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Utilizing Neutrophil Extended Parameters for the Screening of Early-Onset Neonatal Sepsis

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Abstract

Background: Early-onset neonatal sepsis (EOS), occurring within the first 72 hours of life, is a significant cause of morbidity and mortality in newborns. Prompt diagnosis remains a challenge due to the nonspecific nature of clinical signs and the delayed results from standard diagnostic methods such as blood culture. Traditional hematological markers, including total neutrophil count and the immature-to-total neutrophil (I: T) ratio, have shown limited sensitivity and specificity in the early detection of EOS. This study investigates the diagnostic utility of both conventional and novel neutrophil indices, such as absolute neutrophil count (ANC), immature granulocyte (IG) count, I: T ratio, and the advanced scatter-based parameters Neut-Y (neutrophil reactivity index) and Neut-X (neutrophil granularity index), using automated hematology analyzers to improve the early identification of EOS.

Materials and Methods: This prospective study analyzed clinical data from 135 presumed sepsis neonates identified as having maternal and fetal risk factors for early-onset neonatal sepsis. Blood investigations, including full blood counts, differential counts using the Sysmex XE-series (Sysmex Corporation, Kobe, Japan), and blood cultures, were performed. Biochemical markers and neutrophil parameters were analyzed to distinguish sepsis cases from non-sepsis cases.

Results: ANC and neut-Y (neut-RI) were found to be significant biomarkers for early-onset neonatal sepsis ($p < 0.05$). These two parameters can be used to differentiate between sepsis and non-sepsis, with a cut-off value of ANC and neut-Y (neut-RI) as 8.46 x 10^3/ul and 33.85ch, respectively**.**

Conclusion: ANC and neut-Y are promising tools for screening early-onset neonatal sepsis. The clinical history of risk factors supplemented with these two parameters will help to identify neonates who are at risk of sepsis and assist in further and prompt management.

Keywords: Bacterial Infections and Mycoses, Infant, Newborn, Neutrophil Activation, Sepsis

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Introduction

Neonatal sepsis is characterized by infections that occur during the neonatal period. In term neonates, this period spans the first 28 days of life, while for preterm neonates, it extends from birth to 28 days after the expected date of delivery. Neonatal sepsis is categorized into early-onset and late-onset sepsis. Early-onset sepsis (EOS) occurs within the first 72 hours of life (1).

The current EOS investigation guidelines include a full blood count with a differential count, immature granulocyte (IG) to total neutrophil ratio, blood culture, and C-reactive protein testing [1]. However, numerous studies have indicated that the differential count may not be an optimal marker due to variability in the morphological identification of neutrophils and their precursors among morphologists (2). Blood culture is traditionally the gold standard for confirming neonatal sepsis, but it takes a minimum of 3–8 days for results to be obtained (3). Hence, numerous studies have investigated potential biomarkers with high sensitivity and specificity and shorter turnaround times.

While neutrophil count has traditionally served as an infection marker, it is insufficient alone; neutropenia has been observed in 8% of neonates admitted to the neonatal intensive care unit (NICU) (4). Furthermore, the immature to total neutrophil (I: T) ratio, calculated as I: T ratio = [(bands + metamyelocytes + myelocytes) / (segmented neutrophils + bands + metamyelocytes + myelocytes)], has demonstrated predictive value for neonatal sepsis. In various studies, the cutoff value for the I: T ratio was predominantly set at >2.0; however, sensitivities varied between 47% and 52% (5,6). Additional potential markers include neutrophil granulation and the nucleic acid/protein content of neutrophils.

Focusing on neutrophil details is crucial, as such details are integral to the innate immune response, and neutrophil dysregulation can significantly impact the morbidity and mortality of sepsis patients (7). A previous study examined neutrophil granule activation, revealing a notable increase in hypogranulation followed by hypergranulation of neutrophils after exposure to substances mimicking bacterial presence, such as lipopolysaccharide and formyl-methionyl-leucyl-phenylalanine (6, 8). The Sysmex XE or XN (Sysmex Corporation, Kobe, Japan) hematology analyzer measures parameters known as neut-X (neut-GI) and neut-Y (neut-RI) for the granularity and reactivity index, respectively, in neutrophils. The 90° angle side scatter of neutrophils is proportional to the granularity index (9). High granulation among adults with sepsis was associated with high neut-X (neut-GI) measurements (10). High IG count is correlated with higher nucleic acid and protein content, which is represented by neut-Y (neut-RI). High neut-Y (neut-RI) is explained by high nucleic acid and protein content in immature cells. Neut-X (neut-GI) and neut-Y (neut-RI) were significantly higher in adult patients with sepsis than in those non sepsis and healthy groups (11). Therefore, the objective of this study was to evaluate the new and old neutrophil parameters, including automated absolute neutrophil count (ANC), IG, I: T ratio, neutrophil granulation (neut-X/neut-GI), and neutrophil nucleic acid/protein content (neut-Y/neut-RI), as potential screening biomarkers in EOS. In addition, the bacterial profile of EOS was determined.

Materials and Methods

The study received approval from the Ethics Committee (Protocol code: USM/JEPEM/14120506) and was carried out according to the Declaration of Helsinki. It involved 135 neonates born within 72 hours of life who were identified with maternal and fetal risk factors for

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early-onset neonatal sepsis. The inclusion criteria for maternal risk factors included mothers who were (i) carriers of Group B streptococcus (GBS), (ii) had a history of GBS in a previous pregnancy, (iii) experienced prolonged rupture of membranes (<18 hours), (iv) had maternal pyrexia, and (v) had foul-smelling amniotic fluid. Fetal risk factors included neonates with (i) an APGAR score of less than 5, (ii) tachycardia (>160/min) or bradycardia (<100/min), (iii) prematurity (<37 weeks of gestation), and (iv) low birth weight (<2500g). Clinical indicators of sepsis include temperature instability, behavioral changes, abnormal skin appearance (e.g., petechiae, jaundice, sclerema, mottling, or pallor), feeding difficulties, and cardiovascular, respiratory, or metabolic issues. Neonates with any of these risk factors or clinical indicators were classified as high-risk and presumed septic. Healthy neonates without maternal risk factors or clinical indicators served as the control group. Neonates with congenital malformations or congenital infections related to the TORCH complex (toxoplasmosis, rubella, cytomegalovirus, or herpes simplex) were excluded. Clinical data from mothers and neonates were obtained from the hospital's record office, NICU, and labor room. These data included maternal conditions, maternal risk factors for neonatal sepsis, gestational age, and fetal risk factors.

Five hundred microliters of peripheral blood were collected within 24 hours of life by using an EDTA BD microtainer tube ® from neonates who have been identified with a history of maternal and fetal risk. They were then classified as presumed sepsis neonates. Two mL of cord blood was taken at birth for identified healthy neonates, who served as controls.

I. Full blood count using the Sysmex XE-series (Sysmex Corporation, Kobe, Japan). Measurements for neutrophil indices, such as absolute neutrophil count (ANC), immature granulocytes (IG) count, immature granulocytes and total neutrophil (I: T) ratio, neut-X/neut-GI, and neut-Y/neut-RI were recorded. An additional 1 mL of blood was sent for blood culture in Bactec® Peds Plus bottles. These bottles were then incubated in a BACTEC 9240 instrument (Becton Dickinson, Cockeysville, MD, USA). Positive bottles underwent Gram staining processes and species identification using Vitek 2 cards (bioMérieux, Marcy-l'Étoile, France)**.** For clarity, the following operational definitions were applied throughout the study: Presumed sepsis refers to neonates presenting with maternal or fetal risk factors and/or clinical signs of infection, who were treated empirically with antibiotics regardless of blood culture result.

II. Sepsis is defined as neonates with a positive blood culture confirming infection.

III. Non-sepsis refers to neonates with negative blood cultures despite being clinically suspected of infection.

Laboratory parameters were analyzed using independent t-tests for normally distributed data and Mann–Whitney U tests for non-normally distributed data, as determined by normality testing. The choice between parametric and non-parametric tests was based on the data distribution to ensure appropriate statistical handling. For categorical variables, such as CRP and blood culture results, chi-square or Fisher's exact tests were applied. Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated with 95% confidence intervals (CIs). Sensitivity was defined as the ability of the test to correctly identify neonates with sepsis, whereas specificity was the ability to correctly identify those without sepsis. ROC (receiver operating characteristic) curve analysis was used to evaluate the diagnostic performance of

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each laboratory parameter, with predictors considered strong if the area under the curve (AUC) exceeded 0.7.

Results

In this study, 135 neonates were presumed to have sepsis based on maternal and fetal risk factors. The most common maternal risk factors were prolonged rupture of membranes (26%), maternal fever (15%), foul-smelling amniotic fluid (10%), and maternal history of GBS (2%). Fetal risk factors included low APGAR score (95%), low gestational age (67%), and low birth weight (51%). Of these neonates, 18.5% tested positive on blood culture, while 81.5% tested negative.

A total of 25 neonates had positive blood cultures, with 16% (4 out of 25) showing multiple bacterial species (Figure 1). Gram-positive bacteria were found in 11 neonates (38%), Gram-negative bacteria in 16 neonates (55%), and yeast in two neonates (7%). Thirty-two percent (8 out of 25) of these neonates died, predominantly (75%) due to infections from Gram-negative bacteria. The most prevalent bacteria isolated were CoNS (24%), followed by Klebsiella pneumoniae (K. pneumoniae) (18%) and GBS (12%). Methicillin-resistant CoNS (12%) and extended-spectrum beta-lactamases (ESBL) producing K. pneumoniae (3%) were the most common resistant pathogens.

Table 1 presents the initial comparison between presumed sepsis and healthy neonates, emphasizing the hematological parameters. Neonates with presumed sepsis displayed significant neutrophilia and an I: T ratio (automated) derived from the hematology analyzer. Both neut-X/neut-GI and neut-Y/neut-RI were significantly higher. Table 2 shows a comparison between neonates with blood culture positive, defined as sepsis, and blood culture negative as non-sepsis. Only ANC and neut-Y/neut-RI were statistically significant difference parameters between sepsis and non-sepsis, with cut-off value as 8.46 x 10^3/ul and 33.85 ch (channel) respectively, where 'ch' denotes fluorescence intensity channels as measured by Sysmex hematology analyzers." Table 3 provides an evaluation of significant parameters using ROC curve analysis, detailing the area under the curve (AUC), sensitivity, specificity, and cut-off points for various laboratory parameters. Figure 2 illustrates the ROC curve analysis for NRBC count and Neut-Y, with the the area under the curve (AUC), sensitivity, specificity, and cut-off points for NRBC and Neut-Y evaluation. This demonstrates their effectiveness as biomarkers. Both parameters were significantly elevated in neonates with sepsis compared to those without. Elevated NRBC count and Neut-Y values strongly suggest neonatal sepsis.

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Table I: Laboratory parameters for presumed neonatal sepsis.

Predictors of EOS (unit)	Presumed sepsis n = 135 Mean (SD)	Healthy Control n = 25 Mean (SD)	Mean score different (95% CI)	t-statistic (df)	p-value[c]
WBC (10^3/uL)	15.28 (7.67)	13.88 (4.79)	-1.40 (-4.54, 1.74) x10^3/uL	-0.88 (158)	0.234[a]
Neut# (10^3/uL)	9.34 (5.54)	7.41 (3.25)	-1.92 (-4.24,0.39) x10^3/uL	-1.64 (146)	0.013[a]
IG# (10^3/uL)	0.34 (0.48)	0.44 (0.47)	0.10 (-0.11, 0.31) x10^3/uL	0.95 (150)	0.050[b]
IG%	1.83 (1.94)	2.85 (2.24)	1.02 (0.14, 1.89) %	2.31 (153)	0.050[b]
I:T ratio (automated)	0.028 (0.028)	0.059 (0.048)	0.03 (0.02, 0.44)	4.33 (149)	0.001[b]
I:T ratio (manual)	0.018 (0.046)	0.025 (0.075)	0.01 (-0.01, 0.03)	0.65 (151)	0.721[b]
Neut-X (ch)	133.45 (3.20)	131.32 (2.02)	-2.13 (-3.14, -1.11)ch	-4.23 (155)	0.001[a]
Neut-Y (ch)	37.28 (8.59)	35.04 (2.06)	-2.24 (-3.93, -0.55)ch	-2.62 (155)	0.010[a]
RBC parameters					
RBC# (10^6/uL)	4.57 (0.87)	4.68 (0.59)	0.12 (-1.65, 0.40) x10^6/uL	0.83 (157)	0.406[a]
Hb# (g/dL)	15.89 (3.08)	16.48 (1.63)	0.59 (-0.25, 1.43) g/dL	0.937 (158)	0.162[a]
RETIC# (10^6/uL)	0.31 (0.22)	0.19 (0.06)	-0.12 (-0.17, -0.07) x10^6/uL	-4.66 (115)	0.001[b]
RETIC%	5.86 (2.95)	5.47 (0.06)	-0.39 (-2.06, 1.28) %	-0.47 (144)	0.008[b]
NRBC# (10^3/uL)	1.14 (3.43)	0.81 (0.81)	-0.33 (-1.07, 0.41) x10^3/uL	-0.43 (129)	0.005[b]
NRBC%	12.03 (47.27)	4.95 (4.20)	-7.09 (-26.21, 12.04) %	-0.73 (153)	0.017[b]
Platelet parameter					
PLT# (10^3/uL)	196.42 (88.15)	238.55 (112.12)	42.13 (-2.95, 87.21) x10^3/uL	1.85 (114)	0.067[a]

[a] Independent t-test
[b] Mann-Whitney U test
[c] Significance at p-value < 0.05
Abbreviations: WBC, white blood cell count; Neut#, absolute neutrophil count; IG#, immature granulocyte count; IG%, immature granulocyte percentage; I:T ratio, immature to total neutrophil ratio; Neut-X, neutrophil granularity index (channel); Neut-Y, neutrophil reactivity index (channel); RBC#, red blood cell count; Hb#, hemoglobin; RETIC#, reticulocyte count; RETIC%, reticulocyte percentage; NRBC#, nucleated red blood cell count; NRBC%, nucleated red blood cell percentage; PLT#, platelet count.

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Table II: Neutrophil indices between sepsis and non-sepsis neonates.

Neutrophil indices	Sepsis n = 25 Mean (SD)	Not sepsis n = 110 Mean (SD)	Mean score different (95% CI)	t-statistic (df)	p-value
ANC (10^3/uL)	6.48 (5.48)	9.50 (5.27)	3.01 (0.21, 5.82) x10^3/uL	2.14 (69)	0.036[a]
IG# (10^3/uL)	0.33 (0.70)	0.30 (0.43)	-0.03 (-0.29, 0.24) x10^3/uL	-0.22 (72)	0.313[b]
IG%	2.14 (2.67)	1.54 (1.56)	-0.61 (-1.57, 0.36) %	-1.25 (75)	0.684[b]
I:T ratio	0.03 (0.03)	0.02 (0.02)	-0.001 (-0.01, -0.002)	-0.548 (69)	0.333[b]
Neut-X	133.80 (2.91)	133.36 (3.39)	-0.44 (-2.03, 1.15)	-0.55 (76)	0.580[a]
Neut-Y	45.24 (11.13)	32.95 (6.41)	-12.30 (-16.27, -8.32)	-6.16 (76)	0.000[a]

* a Independent t-test b Mann–Whitney U test

Abbreviations: ANC, absolute neutrophil count; IG#, immature granulocyte count; IG%, immature granulocyte percentage; I:T ratio, immature to total neutrophil ratio; Neut-X, neutrophil granularity index (channel); Neut-Y, neutrophil reactivity index (channel).

Table III: ROC curve analysis for neutrophil indices.

Neutrophil indices	AUC	Sensitivity (%)	Specificity (%)	Cut-off point*
ANC (10^3/uL)	0.583	60	57	8.46 x 10^3/uL
Neut-Y/Neut-RI (ch)	0.768	70	65	33.85 ch
NRBC#	0.644	70	51	1.25 x 10^3/uL

* Cut-off for laboratory parameters were chosen to prioritize higher sensitivity over specificity. "ch" = channel; a unit of fluorescence signal intensity measured by Sysmex hematology analyzers for cell complexity.

Abbreviations: ANC, absolute neutrophil count; Neut-Y, neutrophil reactivity index (channel); NRBC#, nucleated red blood cell count; AUC, area under the curve.

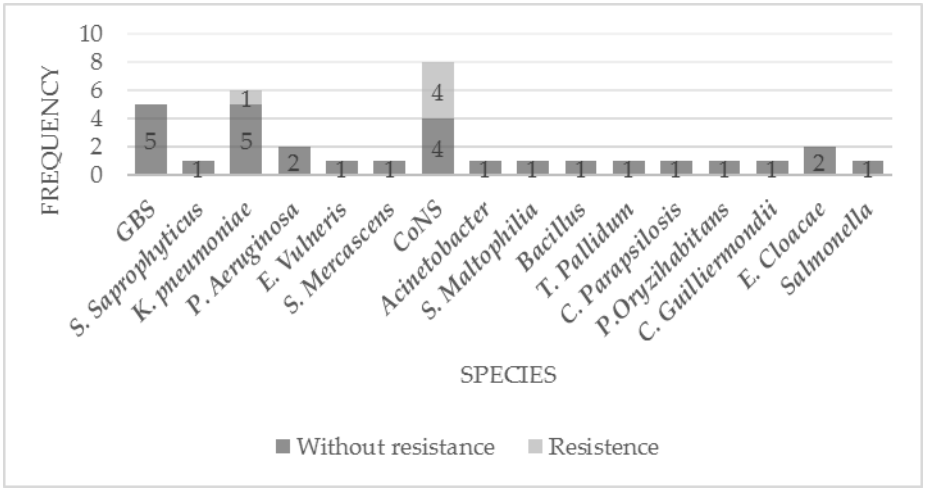


Figure 1. Bacteria profile in early-onset neonatal sepsis.

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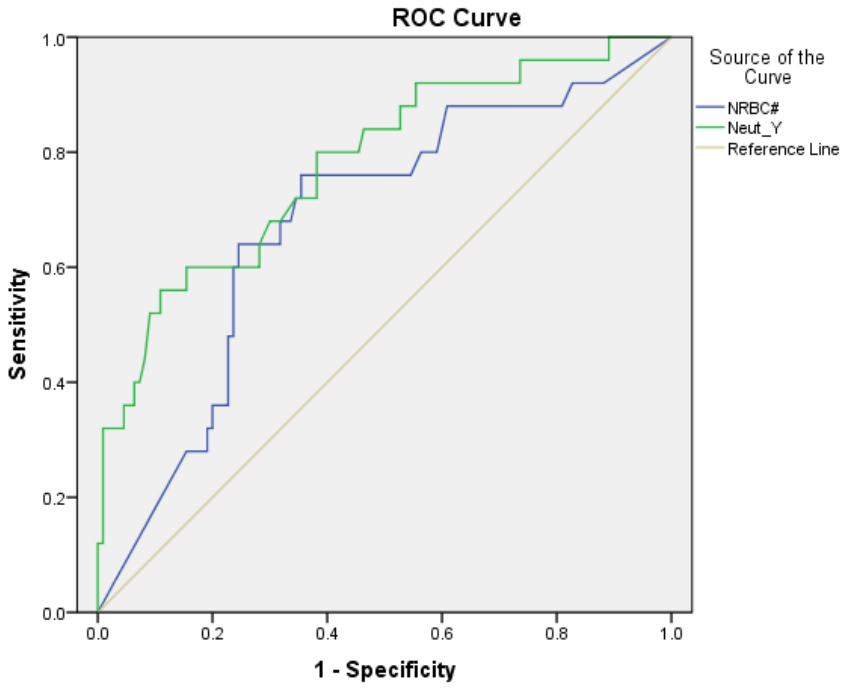


Figure 2. ROC curve analysis for NRBC count and Neut-Y.

Discussion

The study found that despite the promising use of automated IG values as sepsis markers in adults and children, IG values and the I:T ratio were unsuitable for identifying sepsis in neonates born within 72 hours. There was no statistically significant difference in IG values and I:T ratios between sepsis and non-sepsis neonates ($p > 0.05$) (12). These findings are consistent with previous research indicating that IG is not effective for EOS screening in neonates (13). Another study demonstrated that IG values were only significant for predicting neonatal sepsis in infants older than 7 days (14). A critical review highlighted that the "left shift" phenomenon, where immature cells are released into circulation, occurs in phases and may not be captured accurately with a single IG measurement. The insignificant results in this study could be attributed to rapid pathophysiological changes in neonates within the first 72 hours of life (15). Research has shown significant fluctuations in IG values and I:T ratios within the first day of life, with term neonates under 12 hours old having lower values than those of neonates over 12 hours old. This variability underscores the challenges of using IG and I:T ratio as sepsis markers in very young neonates (16).

ANC proved valuable in predicting EOS with a significant p-value (<0.05). Similar findings were reported in another study (17). However, these results contradicted a study that used manual ANC counting, which found no statistical difference between sepsis and non-sepsis neonates (18). Manual counting's subjectivity and dependence on observer skills can affect consistency and reliability. Automated blood analyzers provide more precise results by counting a larger number of cells, ensuring consistency even with unequal cell distribution and low IG counts (19, 20).

The results of this study appear to contradict previous research conducted among adults. Several factors might explain this discrepancy. Firstly, the

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sample in this study was sourced from presumed sepsis neonates. The comparison was made between positive and negative blood culture cases among these presumed sepsis neonates. It is highly likely that many of these neonates were experiencing inflammation due to other health issues common among preterm infants, such as chronic lung disease or necrotizing enterocolitis (21). The mean neut-X/neut-GI value in presumed sepsis cases (133.45±3.20) was almost identical to the mean in neonates with positive blood cultures (133.80±2.91). Therefore, neut-X/neut-GI is not a specific marker for predicting EOS. This contrasts with findings in adults, where neut-X/neut-GI is reported to be significantly higher in sepsis cases (11). In this study, blood samples were taken within the first three days postnatal. Neut-X/neut-GI was not elevated in the neonatal sepsis group, likely because sepsis had not yet progressed to a serious stage. Another possible reason is that neonates have higher levels of IG, and their neutrophil granules are impaired compared to those of adults (22). Neutrophil granules contain bactericidal/ permeability-increasing protein (BPI), which binds to the lipopolysaccharide of Gram-negative bacteria to combat infection. BPI levels are lower in neonates than in adults. Additionally, neonatal neutrophils lack gelatinase and secretory granules that are present in adult neutrophils (23). Since granulation is impaired in neonates, changes in granulation were not significant. Therefore, neut-X/neut-GI cannot be used as a predictive marker for EOS in neonates, unlike in adults.

Assessment of neutrophil activation for sepsis screening involves not only phagocytosis but also the production of neutrophil extracellular traps (NETs) to counteract pathogens. A study showed that NETs are released from neutrophils approximately 10 minutes after activation. NETs consist of DNA, histones, and neutrophil elastase, which work together to bind and kill bacteria (24). In this study, neut-Y/neut-RI was significantly higher in sepsis neonates than in non-sepsis neonates ($p < 0.05$). Neut-Y is a fluorescence-based parameter measured by modern hematology analyzers, reflecting the intracellular nucleic acid and protein content of neutrophils. In sepsis, neutrophils undergo early activation, increasing their transcriptional and translational activity, which elevates fluorescence intensity. This correlates with cellular complexity and activation status. Elevated Neut-Y likely indicates heightened immune responsiveness and NETs formation, characteristic of the early inflammatory phase of sepsis (24). These findings are consistent with previous studies showing increased Neut-Y levels in adults with sepsis (11). The increase in neut-Y/neut-RI might be attributed to elevated protein and DNA content in neonatal neutrophils.

This study demonstrated that toxic granulation can be quantified using the Granulation Index (GI) with a blood analyzer such as the Sysmex XE-5000, making it useful for sepsis screening across a wide age range, from neonates to adults (25). However, this study focused solely on neonates under 72 hours old and found no significant difference in GI between sepsis and non-sepsis cases (p-value > 0.05), which aligns with manual observations of toxic granulations in another study (26).

In this study, 55% (16 cases) of the isolated pathogens were predominantly Gram-negative bacteria. Notably, death cases were also primarily associated with Gram-negative bacteria, with all fatalities involving premature neonates. Several other studies have reported similar findings, indicating that the majority of fatal EOS cases are caused by Gram-negative bacteria (27–29). The detrimental effects of being premature were more pronounced among sepsis neonates with

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Gram-negative bacteria. Since EOS is often associated with maternal colonization or infection, the pathogens involved were frequently colonizer types, such as CoNS, K. pneumoniae, and GBS. K. pneumoniae is typically found in the gastrointestinal tract, CoNS can be present on the skin or in the mucus of the mother, and GBS usually inhabits the genital area (30). It was observed that neonates were at high risk for GBS infection when the mother had intrapartum GBS colonization. One in four neonates with isolated GBS died. Neonates are believed to encounter the pathogen through vertical transmission, such as from contaminated amniotic fluid or during passage through an infected birth canal. Critical care for mothers with a history of GBS colonization is highly recommended to prevent pathogen transmission. K. pneumoniae was the most frequent isolate associated with neonatal mortality in sepsis cases. This finding is not unique to this study, as K. pneumoniae is also a common and concerning pathogen in neonatal sepsis in several Asian countries, including India and Egypt (31–33). A study identified risk factors associated with K. pneumoniae infection as low gestational age, low birth weight, and being within the first 72 hours of life. Given the high mortality rate associated with this pathogen and the frequency of its occurrence, it is crucial to emphasize proper management and preventive strategies for K. pneumoniae infections in neonates.

Study Limitations

The study focused on EOS in neonates within the first three days of life, during which physiological changes affect hematological parameters such as hemoglobin, WBC, ANC, and I:T ratio. To minimize the influence of these changes, future studies could group neonates by sample collection time (e.g., within 24, 48, or 72 hours) and ensure similar sample sizes. Additionally, analyzing term and preterm neonates separately is recommended, as preterm neonates typically exhibit lower ANC, RBC, and hemoglobin levels due to limited iron supply from the mother during the third trimester.

The unequal group sizes, particularly the small number of healthy controls (n=25) compared to the presumed sepsis group (n=135), represent a limitation of this study. This imbalance was primarily due to the difficulty in recruiting suitable healthy neonates without risk factors or clinical symptoms within the study timeframe. To mitigate this issue, statistical methods such as Welch's t-test which accounts for unequal variances and sample sizes were applied where appropriate to ensure more reliable comparisons. Nonetheless, larger and more balanced cohorts in future studies are essential to validate these findings and strengthen statistical power.

Conclusion

This study demonstrates that among conventional and extended neutrophil parameters, absolute neutrophil count (ANC) and Neut-Y/neut-RI provide the most reliable diagnostic performance for screening early-onset neonatal sepsis. While traditional markers such as immature granulocyte count and I:T ratio were not useful in the first 72 hours of life, Neut-Y showed strong discriminatory ability, reflecting early neutrophil activation and intracellular changes during infection. Despite limitations, including unequal group sizes and challenges in control recruitment, our findings highlight the potential of incorporating automated scatter-based parameters into routine hematology analysis for faster and more accurate identification of at-risk neonates. Larger multicenter studies are warranted to validate these results and establish standardized cut-off values before clinical implementation.

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Data availability

The datasets generated and analyzed during the current study are not publicly available due to patient confidentiality restrictions but are available from the corresponding author on reasonable request.

Ethical Considerations

The study was approved by Jawatankuasa Etika Penyelidikan Manusia (JEPeM-USM) Committee. (Protocol code: USM/JEPEM/14120506).

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Authors' Contributions

Conceptualization, R.H; methodology, N.N.F.N.M; validation, R.H.; N.R.I; W.R.W.R..; formal analysis, N.N.F.N.M; writing: original draft preparation, N.N.F.N.M, S.A.H; writing: review and editing, R.H; R.H.Z. S.A.H M.A.R.M.R., S.A, N.I.S.S; supervision, R.H. All authors have read and agreed to the published version of the manuscript. Authors 1 and 2 have equally contributed.

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Conflict of Interest

The authors declare that there is no conflict of interest to disclose.

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