

## RESEARCH ARTICLE

## Epithelial Cells Count and the Ratio of Leukocytes and Epithelial Cells as the Criteria to Determine Qualified Specimen for Community-Acquired Pneumonia (CAP)-causing Pathogens Identification

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### Abstract

**BACKGROUND:** Community-acquired pneumonia (CAP) is the most common infectious with serious rate of morbidity and mortality. Recent conventional method only described 30-50% of CAP etiology. Sputum specimen quality assessment is important to obtain an accessible CAP-causing pathogens identification.

**METHODS:** This was a prospective descriptive study involving 100 specimens from CAP-diagnosed subjects in Budhi Asih Regional General Hospital inpatient care. We assessed three gram-staining criteria for specimen quality determination, and continued by bacterial identification.

**RESULTS:** All specimens were qualified according to criteria II, while only 94 and 96 specimens were qualified according to criteria I and III, respectively. Sixty-five specimens could be identified by culture and pneumoCLART polymerase chain reaction (PCR)

examination, and the 35 specimens remained unknown. Ten out of those 35 specimens were positive after analyzed by Acid-fast Bacilli (AFB) test. The pathogens we identified including *Klebsiella pneumoniae* (29.6%), *Acinetobacter baumannii* (10.2%), *Enterobacter cloacae* (4.6%), *Pseudomonas aeruginosa* (4.6%), *Staphylococcus aureus* (4.6%), *Moraxella catarrhalis* (3.7%), *Enterobacter aerogenes* (2.8%), *Escherichia coli* (2.8%), *Streptococcus pneumoniae* (1.9%), *Mycoplasma pneumoniae* (1.9%) and *Citrobacter koseri* (0.9%).

**CONCLUSION:** There were no significant differences among the three criteria for sputum specimen quality assessment, based on culture and pneumoCLART examination. We suggest that criteria II could be used to avoid many specimen rejections while good quality specimens still attained for accessible bacteria identification.

**KEYWORDS:** community-acquired pneumonia, sputum, gram stain, pathogens, bacteria

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### Introduction

Community-acquired pneumonia (CAP) is the most common infectious disease, and particularly becomes the most life-threatening infection in developing countries.(1,2) Pneumonia is found as one of the 10 highest inpatient care

cases in Indonesia including 53.95% males and 46.05% females, with 7.6% crude fatality rate (CFR), counted as the highest compared to other diseases.(3)

CAP is defined as the lung parenchyma infection that is acquired outside the hospital or healthcare facility, or pneumonia that occurred less than 48 hours before hospital admission.(4,5) Understanding the etiology of CAP is

important to determine the adequate antimicrobial treatment. (6,7) A study in the United States found the most common pathogens for CAP were human rhinovirus, followed by the influenza virus, and *Streptococcus pneumoniae*. (8) Studies in Norway and Iran found *Streptococcus pneumoniae* to be the most common causative pathogen. (7,9) Another study in Semarang, Indonesia, identified influenza virus, followed by *Klebsiella pneumoniae* and *Streptococcus pneumoniae* as the most common causal pathogens for CAP. (6) Meanwhile in Persahabatan Hospital, Jakarta, Indonesia, *Klebsiella pneumoniae* was identified as the most common, followed by *Acinetobacter baumannii*. (3)

The conventional method currently applied to identify CAP-causing pathogens performed by sputum gram staining and culture examination, but so far only 30-50% of the pathogens could be identified. (10) The gram staining is an easy, cheap and simple method. However, the usefulness of gram staining method in CAP patients was still in debate, due to the difficulty in determination for suitable criteria to avoid too many specimen rejection, while good quality specimens were still obtained, so the precise information could be provided for CAP initial therapy. (11-13)

Some criteria that commonly used recently to assess the sputum quality including: 1) Good quality specimen is defined as the number of epithelial cells <10/low-power field (LPF) or white blood cells (WBC) >10 times of epithelial cells, and single bacterial morphology found  $\geq 6$  cells/oil immersion field (OIF) on microscopic examination, as in accordance to The American Society for Microbiology (ASM) criteria (14); 2) Good quality specimen is defined as sputum having a total score  $\geq 1$  when examined at 100x magnification, as in accordance to Bartlett's grading system (13,15); 3) Good quality specimen is defined as sputum contains  $\geq 10$  WBCs for each one epithelial cell with 400x magnification (16); and 4) Good quality specimen is defined as number of epithelial cells <10/LPF or the ratio of leukocytes:epithelial cells ratio is >2.5 (6).

In this study, we evaluated three from four criteria mentioned before we continued for culture examination. The specimens that failed to grow in the culture process will be assessed further using polymerase chain reaction (PCR) by pneumoCLART.

## Methods

### Study Design and Specimens

This was a prospective descriptive study. This study has been approved by Medical Research Ethics Committee of Faculty of Medicine, Universitas Indonesia (No. 607/UN2.

F1/ETIK/2016). Sputum collected from subjects admitted with CAP during September 2016 - February 2017 in Budhi Asih Regional General Hospital. CAP was diagnosed based on Indonesian Society of Respiriology Guideline, including the epithelial cells of sputum <10/LPF, or the ratio of leukocytes:epithelial cells ratio is >2.5. Specimens were sent to Clinical Microbiology Laboratory, Faculty of Medicine, Universitas Indonesia for microbiological study, including the Gram staining, culture examination, and PneumoCLART examination.

There were three criteria used to define the quality of sputum. Criteria I defined as good quality sputum when the number of epithelial cells <10/LPF or WBC >10 times of epithelial cells, and single bacterial morphology found  $\geq 6$  cells/OIF on microscopic examination (14); criteria II defined good quality sputum when number of epithelial cells <10/LPF or the ratio of leukocytes:epithelial cells ratio is >2.5 (6); and criteria III defined good quality as sputum contains  $\geq 10$  WBCs for each one epithelial cell with 400x magnification (16). Any antibiotic therapy initiated before the microbiology study was recorded.

### Bacteria Identification

The quality of sputum was assessed using three Gram stain criteria (I, II and III). All qualified specimens were then inoculated on blood agar, chocolate agar, and MacConkey agar (Oxoid, Thermofisher Scientific, Massachusetts, USA), for bacterial identification using optochin disk test and incubation in Vitex®-2 system (bioMérieux, Marcy-l'Étoile, France). Optochin disk test was performed to presumptive identification of *Streptococcus pneumoniae* from other alpha-hemolytic streptococci, while Vitex®-2 system identified the typical bacteria. Atypical bacteria such as *Mycoplasma pneumoniae*, *Chlamydothilla pneumoniae*, or fastidious bacteria like *Streptococcus pneumoniae* and *Haemophilus influenzae* was then further investigated using pneumoCLART (Genomica, Madrid, Spain). For bacilli co-infection identification, Acid-fast Bacilli (AFB) test was performed using Ziehl-Neelsen stain.

### Data Analysis

A chi-squared distribution data analysis was performed using SPSS v.21.0 (IBM Corporation, New York, USA).

## Results

Total of 100 sputum specimen were collected from subjects based on the inclusion criteria. Sixty-seven subjects (67%) were male and 33 subjects (33%) were female. The age

ranged from 18–83 years old, with mean of 50.40±16.73 years old.

All specimens were qualified after assessed by criteria II, while 94% and 96% specimens were qualified based on criteria I and III, respectively. All specimens were then cultured for CAP-pathogen causing identification. The culture examination after specimens' quality assessment results was shown in Table 1.

**Table 1. Number of specimens after culture examination of specimens' quality assessment.**

Assessment Criteria	Pathogen Identified	No Pathogen Identified	Total
Criteria I	56	38	94
Criteria II	58	42	100
Criteria III	57	39	96

Forty-two specimens failed to be identified by the culture examination were assessed further using pneumoCLART. Seven specimens were identified successfully using this method and the other 35 were unsuccessful (Table 2, Table 3).

**Table 2. Number of specimens with positive pathogen identification after combination assessment of culture examination and pneumoCLART.**

Assessment Criteria	Pathogen Identified	No Pathogen Identified	Total
Criteria I	63	31	94
Criteria II	65	35	100
Criteria III	64	32	96

According to Infectious Diseases Society of America (IDSA) guideline, *Mycobacterium tuberculosis* should be considered as one of CAP-causing pathogens, although Indonesian Society of Respiriology stated the opposite. Due to tuberculosis (TB) endemic in Indonesia, we performed AFB staining using Ziehl-Neelsen method on all specimens and found 25% specimens were AFB positive. The complete workflow on AFB staining can be found in Figure 1.

During the study, any intravenous antibiotic therapy initiated before specimen collection was recorded. Forty-three percent of specimens were collected before antibiotic therapy, and 53% specimens collected less than 24 hours after antibiotic therapy. There were no significant differences between both specimens due to culture growth ( $p=0.664$ ).

**Table 3. Bacterial identification on sputum specimens by combination assessment of culture examination and pneumoCLART (n=100).**

Microorganism	Total n (%)
Normal flora	35 (32.4%)
<i>Klebsiella pneumoniae</i>	32 (29.6%)
<i>Acinetobacter baumannii</i>	11 (10.2%)
<i>Enterobacter cloacae</i>	5 (4.6%)
<i>Pseudomonas aeruginosa</i>	5 (4.6%)
<i>Staphylococcus aureus</i>	5 (4.6%)
<i>Moraxella catarrhalis</i>	4 (3.7%)
<i>Enterobacter aerogenes</i>	3 (2.8%)
<i>Escherichia coli</i>	3 (2.8%)
<i>Streptococcus pneumoniae</i>	2 (1.9%)
<i>Mycoplasma pneumoniae</i>	2 (1.9%)
<i>Citrobacter koseri</i>	1 (0.9%)
Total	108* (100%)

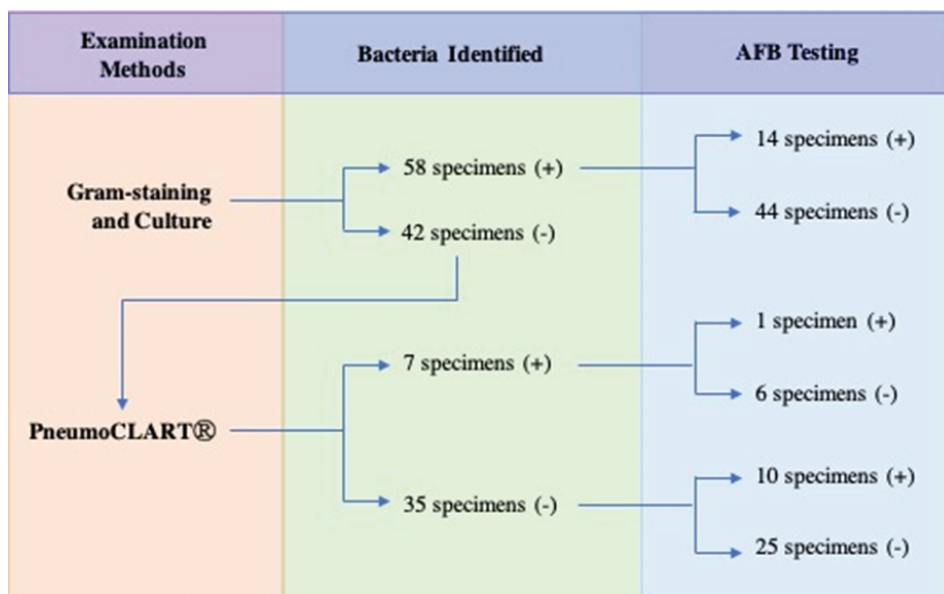
\*co-infections were identified in 8 specimens.

## Discussion

The quality of specimens was assessed by three criteria, and we found no significant difference between those three criteria. All specimens were qualified based on criteria II, suggest that criteria II could be recommended to prevent too many specimen rejection, while the good quality of specimens was still accessed.

After culture examination, 67% specimens (63/94) assessed by criteria I showed a positive identified pathogen, 65% (65/100) by criteria II and 66.7% (64/96) by criteria III. Some studies showed 63% and 57% positive identification on their culture.(12,17) Another study in subjects with bacteremic pneumococcal pneumonia, the specimens' quality was assessed by leukocyte count  $\geq 10$  times of the epithelial cells, and they found 31% gram staining positive result.(16) Whereas qualified sample assessed with LPF magnification (100x) by the number of epithelial cells  $< 10$  and the number of polymorphonuclear neutrophils (PMN) cells  $\geq 25$ , and had 45% and 65% positive results consecutively on culture.(18,19)

The culture examination identified 58% CAP-causing bacteria out of 100 specimens. We further investigated the rest 42 specimens using pneumoCLART and identified



**Figure 1. Workflow of AFB staining performed using Ziehl-Neelsen method on specimens after culture examination and PneumoCLART.** The assessment meant to investigate any co-infection with bacilli. Twenty-five percents of the specimens were found to be TB positive.

another 7 specimens, while the other 35 were remain unknown. A Study in Semarang, Indonesia found 32% CAP specimens were unidentified (6), and study in Iran, found 40% CAP etiology was unknown.(10). Another study even found 76% unidentified CAP specimens.(20)

The combination of culture examination and pneumoCLART identified 57 single CAP-causing bacteria in our specimens, and 8 specimens with co-infections including *Enterobacter cloacae* and *Enterobacter aerogenes*, *Klebsiella pneumoniae* and *Staphylococcus aureus*, *Klebsiella pneumoniae* and *Acinetobacter baumannii*, *Klebsiella pneumoniae* and *Enterobacter cloacae*, *Escherichia coli* and *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*, *Enterobacter cloacae* and *Staphylococcus aureus*, *Klebsiella pneumoniae* and *Escherichia coli*.

Most of the CAP-causing bacteria found in our specimens were dominated by Gram-negative bacteria, such as *Klebsiella pneumoniae* 32 isolates (29.6%), and *Acinetobacter baumannii* 11 isolates (10.2%) from a total of 108 isolates. Another study that was performed in Persahabatan Hospital also found mostly Gram-negative bacteria, such as *Klebsiella pneumoniae* (34%) and *Acinetobacter baumannii* (19.1%) as the CAP-causing pathogen (21), and a study in Semarang found *Klebsiella pneumoniae* (14%) and *Streptococcus pneumoniae* 13% as the most CAP-causing pathogens (6). Similarly, the study in Zainoel Abidin Hospital, Banda Aceh

found *Klebsiella pneumoniae* (47.7%) and followed by *Streptococcus pneumoniae* (20%) (22), and a study in Cambodia on acute lower respiratory tract infections subjects found *Klebsiella pneumoniae* (8%) as the most frequent infection causal (23).

Different results were showed by another studies, who found *Streptococcus pneumoniae* as the highest pathogen causing CAP, ranged from 15-38% while *Streptococcus pneumoniae* was only found 1.9% from all cases (7,9,20,24-26), suggested that different culture media contributed to different bacterial description. In our study, gentamicin blood agar was not performed due to cost limit. Different region of study could also present different pathogens, complicate with the highly frequent antibiotic use in Indonesia, especially in Jakarta, that contributed to change the pattern of pathogen.

The nasopharyngeal bacterial colonization also took part in determining CAP-causing pathogens. Data from a study in Semarang described that 15% adult aged 45-75 are *Klebsiella pneumoniae* carrier and 20% other Gram-negative bacteria while 11% children are *Streptococcus pneumoniae* colonization carrier.(6,27) A smaller prevalence of *Streptococcus pneumoniae* carrier (3%) was shown in a study performed in Jakarta, on older subjects group ( $\geq 60$  years old).(28) As the enteric microflora, *Klebsiella pneumoniae* and other Gram-negative carrier were usually associated with poor sanitation contamination, especially food and water.(29)



Atypical bacteria, such as *Mycoplasma pneumoniae*, cannot be identified by microscopic and culture examination due to its low culture-sensitivity, but requires PCR and serology method.(30) Thus, it was not recommended to be performed in routine.

We found that 25% of our subjects were infected by tuberculosis (TB), showed by AFB positive result. We suggested that AFB or TB culture examination should be considered for specimen with negative microscopic and culture examination results, especially those with negative results of atypical bacteria examination.(8,31)

Antibiotic initiation less than 24 hours before the specimen collection showed no significant effect on bacterial culture examination ( $p>0.05$ ) This was consistent with a study performed in Japan who found 80% vs. 80% positive culture between specimens with or without antibiotic less than 24 hours. However, an antibiotic therapy more than 24 hours before specimen collection will significantly reduce the culture growth (44% vs. 80%).(18) Other data also observed a similar result where the culture growth was significantly reduced on specimens with antibiotic initiation more than 24 hours before microbial procedure ( $p=0.03$ ). (15)

## Conclusion

There were no significant differences among the three criteria for sputum specimen quality assessment, based on culture and PneumoCLART examination, and we suggest that the criteria II could be used to avoid too many specimen rejections while good quality specimens still attained, and bacteria could be well-identified. Specimens could be collected less than 24 hours after antibiotic initiation. We recommend TB assessment (AFB and culture) to be performed on CAP patients, since in our findings 25% of CAP subjects were infected with TB as well.

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