Original Article

Ratios of CD64 expressed on neutrophils, monocytes, and lymphocytes may be a novel method for diagnosis of neonatal sepsis

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Abstract
Introduction: Neutrophil CD64 expression has been demonstrated as an improved diagnostic marker of infection and sepsis. The purpose of this study was to develop a new method to evaluate neutrophil CD64 expression for diagnosis of neonatal sepsis.
Methodology: Eighty neonates with neonatal sepsis (21 culture positive, 59 negative) were enrolled in this prospective study along with 19 neonates with no symptoms or signs of infection as controls. Expressions of CD64 on monocytes, lymphocytes, and neutrophils were evaluated with flow cytometry (FCM). Ratios were calculated with these levels of CD64 expression. Blood culture and other laboratory exams were done at the same time for the diagnosis of neonatal sepsis. Results were compared between the neonatal sepsis and control groups.
Results: CD64 ratios showed significant difference between the groups (p < 0.01). Receiver operating curve (ROC) analysis showed that the CD64 ratios possessed high sensitivity (90%) and specificity (89.5%) in neonatal sepsis identification.
Conclusions: The novel CD64 evaluation method, CD64 ratio, can be used as a supplementary method for diagnosis of neonatal sepsis.

Key words: CD64; CD64 ratio; neonatal sepsis; flow cytometry.

Introduction

CD64, known as Fc-gamma receptor 1 (FcγRI), has high affinity to monomeric IgG-type antibodies, and is constitutively expressed on cells of the monocyte-macrophage lineage [1-3]. CD64 plays a pivotal role in the development of host immune responses to bacterial infections [4]. The expression of CD64 on neutrophils can be induced by bacterial or viral infection [5-7].

Neutrophil CD64 expression is a useful biomarker for improving the diagnosis and management of hospital patients with bacterial infections [4,8]. Also, it can be incorporated as a valuable marker to exclude neonatal sepsis (NS) [9-14]. Studies have showed that the expression of CD64 on neutrophils has high sensitivity and accuracy in identifying sepsis [14-16]. However, diversities of CD64 expression descriptions and various cut-off values exist in different laboratories [9,16]. This may be due to the widely used CD64 index method [17]. CD64 index is a quantification method to evaluate CD64 expression with a relative quantitative beads system (such as

Leuko64 kit or Quantibrite), but no uniform standard has been established for different kit producers.

Microbiological culture is a proven method to confirm sepsis and other infectious diseases. But microbiological culture is time consuming and often negative in patients who are receiving antibiotics [18,19]. The standard laboratory evaluation parameters of bacterial infection, such as leukocyte and neutrophil counts, serum C-reactive protein (CRP) level, and erythrocyte sedimentation rate (ESR), have relatively poor sensitivity and specificity [20]. Thus, a simple and reliable method to evaluate CD64 level is required in the diagnosis of NS and other infectious diseases.

Neutrophil CD64 is highly expressed on cells of the monocyte-macrophage lineage rather than the lymphocyte [1,3]. Recently, Soni *et al*. reported that a high sensitivity was obtained using monocyte CD64 as an internal reference to evaluate neutrophil CD64 in NS [10]. Here, monocytes and lymphocytes were used as internal references to evaluate CD64 expression level on neutrophils in order to find a novel method for the diagnosis of NS.

Methodology

Subjects and setting

Consecutive patients who underwent a sepsis evaluation in the neonatal intensive care unit (NICU) of XuZhou Children's Hospital between August 2012 and December 2012 were enrolled. During the study period, 1,006 neonates were admitted and followed up in the NICU. Among them, 170 (16.9%) neonates undergoing sepsis evaluation were enrolled in the study. Nineteen gestational age and gender-matched healthy neonates without any diseases were included as healthy controls (Table 1).

Patients in any of the following cases was excluded: chromosomal abnormality, lack of informed consent from the parents, inadequate blood sampling for all tests, and ambiguous flow cytometry (FCM) test result. This study was approved by research ethics committee of Xuzhou Children's Hospital. Informed written consent was received from parents of all neonates in this study.

Sepsis evaluations

Babies were investigated whenever a neonatologist suspected neonatal sepsis as previously described [14,21]. Suspected NS patients were microbiologically confirmed with blood culture. All blood cultures were collected using standard sterile techniques, and the Bactec Microbial Detection System (Becton-Dickinson, San Diego, USA) was used to detect positive blood cultures. Following unit protocol, attainment of three blood cultures was attempted. Infants whose culture results had two or three positive bacterial cultures with the same pathogen were diagnosed as having microbiologically confirmed neonatal sepsis (MNS). The infants who only had clinical NS features were grouped into clinically diagnosed neonatal sepsis (CNS).

Methods

Venous blood samples were obtained by venipuncture and collected in EDTA vacuum tubes. Blood samples were immediately transported to the laboratory and processed upon arrival. All the blood samples were taken within the first 24 hours of symptoms. The age when the blood was taken was recorded as the evaluation age.

Phycoerythrin (PE) conjugated anti-human CD64 antibody (clone 10.1, Becton-Dickinson) was used to measure the expression of CD64. A total of 50 μL of well-mixed, anticoagulated whole blood was incubated with CD64-PE antibody for 15 minutes at room temperature, followed by lysis with lysing solution (Becton-Dickinson); the mixture was then washed using phosphate buffer solution (PBS). The mean fluorescence intensities (MFIs) of stained CD64 on monocytes, lymphocytes, and neutrophils were recorded by a FACSCanto FCM (Becton-Dickinson). At least 10,000 events were recorded for each sample. Analyses were performed using FACSDiva software (Becton-Dickinson). The investigators checking and confirming the CD64 results were blinded to the clinical data, including the blood culture results.

Statistical analyses

Statistical Package for the Social Sciences (SPSS) version 15.0 (SPSS, Chicago, USA) was used for statistical analysis. Fluorescence ratios of N to L, N to M, (N-L) to (M-N), (N-L) to (M-L), and N^2 to (L*M) (N: neutrophil; M: monocyte; L: lymphocyte) were calculated first from MFIs. Then ratios were transformed to (or very nearly to) Gaussian distribution by logarithmic transformation. Differences among the three groups were analyzed by one-way analysis of variance (ANOVA) for transformed data and by Kruskal-Wallis tests for untransformed data. Group comparisons after the ANOVA test were carried out using Tukey's multiple comparison technique. Furthermore, comparisons between two groups were also analyzed by independent samples t-test for transformed data and Mann-Whitney U test for untransformed data, in which comparisons between the control group and the all-NS group (which

Table 1. Characteristics of neonatal population according to groups

Variables	CNS	MNS	Con	P
Birth weight (g)	3,308 (294)	3,275 (277)	3,341 (221)	0.757[a]
Gestational age (weeks)	38.9 (2.0)	38.8 (1.8)	39.3 (1.6)	0.611[a]
Male gender, n (%)	39 (66.1)	12 (57.1)	9 (47.4)	0.325[b]
Vaginal delivery, n (%)	45 (76.3)	15 (71.4)	14 (73.7)	0.902[b]
Preterm, n (%)	7 (11.9)	2 (9.5)	1 (5.3)	0.896[c]
Age at evaluation (days)	12.3 (9.2)	11.3 (7.9)	11.1 (7.9)	0.829[a]
Early onset sepsis (< 7 days of age), n (%)	25 (42.4)	8 (38.1)	NA	0.732[b]

[a]Analyzed by ANOVA test; [b]analyzed by Pearson Chi-square test; [c]analyzed by Fisher's exact test; Values are presented as mean with standard deviation (SD) or number (%); Con: healthy control; MNS: microbiologically confirmed neonatal sepsis; CNS: clinically diagnosed neonatal sepsis

contained MNS and CNS) were also analyzed. A value of p < 0.05 was considered statistically significant.

The diagnostic cut-off values were defined using receiver operating curve (ROC) analysis. In order to quantify the predictive value of CD64 ratios, the area under the ROC (AUC, with 95% confidential interval [95% CI]) was calculated, and then the optimum cut-off point of CD64 ratios based on maximizing the Youden index (J = [sensitivity + specificity - 1]) was identified. The sensitivity and specificity at the optimum cut-off point were considered to be the optimum sensitivity and specificity. Patients' CD64 ratios over and under the cut-off point value were defined as true positive (TP) and false negative (FN), and control CD64 ratios over and under the cut-off point value were defined as false positive (FP) and true negative (TN), respectively.

In addition to sensitivity and specificity, the following statistics at the optimum cut-off point were calculated: positive predictive value (PPV): TP/(TP + FP); negative predictive value (NPV): TN/(TN + FN); positive likelihood ratio (PLR): sensitivity/(1 - specificity); and negative likelihood ratio (NLR): (1 - sensitivity)/specificity.

Results

A total of 189 samples (170 suspected NS and 19 healthy controls) were obtained in this study. After exclusion, a total of 80 neonates diagnosed with NS were included for analysis. Subjects were divided into the MNS group (n = 21; 12 males and 9 females), the CNS group (n = 59; 39 males and 20 females), and the control group (n = 19; 9 males and 10 females). Demographic characteristics of the three groups are presented in Table 1.

Figure 1. Box plot distributions of the groups by each ratio
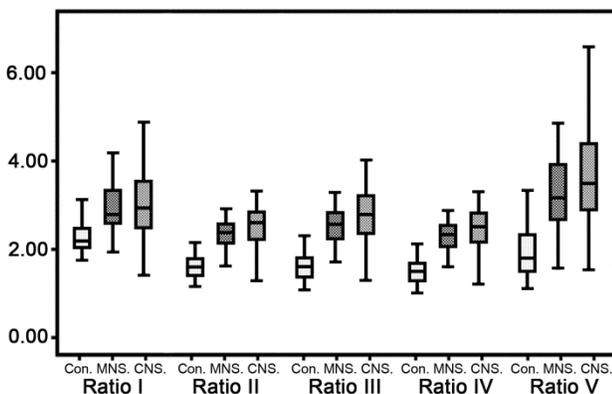
Calculation formula: ratio I, N/L; ratio II, N/M; ratio III, (N-L)/(M-N); ratio IV, (N-L)/(M-L); ratio V, N2/(L*M); Con: healthy control; MNS: microbiologically confirmed neonatal sepsis; CNS: clinically diagnosed neonatal sepsis

Figure 2. Receiver operating characteristic curves of CD64 ratio for MNS and CNS
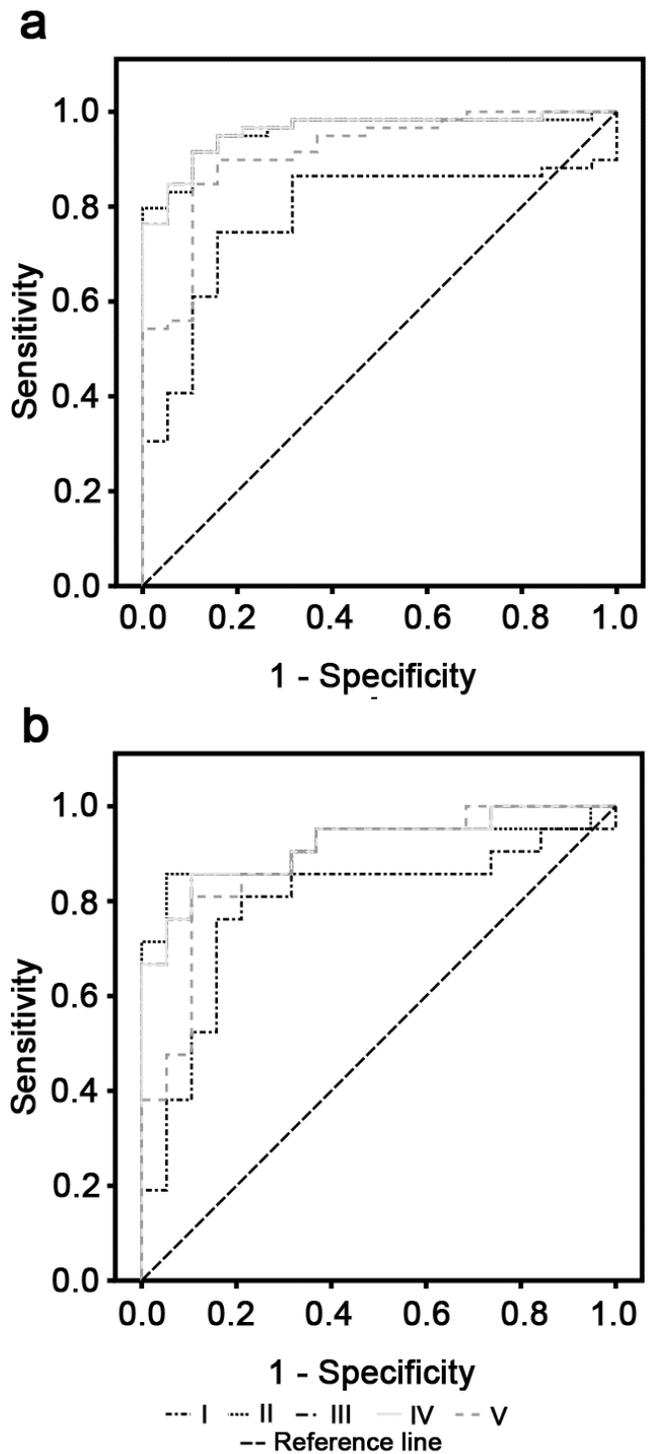
A: ROC for CNS (n = 59). Ratio II, III, and IV had the largest area under the curve (0.96); B: ROC for MNS (n = 21). Ratio III and IV had the largest area under the curve (0.92)

Table 2. Comparison of study groups

	Mean (SD)					P values			
	All patients	**Con**	**MNS**	**CNS**	**ANOVA**		**Turkey's test**		**Kruskal-Wallis**
Ratios	**(n = 99)**	**(n = 19)**	**(n = 21)**	**(n = 59)**		**Con vs. MNS**	**Con vs. CNS**	**MNS vs. CNS**	
I	32.60 (50.44)	11.45 (6.68)	35.72 (61.58)	38.29 (52.86)	0.003	0.033	0.002	0.905	0.002
II	0.22 (0.11)	0.10 (0.03)	0.22 (0.11)	0.25 (0.10)	< 0.001	< 0.001	< 0.001	0.225	< 0.001
III	0.29 (0.22)	0.10 (0.03)	0.30 (0.29)	0.34 (0.20)	< 0.001	< 0.001	< 0.001	0.271	< 0.001
IV	0.20 (0.11)	0.09 (0.03)	0.21 (0.11)	0.24 (0.10)	< 0.001	< 0.001	< 0.001	0.240	< 0.001
V	9.17 (18.41)	1.17 (0.91)	9.17 (18.81)	11.74 (20.57)	< 0.001	< 0.001	< 0.001	0.600	< 0.001

Data of ratios expressed as mean values with standard deviation (SD);Con: healthy control; MNS: microbiologically confirmed neonatal sepsis; CNS: clinically diagnosed neonatal sepsis

Table 3. Sensitivity, specificity, AUC, PPV, NPV, PLR, and NLR of neonatal sepsis ROC analysis

				CNS				
Ratios	**Cut-off [a]**	**Sensitivity (%)**	**Specificity (%)**	**AUC (SE, 95% CI)**	**PPV (%)**	**NPV (%)**	**PLR**	**NLR**
I	12.64	74.6	84.2	0.78 ± 0.06 (0.67-0.89)	91.7	50.0	4.72	0.30
II	0.13	91.5	89.5	0.96 ± 0.02 (0.92-1.00)	94.7	76.2	8.69	0.09
III	0.14	91.5	89.5	0.96 ± 0.02 (0.92-1.00)	96.4	77.3	8.69	0.09
IV	0.12	91.5	89.5	0.96 ± 0.02 (0.92-1.00)	96.4	77.3	8.69	0.09
V	1.81	84.7	89.5	0.91 ± 0.04 (0.84-0.98)	96.2	65.4	8.05	0.17
				MNS				
	Cut-off [a]	**Sensitivity (%)**	**Specificity (%)**	**AUC (SE, 95% CI)**	**PPV (%)**	**NPV (%)**	**PLR**	**NLR**
I	13.17	76.2	84.2	0.79 ± 0.08 (0.64-0.94)	84.2	76.2	4.83	0.28
II	0.15	85.7	94.7	0.91 ± 0.05 (0.82-1.01)	94.7	85.7	16.29	0.15
III	0.13	85.7	89.5	0.92 ± 0.04 (0.83-1.00)	85.7	84.2	2.87	0.14
IV	0.12	85.7	89.5	0.92 ± 0.04 (0.83-1.00)	90.0	85.0	8.14	0.16
V	1.80	81.0	89.5	0.88 ± 0.05 (0.78-0.99)	89.5	81.0	7.69	0.21

[a]Optimum cut-off value; AUC: area under the ROC curve; ROC: receiver operating curve; SE: standard error; CI: confidence interval; PPV: positive predictive value; NPV: negative predictive value; PLR: positive likelihood ratio; NLR: negative likelihood ratio

Table 4. Sensitivity, specificity, AUC, PPV, NPV, PLR, and NLR of overall neonatal sepsis ROC analysis

| **Ratios**	**Cut-off [a]**	**Sensitivity (%)**	**Specificity (%)**	**AUC (SE, 95% CI)**	**PPV (%)**	**NPV (%)**	**PLR**	**NLR**
I	12.64	75.0	84.2	0.78 ± 0.05 (0.68-0.89)	95.2	44.4	4.75	0.30
II	0.13	90.0	89.5	0.95 ± 0.02 (0.90-0.99)	96.0	66.7	8.55	0.11
III	0.13	90.0	89.5	0.95 ± 0.02 (0.91-0.99)	96.1	69.6	8.55	0.11
IV	0.12	90.0	89.5	0.95 ± 0.02 (0.91-0.99)	97.3	68.0	8.55	0.11
V	1.80	83.8%	89.5	0.91 ± 0.04 (0.83-0.98)	97.1	56.7	7.96	0.18

[a]Optimum cut-off value; AUC: area under the ROC curve; ROC: receiver operating curve; SE: standard error; CI: confidence interval; PPV: positive predictive value; NPV: negative predictive value; PLR: positive likelihood ratio; NLR: negative likelihood ratio

For most samples (187 of 189), neutrophils, monocytes, and lymphocytes were clearly separated by forward scatter/side scatter (FSC/SSC) dots. CD64 MFIs of these three populations was recorded. The samples that had incomplete neutrophil, monocyte, or lymphocyte populations and that could not be clearly separated by FSC/SSC dots were excluded. The eighty samples included in statistical analysis had clear FCM test results.

The ratios were respectively numbered as I: N/L; II: N/M; III: (N-L)/(M-N); IV: (N-L)/(M-L); and V: $N^2/(L*M)$. Table 2 shows the differences among healthy control (Con), MNS, and CNS presented by these five ratios. Both ANOVA and Kruskal-Wallis tests show significant differences among the three groups. Figure 1 shows the box plot distributions of these groups by each ratio. Non-parametric comparison using the Kruskal-Wallis test gave results similar to ANOVA. After ANOVA, multiple comparison by Tukey's test showed that between the Con and MNS groups, and between the Con and CNS groups, the difference was highly significant ($p < 0.01$). There was, however, no significant difference between MNS and CNS by all the five ratios. Independent samples t-test and non-parametric Mann-Whitney test were used to confirm the differences between each group and to compare the overall NS patients (which contained MNS and CNS) to the control group. Independent samples t-test and non-parametric Mann-Whitney test showed similar differences in each of the two groups as did Tukey's test. Comparison between overall NS patients and the Con group showed a very significant difference ($p < 0.01$).

ROCs were drawn for testing sensitivity and specificity of these ratios to distinguish NS. In CNS ROC analyses (Figure 2a), ratio II, III, and IV had the largest AUC (0.96 ± 0.02 at 95% CI 0.92 to 1.00), and at the optimum cut-off point (Table 3), the three ratios had the same sensitivity (91.5%) and specificity (89.5%). The ratio III and IV had the highest PPV, NPV, and PLR (96.4%, 77.3%, and 8.69, respectively). In MNS ROC analyses (Figure 2b), ratio III and IV had the largest AUC (0.92 ± 0.04 at 95% CI 0.83 to 1.00), and at the optimum cut-off point (Table 3), the three ratios had the same sensitivity (85.7%), but ratio II had a higher specificity (94.7%). Ratio II had the highest PPV, NPV, and PLR (94.7%, 85.7% and 16.29, respectively). In overall NS ROC analyses (Figure 3), ratio II, III, and IV had the largest AUC (0.95 ± 0.02 at 95% CI 0.91 to 0.99), and at the optimum cut-off point, the three ratios had the same

Figure 3. Receiver operating characteristic curve analysis of CD64 ratio for overall NS
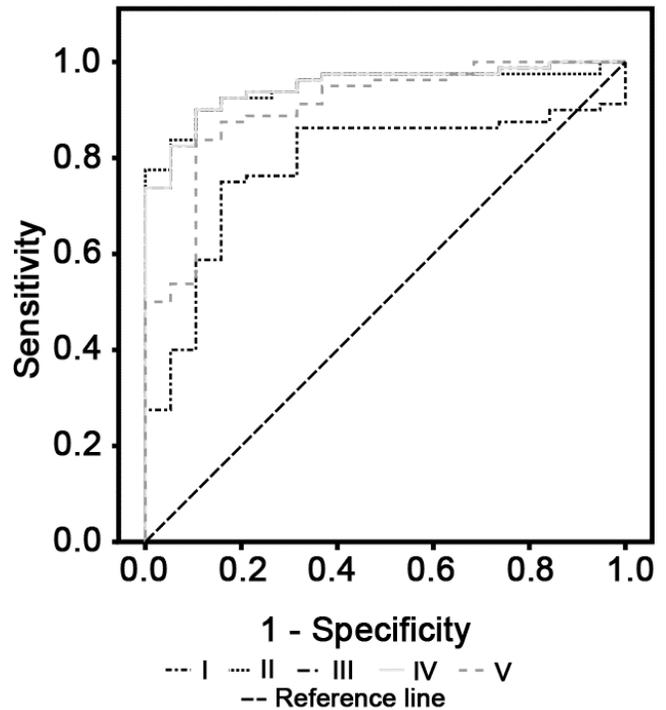
Ratio II, III and IV had the largest AUC (0.95); at the optimum cut-off point, the three ratios had same sensitivity (90%) and specificity (89.5%)

sensitivity (90%) and specificity (89.5%) (Table 4). Ratio III and IV had the same PLR (8.55), but ratio III had higher NPV (69.6%) and ratio IV had higher PPV (97.3%). These ROC analysis results indicate that ratio III and IV were the best markers for CNS, and that ratio II was the best marker for MNS. However, in overall NS, ratios III and IV had differences in PPV and NPV.

Discussion

According to the World Health Organization, approximately four million neonates die annually. Sepsis is the second most frequent direct cause of death among neonates, and it is still a major threat in many developing countries [22,23]. It is extremely important that NS be diagnosed early, because prompt institution of antimicrobial therapy improves outcomes. It is difficult to identify sepsis before knowing the blood culture results. Blood culture results are the current diagnostic gold standard of NS. However, there is a possibility of sepsis even in the presence of negative blood culture results. What is more, blood culture is time consuming and requires a fixed period of at least 48 hours [24]. In addition, the nonspecific signs and symptoms of NS may also be

observed in the absence of infection. Thus, a reliable and rapid method with high sensitivity and specificity for helping diagnose NS is needed.

NS is a severe disease of bacterial infection. Currently, several methods have been developed to identify bacterial infections are used to diagnose NS, such as white blood cell (WBC) count, erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), plasma procalcitonin (PCT), inflammatory mediators, triggering receptor expressed on myeloid cells 1 (TREM-1) protein, as well as phagocyte complement receptors [25]. Individually, they do not possess high specificity or sensitivity and are generally more helpful when considered together [26-29]. As a sensitive marker, neutrophil surface CD64 expression has shown particular promise as an early marker for infection, and has been widely reported in recent years [14,30,31]. Many studies have obtained attractive results using CD64 as a biomarker in the diagnosis of NS. CD64 may become an important supplementary marker for NS diagnosis.

FCM can distinguish slightly abnormal cells from a large cell population, such as minimal residual disease (MRD) detection and stem cell count. Most clinical laboratories in developed and some developing countries have FCM technology. FCM technology is used to quantitatively detect CD64 expression on neutrophils, and many commercial kits are available. But this quantitative method of CD64 detection is relatively expensive, and there is no uniform product standard for different kit producers, which are the biggest barriers to applying this method more widely in clinical practice.

Recently, Soni *et al*. showed that median M/N CD64 ratio is a highly sensitive marker of culture-positive NS [10]. In their study, only monocytes were used as an internal reference. In our study, we used lymphocytes as negative internal reference and monocytes as positive internal reference, and evaluated the level of CD64 expression on neutrophils. Compared to the quantitative method, our method does not need quantitative beads. Thus, it is cheaper and simpler. In addition, our method also showed high sensitivity (90%), specificity (89.5%), PPV (97.3%), NPV (69.6%), and PLR (8.55) in NS diagnosis.

Our study has some limitations. First, some of the patients included in the study might have already received treatment with antibiotics at a clinic before being enrolled in the study. The pretreatment with antibiotics could have potentially influenced the blood cultures and inflammatory responses. Secondly, the MNS patient and control sample sizes were relatively small, so some data had to be transformed to Gaussian distribution for statistical analysis. A small sample is likely to affect the reliability of statistical results. Thirdly, for cost reasons, we did not compare the CD64 ratios to CD64 index assay in this study. Thus, our study could not provide data about the CD64 ratios and CD64 index, which was more reliable in NS diagnosis. Finally, for cost reasons, we did not evaluate the influences of the antibody's clone type, fluorescein type, and FCM type. These factors may be potential confounders of external validity.

The CD64 ratios reflect the relative relationship of different CD64 MFIs. The photo multiplier tube (PMT) voltage of FCM has overall influence on MFIs of a whole channel, rather than on individual MFIs. Therefore, the influence of PMT voltage on CD64 ratios can be ignored. However, the amplification of MFIs by PMT voltage may affect the accuracy of the CD64 ratios. In addition, different types of FCM may have different MFI resolution. In our institute, we found that the CD64 MFI values measured with FACSCanto FCM and FACSCalibur FCM (Becton-Dickinson) were much different, but the CD64 ratios were similar (data not shown). Therefore, the optimum cut-off values obtained in our study may not suitable for other types of FCM. The CD64 ratios may be used for supplementary diagnosis after a laboratory internal testing to find a suitable cut-off.

In summary, we tested a quick, cheap, and easy-to-use method for CD64 evaluation. This method may be a reliable supplement for NS diagnosis.

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