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**Original**

# Development of new endotoxin measurement assay using bioluminescence method

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Abstract

Turbidimetric kinetic assay is currently used for plasma endotoxin measurement. In our previous study, we reported that its measurement accuracy was not high. Because many endotoxins in the blood exist in the bound state to or are taken up by leukocytes, we developed a measurement method that used leukocyte-rich plasma. This method significantly increased the accuracy of endotoxin measurement. In this study, we investigated whether newly developed automated bioluminescence method can be used to measure endotoxins using LRP. Blood was collected from 24 patients with suspected bacterial infection upon their arrival at our hospital and 8 healthy individuals. Comparison of measurement times revealed that the automated bioluminescence method required 20 min in all cases. This time was significantly shorter than that for the turbidimetric kinetic assay method. Comparing of the endotoxin levels revealed a significant correlation between the values from both methods. In addition, the automated bioluminescence method tended to show higher values.

Considering measurement time and accuracy, the automated bioluminescence method may be an excellent diagnostic tool for infectious diseases.

Key words : *endotoxin, turbidimetric kinetic assay, automated bioluminescence methods, leukocyte-rich plasma, sepsis*

## I. Introduction

Endotoxins are a cell wall component present in gram-negative bacteria. For measuring endotoxins in clinical samples, the turbidimetric kinetic assay, which utilizes a Limulus reaction, is used. Because this method uses plasma samples only endotoxins released into the plasma can be measured, thus reducing the measurement sensitivity [1, 2]. Endotoxins operate by binding to leukocytes and activating them in the blood. They are subsequently taken up by the cells. Therefore, most endotoxins are either bound to or are taken up by leukocytes, and it is believed that very little endotoxin is released into plasma. Based on this background, we have developed a method for measuring

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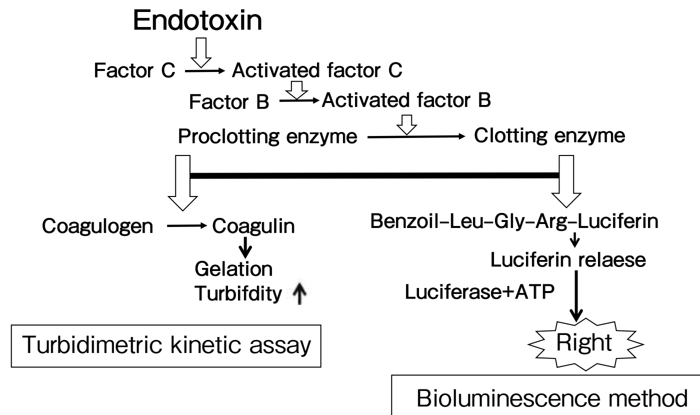


Fig. 1. The clotting enzyme, which is generated by the Limulus reaction, along with the endotoxin activates the luminescent substrate; the released luciferin emits light in the presence of luciferase and ATP.

endotoxins with turbidimetric kinetic assay using leukocyte-rich plasma samples (LRP). In our previous study, we have reported that this new measurement method has higher measurement accuracy than the conventional one [1, 2].

On the other hand, a bioluminescence technique for measuring endotoxin using high-intensity luciferase has been developed by Kuroda et al.[3] This technique was later improved by Onodera et al.[4] In this method, the clotting enzyme, which is generated by the Limulus reaction, along with the endotoxin activates the luminescent substrate; the released luciferin emits light in the presence of luciferase and ATP (Fig. 1). Recently, an automated method was developed, and it is being used clinically for the determination of endotoxin in dialysate [3, 4]. However, at present, no paper has examined endotoxins in human blood using this automated method. In this study, we compared the diagnostic of the turbidimetric kinetic assay using LRP and the automated bioluminescence method using the same samples for detecting infectious diseases.

## II. Materials and methods

### 1. Specimens

Between April 2018 and April 2019, patients who were transported to the Iwate Medical University Advanced Critical Care Center and were suspected of having a bacterial infection were approached for enrollment in this study. Written informed consent was obtained from all the patients or the patients' family members. This study was a prospective study, The "International Consensus Definition of Sepsis and Septic Shock, 3rd Edition (Sepsis-3)" was used to identify bacterial infection, sepsis, and septic shock. The Acute Physiology and Chronic Health Evaluation II (APACHE II) scores [5] and sequential organ failure assessment (SOFA) scores [6] were used as indices of severity.

### 2. Leukocyte-rich plasma (LRP)

After collecting 2 mL of blood from each patient, 0.1 mL of heparin was added to the whole blood sample and incubated at 37℃ for 5-10 min. Next, 800 μL of the 37℃-equilibrated blood and 400 μL of the 37℃-equilibrated 6% dextran T500 (Pharmacosmo, Holdaek, Denmark) were mixed and incubated at 37℃

for 15 min. The pellet contained red blood cells aggregated by dextran, whereas the supernatant layer was collected as the LRP. We have named this method the LRP37 method [7].

Dextran T500 was autoclaved at 121 ℃ for 90 min before use to ensure that endotoxin levels were below the detection limits of the Limulus test (i.e., endotoxin-free).

**3. Endotoxin measurement by turbidimetric kinetic assay**

After 10-fold dilution of the test samples, which was obtained by the LRP37 method, with a plasma pretreatment solution (0.02% Triton X-100 solution, FUJIFILM Wako Pure Chemical Corporation, Osaka), they were mixed well in a vortex mixer to ensure destroying leukocytes. Then, the samples were heated at 70℃ for 10 min and cooled in ice water for 5 min to prepare a pretreated specimen. Endotoxin single test Wako (FUJIFILM Wako Pure Chemical Corporation) was dissolved in 0.2 ml of the pretreated sample, and Toxinometer MT-5500® (FUJIFILM Wako Pure Chemical Corporation, Osaka) was used for measurement. The time taken for the amount of transmitted light to decrease by 8% was defined as the gelation time, and endotoxin concentration was calculated using a calibration curve created from the gelation time of standard LPS (E.coli 0111:B4, FUJIFILM Wako Pure Chemical Corporation) [8, 9].

The maximum set-up time for this instrument is 200 min, and the lowest endotoxin measurement limit in plasma is approximately 0.3-0.5 pg/ml. Moreover, because the endotoxin levels are expressed as endotoxin units (EUs) in the luminescence method, the turbidimetric value was also expressed in EU, where 1 pg/ml = 7 mEU/ml.

**4. Endotoxin measurement by the automated bioluminescence method**

The automated bioluminescence system developed by DKK TOA Corporation (Tokyo, Japan) was used. The samples obtained by LRP37 method was pretreated by dilution and heating method as the same method described above, and the 0.2 ml samples were added into LAL tube. After confirming dissolution, the BL tube containing the luminescent substrate was inserted into LAL tube and the mixture was automatically stirred for 5 second. Then, it was automatically heated to 37℃ for 19 min to release luciferin by the Limulus reaction. ATP and luciferase in the BL tube were then automatically added into the mixture. The bioluminescence reaction occurs immediately, and the amount of luminescence was measured, and the amount of endotoxin was finally calculated using a software that incorporated a calibration curve. Bioluminescence-type endotoxin analyzer Luminitz-ET® (DKK-TOA Corporation) was used for this assay.

**5. Statistical analysis**

Comparisons between the two groups were made using Wilcoxon signed rank test. The systematic bias between the two measurement methods ware investigated using Bland-Altman analysis. The Spearman rank order correlation coefficient was employed for the analysis of correlations. We used at cut-off value obtained through receiver operating characteristic (ROC) analysis. A $p$-value $< 0.05$ was considered statistically significant. Two-group comparisons, correlations, and multiple liner regression analyses were performed

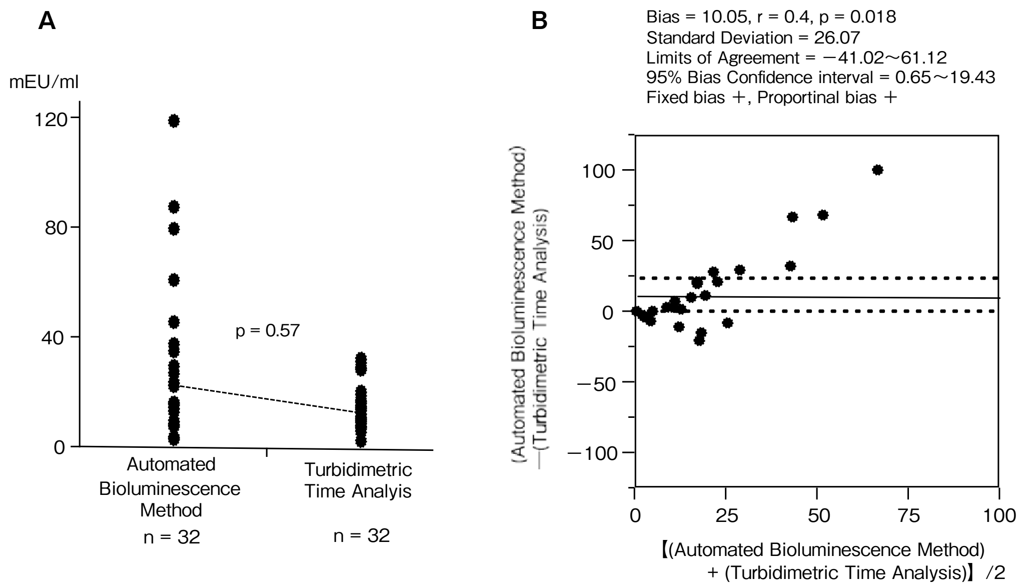


Fig. 2. Comparison and correlation between automated Bioluminescence method and turbidimetric kinetic assay.
A: Comparison of measurement values by each measurement method. The measurement value of automated bioluminescence method tended to be higher.
B: Correlation of both measurements. Significant correlation between both measurements.

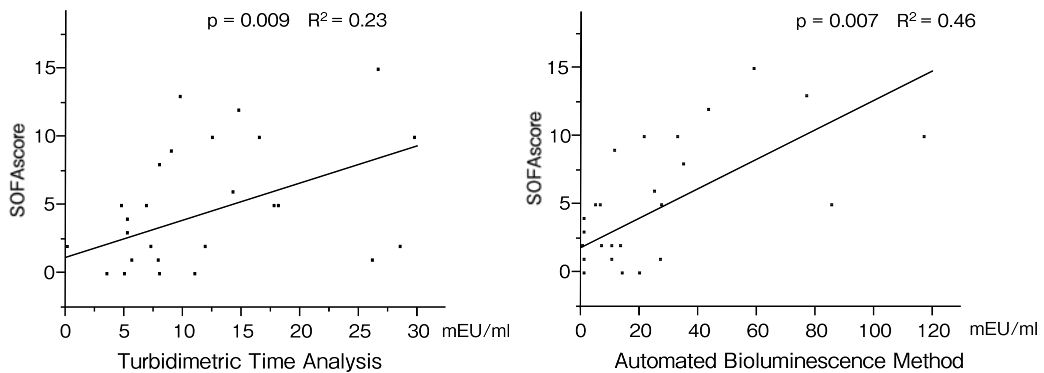


Fig. 3. Evaluated SOFA score to determine severity of sepsis, and also assessed the correlation between these two values.

using JMP software (SAS Institute, Cary, NC, USA). ROC analysis was performed using Dr. SPSS II software (SPSS, Chicago, IL, USA).

### 6. Ethics

Ethical approval for the study (Ethical Committee No. H29-174) was provided by the Ethical Committee of Iwate Medical University, Morioka, Japan (Chairperson: Prof. Y Sato) on March 15, 2018.

## III. Results

Blood samples were obtained from 32 individuals; 24 infected patients and 8 healthy individuals. Results revealed that the endotoxin level was to be 10.61 ± 7.99 mEU/ml using the turbidimetric kinetic assay, whereas the endotoxin levels were found to be 18.8 ± 28.31 mEU/mL using the automated bioluminescence method. The

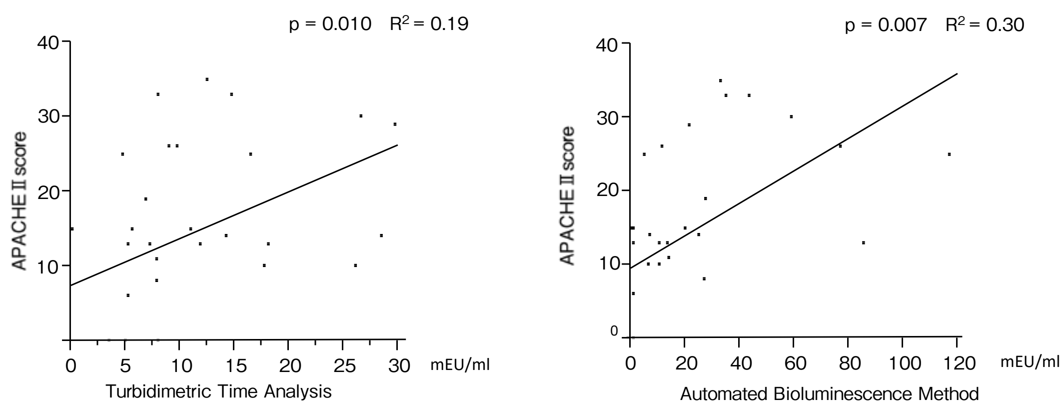


Fig. 4. Evaluated APACHE II score to determine severity of sepsis, and also assessed the correlation between these two values.

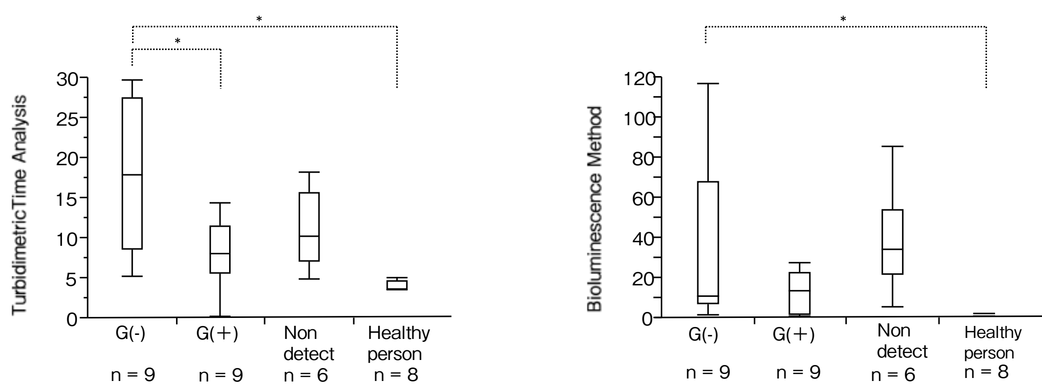


Fig. 5. Examination of measured values based on detected bacterial species.
* $p < 0.05$

automated bioluminescence method showed a slightly higher value and a larger standard error. The Bland-Altman analysis showed a significant correlation between the two measurement methods. In addition, systematic bias was also recognized, and it became clear that the difference between both measured values tended to increase as the average of both measured values increased (Fig. 2). The measurement time was 138.01 ± 8.58 min for the turbidimetric kinetic assay and 20.0 min for the automated bioluminescence method; thus, the automated bioluminescence method was significantly faster ($p < 0.01$) (data not shown). The diagnostic accuracy of infectious diseases using both measurement methods was compared. For turbidimetric kinetic assay, sensitivity was 0.91, specificity was 0.89, and AUC was 0.91 when the diagnostic cutoff value was 5.22. For the automated bioluminescence method, sensitivity was 0.95, specificity was 1.0, and AUC was 0.95 when the diagnostic cutoff value was 1.21 (data not shown). Additionally, we evaluated SOFA (Fig. 3) and APACHE II (Fig. 4) scores to determine the severity of sepsis, and also assessed the correlation between the severity and endotoxin values. The correlation between turbidimetric kinetic assay and SOFA score ($p = 0.009$, $R^2 = 0.23$) or APACHE II score ($p = 0.010$, $R^2 = 0.19$) and

that between the automated bioluminescence method and SOFA (p =0.007, $R^2$ = 0.46) or APACHE II scores (p = 0.007, $R^2$ = 0.30) were observed, and the automated bioluminescence method demonstrated a greater correlation.

The measured value of the bacteria type was compared (gram-negative bacteria; 9, gram-positive bacteria; 9, Non-detect; 6, Healthy individuals; 8). In both measurement methods, the measurement value was highest in cases of gram-negative bacteria detection. There was a significant difference between the cases of gram-negative bacteria detection and healthy people. None of the healthy individuals showed a value that exceeded the cut-off value for infectious diseases (Fig. 5).

## IV. Discussion

Currently, the turbidimetric kinetic assay method is used for measuring endotoxin levels in a plasma sample. Recently, we evaluated the accuracy of infectious disease diagnosis using this method, and we found a sensitivity of 0.58 and a specificity of 0.97 [10], which was not sufficiently high. To solve this problem, we added dextran to the whole blood sample and separated the layer containing a large amount of white blood cells while heating the sample to 37℃ before subjecting the sample to turbidimetric kinetic assay. This modification, named the LRP37 method [7], significantly increased measurement accuracy. In this study, we compared endotoxin levels in samples obtained by the LRP37 method using the turbidimetric kinetic assay and the automated bioluminescence method. The automated bioluminescence method, which has recently been used in Japan as a method for measuring endotoxin in dialysate in hemodialysis, has been applied to blood samples for the first time in this study.

Our results indicate the following: (1) there was a significant correlation between the values measured by both methods, even though the values measured by the automated bioluminescence method tended to be slightly higher; (2) the measurement time was significantly shorter with the automated bioluminescence method; (3) measurement accuracy of infectious diseases was significantly high for both methods, but it was higher for the automated bioluminescence method; (4) in both methods, the value of gram-negative bacteria was significantly higher than that of healthy individuals; and (5) the values of both methods were significantly correlated with patient severity.

Because both assays use the Limulus reaction as the measurement principle, the correlation of the measurement values was in line with our prior expectations. On examining the measurement values for each case, the standard error of the measurement values was larger in the automated bioluminescence method, and it is our opinion that further studies are necessary to accurately capture the kinetic characteristics of the luciferase reaction. With respect to measurement time, the automated bioluminescence method required 20 min in all cases. However, in the turbidimetric kinetic assay, turbidity develops with difficulty at low endotoxin concentrations; therefore, the assay may take 2 hour or more. Thus, the automated bioluminescence method will be particularly useful for quickly measuring low endotoxin concentrations.

In this study, measurement sensitivity was 0.95 when the specimen made by

LRP37 method was measured using the automated bioluminescence method. This value was dramatically superior to the endotoxin measurement method currently used in clinical practice. When comparing bacterial species detected in blood, ascites, urine, sputum, and cerebrospinal fluid, both methods demonstrated high values in cases of detection of gram-negative bacteria. However, in cases where only gram-positive bacteria were detected and in cases where bacterial infection was suspected but bacteria were not detected, endotoxins increased slightly. In particular, in the automated bioluminescence method, the measurement value in the case of detection of gram-positive bacteria was slightly higher, and did not differ significantly from the measurement value of gram-negative bacteria. The reasons may be that both endotoxin assay methods demonstrated false positives, and the sensitivity of the bacterial culture test may be insufficient, which may have led to gram-negative bacteria not being detected. However, for healthy subjects, both tests did not increase endotoxin levels. Considering these facts, there is the possibility that a small amount of endotoxin leaked from the intestinal tract into the blood and was detected in severe conditions. The automated bioluminescence method may have detected the leaked endotoxin more sensitively.

The usefulness of direct hemoperfusion with polymyxin B immobilized fiber (PMX-DHP), an endotoxin adsorption therapy, has also been reported in sepsis [11] and sepsis ARDS [12] caused by gram-positive infections without endotoxin production. It has been reported that the removal of anandamide with peripheral vasodilatory effect [13], the removal of activated monocytes [14] and neutrophils [15], and the recovery of HLA-DR antigen by adsorption of human leukocyte antigen [16] act as mechanisms. Therefore, we believe that PMX-DHP was effective even in cases that appeared to be endotoxin-free. However, the turbidimetric kinetic assay using plasma with few blood cell components may have low endotoxin detection sensitivity. We must re-examine these cases using new measurements.

There are no reports that show a correlation between disease severity and endotoxin levels, and the significant correlation between the endotoxin level and SOFA or APACHE II score reported here suggests that endotoxin levels can serve as an effective marker of severity as well as a diagnostic marker for infectious diseases in the future. Simultaneously, it may also provide sufficient evidence for the treatment of sepsis using endotoxin adsorption therapy.

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Conflict of interest: The authors have no conflict of interest to declare.

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# 生物発光法を用いた新しいエンドトキシン測定法の開発

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要旨

エンドトキシン測定法は少量の血球を含んだ血清を用いて測定する比濁時間法が利用されるが，高い測定精度を報告できていない．我々はこれまで多くのエンドトキシンは白血球と結合あるいは白血球内に取り込まれた状態で存在することに着目し，多白血球血漿を用いた測定で測定値が高くなることを報告してきた．今回は多白血球血漿を用いて比濁時間法と生物発光法を用いたエンドトキシン測定について比較した．

測定は当院に搬送された感染が疑われる患者検体 24 検体と健常者 8 検体を使用し，多白血球血漿検体を作成して比濁時間法と生物発光法で行った．

測定時間は生物発光法において比濁時間法に比較して有意に短時間で測定値を得た．エンドトキシン測定値は，生物発光法において高値となった．また多白血球血漿検体を用いることで重症度と測定値が相関した．

生物発光法は測定時間と測定精度で感染症の診断マーカーとして有意に優れているという結果であった．