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[原著]

Short-term follow-up analysis of plasma endotoxin levels to predict survival after surgical correction of displacement of the colon in horses: A retrospective case note analysis

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Summary

Objective: Measurement of endotoxin levels in equine colic has been used to predict their outcome in previous studies, but current studies indicate that endotoxin0induced systemic inflammatory response (SIRS) is the most clinically critical and relevant issue rather than endotoxin levels in blood and other biomarkers have been sought to characterize the SIRS. This study aimed to re-assess the clinical efficiency of the measurement of plasma endotoxin levels in equine colic cases by the kinetic chromogenic limulus amebocyte lysate assay.

Results: We retrospectively analyzed previous data regarding the plasma endotoxin levels obtained at the time of the first admission for 21 horses diagnosed with a displacement of the large colon, which was surgically corrected. The relationships between endotoxin levels and outcomes were statistically examined. A total of 9 nonsurvivors exhibited significantly higher endotoxin levels than12 survivors. Statistical analysis showed that the endotoxin levels had significant diagnostic power for determining outcomes. Measurement of endotoxin levels at admission appears to have prognostic predictive value in horses with colic within a period of 3 days of hospitalization for surgery; however, the study was unable to draw clear conclusions since of the evidence base was still limited.

Keywords: Colic, horses, large colon displacements, measurement of plasma endotoxin levels, mortality prediction, predictive prognostic value

Introduction

To prevent death due to endotoxemia in horses, early laboratory diagnosis of equine endotoxemia (EET) is an essential step (Moore et al., 2014). Previous studies of EET have focused primarily on the detection and measurement of endotoxin levels in the blood of horses (Senior et al.,

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2011). However, current studies indicate that endotoxin-induced systemic inflammatory response (SIRS), which can originate from other pathogen-associated molecular patterns besides endotoxin, is the most clinically critical and relevant issue rather than endotoxin levels (Moore et al., 2014, Sheats, 2019). Thus, the measurement of endotoxin levels seems to become less significant in the field of equine medicine, and the search for more reliable biomarkers to replace endotoxin levels is hoping for the development of a precise diagnostic method as the gold standard (Epstein et al., 2011, Fogle et al., 2017, Muko et al., 2021, Nocera et al., 2021).

The authors previously used a kinetic chromogenic limulus amebocyte lysate (LAL) assay and a commercially available kit to measure blood endotoxin levels in horses affected with fatal colic when the histopathological study was conducted (Oikawa et al., 2004). With this commercially available kit, most measurements of the LAL assay were automated, endotoxin-specific detection was possible, and testing was relatively simple and required little time from blood collection to completion of the measurement (approximately 90 min). This study aimed to retrospectively re-examine the previously unpublished data measured using a commercially available kit and the kinetic chromogenic LAL assay to study the relationship between blood endotoxin levels obtained at the time of admission and outcomes at 3 days after surgery for horses that were clinically diagnosed with colic and to reassesses the prognostic predictive value of the LAL assay for measuring endotoxin levels in horses with colic within a period of 3 days of hospitalization for surgery.

Materials and Methods

Case description

Data used in this study (cases, blood materials, and endotoxin measurement values) were obtained from the Equine Research Institute of the Japan Racing Association by the authors between 2004 and 2006. The study included 21 adult thoroughbred horses, aged 1-16 years, who presented to an equine veterinary hospital for surgical correction of acute left or right displacement of the colon (Table S1).

Sample collection

Sample collection and measurement reagents (buffered solution, LAL reagent, distilled water, control standard endotoxin, and LAL reagent water [LRW]), instruments, and the equipment used were included in kits or commercially available items by Seikagaku Corporation (Tokyo, Japan). Blood collected at the time of the first admission was used to measure the blood endotoxin levels. To avoid endotoxin contamination of the blood during collection, blood samples from the left or right jugular vein were obtained from horses after scrubbing the skin at the venipuncture site with alcohol and iodine solution, at least three times. If blood was collected aseptically from the jugular vein with a heparin-coated, endotoxin-free (EF) plastic syringe, a syringe needle was used to transfer the blood to a 15-mL EF centrifuge tube (Falcon, Corning, Corning, NY). Blood was centrifuged (4˚C, 150 × g, 10 min) in a cooled centrifuge to prepare platelet-rich plasma to determine the endotoxin concentration. The supernatant was aspirated with a 1-mL disposable injector equipped with a gamma-sterilized EF 26-G syringe needle and transferred to a gamma-sterilized, EF serum tubes made of polypropylene, which were sealed with sterilization tape for storage at -80˚C until measurement.

Endotoxin measurement

After thawing the supernatant at room temperature, a kinetic chromogenic LAL assay using a commercial product (Endospecy® ES-50M kit; Seikagaku Corporation) was performed, according to the manufacturer s instructions (), for platelet-rich plasma to quantify the endotoxin concentration in U/mL of plasma.

Pretreatment of plasma

Plasma was pretreated by adding 200 µL of 0.36 M perichloric acid to 100 µL of supernatant and heated at 37°C for 30 min. It was centrifuged at 3000 rpm for 10 min to remove the denatured protein precipitate produced during this process. The test tubes were wrapped in parafilm to prevent contamination during centrifugation. After centrifugation, 50 µL of supernatant was placed in a separate test tube and 50 µL of 0.18 M NaOH was added to it.

Reconstitution of LAL reagent

The total volume (2.8 mL) of the buffer solution was added to the vial of LAL reagent using a syringe, and the top of the vial was covered with aluminum foil (dry-heat sterilization) and mixed until the contents were dissolved.

Reconstitution of control standard solution

To reconstitute the control standard endotoxin solution, 5 mL LRW was added, the top of the vial was covered with a sheet of aluminum foil (dry-heat sterilized), and the contents were mixed vigorously mixed using a test tube mixer for at least 1 minute. Dilutions of 0.5, 5, and 50 pg/mL were made with LRW as reference according to the US Pharmacopeia Endotoxin Reference Standard.

LAL assay

To perform the LAL assay, 50 µL of each of the supernatants of the sample, the control standard endotoxin solutions (serial solutions), and endotoxin-distilled water as the blank (negative control) were transferred to EF wells using EF pipette tips. Then, 50 µL of the reconstituted LAL reagents was added to each well containing the sample, the control standard endotoxin solutions (serial solutions), and the blank by using EF distilled water. The microplate was then covered and placed on a dedicated microplate reader. After stirring, the measurement was started when the temperature reached 37°C (reading wavelength, 405 nm; reference wavelength, 492 nm), and the endotoxin concentrations in the samples were automatically calculated after the reaction was completed. The standard assay reaction required 30 min to complete. The detection range was 1-10,000 pg/mL. Generally, the LAL assay requires approximately 90 min to perform and interpret the results.

Statistical Analysis

The relationship between blood endotoxin levels (pg/mL) and outcomes (survival and mortality; age) in the survivor (n = 12) and nonsurvivor (n = 9) groups statistically analyzed. Next, the median, first quartiles, and third quartiles of age, endotoxin levels and were aggregated and calculated. The Mann-Whitney U-test was used to compare significant differences in median values between survivors and nonsurvivors. To verify the blood endotoxin concentration in terms of sensitivity, specificity, and cutoff value, the data were computed and visualized by a receiver-operating characteristics (ROC) curve, using “survival” and “death” as dependent variables and endotoxin level as the independent variable. ROC analysis was used to assess the diagnostic power for determining the outcomes with endotoxin levels. Based on

the Youden index, the cutoff values for maximum sensitivity (the probability that a truly affected individual would test positive) and specificity (the probability of a survivor having a negative test result) when determining death as the outcome was calculated. The cutoff value was 1 pg/mL. With 1 pg/mL as the reference cutoff value for blood endotoxin, all horses were broadly divided into the high-level group (n=10; ≥ 1 pg/mL) and low-level group (n=11; ≤ 1 pg/mL). A Kaplan-Meier survival curve showing the relationship between the 3-day survival rate and length of hospital stay between the two groups was created. The outcome was used as the dependent variable, and the number of days from admission until death or discharge was used as the independent variable during the analysis. Cox regression was used to calculate the hazard ratio of blood endotoxin levels to the mortality rate. The level of statistical significance was set at $p < 0.05$. Statistical analyses were performed using EZR version 1.51 (Kanda, 2013).

Results

The median age was 9 years (range: 1-12 years) for survivors (n=12) and 10 years (range: 6-12 years) for nonsurvivors (n = 9); no significant difference in the median age was observed between these groups. The median endotoxin levels were 0 pg/mL (range: 0-1 pg/mL) for survivors and 8 pg/mL (range: 2-23 pg/mL) for nonsurvivors (Figure 1). Nonsurvivors had significantly higher median endotoxin levels (p = 0.003) (Figure 1). Figure 2 shows the results of the ROC analysis using the outcome as the dependent variable and endotoxin level as the independent variable. Endotoxin levels had significant diagnostic power for the outcome, with an area under the curve of 0.87 (95% confidence interval

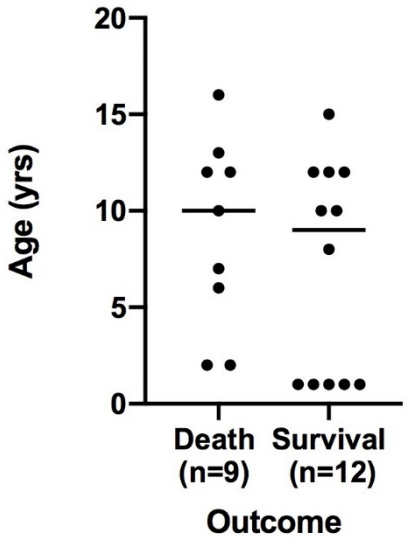

Figure 1. Relationship between outcomes and plasma endotoxin levels in nonsurvivors and survivors. Horizontal solid lines represent median endotoxin levels. Endotoxin levels in the deceased horses were significantly higher than those in the survivors (p = 0.003).

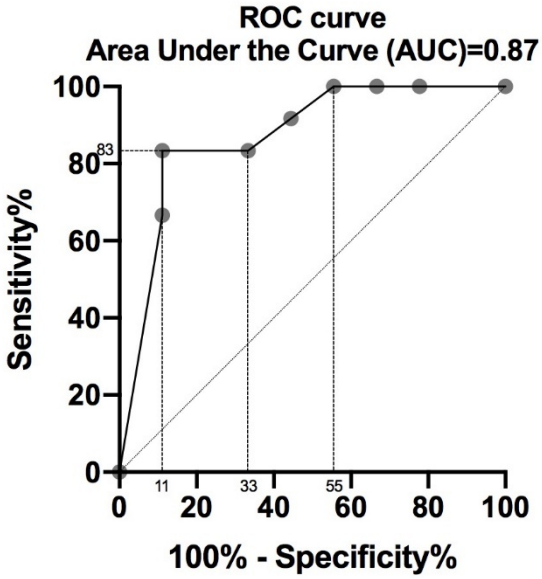

Figure 2. Receiver operating characteristic curve and area under the curve for the diagnostic power of the plasma endotoxin level. The area under the curve was 0.87, which indicated moderate to high diagnostic accuracy.

[CI], 0.71-1.00; $p < 0.001$), which indicated moderate to high diagnostic accuracy. A cutoff value of ≥1 pg/mL was used to predict death (death occurred at ≥ 1 pg/mL), with a sensitivity of 88. 9% and

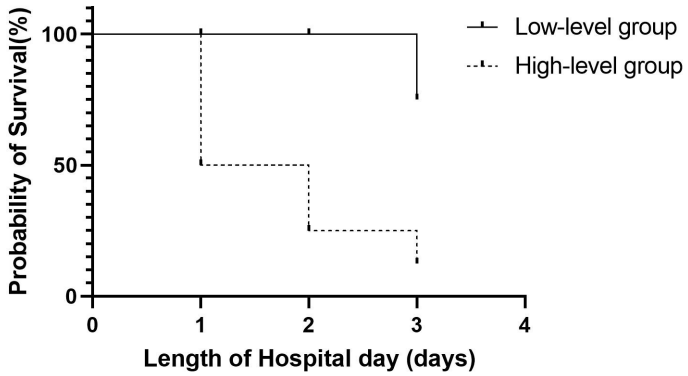

Figure 3. Survival curves and survival rate based on hospitalization of groups with high and low blood endotoxin levels. The dotted line represents the high-level endotoxin group (>1 pg/mL) and the solid line represents the low-level endotoxin group (≤1 pg/mL)

specificity of 83. 3%. Figure 3 shows the Kaplan-Meier survival curve and survival rate for the high-level (>1 pg/mL) and low-level (≤ 1 pg/mL) groups during 3 days of hospitalization. The high-level group had a significantly lower survival rate than the low-level group (Figure 3), which indicated that horses with blood endotoxin values >1 pg/mL were at high-risk of dying within 3 days after admission. The hazard ratio for the mortality rate was 1.11 when endotoxin levels increased by 1 pg/mL (95% CI, 1.03-1.20; p = 0.008) (Table S2). The hazard ratio for the mortality rate in age was 1.05 (95% CI, 0.92-1.21; r = 0.09; p = 0.437) (Table S3).

Discussion

This study indicated that nonsurvivors had significantly higher endotoxin levels than survivors, which suggested that even continuous or intermittent inflow of endotoxin into the blood occurred and that the likelihood of death is higher if large amounts of endotoxin enter the bloodstream (Kiku et al., 2003, Peek et al., 2004). Fluctuations in endotoxin levels in each case could have happened because blood collection was not performed at regular intervals from the time of onset in all cases. Studies involving predictions of horse outcomes using measurements of blood endotoxin levels have reported that horses with high blood endotoxin levels have a poor prognosed and that the mortality cases had significantly higher endotoxin levels than survival cases (Senior et al., 2011). Consistent with these findings, the nonsurvivors in this study also exhibited higher endotoxin levels than survivors. Particularly, endotoxin level showed significant diagnostic power to predict death, with favorable sensitivity and specificity; however, the cases examined were a small sample size. This implies that the measurements of endotoxin levels in cases of colon displacement on admission are of adjunctive importance in making a prognosis diagnosis. The survival curve and hazard ratio analyses also suggest that the prognosis diagnosis of this disease may be aided by the measurements of endotoxin on admission.

Conclusions

In conclusion, the measurement of endotoxin levels at admission appears to have prognostic predictive value in horses with colic within a period of 3 days of hospitalization for surgery; however, the study was unable to draw clear conclusions since of the evidence base was still limited as shown below.

Limitations

This study was unable to draw clear conclusions from the obtained data because of the limited evidence base and inadequate study design involving a small sample size and endotoxin level at 1 time point (not the kinetics of endotoxin release, i.e., no data of the interval between the time of onset and the time of

material collection was provided). Additionally, a comparison to a group of healthy control horses to document the usefulness of the assay and clinical data (vital signs, clinical-pathological data, etc.) to support the assay was not provided. Moreover, the short follow-up period of only 3 days and no consideration on the difference between mortality and survival rates due to great advances in the recent surgical treatment are also the reasons for which clear conclusions could not be made. This assay does not directly measure biomarkers to characterize the severity of SIRS.

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Conflict of interest
The author declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethical statement
The author confirms that the ethical policies of the journal.

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Manufactures' wares used in this study
Endospecy ES-50M Kit®, Seikagaku Corporation, Tokyo, Japan
Control standard endotoxin®, Seikagaku Corporation, Tokyo, Japan
LAL reagent water®, Seikagaku Corporation, Tokyo, Japan
0.36M perchloric acid®, Seikagaku Corporation, Tokyo, Japan
0.18M NaOH®, Seikagaku Corporation, Tokyo, Japan
Toxipet Dispenser Syringe®, Seikagaku Corporation, Tokyo, Japan
Dry-heat sterilized aluminum foil®, Seikagaku Corporation, Tokyo, Japan
Toxipet tip 200® and Toxipet tip 1000®, Seikagaku Corporation, Tokyo, Japan
Toxipet Plate LP®, Seikagaku Corporation, Tokyo, Japan
Well reader SK600®, Seikagaku Corporation, Tokyo, Japan

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Table S1. Total of 10 cases were examined

Horse no.	Age (years)	Gender	Plasma endotoxin concentration (pg/ml)	Outcome	Duration of hospitalization (day)
1	10	F	1	S	3
2	10	F	0	S	2
3	15	M	0	S	2
4	12	M	8	S	1
5	12	F	0	S	2
6	8	F	1	S	3
7	12	M	0	S	3
8	1	M	0	S	2
9	1	M	7	S	3
10	1	F	0	S	2
11	1	M	0	S	1
12	1	F	0	S	2
13	10	F	8	D (Di)	3
14	6	F	7	D (Di)	2
15	12	F	27	D (Di)	1
16	2	M	12	D (E)	2
17	17	F	2	D (E)	1
18	12	F	2	D (E)	1
19	7	F	0	D (E)	2
20	16	M	23	D (E)	1
21	2	F	27	D (E)	1

M: male, F: female, S: survivor, D: death, Di: died in hospital, E: euthanatized in hospital

Table S2. Groups with high and low blood endotoxin levels

Outcome				
Group	Blood endotoxin levels	Nonsurvivors	Survivors	Total
High-level group	>1 pg/ml	8 (88.9%)	2 (16.7%)	10 (47.6%)
Low-level group	≤1 pg/ml	1 (11.1%)	10 (83.3%)	11 (52.4%)
Total		9 (100%)	12 (100%)	21 (100%)

Table S3. Hazard ratio for the mortality rate in the blood endotoxin levels and age (Cox regression)

		95% confidential interval		
	Hazard ratio	Lower limit	Upper limit	*p*-value
Blood endotoxin level	1.11	1.03	1.2	0.008
Age	1.05	0.92	1.21	0.437

Dependent variables: outcome (deaths), time since admission

馬の結腸変位疝罹患例の血中菌体内毒素濃度と転帰について：遡及的分析記録

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要旨

馬の変位疝は菌体内毒血症 (ET)を誘発し、致死的経過を招きやすい病態である。疝痛罹患馬の血中菌体内毒素濃度測定は、従前から早期診断指標としての意義を探る目的で種々試みられてきたが、測定に要する操作の複雑さ等により、未だ実験室レベルの検査法の域にとどまっている。近年、ET は菌体内毒素による直接的組織傷害の結果としての病ではなく、毒素に誘発されて起こる全身性炎症反応症候群（SIRS）の病態として認識されるに至ったことから、血圧や心拍数などの生体由来のデータ、或は、血中タンパクの分子バイオマーカーの探索から SIRS の病態を把握する方向へ研究が展開している。しかしながら著者らは、2000 年代初頭に、市販の ET 診断キットを用いて測定した馬のデータの中から、結腸変位疝罹患馬の初診時の血中菌体内毒素濃度と結腸変位整復術後 3 日間の転帰の関係のデータを遡及的に再度分析したところ、初診時における血中菌体内毒素濃度と馬の転帰の関連性を示唆する成績が得られた。この結果の解釈には慎重を要するものの、血液中への当該毒素の曝露の量的程度の差が ET 罹患馬の予後に影響する可能性が示唆された。

キーワード：馬、変位疝、菌体内毒血症、血中菌体内毒素濃度測定、予後、転帰