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RESEARCH ARTICLE

Validation of sputum gram stain and culture for diagnosis of pneumonia in critically iii patient: a retrospective observational study

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ABSTRACT

Validation of culture results based on direct gram stain plays a vital role in differentiating pathogens' cause of infection or colonization in pneumonia cases. Our study aims to evaluate the validation of diagnostic microbiology based on gram stain and culture compared to chest radiography in critically ill patients with suspected pneumonia. This was a single-center retrospective data analysis in the Intensive Care Unit at Secondary Care Hospital in Central Java. The quality of sputum was determined by using a Modification Criteria of Bartlett and a Semi-Quantitative Score. The results of sputum culture with neutrophils count > 10 and bacterial count > 2 per field in Gram stain were considered the presumptive pathogen. Seventy sputum specimens were collected; however, only 58 were selected for further analysis in this study. In sputum specimens with Bartlett score +2 and Semi-Quantitave score 3 or 4, the chest x-ray results of all patients had positive infiltrates (100%). Diagnostics accuracy of the results of clinical microbiologist examination with chest X-ray had a sensitivity of 90%, specificity of 60.71 %, accuracy of 65.5%, a positive predictive value of 61.36%, a negative predictive value of 78.6%. It was concluded that validation of diagnostic microbiology based on gram stain and culture had susceptible and adequately specific results to differentiate between presumptive pathogens from colonization in pneumonia cases.

1. Introduction

Pneumonia remains a worldwide health problem, with a significant morbidity and mortality rate, especially among the elderly and patients with comorbidities (Del Rio-Pertuz *et al.,* 2019; Kaffashian *et al.,* 2022). Determining whether pneumonia is the root of clinical symptoms such as sepsis (Upchh *et al.,* 2018) is crucial. Clinical pneumonia resulted in 6.8 million hospitalizations worldwide and about 1.1 million in hospital Dhs 2015 (Chebib *et al.,* 2021). In Indonesia, pneumonia is one of the ten most common inpatient care cases, with a crude fatality rate (CFR) of 7.6% compared to other diseases (Dharmawan *et al.,* 2020).

Gram stain and sputum culture are routine tests in microbiology laboratories for critically ill patients with suspected pneumonia (Huang *et al.,* 2020). The sputum gram stain is an inexpensive, fast, and convenient laboratory method that predicts the bacterial pathogen. It can also guide the clinician in choosing appropriate antibiotics for prompt treatment (Huang *et al.,* 2020). Direct gram stain of clinical specimens is used to determine whether a sample is representative of the site of infection (Mariraj *et al.,* 2011). Inappropriate specimen collection, specimen processing, smear preparation, and prior antibiotics therapy are all factors that can influence gram stain results (Samuel *et al.,* 2016). Sputum specimens are frequently contaminated

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Table 1. Bartlett's Criteria and Semi-quantitative scoring of Gram stain (the number of bacteria per high-power (×1.000) oil immersion field)

Parameter	Criteria	Score
Neutrophil (PMN) count based on Bartlett's Criteria	< 10 Neutrophils /10 x field	0
	10 – 25 Neutrophils /10x field	+1
	>25 Neutrophils /10 x field	+2
Bacterial count based on Semi-quantitative scoring of Gram stain (the number of bacteria per high-power (×1.000) oil immersion field)	No bacteria per field	0
	≤1 bacteria per field	1
	2 – 5 bacteria per field	2
	6 – 30 bacteria per field	3
	>30 bacteria per field	4

by resident flora of the oropharynx. Sputum is often watery saliva, leading to mistaken results (Chinnnusamy *et al.,* 2016).

Consequently, identifying microbial pathogens causing pneumonia is challenging for conventional microbiological methods (Torres *et al.,* 2016). The interpretation of Gram stain and culture results is prone to variability and differences between clinical microbiologists. A sputum culture is another diagnostic test to identify pathogens and guide antibiotic therapy (Huang *et al.,* 2020).

Neutrophils are indirect indicators of an acute bacterial infection, which can be observed from microscopic examination. A high neutrophil count will increase the likelihood of bacterial infection. (Huang *et al.,* 2020) When clinical findings suggest pneumonia, various tests are performed to determine the causative pathogen and chest radiography (Nambu *et al.,* 2014). Our study aims to evaluate the validity of diagnostic microbiology based on Gram stain and culture compared to chest radiography in critically ill patients with suspected pneumonia.

2. Materials and Methods

This research was a single-center retrospective study in the Intensive Care Unit at Secondary Care Hospital in Central Java. This study was conducted after obtaining an ethical clearance certificate from the Health Research Ethics Committee of Teaching Hospital Sultan Agung with certificate number No.64/KEPK-RSISA/VII/2022). The inclusion criteria were adult patient (≥18 years), hospitalized with suspected pneumonia (the presence of a new pulmonary infiltrate on chest radiograph at the time of hospitalization associated with 1 of the following: (1) new or increased cough with/without sputum production; (2) fever (>37.8° C) or hypothermia; (3) pathological lung auscultation). The exclusion criteria were incomplete medical records, poor quality of the sputum (epithelial cell >10 per LPF), and the specimen either showing no growth on culture or mixed culture. The medical records of all patients suspected of pneumonia were enrolled in the study between August and December 2022. Pneumonia diagnosis was based on clinical signs and symptoms of lower respiratory tract infection and the finding of a new infiltrate on the chest radiographs. Sputum samples were collected in a sterile container and then transported and processed immediately in the microbiology laboratory for gram staining and culture to determine the bacterial pathogen. Gram-stained sputum smears were observed under a microscope to count the epithelial cells, neutrophils, and bacteria. The quality of the sputum was determined based on the Modification Criteria of Bartlett and the Semi-Quantitative Score on the gram-stained smear result (Tables 1). The sputum was processed when the squamous epithelial cells (SECs) were < 10 per Low Power Field (LPF).

The sputum specimens were inoculated on Blood Agar and Mac Conkey Agar, then incubated overnight at 37^0C. After 24 hours, the inoculated plates were observed for bacterial growth. Plates with pure isolate and predominant isolate were included. Kirby Bauer's disc diffusion method on Mueller Hinton agar was performed for antibiotic susceptibility testing.

The clinical characteristics, including age, gender, outcome, Bartlett Criteria, and Semi-Quantitative Score in patients with positive and negative infiltrates, were compared. An acute infiltrate on a chest X-ray defined pneumonia. Sputum specimens were considered of good quality when they had <10 squamous epithelial cells (SEC) per low-power field (LPF) and >10 Neutrophils (PMN) per oil immersion field. The presumptive pathogen's determination criteria were the neutrophils count ≥ 10 and bacterial count ≥ 2 per field in Gram stain.

The validity of the current diagnostic method was measured using a diagnostic accuracy test. The sensitivity, specificity, positive predictive value, negative predictive value, and test accuracy were calculated according to standard equations. All data were processed in SPSS 24.0 (SPSS Inc., Chicago, IL, USA).

Table 2. Basic characteristics of the study population

Description	Negative Infiltrates (n = 28)	Positive Infiltrates (n=30)
Gender		
➢ Male (n = 32)	15 (53.6%)	18 (60.0%)
➢ Female (n= 28)	13 (46.4%)	12 (40.0%)
Age (Year-Old)		
➢ 18 - 60	20 (71.4%)	19 (63.3%)
➢ > 60	8 (28.6%)	11 (36.7%)
Outcome		
➢ Survival	18 (64.3%)	12 (40.0%)
➢ Death	10 (35.7%)	18 (60.0%)
Bartlet Criteria		
➢ < 10 Neutrophils /10 Field (Score 0)	9 (32.1%)	3 (10.0%)
➢ 10 - 25 Neutrophils /10 Field (Score +1)	5 (17.9%)	6 (20.0%)
➢ >25 Neutrophils /10 Field (Score +2)	14 (50.0%)	21 (70.0%)
Semi-Quantitative Score		
➢ No Bacteria Per Field (Score 0)	5 (17.09%)	0 (0.0%)
➢ <= 1 Bacteria Per Field (Score 1)	15 (53.06%)	15 (50.0%)
➢ 2 - 5 Bacteria Per Field (Score 2)	6 (21.04%)	7 (23.3%)
➢ 6 - 30 Bacteria Per Field (Score 3)	2 (7.1%)	7 (23.3%)
➢ > 30 Bacteria Per Field (Score 4)	0 (0.0%)	1 (3.3%)

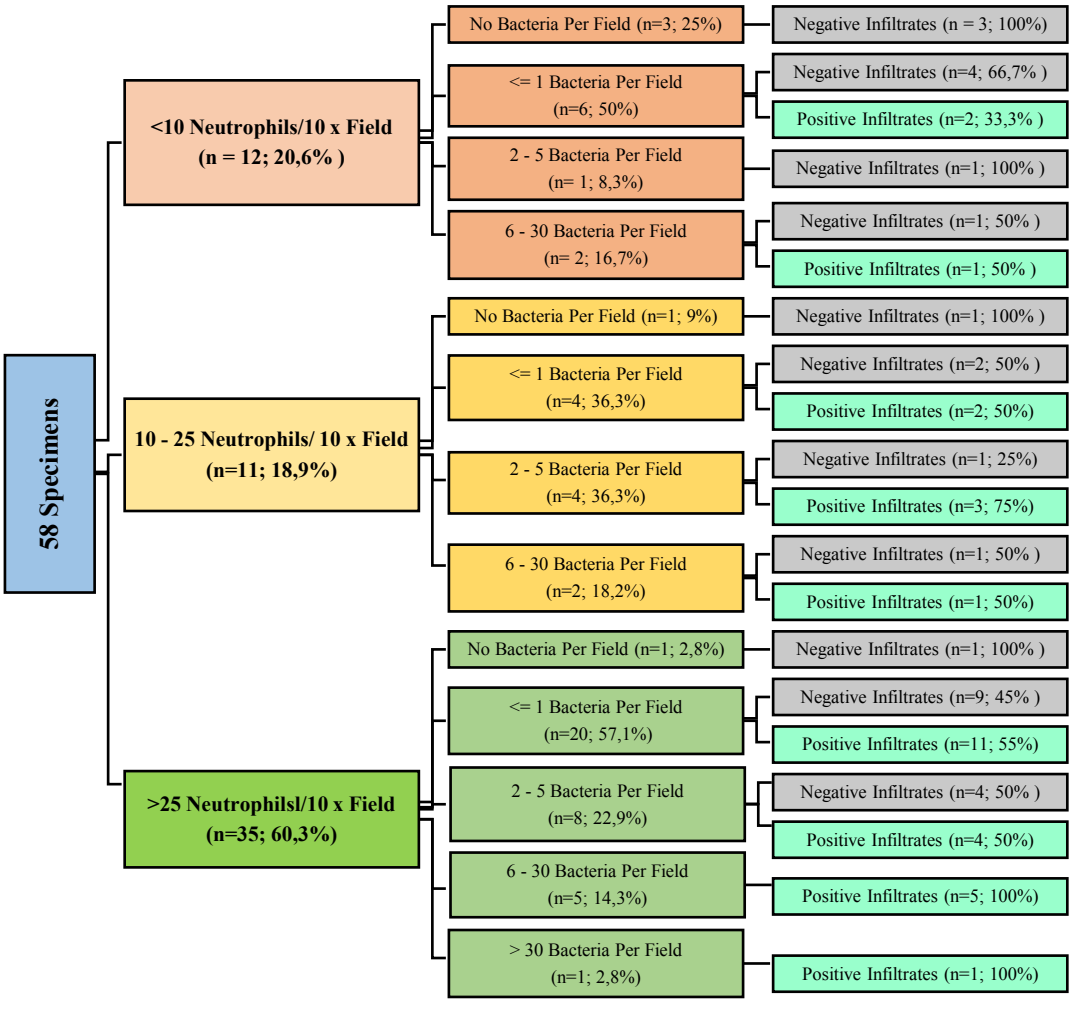

Figure 1. Flow chart of this study

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Table 3. Diagnostic performance of Validation Results of Clinical Microbiologist with Chest X-Ray

Validation Result of Clinical Microbiologist	Chest X-Ray		Total
	Positive Infiltrate	Negative Infiltrate	
Presumptive Pathogen	27	17	44
Colonization	3	11	14
Total	30	28	58

Table 4. Result of sputum culture

Microorganism	Number of Positive Culture (%)
Klebsiella pneumoniae	19 (32.8%)
Acinetobacter baumannii	14 (24.1%)
Pseudomonas aeruginosa	12 (20.7%)
Escherichia coli	7 (12.1%)
Stenotrophomonas maltophilia	4 (6.9%)
Aeromonas hydrophilia	1 (1.7%)
Serratia marcescens	1 (1.7%)

3. Results

A total of 70 sputum specimens were collected during the study period. However, 12 specimens were excluded (8 specimens had squamous epithelial cells count > 10 per field, two had no growth on culture, and two showed mixed cultures). Only 58 sputum specimens were selected for further analysis in this study. Patients were classified based on chest radiographs into positive infiltrates (n=30) and negative infiltrates (n=28). The characteristics of the study population are summarized in Table 2.

Most of the suspected pneumonia patients in the negative and positive infiltrate groups were male (53.6% vs 60%) and aged 18 – 60 (71.4% vs 63.3%). The fatality rate in the positive group was almost twice as much as the negative group (60% vs 35.7%). In the positive infiltrate group, the scores based on Bartlett's Criteria were as follows: scores 0, score +1, and score + 2 were 10%, 20%, and 70%, respectively. In the negative infiltrate group, score 0 was 32,1 %, score +1 was 17,9%, and score +2 was 50%. Based on the semi-quantitative score, the number of bacteria showed that 17.9% of samples in the negative infiltrate group had a score of 0. In contrast, no specimen in the positive infiltrate group scored 0.

As shown in Figure 1, fifty-eight sputum specimens were assessed using Bartlett criteria and semi-quantitative scores. Twelve specimens (20,6%) showed < 10 Neutrophils /10 Field (score 0); 11 (18,9%) showed 10 - 25 Neutrophils/10 Field (score +1); and 35 (60.3%) showed >25 Neutrophils /10 Field (score +2). In sputum specimens with Bartlett score +2 and Semi-quantitative score 3 or 4, the chest x-ray results of the patients were all positive for acute infiltrates (100%). This result indicates that the sputum score positively correlates with the acute infiltrate in the chest x-ray. Table 3 shows the diagnostics performance of clinical microbiologists' validation results compared to chest X-ray results. It has a sensitivity of 90%, specificity of 60,71 %, accuracy of 65,5%, Positive Predictive Value (PPV) of 61,36%, and Negative Predictive Value (NPV) of 78,6%; $p = 0.009$ ($p < 0.05$). The relatively low specificity of the sputum analysis compared to the chest X-ray (CXR) finding was because a considerable portion of negative CXR had positive sputum analysis, reflecting the lower specificity of microbiological testing we performed. This study found that the most common isolates of the sputum cultures were *Klebsiella pneumonia* (32,76%), *Acinetobacter baumanii* (24,14%), and *Pseudomonas aeruginosa* (20,69%) (Table 4).

4. Discussions

Most patients with positive infiltrates in this study were male and aged 18-60 years old. Older people are more susceptible to pneumonia and more likely to die from infection than younger populations. Elderly pneumonia patients who require hospitalization are more likely to develop complications that require extended hospital stays (Wei *et al.,* 2015). A total of 51% of patients with a diagnosis of pneumonia had positive infiltrates on X-ray chest result. Manifestation of pneumonia in various forms and their imaging finding are often non-specific. When a patient has clinical symptoms suggestive of infectious pneumonia, such as fever, cough, or sputum, and the imaging findings are consistent with pneumonia, a definitive diagnosis can be made (Metlay *et al.,* 2019; Nambu, 2014). Chest radiography is the first-line imaging test for identifying pneumonia. Radiographic findings associated with pneumonia include consolidation, opacity, airspace disease, density, haziness, ground glass, and infiltrate. Although imaging is required for diagnosing pneumonia, studies have shown varying degrees of statistical measures of the performance of chest radiography for identifying pneumonia. Sensitivity has ranged from 32% to 77.7%, and specificity has ranged from 58.8% to 94% (Makhnevich *et al.,* 2019). Chest radiography is advised by international recommendations as a standard evaluation of a patient suspected of having pneumonia, although it is an insensitive approach with only moderate accuracy. Wesley *et al.* assessed the effectiveness of using computed tomography (CT) scanning in combination with chest radiography to diagnose pulmonary opacities. The sensitivity of chest radiography was 43.5% (95% CI, 36.4%-50.8%). PPV was 26.9% (95% CI, 22.1%-32.2%)

of the final radiologist reports of noted opacity, infiltrate, consolidation, pneumonia, or bronchopneumonia in 3,423 adult emergency department patients with acute cardiopulmonary symptoms (Rider & Frazee, 2018). When the clinical presentation indicates pneumonia but the initial chest radiograph does not show radiological evidence, Cameron *et al.* advise using CT imaging to evaluate for pneumonia (Upchurch *et al.,* 2018; Ye Xiong *et al.,* 2015). Although a thoracic CT scan is the "gold standard" for detecting pneumonia and other lung diseases, it cannot be used as the first-line radiological assessment in all patients with suspected pneumonia. This is primarily because it is frequently expensive, unavailable, and involves significant radiation exposure. CT-only pneumonia appears more prevalent in obese patients, possibly due to reduced chest radiography sensitivity caused by adipose tissue attenuation (Ye Xiong *et al.,* 2015). A trained radiologist and confirmation by clinical history, vital signs, and laboratory evaluation strongly impact the diagnosis of pneumonia on chest radiography (Shih *et al.,* 2019). Infiltrates on radiographs can also be subtle: a single radiologist can overlook infiltrates up to 15% of the time, and two radiologists examining the same chest radiograph disagree 10% of the time (Wunderink & Waterer, 2014).

Our study assessed sputum specimens using Bartlett criteria and semi-quantitative scores. Sputum Gram stain still plays a significant role in diagnostic testing in the microbiology laboratory for patients with community-acquired pneumonia and other lower respiratory tract infections. Incorrectly interpreted Gram stains can lead to inappropriate therapy and potentially deteriorate patient outcomes (Samuel *et al.,* 2016). Good quality sputum specimen is essential to detect diseases. The Bartlett criterion was chosen due to its convenience in interpretation and lower rejection rate than other criteria. The limitation of human vision is one of the obstacles in the interpretation of Gram staining, which causes a high error rate (Azman *et al.,* 2014). The diagnostic performance of sputum Gram stain in CAP varies in different studies. The meta-analysis evaluating the sputum Gram stain in community-acquired pneumococcal pneumonia showed that the sensitivity ranged from 15-100% and specificity from 11-100%. The IDSA/ATS guidelines recommend that Gram stain be performed only if quality performance measures for collecting, transporting, and processing samples can be met. Previous studies reported that receiving antibiotics before sputum sample collection adversely affects the performance of Gram stain. Not detecting these bacteria on sputum Gram stain does not mean the absence (Fukuyama *et al.,* 2014). Therefore, the value of sputum samples might be dubious in persons who have been taking antibiotics before admission (García-Vázquez *et al.,* 2004). The sputum samples are often contaminated saliva and contain resident oropharyngeal microbial flora. When a potential pathogen is isolated from the sputum sample, it is often difficult to decide whether the potential pathogen is an etiological agent or represents oropharyngeal contamination. The microbiology laboratory must use objective criteria by Gram stain screening for purulence before inoculation into culture media (Chinnnusamy *et al.,* 2016; Mariraj *et al.,* 2011). Without microscopy, culture results are of unknown relevance and may be misleading. Hence, diagnosing respiratory infection by sputum culture without microscopic examination invites confusion and misinformation. To minimize the effect of oropharyngeal contamination on the lower respiratory tract, Bartlett, Murray, and Washington devised screening criteria based on the quantitation of leucocytes and squamous epithelial cells (Mariraj *et al.,* 2011). Due to the inevitable oropharyngeal bacterial contamination that occurs in the collection of all respiratory secretion samples, quantitative culture techniques are always needed to differentiate oropharyngeal contaminants present at low concentrations from higher-concentration infecting organisms (Miyashita *et al.,* 2008).

In this study, sputum specimens with Bartlett score +2 and Semi-Quantitave score 3 or 4 had positive infiltrates in the CXR (100%), indicating a good correlation between sputum scoring and CXR result. Based on the Bartlett Score, the neutrophils (PMN cells) and epithelial cells were observed under a microscope in 20-30 low-power fields. An average number of epithelial cells and pus cells was calculated. The final score value of less than or equal to 0 indicates salivary contamination of the sputum sample or lack of active inflammation (non-acceptable sputum sample). Chinnnusamy (2016) reported that in 130 sputum samples, 72 (55.4%) were acceptable, and 8 (44.6%) were non-acceptable based on Bartlett's screening criteria. In contrast, Daniel Musher *et al.* reported a low percentage of 31% acceptability. Also, Ravich and Ran *et al.* said a low percentage of acceptability that all 74 (100%) of their sputum samples were in the non-acceptable category. Bartlett's sputum grading system is not applicable for lower respiratory tract infections caused by viruses, fungi, *Mycobacterium tuberculosis*, and Legionella species (Chinnnusamy *et al.,* 2016).

The pathogens of pneumonia are a wide variety of microorganisms, including not only ordinary bacteria but also mycobacteria, viruses, and fungi (Samuel *et al.,* 2016). This study found that the most common pathogen isolates result of sputum culture obtained were *Klebsiella pneumonia* (32,76%), *Acinetobacter baumanii* (24,14%), and *Pseudomonas aeruginosa* (20,69%)

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(Table 4). Chinnnusamy *et al.* reported similarly in their study that the most common isolates obtained were *Klebsiella pneumonia* (31.71%) (Chinnnusamy *et al.,* 2016). Another study in Persahabatan Hospital also showed that Gram-negative bacteria, such as *Klebsiella pneumoniae, Acinetobacter baumannii*, *and Pseudomonas aeruginosa,* as the most common causal pathogens for Community-Acquired Pneumonia (CAP) (Dharmawan *et al.,* 2020). Another study showed different results. Holter *et al.* found *Streptococcus pneumoniae* and Rhinovirus to be the highest pathogen causing CAP (Holter *et al.,* 2015). The high prevalence of *Klebsiella pneumonia* and other Gram Negatives may be due to the tropical climate in Semarang, which has higher temperatures and humidity (Farida *et al.,* 2013).

This study had several limitations that have to be pointed out. First, this study is a single center with limited subjects. Second, the limitations of Gram staining depend on the observer's skill and experience. Third, our study only examined culture from aerobic microorganisms, not anaerobic microorganisms, atypical microorganisms, and viruses. Fourth, validation of diagnostic microbiology based on gram stain and culture, besides being compared with chest radiography, can also be confirmed by a CT scan. Future studies are suggested to verify this study's results.

5. Conclusions

In conclusion, neutrophils count $\geq$ 10, and bacterial count $\geq$ 2 per field in sputum Gram stain were highly sensitive and adequately specific to differentiate presumptive pathogens of infection or colonization in pneumonia cases. In this study, the authors recommended processing good-quality sputum, firming the clinical history, and imaging examinations to decide on the potential pathogen in case of pneumonia. The Gram stain and culture of sputum samples can be helpful for the diagnosis of bacterial pneumonia. They may be beneficial in guiding pathogen-directed antimicrobial therapy only when a good-quality sputum sample is available.

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Conflict of interest

All authors have no conflict of interest in this article.

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