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The significance of bacterial engulfment in Gram-stained sputum in patients with respiratory infections

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Abstract

In general, physicians believe that the presence of bacterial engulfment in white blood cells (WBCs) on Gram-stained sputum is a hallmark of lower respiratory infection. However, no studies have described the significance or diagnostic accuracy of engulfment in lower respiratory tract infections.

We prospectively studied sputum samples by Gram staining (Favor method) for their quality and engulfment score in WBCs obtained from patients with respiratory symptoms at inpatient and outpatient settings at Kyorin University Hospital between December 2012 and April 2015.

A total of 163 patients were enrolled. The patients were classified into an infection (n = 93) or non-infection (n = 70) group based on clinical or radiological findings prior to the evaluation of sputum samples. The proportion of engulfment-positive cases was equal in the infection and non-infection groups (49.5% vs 35.7%, $P = 0.11$). In the infection group, the engulfment score (%) for *Streptococcus pneumoniae* was significantly lower (median 3%, interquartile range [IQR]: 2% to 5%, $P = 0.005$) than that of the non-*S. pneumoniae* bacteria (*H. influenzae, M. catarrhalis*, and methicillin-susceptible *Staphylococcus aureus* (MSSA))(median 22.5%, IQR: 17% to 35.5%). The engulfment score of *S. pneumoniae* in the WBC was low in the infection group, and no cases were recognized in the non-infection group. Using a cut-off value of 3%, the diagnostic accuracy for infection was as follows: sensitivity: 50%, specificity: 65.7%, and area under the curve (AUC): 0.579 (95% CI 0.464 to 0.694). For the non-*S. pneumoniae* bacteria (*H. influenzae, M. catarrhalis*, and MSSA), the engulfment score was significantly higher in the infection group (median 22.5%, IQR 17 to 35.5%) than in the non-infection group (median 6.0%, IQR: 3 to 13%, $P = 0.011$), and the diagnostic accuracy for infection was as follows: sensitivity: 75%, specificity: 85.7%, and AUC: 0.902 (95% CI 0.75 to 1.00) when the threshold for the engulfment score was defined as 18%.

This study provides the first evidence that the engulfment of bacteria in WBCs is not always indicative of infection and that the engulfment score can fluctuate according to the pathogen.

Abbreviations: AUC = area under the curve, CAP = community-acquired pneumonia, COPD = chronic obstructive lung disease, *H. influenzae* = *Haemophilus influenzae*, IQR = interquartile range, *M. catarrhalis* = *Moraxella catarrhalis*, MAC = *Mycobacterium avium* complex, MSSA = methicillin-susceptible *Staphylococcus aureus*, ROC = receiver operating characteristic, *S. pneumoniae* = *Streptococcus pneumoniae*, WBCs = white blood cells.

Keywords: engulfment, gram stained sputum, phagocytosis

1. Introduction

Generally, with regard to Gram-stained sputum, the presence of the engulfment (phagocytosis) of bacteria by white blood cells (WBCs) is considered a sign of infection caused by those organisms. Phagocytic clearance by alveolar macrophages or polymorphonuclear and mononuclear cells is an important component of the host's lung defense system.[1] However, no reports have described the significance of the engulfment of bacteria in Gram-stained sputum. Therefore, we aimed to demonstrate that the engulfment of bacteria in Gram-stained sputum can be used as a diagnostic tool for respiratory infections.

2. Methods

2.1. Patients and study design

We prospectively studied patients with respiratory symptoms from inpatient and outpatient settings at Kyorin University Hospital (an 1100 bed tertiary center in Tokyo) between December 2012 and April 2015. Sputum samples were obtained from the patients, and a diagnosis of respiratory infection was made based on the deterioration from their usual status, need for antibiotics, physical examinations, and radiological or laboratory findings. All patients with bronchiectasis were enrolled in the study regardless of the presence of *Mycobacterium avium* complex (MAC). Sputum samples were obtained at the time of the initial referral to our

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The authors report no conflicts of interest.

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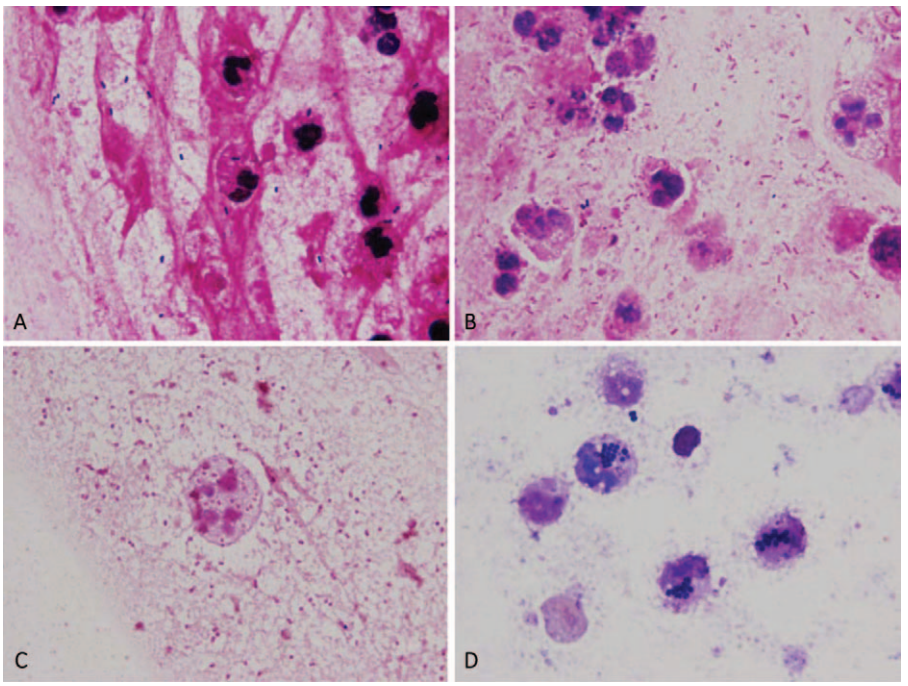

Figure 1. Representative image of bacterial engulfment on a sputum Gram stain. (A) *Streptococcus pneumoniae*. (B) *Hemophilus influenzae*. (C) *Moraxella catarrhalis*. (D) *Staphylococcus aureus*. Each image was taken at 1000 × magnification.

department or upon admission, and prior to the use of antibiotics. After Gram staining with Favor method,[2,3] samples were categorized into 6 groups according to the Geckler criteria,[4] in which only groups 4 and 5 are defined as high quality.[5]

2.2. Definition of bacterial engulfment in WBCs

Two physicians examined the presence of bacterial engulfment in the cytoplasm of WBCs (Fig. 1) by counting up to 100 WBCs 3 times using a consensus interpretation, in which the median value was adopted as the engulfment score (%) for the 100 WBCs.

2.3. Ethics approval

Samples were collected after written informed consent was obtained from the patients. The Ethics Committee on Human Research of Kyorin University Hospital approved the study protocol (H24-095) on November 7, 2012. The protocols were carried out in accordance with the approved guidelines.

2.4. Statistical analysis

Categorical data are presented as percentages of the total or numerically, as appropriate. Statistical comparisons of nonparametric data were performed using Mann–Whitney or Wilcoxon's signed-rank tests. Comparisons of categorical data were made using Pearson's chi-squared tests. All tests were two-sided. A value of $P < .05$ indicated statistical significance. All statistical analyses were performed with *EZR, version* 1.35.[6] (Saitama Medical Center, Jichi Medical University, Saitama, Japan), which is a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria). More precisely, it is a modified version of R commander designed to add statistical functions frequently used in biostatistics.

3. Results

3.1. Clinical characteristics of enrolled patients

A total of 163 sputum samples were obtained from 98 inpatients and 65 outpatients. The patients were classified into an infection group (n = 93) and a non-infection group (n = 70). The median age and gender ratio of each group was similar, with no significant differences (Table 1). Comparisons between the infection and non-infection groups showed that the underlying diseases were similar, except for chronic obstructive lung disease (COPD) (20.3% vs 5.8%, $P = .031$) and bronchiectasis with or

Table 1

Patients' background.

	Infection (+)	Infection (−)	*P* value
Number of patients	93	70	
Age (median, interquartile range)	69.7 (65.0–78.0)	69.0 (65.3–78.0)	0.534
Gender (male/female)	47/46	40/30	0.431
Diagnosis at enrollment in study			
Pneumonia	64 (68.8)	–	–
Bronchitis	24 (25.8)	–	–
Aspiration pneumonia	5 (5.4)	–	–
Seasonal influenza virus infection	4 (4.3)	–	–
Underlying disease	84 (90.3)	58 (82.9)	0.167
COPD	16 (20.3)	4 (5.8)	0.031
Bronchial asthma	7 (8.9)	9 (13.0)	0.295
Interstitial lung disease	6 (7.6)	11 (15.9)	0.070
Old pulmonary tuberculosis	6 (7.6)	2 (2.9)	0.468
Bronchiectasis with or without MAC	7 (8.9)	22 (31.9)	<0.001
Lung cancer	6 (7.6)	6 (8.7)	0.764

COPD = chronic obstructive pulmonary disease, MAC = mycobacterium avium complex.

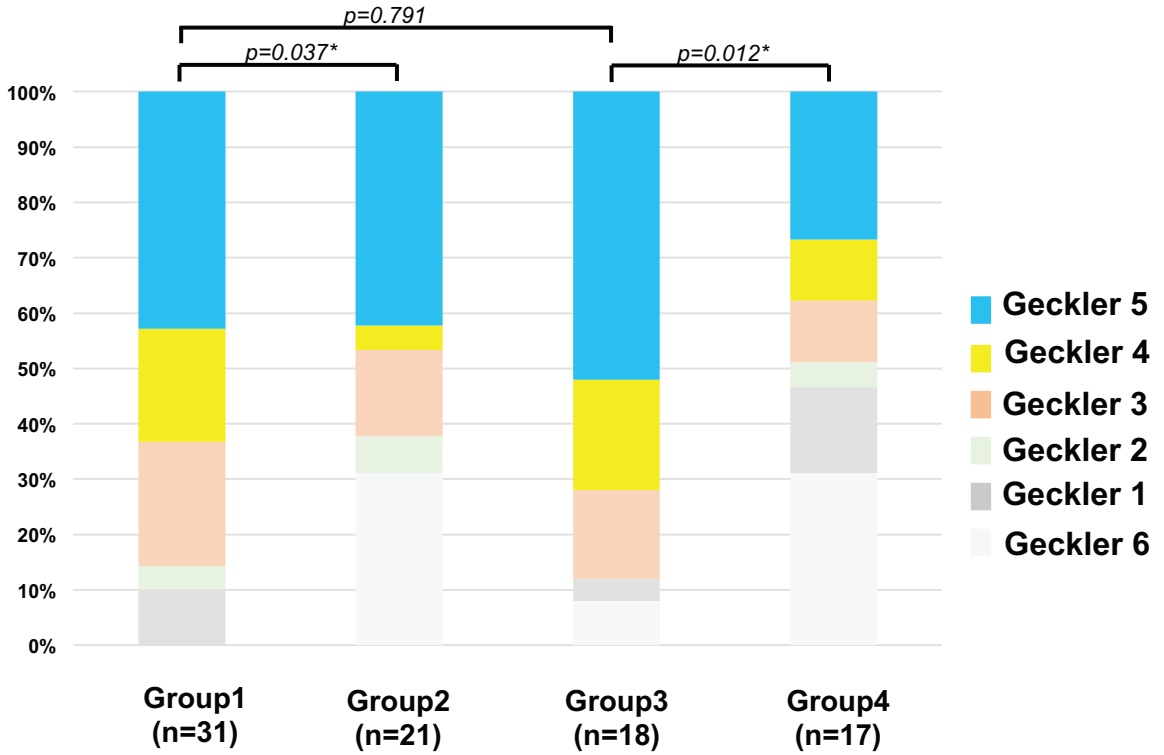

Figure 2. Geckler classification based on the presence of infection and engulfment. Group 1 (n=31): infection (+)/engulfment (+). Group 2 (n=21): infection (+)/engulfment (−). Group 3 (n=18): infection (−)/engulfment (+). Group 4 (n=17): infection (−)/engulfment (−). The figure depicts the proportion of Geckler classification levels in Groups 1–4. With respect to the proportion of high quality samples (Geckler 4 or 5), Group 1 had significantly higher values than Group 2 (67.4% vs 44.7%, $P=.037$). Group 3 had a higher proportion of high quality samples than Group 4 (72.0% vs 37.8%, $P=.012$). The ratio of high quality samples was not significantly different between Groups 1 and 3 (67.4% vs 72.0%, $P=.79$).

without MAC (8.9% vs 31.9%, $P<.001$). At the time of enrollment into the study, the infection group comprised individuals with pneumonia (n=64, 68.8%), bronchitis (n=24, 25.8%), aspiration pneumonia (n=5, 5.4%), and seasonal influenza virus infection (n=4, 4.3%).

3.2. Evaluation of the quality of sputum samples, with a focus on the presence of infection or engulfment

All enrolled patients were divided into 1 of 4 groups: Group 1 (n=31): infection (+)/engulfment (+), Group 2 (n=21): infection (+)/engulfment (−), Group 3 (n=18): infection (−)/engulfment (+), or Group 4 (n=17): infection (−)/engulfment (−). With respect to the proportion of high-quality samples (Geckler 4 or 5), Group 1 had significantly higher values than Group 2 (67.4% vs 44.7%, $P=.037$). Similarly, Group 3 had a higher proportion of high-quality samples than Group 4 (72.0% vs 37.8%, $P=.012$). Importantly, the proportion of high-quality samples was not statistically significant between Groups 1 and 3 (67.4% vs 72.0%, $P=.791$), indicating that the frequency of bacterial engulfment in WBCs did not seem to depend on sample quality (Fig. 2).

3.3. Profile of isolated bacteria in sputum samples

The pathogen profile of the infection groups (Groups 1 and 2) was: *Streptococcus pneumoniae* (n=15), *Haemophilus influenzae* (n=7), *Moraxella catarrhalis* (n=3), Methicillin-susceptible *Staphylococcus aureus* (MSSA)(n=1), anaerobic bacteria (n=5), other pathogens (n=10), and unknown (n=46).

3.4. Frequency of engulfment in the infection and non-infection groups

Among the 163 patients, the proportion of engulfment positive cases was not significantly different in the infection (n=46, 49.5%, $P=.11$) and non-infection (n=25, 35.7%) groups (Table 2). Even when only high quality sputum samples (Geckler 4 or 5) were considered, the proportion of engulfment in the infection (n=31, 63.3%, $P=.51$) and the non-infection (n=18, 55.3%) groups was not significantly different (Table 3).

3.5. Comparison of the infection and non-infection groups, with a focus on the bacterial engulfment score in WBCs

In the infection group (Fig. 3A), the engulfment score (%) of *S. pneumoniae* was significantly lower (median 3%, interquartile range [IQR]: 2 to 5%, $P=.005$) than that of non-*S. pneumoniae* bacteria (*H. influenzae*, *M. catarrhalis*, and MSSA) (median 22.5%, IQR: 17 to 35.5%). The non-infection group (Fig. 3B) had no samples of *S. pneumoniae* with engulfment, and the non-*S. pneumoniae* bacteria consisted of *H. influenzae* (n=2),

Table 2

Frequency of engulfment in the infection (+) and infection (−) groups.

	Infection (+)	Infection (−)	Total
Engulfment (+)	46	25	71
Engulfment (−)	47	45	92

The proportion of engulfment in the infection and non-infection groups was not significantly different ($P=.11$).

Table 3

Frequency of engulfment in the infection (+) and infection (−) groups (high quality samples, that is, G4 or G5, only).

	Infection (+)	Infection (−)	Total
Engulfment (+)	31	18	49
Engulfment (−)	21	17	38

The proportion of engulfment in the infection and non-infection groups was not significantly different (P=.51).

M. catarrhalis (n=2), and MSSA (n=3), indicating that the engulfment of those bacteria can be found in non-infected individuals.

In regard to the non-*S. pneumoniae* bacteria (*H. influenzae, M. catarrhalis*, and MSSA), the engulfment score (%) was significantly higher in the infection group (median 22.5, IQR 17 to 35.5%) than in the non-infection group (median 6.0, IQR: 3 to 13%, P=.011).

3.6. Receiver operating characteristic curve for the diagnosis of infection based on the engulfment score in all pathogens, with a focus on H. influenzae, M. catarrhalis, and MSSA

When the cut-off value was defined as 3%, the diagnostic yield of the engulfment score for infection, including all pathogens, had a sensitivity as low as 50% and a specificity of 65.7%, with an AUC of 0.579 (95% CI 0.464 to 0.694)(Fig. 4A). For non-*S. pneumoniae* pathogens (*H. influenzae, M. catarrhalis*, and MSSA), the diagnostic yield for infection dramatically increased, with a sensitivity of 75%, specificity of 85.7%, and AUC of 0.902 (95% CI: 0.75–1.0) at a cutoff point of 18% for the engulfment score (Fig. 4B).

4. Discussion

Physicians generally believe that the presence of bacterial engulfment in the cytoplasm of WBCs in high-quality sputum samples indicates infection. Previous studies have demonstrated the usefulness of sputum Gram stain in community-acquired pneumonia (CAP) for pathogen-targeted treatment.[7–10] However, no studies have reported the diagnostic accuracy and/or significance of engulfment itself. In this regard, we have provided the first evidence that engulfment is not always indicative of infection, even in high quality sputum samples. In other words, patients without infections can have bacterial engulfment in WBCs to some extent. More importantly, this study clearly demonstrated the difference in engulfment scores between *S. pneumoniae* and non-*S. pneumoniae* pathogens such as MSSA, *H. influenzae*, and *M. catarrhalis*, which suggests that the interpretation of engulfment should be performed separately for each pathogen.

Pugin et al[11] found that 11 of 15 bronchoalveolar lavage fluid samples (73%) obtained from patients with ventilator-associated pneumonia in an intensive care unit found bacterial engulfment in WBCs with a sensitivity of 73% and specificity of 100%. The diagnostic accuracy of engulfment in our study was lower than that of Pugin et al, which might have been due to the difference in respiratory samples and/or enrolled patients. Ehara et al[12] found that the frequency of bacterial engulfment in sputum that was associated with CAP due to *S. pneumoniae* was 55.5%, which would have been comparable to our results if our analysis of infected patients had been confined to *S. pneumoniae* (46.7%).

In terms of the vulnerability of phagocytosis by WBCs, the precise differences between each pathogen are unknown;[13] however, the *S. pneumoniae* capsule affects multiple aspects of complement and neutrophil-mediated immunity, resulting in the profound inhibition of opsonophagocytosis.[14] This may explain the reason for the relatively small engulfment count noted in cases

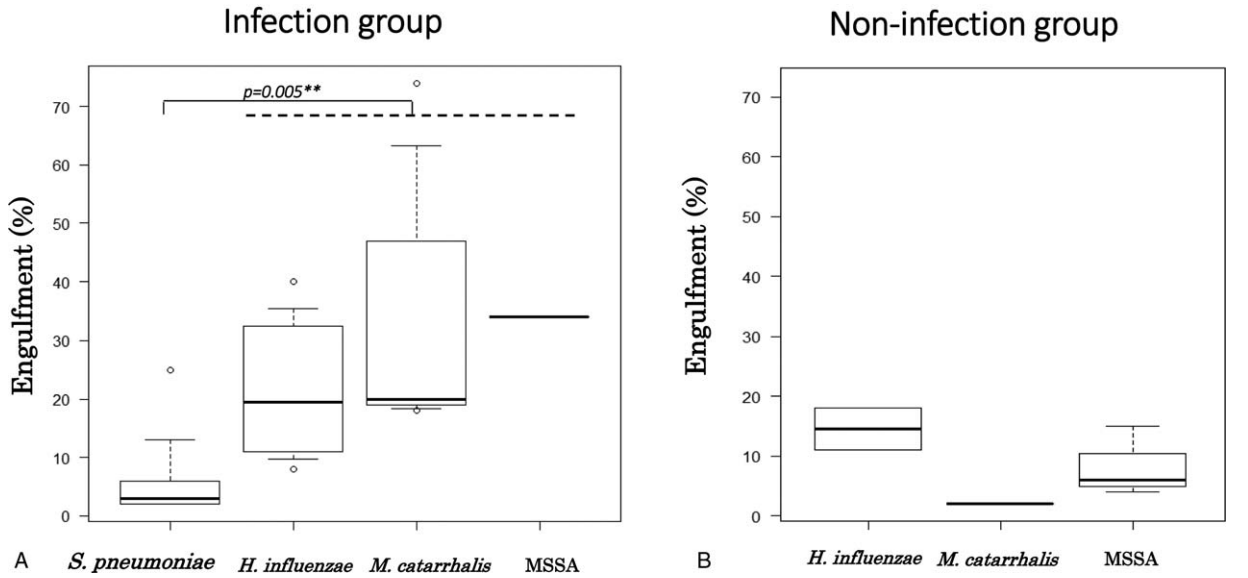

Figure 3. The pathogen profile using only high quality sputum (G4 or G5), and the percentage of engulfment according to the pathogen's profile. In the infection group (Fig. 3A), the engulfment score (%) of *S. pneumoniae* was significantly lower (median 3%, IQR: 2% to 5%, P=.005) than that of non-*S. pneumoniae* bacteria (*H. influenzae, M. catarrhalis*, and MSSA)(median 22.5%, IQR: 17% to 35.5%). The non-infection group had no samples with *S. pneumoniae* with engulfment (Fig. 3B). Comparison of the engulfment score between the infection and non-infection groups, with a focus on the non-*S. pneumoniae* bacteria (*H. influenzae, M. catarrhalis*, and MSSA). The engulfment score was significantly higher in the infection group (median 22.5, IQR 17% to 35.5%) than in the non-infection group (median 6.0, IQR: 3% to 13%, P=.011).

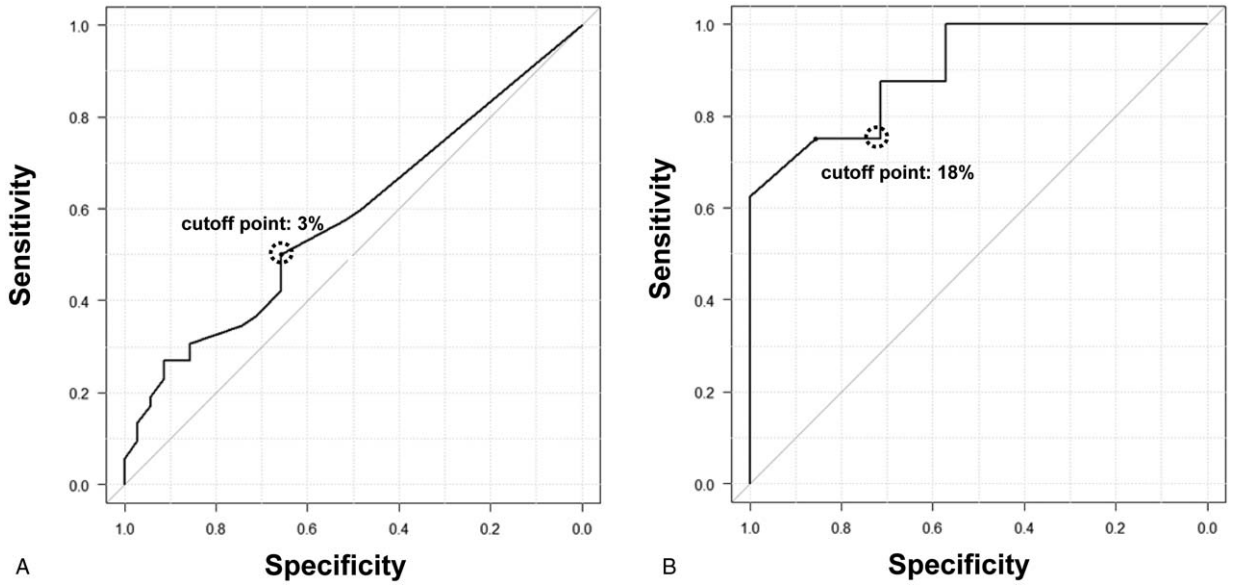

Figure 4. The receiver operating characteristic curve for the diagnosis of infection based on the engulfment score in all pathogens (A) and in MSSA/*H. influenzae/M. catarrhalis* (B). (A) For all pathogens, the diagnostic accuracy for infection at a cut off value of 3% was: sensitivity: 50%, specificity: 65.7%, and area under the curve (AUC): 0.579 (95% CI 0.464 to 0.694). (B) For *H. influenzae, M. catarrhalis*, and MSSA, the diagnostic accuracy for infection at a cut off point of 18% was: sensitivity: 75%, specificity: 85.7%, and AUC: 0.902 (95% CI 0.75 to 1.00).

of *S. pneumoniae* infection in comparison with non-*S. pneumoniae* bacteria in this study.[13] Regarding with staphylococcal infection, laboratory-based observation demonstrated that staphylococci are versatile pathogens with a high capacity of adhesion to eukaryotic cells and cluster adhesion which towards successful intracellular replication as well as tetrad morphology.[15] Those facts might be associated with vulnerability of engulfment of staphylococci in WBCs. Taken together, the significance of engulfment should be interpreted based on the pathogen.

The present study had some limitations. First, a relatively small number of patients were enrolled in the study. Second, the prevalence of COPD and bronchiectasis differed in the infection and non-infection groups. Third, the quality of samples in the true-positives (Group 1) was higher than in the false-negatives (Group 2). Similarly, the false positive Group (Group 3) has high-quality samples than in the true negative group (Group 4), which might be a selection bias.

Thus, those limitations might have affected the engulfment score. To confirm our findings, a greater number of index cases are required.

5. Conclusions

The present study provided the first evidence of the diagnostic accuracy and significance of the presence of bacterial engulfment, which might be useful in physicians' interpretations of results based on the pathogen's profile.

Author contributions

Data curation: M. Shimoda, T. Saraya, S. Yonetani, K. Araki.
Formal analysis: M. Shimoda.
Investigation: M. Shimoda, T. Saraya, S. Yonetani, K. Araki.
Methodology: T. Saraya, H. Takizawa.
Software: M. Shimoda.
Writing – original draft: M. Shimoda, T. Saraya.
Writing – review & editing: T. Saraya, H. Takizawa.

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