxitin, PIT- piperacillin tazobactam, CAZ- cefazolin, AT-, IPM- imipenem, CPM- cefepime, CL- colistin, PB- polymyxin b, TG-, P- penicillin, RIF-, E- erythromycin, LE- levofloxacin, DO- doxycycline, TR- tetracycline, CD- clindamycin, VA- vancomycin, TEI- teicoplanin, HLG-, LZ- linezolid,

Discussion

Among the most common infectious diseases respiratory tract infections pose a significant morbidity and mortality cases. Because of expanded variety of emerging pathogens diagnosis always remain a challenges for the microbiology laboratory. In recent years, there has been substantial rise in antibiotic resistance among respiratory pathogens. [11]

This study was conducted for a period for one year from March 2022 to March 2023 in Trichy SRM Medical College and research centre. During this period we received a total of 1058 sputum samples for culture and sensitivity testing. All the samples were of patients with lower respiratory tract infections. Examination of expectorated sputum has been one of the the primary testing methods for diagnosis of bacterial pneumonia. .But most of the times lower respiratory tract secretions are always contaminated with upper tract flora present in the saliva, thereby sputum being the least clinically relevant specimens received for culture in the Microbiology laboratory, even though it is one of the most numerous and time consuming specimens. Good sputum samples depend mainly on healthcare worker and how you educate the patient to achieve a good sputum sample. [12] In our study we assessed the quality of sputum sample using Barletts grading system. According to the Barletts grading score which has one and more than one were considered accepted samples. Out of 1058 samples 465 samples were considered as acceptable samples accounting to about 44% and 56% as unacceptable/salivary samples. Other study showing similar percentage reported by Daniel Musher et al low percentage of 31% of acceptability. [13] Others studies reporting acceptability percentages include Anevlavis et al of 63% and Mariraj et al reported 79%. [14,15] In other study Ravichandran et al had reported that all 74 (100%) of their sputum samples so screened came in the non-acceptable category, which contained only mixed flora not acceptable for culture.[16] Anuradha et al have reported an acceptability of 65 %. [19] Grading system for sputum cannot be assessed for lower respiratory tract infections by Legionella species, Mycobacterium tuberculosis, fungi and viruses. The microorganisms which have been isolated from the culture should be correlated clinically always. In our study score less than 1 was most frequently observed in age group of above fifty years of age.

 Total culture positivity in our study was 30% (316/1058). , Aroma Oberoi et al have reported a culture positivity of 32% & Nihan Ziyade et al 44.7% culture positivity.]17,18].Other studies reported were Jean J Lloveras et al 57%, Daniel Musher et al- 79%, Somporn et al- 40.95%, Nawfal et al 41.7% respectively.[19,20,21]. In contrary

Ravichandran et al have reported a percentage of only 5% culture positivity. Anuradha et al have reported in her study a culture positivity of 61%. M R Shariatzadeh et al grew potential pathogens only in 33.7% of their acceptable samples, similar to our study. Mariraj et al grew in 63.2% of their acceptable samples. [15,23]. In our study out of thirty percent of culture positivity fifteen percent (48 samples)showed growth . Anuradha et al in their study reported 42 samples in the non-acceptable category showed growth. [23] Mariraj et al had reported 2 out of their 21 non acceptable samples growing potential pathogens (9.5%).[15] Akansha et al in their study reported that potential pathogens were obtained from 183 of 233 samples, of which 141 are from acceptable samples (77.05%), and 42 are from nonacceptable samples (22.92%). [8]

In the present study gram negative bacilli(90%) showed predominant growth and gram positive cocci Among the gram negative bacilli Klebsiella pneumoniae in 156 samples(49%) were the highest followed by Pseudomonas species 80 samples.(25%).Among the gram positive cocci Staphylococcus aureus was highest with only sixteen samples(5%) which is contrast to other study which have higher growth of gram positive organism [25] This may be due to the fact that samples we received were from admitted patients ,many of them have hospital acquired infections which is most prevalent nowadays. In our study we were not able to speciate streptococcus species which is one of the drawback of the study. It has been observed that even in patients with S. pneumoniae , the usual laboratory methods cannot detect the pathogen in 45-50% of cases even when large numbers of organisms have been noted on gram stain.[28] Among the culture positives eight fungal growth was also observed. Anuradha et al also reported 68.57% of gram negative bacilli growth in their study, Somporn et al had reported 76% gram negative bacilli growth in their study. Similar to our studies Anuradha et al also reported predominance of Klebsiella pneumoniae growth in their study. [19,21] This goes well with the fact that in lower respiratory tract infections predominantly we observe Klebsiella pneumoniae in India [24]. The second predominant organism isolated was Pseudomonas spp which is also correlating with other studies conducted in India.[26] Pseudomonas spp is a ubiquitous organism that it could affect individual with immunocompromised situation and one of major reasons for nosocomial infection.[27] In our study Staphylococcus aureus was the most frequently isolated gram positive bacteria from respiratory samples which was similar to other studies.[29,30] Out of sixteen of the isolated Staphylococcus aureus four were methicillin resistant Staphylococcus aureus (MRSA). The antimicrobial resistance among the respiratory

pathogens is one of the crucial factors interfering the patient treatment. In the present study gram negative isolates showed higher resistance patterns to Amoxyclav, followed by aminoglycosides and third generation cephalosporins which is similar to other studies. [31,32]. In Klebsiella pneumoniae and E,coli 50% resistance was observed for third generation cephalosporins.

In appropriate and widespread use of antibiotics is one of the factors for emerging resistance. The emergence of fluoroquinolones resistance among RTIs has now been documented in many countries. [34,35] Nowadays, fluoroquinolones are substituted by the 3rd generation cephalosporins, which are frequently used by clinicians. In this study higher resistance to fluoroquinolones are observed for Acinetobacter sps (75%) which is similar to other studies.[33] and Most of the gram negative bacilli showed good sensitivity to amikacin in the present study which is in accordance to other studies conducted in India. [29] This may be due to selective use of amikacin because of higher adverse effects caused by injectable antibiotic.

Carbapenems are very effective antibiotics and are most widely used against gram negative bacilli nowadays. Sensitivity to Imipenem were tested for gram negative bacilli in this study .We observed 50% resistance in Acinetobacter sps and Citrobacter sps in our study. Klebsiella oxytoca gave a sensitivity of 100 % to imipenem. Carbapenems resistance is increasing day by day and it is a matter of great concern in management of infection. [36]. Among antipseudomonal drugs Cefepime showed a increasing resistance percentage of 47% Higher resistance of P.aeruginosa in a range of 57-65% against Cefepime were recorded by other authors also. [37,38]. Colistin was only tested for Klebsiella pneumoniae which showed a 50% resistance . (Table no: 6) Gram positive organisms (Enterococcus and Sreptococcus sps) showed highest sensitivity towards Vancomycin followed by Linezolid. Only four isolates out of 12 were MRSA (15.4%) which is lower and similar to other study.[24].In a study from Chennai it was 25%, from Jharkhand it was 23.29%, from Manipur it was 62.06%, 82.7% in Iran and 55.6% in Nagpur. [37,38]

Table 6: Comparison of resistance pattern of Klebsiella and Pseudomonas with other study

Studies	Klebsiella pneumoniae (resistance %)				P.aeruginosa (resistance %)				
	IPM	PTZ	AK	CIP	IPM	PTZ	AK	CIP	
Our study	30	28	35	35	20	18	50	7	
Amutha et al	0	10	13	55	3	4.4	30	42	
Elumalai et al	2.6	9.3	44.2	54	0	0	10.8	37.5	
Thomas et al	40	70	70	80	33.3	41.7	25	33.3	
Ratna et al	5	42.62	NT	18.03	7.14	42.85	NT	14.28	
Regha et al	8.9	34.4	30	51.1	3.4	21.8	20.7	36.8	

The emergence of multidrug resistant strains is one of the major threat to the treatment of patients globally. Among the gram negative isolates, various mechanisms of drug resistance have been attributed. [31] Moreover not all the bacteria causing RTIs are identified by conventional culture and sensitivity method. One of the reasons are ineffective antimicrobial resistance containment program including antimicrobial stewardship .As a matter of fact the multidrug resistant strains are increasing. This is a new study conducted in our institution as a small effort to find the antimicrobial susceptibility pattern thereby establishing an antibiogram for a better treatment outcome. Moreover laboratory testing methods especially gram stain which is the basis of diagnosis of respiratory tract infections should be meticulously followed for better diagnosis, identification of true pathogen, thereby avoiding inappropriate treatment and thereby reducing the antibiotic misuse.In a study by Mariraj et al, the authors had concluded that Microbiology laboratories may reject for

culture, those sputum samples which fail to meet the criteria of Bartlett for purulence, and sputum cultures must be ordered judiciously for documented episodes of LRTIs to provide a meaningful output which highlights the importance of the gram stain. Clinical relevance should be applied in diagnostic Microbiology thereby giving the physicians to give best care for their patients

Conclusion

Sputum Gram stain test is highly sensitive and specific for identifying causative pathogens in adult patients with lower respiratory tract infections. Studies evaluating the impact of the use of sputum Gram stain in treatment decision-making on outcomes in hospitalized patients with CAP are now much needed. Sputum cultures must be ordered judiciously for documented episodes of lower respiratory tract infection to provide meaningful report. The microbiology laboratory must use objective criteria like Barletts grading system for Gram stain screening for purulence

before inoculation in to culture media. Hence the routine sputum Gram stain is essential to provide meaningful culture report.

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