“A Four-Leaf Clover” Sign for Rapid Identification of Coagulase-Negative Staphylococci by Gram Staining From Positive Blood Culture: A Cross-Sectional Study

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Abstract

**Background**: Coagulase-negative staphylococci (CoNS) are one of the most common contaminant microorganisms isolated from blood cultures. Few studies exploring the use of Gram staining to distinguish between *Staphylococcus aureus* (SA) and CoNS have been reported. Here, this study aimed to explore whether morphological features of Gram staining could identify SA or CoNS.

**Methods**: This study was conducted at St. Luke’s International Hospital from November 2016 to September 2017. The positive blood cultures for which the Gram staining showed gram-positive cocci (GPC) in clusters were included in our study. The direct smear of Gram staining obtained from positive blood culture bottles were examined within 24 hours of positivity. We have identified and characterized the following two signs: “four-leaf clover (FLC)” if 4 GPC gathered like a planar four-leaf clover and “grapes” if the GPC gathered like grapes in a three-dimensional form. The number of fields with FLC and grapes signs in 10 fields per slide with ×1,000 power was counted, and the results in a total of 20 fields with ×1,000 power were combined. We performed a logistic regression analysis to assess whether these signs could serve as factors distinguishing between SA and CoNS. The predictive ability of these signs was evaluated based on the sensitivity, specificity, positive predictive value, and negative predictive value for CoNS via receiver operating curve analysis.

**Results**: In total, 106 blood cultures for which Gram staining showed GPC in clusters were examined; 46 (43%) were SA, and 60 (57%) were CoNS samples. The result of multivariate logistic regression analysis showed that the FLC sign was a statistically significant marker of CoNS with an odds ratio of 1.31 (95% confidential interval (CI): 1.07–1.61, p<0.05). In aerobic bottles, sensitivity, specificity, positive predictive value, and negative predictive value for CoNS were 0.67, 0.91, 0.92, and 0.65, respectively, and the value of area under the curve was 0.79 (95% CI: 0.67–0.91).

**Conclusions**: To our knowledge, this is the first study to show that the FLC could be a rapid and useful indicator to identify CoNS in aerobic bottles. Thus, the presence of FLC sings could help clinicians to suspect the possibility of CoNS before the final identification by cultures.

**Background**

Coagulase-negative staphylococci (CoNS) are one of the most common contaminant microorganisms isolated from blood cultures.\(^1\) Prior to the final identification of CoNS, clinicians often face a dilemma whether to start antimicrobial therapy if the blood culture turns out to be positive for Gram-positive cocci (GPC) in clusters. Undoubtedly, if the result comes out to be *Staphylococcus aureus* (SA) positive, SA must be considered as a true causative organism and the treatment should be immediately initiated as SA bacteremia is associated with high mortality and morbidity.\(^2\) However, ambiguous culture results often promote unnecessary prescription of antimicrobial agents leading to a selection of antimicrobial-resistant organisms such as vancomycin-resistant enterococci,\(^3\) as well as unnecessary testing,\(^4\) and longer hospitalization, which ultimately increases the treatment costs significantly.\(^5\) Hence, it is important
for clinicians to know if the observed Gram-positivity is a result of true infection or just contamination. Clinicians often try to differentiate true infection from contamination by going through patient’s status, counting the number of bottles of positivity, considering the time to positivity of blood culture, or by establishing systemic inflammatory response syndrome criteria.

There are some rapid identification methods reported to distinguish SA from CoNS. These methods, however, use peptide nucleic acid fluorescence in situ hybridization and real-time PCR, which is not a cost-effective alternative. The laboratory technicians often characterize the morphological features to distinguish SA and CoNS. Still, there are only a few studies in which the optimal findings have been reported to differentiate between them by Gram stain. Therefore, the current study aims to investigate the contributable findings in Gram stain to identify CoNS and distinguish it from SA.

**Methods**

We conducted a single-center, retrospective, and cross-sectional study at St Luke’s International Hospital, a 520-bed teaching hospital in Tokyo, from November 2016 to September 2017. The following inclusion criteria were applied: positive blood cultures (BacT/ALERT system with PA plus and PN plus [bioMérieux, Inc., Durham, NC]), in which Gram stain showed GPC, were first identified. Then, two infectious diseases fellows examined the Gram staining obtained from positive blood culture within 24 hours of blood culture positivity, under supervision of the chief medical technician in the laboratory. Predominantly, two signs were identified, which we have defined as (i) “four-leaf clover (FLC)” sign if 4 GPCs gather like planar four-leaf clover (Figure 1) and (ii) “grapes” sign if the edge of GPCs cluster appears blurry when focusing the center of the objects on the microscope and GPCs gather like grapes in a three-dimensional manner (Figure 2). The examiners counted the number of fields that had FLC and grapes signs out of 10 fields per slide with ×1,000 power, and the results from a total of 20 fields with ×1,000 power were combined. If there were positivity in both aerobic and anaerobic culture bottles, both were analyzed. The examiners, however, were kept oblivious of whether smear slides of Gram staining in positive blood cultures came from aerobic or anaerobic cultures.

**Statistical analyses**

A logistic regression analysis was performed to assess whether these signs could indeed be the identifiable factors for SA or CNS. The predictive characteristics imparted by these signs were evaluated based on the sensitivity (Sen), specificity (Spe), positive predictive value (PPV), and negative predictive value (NPV) for CoNS using receiver operating curve (ROC) analysis. Based on the Youden index, the best values for Sen and Spe were calculated and the optimal cut-off point was identified in terms of FLC sign based on the above developed logistic regression model. All analyses were performed using SPSS 19.0J statistical software (IBM Japan, Tokyo, Japan).

**Results**
In total, 106 blood cultures were examined, in which a direct Gram stain showed GPC in cluster, and they were finally identified as 46 (43%) were SA, and 60 (57%) were CoNS. The median values of the number of fields in SA, with FLC sign and grapes sign were 5.5 and 11, respectively, out of total 20 fields. In CoNS, the median values of the number of fields with FLC sign and grapes sign were 13.5 and 7 respectively, out of total of 20 fields. There were no non-staphylococcal GPC observed in cluster. In aerobic bottles, the median number of fields that accounted for grapes sign was higher in SA than in CoNS (18 versus 8 out of 20 fields). On the contrary, the median number of fields that accounted for the FLC sign was higher in CoNS than in SA (17 versus 3.5 out of 20 fields). There were no significant differences observed between SA and CoNS in anaerobic bottles. The result of multivariate logistic regression analysis showed that FLC sign was a statistically significant variable of CoNS with odds ratio (OR) of 1.31 (95%CI 1.07–1.61, p < 0.05). In aerobic bottles, Sen, Spe, PPV, NPV were 0.67, 0.91, 0.92, and 0.6 respectively (Table 3). Figure 3 shows a ROC curve of FLC sign for CoNS. The value of the area under the curve was 0.79 (95%CI 0.67–0.91). Based on the Youden index, the optimal cut-off number for the number of fields was observed to be 6.5 out of 20 fields.

### Table 1
The median number of fields out of 20 fields that contained each sign.

| Sign                    | S. aureus, N = 46 | CoNS, N = 60 |
|-------------------------|--------------------|--------------|
|                         | Aerobic, N = 22    | Anaerobic, N = 24 | Aerobic, N = 33 | Anaerobic, N = 27 |
| Grapes sign, median number of fields | 18 | 6 | 8 | 5 |
| FLC sign, median number of fields   | 3.5 | 10.5 | 17 | 12 |

Abbreviations: CoNS, coagulase-negative staphylococci; FLC sign, Four-leaf clover sign

### Table 2
Logistic regression analysis of each sign for CoNS.

| Bottles | Aerobic | Anaerobic |
|---------|---------|-----------|
| Sign    | Odds ratio | P value | 95%CI | Odds ratio | P value | 95%CI |
| Grapes sign | 1.09 | 0.33 | 0.917–1.29 | 1.05 | 0.40 | 0.972–1.24 |
| FLC sign | 1.31 | 0.008 | 1.07–1.61 | 1.10 | 0.13 | 0.933–1.19 |

Abbreviations: FLC sign, Four-leaf clover sign
Table 3
Sensitivity, specificity, positive predictive value, and negative predictive value of “Four-leaf clover sign” for CoNS.

|                | Sen | Spe  | PPV  | NPV  |
|----------------|-----|------|------|------|
| Aerobic bottles| 0.67| 0.91 | 0.92 | 0.65 |
| Anaerobic bottles | 0.74 | 0.54 | 0.65 | 0.65 |

Abbreviations: CoNS, coagulase-negative staphylococci; Sen, sensitivity; Spe, specificity; PPV, positive predictive value; NPV, negative predictive value

Discussion

In this study, we have identified that the FLC sign observed in the blood cultures, in which Gram staining showed GPC in cluster, could be an identifiable feature of CoNS. To the best of our knowledge, this is the first study suggesting that the FLC sign could help in the rapid identification of CoNS from SA, in aerobic bottles. This Gram stain-based approach is a convenient, cost-effective, and time-effective method to rapidly identify the causative pathogens. In Japan, for many decades, infectious diseases clinicians and primary care providers have been using Gram staining in the emergency room or ward to determine the appropriate antimicrobial regimen.\(^{12,13}\) Traditional use of Gram stain is not prevalent in many countries,\(^{14}\) but this method could be useful for low-cost and rapid estimation of the causative agent in such countries where Gram staining is used as a traditional method immediately after a positive blood culture as well as sputum or urine samples.\(^{15}\) Gram stain has contributed immensely towards curbing the overuse of broad-spectrum antimicrobials without affecting the effectiveness of the treatment.\(^{13}\)

It has been reported previously that the GPCs tend to differ in sizes between aerobic and anaerobic bottles.\(^{16}\) The size of CoNS was reported to be < 1 µm in size in aerobic and > 1 µm in size in anaerobic bottles. These findings are consistent with the current study showing that the FLC signs in aerobic bottles are of a relatively smaller size than those in anaerobic bottles; however, the reason behind this observation is still unknown.

No statistically significant associations were observed between SA and grapes sign, although the fraction of grapes sign in SA group was higher than that in the CoNS group. This might be because of the small sample size. Further clinical studies including larger sample size are therefore required. By combining this study along with the other useful finding i.e. the “Oozing sign” to identify SA in Gram staining,\(^ {15}\) more contributable identification between SA and CoNS might be possible by observing Gram staining. This will be a more promising and cost-effective option.

It is true that the sensitivity of this method is lower than the tube coagulase test, peptide nucleic acid fluorescence in situ hybridization (PNA-FISH), and real-time PCR.\(^ {7,8,9,10}\) Based on the current study only, clinicians cannot rule out CoNS in the absence of the FLC sign with low sensitivity. However, the high specificity of FLC signs could help clinicians predict the presence of CoNS with higher confidence while
awaiting the culture results. Furthermore, we believe that this cost-effective and easy to perform a method, which uses only Gram staining, could be an effective way in the facilities where PNA-FISH or real-time PCR are not available. Most importantly, by using this methodology, inappropriate empirical antimicrobial therapy used for SA could be minimized.

This study has several limitations. First, this was a relatively small observational study performed with samples taken from a single institution. For further validation, it is essential to compare the results with blood cultures taken from other hospitals. Second, this study did not include non-Staphylococcal GPC in cluster such as Micrococcus spp., or Aerococcus spp. Hence, the characterization of specific signs in Gram staining for these microorganisms could not be done. Third, this study did not assess the comparison between methicillin-susceptible SA (MSSA) and methicillin-resistant SA (MRSA). It remains unknown whether there are differences in size or number of FLS signs between MSSA and MRSA. Although the treatment varies between the two, this finding could help clinicians predict whether they should start the empirical treatment for SA with consideration about whether MSSA or MRSA could be the causative pathogen based on its regional prevalence and risk factors. Fourth, the respondents analyzing Gram stain slides in this study were fellows under training, which has the potential for less accurate responses compared to those from experienced microbiologists or laboratory technicians. However, it is noteworthy that this highly specific result was obtained by a non-skilled microbiologist. Finally, this study did not compare by species among CoNS. It also remains unknown whether there are differences between S. epidermidis and methicillin-susceptible CoNS such as S. lugdunensis or S. schleiferi. Although the application of this method should be carefully based on the clinical status of patients, we believe that the presence of the FLC sign could contribute to the identification of CoNS by clinicians. Further studies with larger sample size and diverse blood culture systems are also warranted.

Conclusions

In summary, a “four-leaf clover” sign could enable clinicians to rapidly identify CoNS species by Gram stain of positive blood culture in aerobic bottles. If the FLC sign is observed, CoNS could be highly suspected with the specificity of 91% before the final identification can be made.

List Of Abbreviations

Coagulase-negative staphylococci: CoNS; Staphylococcus aureus: SA; gram-positive cocc: GPC; four-leaf clover: FLC; confidential interval: CI; sensitivity: Sen; specificity: Spe; positive predictive value: PPV; negative predictive value: NPV; receiver operating curve: ROC; odds ratio: OR; peptide nucleic acid fluorescence in situ hybridization: PNA-FISH; Methicillin-sensitive Staphylococcus aureus: MSSA; Methicillin-resistant Staphylococcus aureus: MRSA.

Declarations

Ethics approval and consent to participate
This study was approved by the Institutional Review Board of St. Luke’s International Hospital in Tokyo, Japan (Number: 19-J128). The requirement for patient consent was waived because the study was based on a retrospective analysis of routinely collected data.

**Consent for publication**

Not applicable.

**Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Competing interests**

None

**Funding**

None

**Authors’ contribution**

TM collected the data and wrote the initial draft of the manuscript. NM and AS contributed to the study design. NM and YU assisted in writing the manuscript. KH contributed to the statistical analysis and manuscript editing. MS and MM contributed to collecting the data. All authors read and approved the final manuscript.

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**Figures**
Figure 1

“Four-leaf clover sign”
Figure 2

“Grapes sign”
Figure 3

The receiver operating characteristic curve of the “Four-leaf clover sign”