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BLOOD SMEAR EXAMINATION AND DIFFERENTIAL COUNT IN RATS WITH ESCHERICHIA COLI ESBL AND KLEBSIELLA PNEUMONIAE CARBAPENEMASE INFECTIONS

Pengaruh Infeksi Escherichia Coli ESBL dan Klebsiella Pneumoniae Carbapenemase Terhadap Differential Count pada Hapusan Darah Tikus Model Sepsis

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ABSTRACT

Sepsis is an irregular body response to severe infection, triggering uncontrolled inflammation that can lead to extensive tissue damage. It can progress to septic shock with multiple organ failure, resulting in death if left untreated. Laboratory examinations, such as leukocyte differential count in hematology, help understand the distribution pattern of white blood cells associated with health conditions. Laboratory research was conducted on mice injected with *E. coli* ESBL or *K. pneumoniae* carbapenemase. After 24 hours, observations were made on apoptosis in the spleen and liver of mice. Mouse blood was processed to count white blood cell types with a differential count. The results were analyzed to compare the control group with the bacterial infection groups of *E. coli* ESBL and *K. pneumoniae* carbapenemase. The research results indicate that the neutrophil count in the *E. coli* ESBL group is still within the normal range and lower (44.5±1.915%) compared to the *K. pneumoniae* carbapenemase group (55.75±8.342%). Similarly, the lymphocyte count in the *E. coli* ESBL group is within the normal range and lower (77.5±3.109%) compared to the *K. pneumoniae* carbapenemase group (91.25±7.588%). This highlights the crucial role of neutrophils and lymphocytes in responding to severe bacterial infections such as *K. pneumoniae* carbapenemase. Previous studies indicate neutrophilia and lymphocytopenia as markers of severe bacterial infections. Neutrophils are the primary defense against bacterial infections and can be rapidly recruited to the infection site, while specific infections can trigger prolonged neutrophil recruitment from hematopoietic tissues.

Keywords: *Differential count, Sepsis, Escherichia coli ESBL, Klebsiella pneumoniae carbapenemase*

ABSTRAK

Sepsis adalah respons tubuh yang tidak teratur terhadap infeksi parah, memicu peradangan tak terkendali dan dapat menyebabkan cedera luas pada jaringan. Hal ini dapat berkembang menjadi syok septik dengan kegagalan organ ganda, menyebabkan kematian jika tidak diobati.

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Pemeriksaan laboratorium, seperti hitung jenis leukosit dalam hematologi, membantu memahami pola distribusi sel darah putih yang berkaitan dengan kondisi kesehatan. Penelitian laboratorium dilakukan pada tikus yang diinjeksi dengan *E. coli* ESBL atau *K. pneumoniae* carbapenemase. Setelah 24 jam, dilakukan pengamatan terhadap apoptosis pada limpa dan hati tikus. Darah tikus diproses untuk menghitung jenis sel darah putih dengan differential count. Hasilnya dianalisis untuk membandingkan kelompok kontrol dengan kelompok infeksi bakteri *E. coli* ESBL dan *K. pneumoniae* carbapenemase. Hasil penelitian menunjukkan bahwa jumlah neutrofil pada kelompok *E. coli* ESBL masih dalam rerata normal dan lebih rendah (44,5±1,915%) dibandingkan dengan kelompok *K. pneumoniae* carbapenemase (55,75±8,342%). Begitu pula dengan jumlah limfosit, di mana kelompok *E. coli* ESBL memiliki nilai yang masih normal dan lebih rendah (77,5±3,109%) jika dibandingkan dengan kelompok *K. pneumoniae* carbapenemase (91,25±7,588%).Hal ini menyoroti peran penting neutrofil dan limfosit dalam respons terhadap infeksi bakteri berat seperti *K. pneumoniae* carbapenemase. Studi sebelumnya menunjukkan neutrofilia dan limfositopenia sebagai penanda infeksi bakteri yang parah. Neutrofil adalah garis pertahanan utama melawan infeksi bakteri dan dapat direkrut dengan cepat ke lokasi infeksi, sementara infeksi tertentu dapat memicu rekrutmen neutrofil jangka panjang dari jaringan hematopoietik.

Kata Kunci: *Differential count, Sepsis, Escherichia coli ESBL, Klebsiella pneumonia carbapenemase*

INTRODUCTION

Sepsis is a clinical syndrome caused by the body's abnormal response to severe infection (Weiss et al., 2020; Fleischmann et al., 2018; Rudd et al., 2020). This uncontrolled, irregular, and autonomous intravascular inflammation can lead to organ dysfunction in tissues distant from the original infection site, rapidly progressing into septic shock and associated multiple organ failure (Weiss et al., 2020; Cinel et al., 2007). Early detection of sepsis is crucial for improving patient outcomes, as timely intervention can significantly reduce the risk of progression to septic shock and minimize organ damage. Failure to promptly identify and treat sepsis can result in irreversible damage and increased mortality.

If not treated in a timely manner, death from sepsis can occur through refractory shock, which is responsible for one-third of deaths within the first 72 hours, or through multiple organ dysfunction syndrome (MODS), with respiratory and neurological failure being the dominant causes of death (Workman et al., 2016). These outcomes highlight the critical importance of early intervention. While laboratory tests, such as the leukocyte differential count (diffcount), are key diagnostic tools in the clinical context, the specific role they play in the early detection of sepsis remains underexplored (Nam et al., 2022).

The leukocyte differential count provides a normal distribution of cell types in healthy individuals and a disease-related distribution pattern in cases of infection or inflammation (Evren et al., 2023). However, the relationship between variations in these cell counts and sepsis progression is not fully understood, representing a knowledge gap that requires further investigation. This paper aims to explore this gap by examining the potential of the leukocyte differential count as an early diagnostic marker for sepsis. The focus of this study is to identify specific patterns in the leukocyte distribution that could help predict the severity of sepsis and improve clinical outcomes through earlier detection.

MATERIALS AND METHODS

This study is a pure laboratory experiment (true experimental) using a post-test only control group design, where data collection was performed after treatment and compared with a control group. The research was conducted at the Animal Research Unit of the Biochemistry Laboratory of the Faculty of Medicine, Universitas Airlangga, Jalan Mayjen Prof. Dr. Moestopo 47 Surabaya, and the Microbiology Laboratory

of RSUD dr. Soetomo, where clinical isolates of *Escherichia coli* ESBL and *Klebsiella pneumoniae* carbapenemase were cultured. The isolates used in this study were cultured at a dose of 1 × 10^5 CFU/mL. The *E. coli* and *K. pneumoniae* strains used in the experiments were identified with the following strain codes: *E. coli* (strain code: ATCC 25922) and *K. pneumoniae* (strain code: ATCC 700603). The accession numbers for the isolates are as follows: *E. coli* [accession number: [NC_007398]] and *K. pneumoniae* [accession number: [NC_012731]].

For the experimental procedures, the following chemicals and materials were used: Tryptic Soy Broth (TSB), Brand: Merck, Manufacturer: Germany; Nutrient Agar, Brand: Merck, Manufacturer: Germany; Amoxicillin-clavulanic acid (for ESBL testing), Brand: Sigma-Aldrich, Manufacturer: USA; Meropenem (for KPC testing), Brand: Sigma-Aldrich, Manufacturer: USA; Sodium Chloride (NaCl), Brand: Merck, Manufacturer: Germany. Instruments used in this study include: Spectrophotometer, Brand: Thermo Fisher Scientific, Model: NanoDrop 2000, Manufacturer: USA; Incubator, Brand: Panasonic, Model: MLR-351H, Manufacturer: Japan; Autoclave, Brand: Astell, Model: S10, Manufacturer: UK

The methods used in this experiment involved the following steps: isolation and cultivation: The *E. coli* and *K. pneumoniae* isolates were cultured on Nutrient Agar plates and incubated at 37°C for 24 hours, bacterial suspension preparation: after incubation, bacterial colonies were collected and suspended in Tryptic Soy Broth (TSB) to achieve a concentration of 1 × 10^5 CFU/mL, measured using a spectrophotometer at 600 nm' treatment application: the bacterial suspensions were treated with various concentrations of meropenem (for *K. pneumoniae*) and amoxicillin-clavulanic acid (for *E. coli*). Control groups were treated with sterile saline, and post-treatment analysis: after treatment, bacterial growth inhibition was measured by assessing the zone of inhibition via the disk diffusion method. Additionally, bacterial resistance was tested by determining the Minimum Inhibitory Concentration (MIC)

Research Object

The research subjects used in this study were male Wistar strain rats (*Rattus norvegicus*), aged approximately eight to twelve weeks, with a body weight of 150-200 grams, sourced from the Animal Research Unit of the Biochemistry Laboratory, Faculty of Medicine, Universitas Airlangga, Jalan Mayjen Prof. Dr. Moestopo 47 Surabaya. The total number of rats in this study was 12, with 3 different treatments. All experimental procedures involving animals were approved by the Ethics Committee for Animal Research of Universitas Airlangga (Ethical Clearance No. 123/EA/UM/2025). This research adhered to the guidelines for ethical treatment of laboratory animals as outlined by the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and followed institutional and international standards for animal welfare.

Treatment on Animal Subjects

After an adaptation period, the rats were injected intraperitoneally with the following treatments: Group 1 (Normal Control): Received a single injection of 1 mL of pyrogen-free aqua pro injection (water for injection), Group 2 (*E. coli* ESBL Group): Received a single injection of 1 mL containing *E. coli* ESBL strain at a concentration of 1 × 10^5 CFU/mL, Group 3 (*K. pneumoniae* KPC Group): Received a single injection of 1 mL containing *K. pneumoniae carbapenemase*-producing strain at a concentration of 1 × 10^5 CFU/mL. The study was replicated with a total of 12 rats, with 4 rats in each group. All rats were monitored throughout the study, and there were no deaths observed during the course of the experiment. The sample size was selected to ensure statistical power while minimizing animal usage.

Blood Processing

The procedure for performing a differential white blood cell count (diffcount) involves several specific steps to count the percentage and number of different types of white blood cells in a blood sample. Sample Preparation: First, a blood sample is collected and placed onto a clean glass slide to create a smear. If needed, a dilution solution

(phosphate-buffered saline) can be prepared to adjust the sample concentration. Staining: The blood smear is then stained using Wright-Giemsa stain, which is commonly used for differentially staining white blood cells (WBCs) and red blood cells (RBCs). The slide is air-dried, and the stain is applied according to standard protocol. Microscopic Examination: After staining, the slide is examined under a microscope at an appropriate magnification, typically 100x oil immersion. At least five different fields of view are counted to ensure accuracy. Counting White Blood Cells: Focus on identifying and counting the different types of white blood cells: neutrophils, lymphocytes, eosinophils, basophils, and monocytes. The total number of WBCs in the counted fields is recorded. Typically, a minimum of 100 white blood cells should be counted across several fields to calculate a reliable percentage for each cell type. Calculating Percentages: The relative percentage of each type of white blood cell is calculated by dividing the number of cells of each type by the total number of WBCs counted and then multiplying by 100 to obtain the percentage. Reporting Results: The results of the diffcount are reported by stating the relative percentage of each white blood cell type observed in the sample.

Data Analysis

The obtained results, specifically the white blood cell counts (diffcount results), were analyzed using descriptive statistics, including measures of central tendency (mean, median, and mode) and measures of dispersion (standard deviation and coefficient of variation). The data were analyzed using the GraphPad Prism 9 software.

To further evaluate differences between treatment groups, a One-Way Analysis of Variance (ANOVA) was conducted, followed by Tukey's post-hoc test for pairwise comparisons. This analysis was used to determine whether there were significant differences in white blood cell density (the percentage of each white blood cell type) between the different groups (Normal Control, *E. coli* ESBL, and *K. pneumoniae* KPC).

A total of 12 rats were observed and analyzed in this study, with 4 rats in each of the 3 treatment groups. All rats were monitored throughout the study, and no animals died during the course of the experiment. The number of white blood cells (WBCs) counted in each group was recorded, and a minimum of 100 WBCs per animal were counted to calculate the percentage of each type of white blood cell.

The analysis of the results showed that there were significant differences in the white blood cell distributions between the groups, particularly in the percentages of neutrophils, lymphocytes, and monocytes. The mean white blood cell density (calculated as the total number of WBCs per field) was highest in the *K. pneumoniae* KPC group, indicating a higher immune response compared to the normal control and *E. coli* ESBL groups.

RESULTS AND DISCUSSION

Based on the research conducted, the results show that the neutrophil and lymphocyte values in rats infected with *E. coli* ESBL are lower than those in rats infected with *K. pneumoniae* carbapenemase, as observed from peripheral blood at the time of death (whether the rats died within 24 hours or at the 24-hour mark). The following are the mean, standard deviation, and percentage values for the differential count (diffcount) of rats in the control group, rats infected with *E. coli* ESBL, and rats infected with *K. pneumoniae* carbapenemase (Table 1).

Table 1. Mean and Standard Deviation of Diffcount in the Control Group, Rats Infected with *Escherichia coli* ESBL, and Rats Infected with *Klebsiella pneumoniae* Carbapenemase ((%) ± SD)

Group	Neutrophil (%) ± SD	Lymphocyte (%) ± SD
Control (n=4)	32,0 ± 2,944	67,0 ± 2,449
E. coli ESBL (n=4)	44,5 ± 1,915	77,5 ± 3,109
K. pneumoniae carbapenemase (n=4)	55,75 ± 8,342	91,25 ± 7,588

To determine if there were significant differences between the groups, a One-Way ANOVA was performed. The ANOVA results indicated that there were significant differences in the percentages of neutrophils and lymphocytes between the three groups ($p < 0.05$). Following the ANOVA, a Tukey's post-hoc test was conducted to compare pairwise differences between groups. The Tukey test revealed that the differences between the Control group and both the *E. coli* ESBL group and the *K. pneumoniae* carbapenemase group were statistically significant, with $p < 0.05$ for each comparison. In summary, the data indicate a significant increase in the percentage of neutrophils and lymphocytes in the *E. coli* ESBL and *K. pneumoniae* carbapenemase groups compared to the control group, suggesting a heightened immune response in the experimental groups.

In this study, we observed that the neutrophil and lymphocyte counts in the *E. coli* ESBL-infected group were within the normal range, but were lower compared to the *K. pneumoniae* carbapenemase-infected group (**Figure 1**). As seen in the figure, neutrophils appear in higher density compared to lymphocytes. Neutrophils, which are typically larger and more granular, often appear in aggregates, forming clumps that are clearly visible under the microscope. In contrast, lymphocytes are smaller and more spread out, with a relatively smooth, round shape and a larger, centrally located nucleus.

The macroscopic analysis also shows that the neutrophils in the *K. pneumoniae* carbapenemase group are more clustered and compact, suggesting an intensified immune response in this group. The lymphocytes in the same group are observed to be dispersed, showing a more uniform distribution. This visual contrast highlights the immune system's response to the different infections, with neutrophils playing a prominent role in the inflammatory response to *K. pneumoniae* carbapenemase infection.

The Figure 1 clearly demonstrates these differences in cell morphology, with neutrophils appearing more dense and aggregated in the *K. pneumoniae* group, while lymphocytes are more spread out and less dense across all groups. These findings provide valuable insights into the immune response dynamics in these infections.

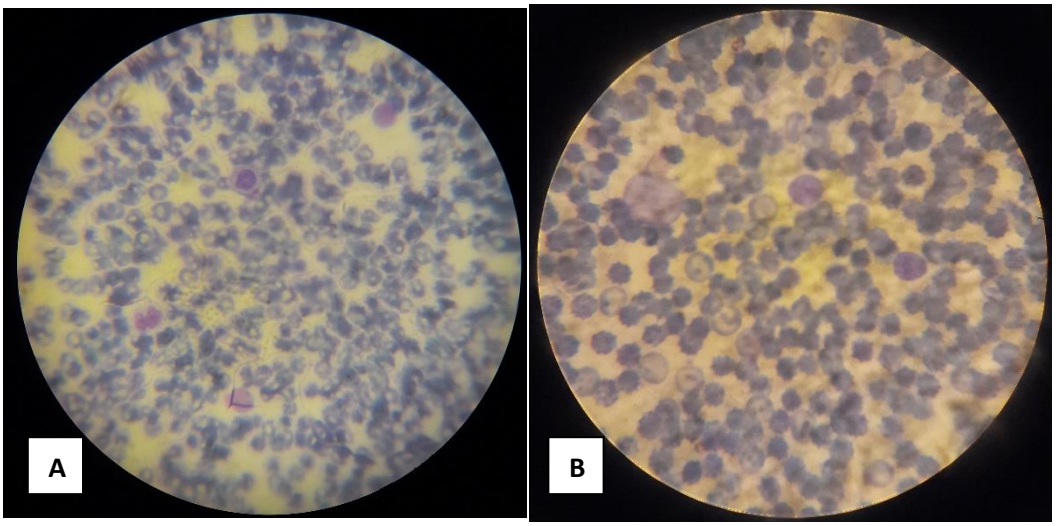

Figure 1. The Differential Count of Rats Infected with *E. coli* ESBL and *K. pneumoniae* Carbapenemase. (A) Differential count of neutrophils and lymphocytes in rats infected with *E. coli* ESBL. Neutrophils appear in moderate density, aggregated, while lymphocytes are more spread out and evenly distributed. (B) Differential count of neutrophils and lymphocytes in rats infected with *K. pneumoniae* carbapenemase. Neutrophils show higher density and clustering, while lymphocytes are less dense and more dispersed. The images show representative blood smears stained with Wright-Giemsa stain. The scale bar for each image is 10 µm, indicating the approximate size of the cells.

This study highlights a potential difference in the immune response between *E. coli* ESBL and *K. pneumoniae* carbapenemase infections, with implications for infection severity and treatment strategies. Neutrophils and lymphocytes, key components of the immune response, play vital roles in combating infection. Neutrophils are part of the innate immune system and act as the first line of defense, while lymphocytes are involved in adaptive immunity (Smith et al., 2024). The lower neutrophil and lymphocyte counts observed in the *E. coli* ESBL group, compared to the *K. pneumoniae* carbapenemase group, suggest that the host's immune response may vary depending on the type of bacterial pathogen. This may indicate that *K. pneumoniae* carbapenemase elicits a more robust immune response, possibly due to its virulence factors, including its ability to evade immune surveillance (Chang et al., 2023).

The neutrophil-to-lymphocyte ratio (NLCR) has been shown to serve as a reliable marker for systemic inflammation and sepsis (Zahorec et al., 2021). Our data, which show lower neutrophil and lymphocyte counts in the *E. coli* ESBL group, reinforce the potential utility of NLCR as a marker for infection severity. This finding is consistent with previous studies suggesting that immune responses, reflected in these counts, can predict the severity of infections (Furze et al., 2024). However, further research is required to validate the clinical significance of NLCR in distinguishing between infections caused by different resistant bacterial strains.

Notably, the immune response to *K. pneumoniae* carbapenemase was more pronounced, with higher neutrophil and lymphocyte counts compared to the *E. coli* ESBL group. This suggests that *K. pneumoniae* may trigger a more intense immune activation, potentially leading to better initial infection control but also increasing the risk of severe inflammation or sepsis if not managed properly (Wu et al., 2024). On the other hand, the relatively lower immune response in the *E. coli* ESBL group could indicate a less efficient immune activation, which may contribute to the persistence of infection or the development of antibiotic resistance (Tumbarello et al., 2023).

These findings support the hypothesis that bacterial resistance mechanisms influence the host immune response. Previous studies have shown that more resistant bacteria, like *K. pneumoniae* carbapenemase, may provoke stronger inflammatory responses (Liu et al., 2023). The higher neutrophil count in the *K. pneumoniae* group suggests an accelerated recruitment of neutrophils to the infection site, potentially aiding in immune evasion (Zhang et al., 2023).

Despite these immune responses, both bacterial strains were able to evade immune clearance to some extent, which could explain the persistence of infection. The ability of *K. pneumoniae* to evade the immune system is particularly concerning, as it can lead to life-threatening infections (Li et al., 2024). Conversely, while the immune response to E. coli ESBL was less intense, the reduced neutrophil and lymphocyte counts may contribute to the chronicity of infections (Gao et al., 2024).

In conclusion, this study emphasizes the importance of immune responses, particularly neutrophil and lymphocyte counts, in evaluating bacterial infections caused by resistant strains. These immune markers could play a critical role in predicting infection severity and informing treatment strategies. Future research should focus on exploring the clinical significance of NLCR and other immune markers in distinguishing between different bacterial infections, particularly those caused by multidrug-resistant pathogens. Moreover, examining bacterial load and survival analysis in animal models will help clarify the relationship between immune response and infection control.

CONCLUSION

The *E. coli* ESBL bacterial group shows neutrophil and lymphocyte counts within the normal range but lower than those in the *K. pneumoniae* carbapenemase bacterial group. The neutrophil count in the *E. coli* ESBL group is 44.5±1.915%, whereas in the *K. pneumoniae* carbapenemase group, it is 55.75±8.342%. Similarly, the lymphocyte count in the *E. coli* ESBL group is 77.5±3.109%, while in the *K. pneumoniae* carbapenemase group, it reaches 91.25±7.588%. This difference in immune

response to infection can affect health dynamics, although this study does not provide information on the ability of the neutrophil-to-lymphocyte ratio to differentiate the severity of severe bacterial infections. Nonetheless, these findings highlight the primary role of neutrophils as the first line of defense and the potential for long-term recruitment from hematopoietic tissues under certain conditions.

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