ORIGINAL RESEARCH

IMPROVING SPUTUM CULTURE RESULTS FOR DIAGNOSIS OF LOWER RESPIRATORY TRACT BY SALINE WASHING

Nihan Ziyade, Aysegul Yagci
Department of Clinical Microbiology Marmara University School of Medicine, Istanbul, Turkey

ABSTRACT

Objective: To evaluate the value of Gram staining and bacteriological culture of sputum by the saline wash method for diagnosis of lower respiratory tract infections (LRTI).
Methods: All samples containing fewer than 10 squamous epithelial cells per low power microscopic field (10x) were cultured both directly and quantitatively.
Results: 620 sputum specimens from 489 patients clinically diagnosed as having LRTI were evaluated. Sensitivity of Gram stain was 78.6% and specificity was 82%, reaching to 100% for H. influenzae and S. pneumoniae. Quantitative method increased overall culture positivity from 52% to 63.5% of inoculated samples. The three most commonly isolated pathogens were *Haemophilus influenzae, Pseudomonas aeruginosa* and *Streptococcus pneumoniae*.
Conclusion: The collection of expectorated sputum is a non-invasive process and saline washing and subsequent Gram stain and culture can provide a high diagnostic yield. Initial Gram examination of sputum samples, especially for *H. influenzae* and *S. pneumoniae* is advisable when experienced microbiologists interpret the slides, since Gram stain is almost as effective as cultivation and the results are available 48 hours sooner.

Keywords: Sputum, Quantitative culture, Lower respiratory tract infection

ALT SOLUNUM YOLU İNFEKSİYONLARININ TANISINDA BALGAM ÖRNEKLERİNİN YIKANMASININ KÜLTÜR SONUCUNA ETKİSİ

ÖZET

Amaç: Alt solunum yolu infeksiyonlarında (ASYİ) Gram boyamanın ve balgam örneklerinin salin ile yıkanmasının katkısının değerlendirilmesi.
Metod: Her bir küçük mikroskop alanı (10x) için 10 dan az yassı epitel hücresi içeren tüm örnekler kalitatif ve kantitatif olarak ekildi.
Bulgular: ASYİ tanısıyla gönderilen 489 hastadan alınan 620 örnek değerlendirildi. Gram boyamanın duyarlılığı %78.6 , özgüllüğü %82 olarak değerlendirilirken, bu değerler *H. influenzae* ve *S. pneumoniae* için %100' e ulaştı. Kantitatif yöntem kültür pozitifliğini %52' den % 63.5' a yükseltti. En sık izole edilen patojenler sırasıyla *Haemophilus influenzae, Pseudomonas aeruginosa* ve *Streptococcus pneumoniae* oldu.
Sonuç: Ekspoktere balgam örneklerinin toplanması invaziv olmayan bir işlem olup salin ile yıkama sonrası yapılan Gram boyama ve kültür laboratuvar tanısını kolaylaştırabilir. Kültür öncesi Gram boyamada deneyimli bir personel *H. influenzae* and *S. pneumoniae* için ön değerlendirmeyi kolaylıkla yapabilir ve kültür sonuçları için gereken 48 saat öncesinde tanıya yardımcı olabilir.

Anahtar Kelimeler: Balgam, Kantitatif kültür, Alt solunum yolu infeksiyonu

İletişim Bilgileri:
Ayşegül Yağcı, M.D.
Department of Clinical Microbiology Marmara University School of Medicine, Istanbul, Turkey
e-mail: ayagci@marmara.edu.tr

Marmara Medical Journal 2010;23(1);30-36

INTRODUCTION

Lower respiratory tract infections (LRTI) are common causes of morbidity and mortality worldwide. Accurate identification of respiratory pathogens is the center of the management of the patients and initial appropriate treatment is associated with a lower mortality rate[1]. For the diagnosis of LRTI, expectorated sputum is the most commonly used sample which can be obtained easily and non invasively. Invasive methods including bronchoalvelaoar lavage (BAL) and/or protected specimen bronchial brushing (PSB) permit the collection of distal pulmonary secretions and improve the identification of causative agents. Normal resident bacteria of the oropharynx may contaminate samples and a large number of different species may overgrow, preventing the determination of the true epidemiologic agent. Although invasive samples have a higher diagnostic yield since they bypass oropharyngeal flora, determination of the bacterial number using accepted thresholds (10^4 cfu/ml for BAL and 10^3 cfu/ml for PSB) increases the reliability of those specimens for diagnosis of LRTI[2,3]. Using a wash technique and quantitative culture of sputum has been shown to decrease the number of contaminants by 100 to 1000 fold and has enhanced the value of sputum samples. However, there are few articles supporting the importance of this approach and suggesting routine use[4-6].

Among the diagnostic methods of LRTI, the most controversial is the Gram stain examination. Gram smear of the sample does not require sophisticated equipment, it is an inexpensive method and results can be obtained in a short period of time. However, the Infectious Diseases Society of America (IDSA) and American Thoracic Society (ATS) guidelines have contradictory suggestions about Gram staining and the cultivation of sputum[3]. Gram stain can vary in sensitivity and specificity, depending on the specimen and the skill of the reader, whereas culture has a lower rate of variability than direct examination. ATS recommends performing bacterial culture where drug resistance is suspected or an unusual pathogen is being considered. Clinical diagnosis of LRTI is associated with the use of multiple, empiric antibimicrobial agents compared to when therapy decisions are based on microbiological findings[7]. The management of LRTI is remarkably simplified when the responsible pathogen is accurately determined. However, neither a standardized laboratory method nor a standard timing for specimen collection exists. Considering the comparatively wider availability of Gram examination and the cultivation of sputum rather than blood culture, antigen detection, or nucleic acid amplification tests, improved evaluation of sputum samples could be a focus for laboratory diagnosis. In this study, we aimed to evaluate the sensitivity and specificity of Gram staining and of quantitative inoculation of washed sputum samples from patients clinically diagnosed as having LRTI.

MATERIAL AND METHOD

Study Population: This prospective study was carried out for a period of one year, from July 2006 to June 2007 in Marmara University Hospital, Istanbul. All sputum samples taken from patients clinically suspected as having LRTI were included in the study and written consent was obtained from the patients. The study was approved by the Local Institutional Review Board.

Direct examination of samples: A fresh sputum sample was obtained and placed in a sterile container and all samples were screened by Gram staining regardless of the macroscopical appearance without grading as mucoid, mucopurulent or purulent. Sputum validity was assessed by means of theGeckler criteria and samples containing over 10 squamous epithelial cells (SEC) per low power microscope field (LPMF) were rejected[8]. Accepted samples were then mixed with physiological saline (1:10 vol), vortexed and centrifuged for 10 minutes at 1500 rpm. An equal volume of N- acetyl- L ¡Vcysteine was added to the pellet for homogenization and the mixture was incubated at 37°C for 15

minutes. A second Gram stain was prepared from washed specimens and smears were evaluated by two microbiologists in a blind manner. Samples containing less than 10 SEC per LPMF were divided into three groups: a. <10 SEC, >25 leukocyte, b. <10 SEC, 10-25 leukocyte, c. <10 SEC, <10 leukocyte and were screened for a predominant bacteria at oil immersion field (x100) by Gram stain.

Sputum culture: Samples accepted after microscopic evaluation were inoculated directly onto sheep blood agar, Mac Conkey agar and chocolate agar plates within 30 minutes of sample collection. Washed samples as described above were further diluted 1: 10 in sterile saline and 10 ul aliquots were inoculated on sheep blood agar, Mac Conkey agar and chocolate agar plates for quantitative evaluation[5,9-11]. After incubation for 48 hours at 37ºC microorganisms present in counts of $>10^6$ cfu /ml in the sputum were accepted as causative pathogens[10,11]. Isolated bacteria were identified by using conventional methods.

Statistical analysis: Comparisons were performed using the chi-square test with SPSS for Windows version 7.0, 2001 (SPSS Inc, Chicago). The difference between two percentage significance tests was used for qualitative and quantitative cultivation methods. P values of <0.05 were regarded as significant. The Kappa statistic was used to measure agreement on the quantity of cells and bacteria between the raters[12].

RESULTS

During the study period, 620 sputum specimens from 489 patients attending our hospital (median age: 49 years) of whom 234 (47.9%) were female and 255 (52.1%) were male were evaluated. Hospitalized patients constituted 21.1% of the study population. The remaining patients were outpatients, and 46.8% of all samples were sent from pediatric and adult chest diseases outpatient's clinics.

The presence of less than 10 SEC and more than 25 leukocytes per low power field was taken as acceptance criteria. Only 22.1% of the samples were found suitable for culture (Table I). This very low ratio may suggest that clinicians who send sputum to the laboratory without having a proper clinical diagnosis are asking for misleading information. A previous study indicated that only 25% of purulent sputum samples of hospitalized patients for exacerbation of chronic obstructive pulmonary disease satisfied the quality criteria, however sputum culture was positive in half of the patients with > 25 leukocyte but >10 SEC per low power field[9]. We wanted to evaluate all samples containing < 10 SEC by Gram staining and cultivation. Direct examination was done by two separate microbiologists and agreement between observers showed a good correlation (k: 0.64). Table II shows correlation between Gram stain and culture results. The sensitivity of Gram stain was 78.6% and specificity was 82% (positive predictive value 87.74%, negative predictive value 69.67%).

We inoculated all the samples containing less than 10 SEC per low power field (277/620, 44.7%) by direct and quantitative inoculation methods. Bacterial pathogens were isolated from 144 (52%) of the samples by direct inoculation and from 176 (63.5%) of the samples by quantitative inoculation ($t=5.75$, $p<0.05$) . This result may be related with the decreased number of contaminants that would overgrow the true etiologic agents (Table I). Among culture positive samples a single pathogen was isolated in 158 and multiple pathogens from 18 of the samples (Table III). The three most commonly isolated pathogens were *Haemophilus influenzae* (78), followed by *Pseudomonas aeruginosa* (29), and *Streptococcus pneumoniae* (21). In 32 (11.5%) of the accepted samples bacterial pathogens were detected only in quantitative plates (Table IV). Gram stain of these samples prior to culture indicated the predominance of bacteria correlated with the culture results in 9/10 samples in group a, in 12/15 samples in group b, and in 3/7 samples in group c.

Table I. Culture positivity of sputum samples graded according to their microscopic examination

Microscopic (10x) grading of sputum samples stained with Gram stain	Gram smear	Culture positivity	
		Direct inoculation	Quantitative inoculation
a. <10 SEC*, >25 leukocyte	137 (22.1%)	88 (61.1%)	98 (55.8%)
b. <10 SEC , 10-25 leukocyte	76 (12.3%)	32(22.2%)	47 (26.8%)
c. <10 SEC , <10 leukocyte	64 (10.3%)	24 (16.7%)	31 (17.4%)
d. >10 SEC	343 (55.3%)	ND**	ND
Total	620	144	176

*Squamous epithelial cells, **not done

Table II.Gram smear and culture results of sputum samples

Gram smear	**Culture**		
	Positive	**Negative**	**Total**
Positive	136	19	155
Negative	37	85	122
Total	173	104	277

Table III. Distribution of bacterial pathogens isolated from quantitatively inoculated samples

	n
Single pathogen	
H.influenzae	66
P.aeruginosa	29
S.pneumoniae	15
E.coli	10
S.marcescens	7
M.catarrhalis	6
K.pneumoniae	6
S.maltophlia	4
S.aureus	3
K.oxytoca	2
Pantoea sp.	2
A.baumanni	2
E.cloacae	1
E.aerogenes	1
H.parainfluenzae	1
P.fluorescens	1
K.ornytolitica	1
E.faecalis	1
	158
Multiple pathogens	
H.influenzae + S.pneumoniae	6
H.influenzae + M.catarrhalis	2
H.influenzae + K.pneumoiae	1
H.influenzae + E.aerogenes	1
H.influenzae + E.cloacae	1
H.influenzae + S.maltophlia	1
S.pneumoniae + E.cloacae	1
S.pneumoniae + K.ozanae	1
E.coli + K.pneumoniae	1
E.coli + S.maltophlia	1
E.coli + C.koserii	1
E.coli + A.baumanni	1
	18
	176

Table IV. Distribution of bacterial pathogens isolated only in quantitative samples

Gram stain	Microorganism	n
	H.parainfluenzae	1
	K.oxytoca	1
a	*S.maltophlia*	1
	H.influenzae	4
	M.catarrhalis	2
	P.aeruginosa	1
		10
	S.maltophlia	1
	H.inluenzae	4
	M.catarrhalis	3
	S.pneumoniae	2
b	*A.baumanni*	1
	K.pneumoniae	1
	E.coli	1
	E.cloacae	1
	Pantoea sp	1
		15
	E.coli	3
	A.baumanni	1
c	*S.marcescens*	1
	K.pneumoniae	1
	E.faecalis	1
		7
	TOTAL	**32**

DISCUSSION

The main obstacle for using sputum as a diagnostic tool for LRTI is obtaining a good quality specimen. In our study, only 22.1 % of the samples were suitable when fewer than 10 SEC and >25 leukocyte per low power field was taken as acceptance criteria. This rate changes from 25% to 55% in different studies[9-13]. One of the major limitations of this paper is the lack of information of previous antibiotic use and final diagnosis of the patients. We wonder if there was an over-diagnosis of cases rather than obtaining low quality sputum. It has been suggested that the value of Gram stain and culture results are dependent upon the pretest probability that the patient has bacterial pneumonia and upon whether the patient has received antibiotics[13]. Although more than 55% of the samples were recruited from what is presumed to be a specialty clinic for chest diseases, clinicians might be sending sputum samples to the laboratory without having supporting clinical and radiological data.

All accepted samples were examined microscopically by two microbiologists in our study and there was a good agreement between observers. Previous studies indicated a low concordance of Gram-stained specimens examined by different technicians, whereas others found the results to be reproducible[12,14-17]. Overall sensitivity of Gram smear in our study was 78.6% with a specificity rate of 82%. Ewig et al[15] did not recommend sputum collection for diagnosis of community acquired pneumonia and suggested that Gram stain had a low diagnostic yield and a low number of positive samples had a corresponding growth in culture. Whereas Parry et al[16] suggested that sputum Gram smear can be a guide to the etiology of pneumonia, particularly pneumococcal pneumonia. Reed et al[17] revealed that, in good-quality sputum samples the sensitivity and specificity of Gram was 35.4% and 96.7% for *S. pneumoniae* and 42.8% and 99.4% for *H. influenzae*, respectively. The specificity of Gram smear was reached as 100% for *H. influenzae* and *S.pneumoniae* in our study. Previous studies showed that the washing procedure of sputum decreased the mean concentration of contaminants by 100 to 1000 fold and enhanced the value of the sputum samples[2,6].

We theorized that washing procedure facilitates the detection of Gram negative bacteria, mainly thin *H. influenz*ae bacilli in smears, probably related to the removal of mucus and contaminants from sputum related with saline washing and homogenization. Quantitative inoculation of sputum samples significantly increased overall culture positivity from 52% to 63.5% of our samples. In 32 samples, pathogenic bacteria were isolated only in quantitative cultures and in 24 of them the predominance of indicative bacteria was detected in the Gram smear. If bacterial count is greater than 106 cfu /ml in sputum with a predominance of related bacteria in Gram smear, with under 25 leukocytes per LPMF, this should improve the clinical management of the pneumonia.

The three most commonly isolated pathogens were *Haemophilus influenzae , Pseudomonas aeruginosa* and *Streptococcus pneumoniae* . These organisms were isolated at different rates in most outpatient studies of acquired LRTI in most series[5,10,16]. In 18 samples, multiple pathogens were isolated in significant numbers. However, it is not clear whether these agents act as co-pathogens or not.

The management of LRTI is remarkably simplified when the responsible pathogens are accurately identified[1,18-20]. The main reason for detecting a microbiological cause of symptoms would be to select patients who could benefit from narrow-spectrum antibiotic treatment and to decrease bacterial resistance, side-effects and costs. Obtaining expectorated sputum samples is a non-invasive procedure and if done, it is best to use saline wash and then Gram stain and culture. Initial Gram staining of sputum samples, especially *for H. influenzae* and *S. pneumoniae* is advisable when experienced microbiologists interpret the slides, since Gram stain is almost as effective as cultivation and results are available 48 hours sooner. Culture results are most convincing when the organism isolated in the culture is compatible with the morphology of organisms present in the Gram smear[21]. One should keep in mind that discrepancy between direct examination and culture might also be associated to the fact that culture detects only viable bacteria whereas Gram staining may also detect non viable bacteria which might be related with previous antimicrobial consumption. On the other hand, it is mandatory to prepare guidelines for appropriate antimicrobials for empirical therapy and to reduce mortality with proper treatment cultivation and antimicrobial susceptibility data.

Acknowledgement. Major financial support for this study was provided by a research grant of Marmara University Research Fund (Project number SAG-TUS-200906-0168).

REFERENCES

1. Winchester CC, Macfarlane T, Thomas M, Price D. Antibiotic prescribing and outcomes of lower respiratory tract infection in UK primary care. Chest 2009, 135: 1163-1172.
2. Baselski V, Wundericink R. Bronchoscopic diagnosis of pneumonia. Clin Microbiol Rev 1994; 7: 533- 558.
3. Mandell LA, Wunderink RG, Anzueto A, Bartlett JG, Campbell D. Infectious Diseases Society of America/American Thoracic society consensus guidelines on the management of community-acquired pneumonia in adults. Clin Infect Dis 2007; 44: 27- 72.
4. Bartlett JG, Finegold SM. Bacteriology of expectorated sputum with quantitative culture and wash technique compared to transtracheal aspirates. Am Rev Respir Dis 1978; 7:1019-1027.
5. Kamruddin A, Wilson S, Jamal WY, et al. Causative bacteria of respiratory tract infections in Kwait by quantitative culture of sputum. J Infect Chemother 1999;5:217-219.
6. Cao LD, Ishiwada N, Takeda N, et al. Value of washed sputum Gram stain smear and culture for management of lower respiratory tract infections in children. J Infect Chemother 2004; 10:31-36.
7. Woodhead M, Blasi S, Ewig S, et al. Guidelines for the management of adult lower respiratory tract infections. Eur Respir J 2005; 26:1138-1180.
8. Geckler RW, Gremillion DH, MacAllister CK, Ellenbogen C. Microscopic and bacterial comparison of paired sputa and transtracheal aspirates. J Clin Microbiol 1977; 6: 366- 369.
9. Roche N, Kouassi B, Rabbat A, Mounedji A, Lorut C, Huchon G. Yield of sputum microbiological examination in patients hospitalized for exacerbations of chronic obstructive pulmonary disease with purulent sputum. Respiration 2007; 74: 19-25.
10. Miravitlles M, Espinosa C, Fernandez-Laso E, Martos JA, Maldonado JA, Gallego M. Relationship between bacterial flora in sputum and functional impairment in patients with acute exacerbations of COPD. Study Group of Bacterial Infection in COPD. Chest 1999;116:40¡V46.
11. Allegra L, Blasi F, Diano P, et al. Sputum color as a marker of acute bacterial exacerbations of chronic

obstructive pulmonary disease. Resp Med 2005; 99: 742- 747.
12. Cooper GM, Jones JJ, Arbique JC, Flowerdew GJ, Forward KR. Intra and inter technologist variability in the quality assessment of respiratory tract specimens. Diagn Microbiol Infect Dis 2000; 37: 231¡V235.
13. Carrol K. Laboratory diagnosis of lower respiratory tract infections: controversy and conundrums. J Clin Microbiol 2002; 40: 3115- 3120
14. Nagendra S, Bourbeau P, Brecher S, Dunne M, LaRocco M, Doern G. Sampling variability in the microbiological evaluation of expectorated sputa and endotracheal aspirates. J Clin Microbiol 2001; 39:2344¡V2347.
15. Ewig S, Schlochtermeter M, Göke N, Niederman MS. Applying sputum as a diagnostic tool in pneumonia. Limited yield, minimal impact on treatment decisions. Chest 2002; 121:1486-1492.
16. Parry CM, White RR, Ridgeway ER, Corkill JE, Smith GW. The reproducibility of sputum Gram film interpretation. J Infect 2000; 41:55-60.
17. Roson B, Carratala J, Verdaguer R, Dorca J, Manresa F, Gudiol F. Prospective study of the usefulness of sputum Gram stain in the initial approach to community-acquired pneumonia requiring hospitalization. Clin Infect Dis 2000;31:869¡V874
18. Reed WW, Byrd GS, Gates RH Jr, Howard RS, Weaver MJ: Sputum Gram¡¦s stain in community-acquired pneumococcal pneumonia- A meta-analysis. West J Med 1996; 165:197-204.
19. Van der Eerden MM, Vlaspolder F, Graaff De CS, Groot T, Jansen HM, Boersma VG. Value of intensive diagnostic microbiological investigation in low- and high- risk patients with community-acquired pneumonia. Eur J Clin Microbiol Infect Dis 2005; 24: 241-249.
20. Kuijper EJ, Van Der Meer J, de Jong MD, Speelman P, Dankert J. Usefulness of Gram stain for diagnosis of lower respiratory tract infection or urinary tract infection and as an aid in guiding treatment. Eur J Clin Microbiol Infect Dis 2003; 22:228-234.
21. Skerrett SJ. Diagnostic testing for community-acquired pneumonia. Clin Chest Med 1999;20:531¡V548.