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ENDOTOXIN ACTIVITY ASSAY FOR THE DETECTION OF WHOLE BLOOD ENDOTOXEMIA IN CRITICALLY ILL PATIENTS

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TITLE:

Endotoxin Activity Assay for the Detection of Whole Blood Endotoxemia in Critically Ill Patients

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KEYWORDS:

endotoxin activity, sepsis, shock, lipopolysaccharide, chemioluminescence, neutrophils

SUMMARY:

We hereby present a protocol to measure at the bedside the endotoxin activity of human whole blood samples. The Endotoxin Activity assay is a simple test to perform and may be a useful biomarker in critically ill patients with sepsis.

ABSTRACT:

Lipopolysaccharide, also known as endotoxin, is a fundamental component of gram-negative bacteria and plays a crucial role in the development of sepsis and septic shock. The early identification of an infectious process that is rapidly evolving to a critical illness might prompt a quicker and more intensive treatment, thereby potentially leading to better patient outcomes. The Endotoxin Activity (EA) assay can be used at the bedside as a reliable biomarker of systemic endotoxemia. The detection of elevated endotoxin activity levels has been repeatedly shown to be associated with an increased disease severity in patients with sepsis and septic shock. The assay is quick and easy to perform. Briefly, after sampling, an aliquot of whole blood is mixed with an anti-endotoxin antibody and with added LPS. Endotoxin activity is measured as the relative oxidative burst of primed neutrophils as detected by chemioluminescence. The assay's output is expressed on a scale from 0 (absent) to 1 (maximal) and categorized as “low” (< 0.4 units), “intermediate” (0.4–0.59 units), or “high” (≥ 0.6 units). The detailed methodology and rationale for the implementation of the EA assay are reported in this manuscript.

INTRODUCTION:

The Lipopolysaccharide (LPS), also known as endotoxin, is a key component of the membrane structure of Gram-negative (GN) bacteria. It makes up about 10% of the cell wall, being vital for the outer membrane integrity and homeostasis. Moreover, it is a potent activator of the host innate immune system[1,2].

In vitro exposure of innate immune system cells to LPS leads to changes in the expression of multiple genes[3]. Administration of very small quantities of LPS in healthy human volunteers triggers the cascade of acute systemic inflammation, whereas sepsis and septic shock may arise with higher endotoxin concentrations[4,5].

Sepsis is a life-threatening condition which, if not promptly recognized, can lead to multi-organ failure and death. Septic patients must be treated in a timely manner, with aggressive resuscitation, adequate antibiotic therapy, optimal source control, and prompt organ support strategies. The diagnosis of the etiology of sepsis is primarily based on clinical recognition and culture-based pathogen detection[6]. However, results of microbial cultures may take up to 48 h and are inconclusive in up to 30% of cases[7]. Early identification and intervention may lead to better patient outcomes. In patients in whom sepsis is suspected, decisions are often made on the basis of physiological and biochemical parameters, without a clear sign of endotoxemia.

The measurement of the Endotoxin Activity (EA) can be obtained by means of a commercial assay (see **Table of Materials**) in whole blood. It can be used as a biomarker of systemic endotoxemia for the early stratification of disease severity, particularly in patients at risk for developing septic shock[8]. The assay was used to guide Polymyxin B hemoperfusion therapy in a recently published double-blind randomized-controlled clinical trial in patients with septic shock[9]. In critically ill patients, the MEDIC study showed increased EA levels to be associated with multiple organ dysfunction, intensive care unit (ICU) length of stay, and mortality[10].

Different assays have been developed to detect endotoxin. The Limulus Amoebocyte Lysate (LAL) assay, either as a gel-clot, turbidimetric, or chromogenic test, has been so far the most frequently adopted for the estimation of serum endotoxin. It is based on the ability of endotoxin to induce coagulation of the hemolymph of the horseshoe crab, *Limulus polyphemus*. However, this assay has some limitations in terms of specificity. In particular, it can also be activated by microbial products other than endotoxin, such as components of the fungal cell wall, and it can be inhibited by various human plasma proteins[11].

During the last decade the measurement of EA has been developed and validated as a biomarker of circulating endotoxemia. Compared to the LAL test, EA is quicker and easier to implement in the clinical setting. Moreover, it has been shown to be more accurate than LAL in whole blood, with increased sensitivity and specificity, both in vitro and in vivo[12].

Despite its initial implementation as an early diagnostic tool for the rapid identification of GN bacteria as sepsis causative agents, the EA level has also been studied as a biomarker of disease severity. In this context, it has been shown to be particularly useful to assess the hypoperfusion

state due to ongoing critical illness, such as septic shock or post-cardiac arrest syndrome[13]. More recently, since the development of hemopurification systems, a positive EA result has also been proposed as a screening tool to accurately identify potential candidates for such therapy[14]. We recently conducted an observational retrospective study on the prevalence and clinical significance of early high levels of EA in 107 patients with septic shock. In line with other recent results, we found that EA is a promising marker of disease severity in patients with septic shock[15].

The aim of the present manuscript is to describe the method to perform the EA assay, either at the bedside or in the laboratory, and to describe its potential use in a representative scenario of septic shock. This technique can detect LPS activity by measuring the enhanced oxidative burst in neutrophils following their priming by complexes of an anti-endotoxin antibody and LPS. The increased respiratory burst is detected by a chemiluminometer and the amount of light emitted is considered proportional to the amount of endotoxin in the blood sample. The assay requires few reagents, takes about 30 min to perform and uses as little as 40 µL of whole blood[12].

PROTOCOL:

The protocol is conducted according to institutional guidelines relating to the handling of human biospecimens and following the current standard operative procedures of our clinical laboratory. The use of EA data and clinical information of patients being tested follows the guidelines of our institution's human research ethics committee.

1. Laboratory equipment and assay kit contents

1.1. Store the EA kit at 2–8 °C when not in use.

1.2. Each EA test consists of 5 different kinds of tubes; use each one for a different portion of the test (see section 2).

1.2.1. Use tube #1 (the "Control" tube) to measure the basal activity of the non-specific oxidative burst of patient's neutrophils in the absence of a specific antibody.

1.2.2. Use tube #2 (the "Sample" tube) to measure the oxidative burst in response to the LPS-antibody complex.

1.2.3. Use tube #3 (the "Max" tube) to measure the maximal oxidative burst of patient's neutrophils in response to an excess of endotoxin.

1.2.4. Use tube #4 (the "LPS" tube) as a source of exogenous endotoxin.

1.2.5. Use tube #5 ("Aliquot" tube) for blood storage.

NOTE: Duplicates of tubes #1, #2 and #3 are provided for a total of 8 tubes to be used for each blood sample being tested (the EA reagent bottle and quality control test can be used for all the tests contained in a pouch).

1.3. Collect patient blood samples in sterile tubes containing EDTA anticoagulant. Store blood samples at room temperature before running the EA test.

1.4. Before starting the test, turn on the chemiluminometer and incubator shaker. Warm the incubator to the temperature of 37 °C.

1.5. Ideally, start processing the sample within 30 min from blood collection.

2. Endotoxin Activity Assay

2.1. Prepare the EA test tubes for each patient's blood sample you need to test. Put the tubes in tube racks. Then remove the caps.

2.2. Using a combipipette, pipette a 1 mL volume of the EA reagent from the bottle into tubes #1 (Control tube), #2 (Sample tube) and #3 (Max tube), each one in duplicate.

NOTE: Pipette down the side of the tube to avoid solution splashing back up.

2.3. Mix the patient blood sample by gently inverting the blood collection tube for 20 times. Then, pipette 0.5 mL of patient blood into tube #4 (LPS max tube) and tube #5 (Aliquot tube). Vortex tube #4 for 10 s.

2.4. Put the tube racks with all the EA test tubes in the incubator shaker. Close the lid and incubate for 10 min at the temperature of 37°C.

2.5. Open the lid and remove the tube racks from the incubator shaker. Vortex tube #5 (Aliquot tube). Using a sterile tip, pipette 40 µL of blood into tubes #1 and #2, in duplicate.

2.6. Vortex tube #4 (LPS tube). Using the same pipette tip, pipette 40 µL of blood from tube #4 into tube #3 (Max tube), in duplicate.

2.7. Vortex the six final test tubes (#1, #2, #3, and respective duplicates), then place them back into their racks.

NOTE: Ensure that all the tubes are vortexed for the same amount of time.

2.8. Put the tube racks back into the incubating shaker and close the lid. Set the incubating shaker at 100 rpm, then start the motion for 14 min.

2.9. Insert the EA labeled chipcard in the chemiluminometer and press start. After the 14-min

incubation, follow the instructions displayed on the chemiluminometer to read the EA tubes in the correct order.

2.10. Gently vortex each tube for 10 s before placing it onto the sample holder of the chemiluminometer. Open the sample drawer and place tube #1 in the sample holder. Then, close the sample drawer and wait for the Relative Light Unit (RLU) reading.

2.11. Repeat step 2.10 for tube 2 and tube 3.

2.12. Repeat step 2.10 for duplicate tubes 1, 2, and 3.

NOTE: Try to vortex all tubes for the same amount of time during steps 2.10–2.12.

2.13. After all the tubes have been processed, note that the EA results will be calculated and printed automatically. Levels are expressed as EA units and represent the mean of duplicate determinations from the same samples.

2.14. Repeat steps 2.2 to 2.13 for every blood sample that needs to be tested.

2.15. Once the assay has been completed, store the remaining test tubes and EA reagents at 2–8 °C for up to 30 days.

REPRESENTATIVE RESULTS:

A 72-year-old man was admitted to the Emergency Department (ED) of an academic urban hospital. A few days earlier he had presented to his primary care physician complaining of burning on urination. A short-course therapy with oral phosphomycin was recommended. His medical history included hypertension, uncomplicated type-2 diabetes and benign prostatic hyperplasia. His medications included enalapril, atorvastatin, tamsulosin and metformin.

In the ED, he was lethargic, confused when awakened. His temperature was 39.1 °C, heart rate was 125 beats per minute, blood pressure was 80/40 mmHg, respiratory rate was 20/min, and SpO_2 was 94% in room air, raising to 99% with 4 L/min oxygen through a nasal cannula. Abdominal exam was normal, except for poorly-localized mild suprapubic tenderness. With difficulty, a Foley catheter was placed. A low amount of dark-colored purulent urine was drained.

A complete blood count revealed a white cell count of 18.5 x 10^3. Creatinine was 2.7 mg/dL, glucose was 250 mg/dL, and the lactic acid level was 4.5 mmol/L. Arterial blood gas analysis revealed mixed acidosis, with pH 7.23, pCO2 48 mmHg, pO2 88 mmHg, and $HCO3^-$ 15 mmol/L.

A central venous catheter was placed, with ultrasound guidance, into the right internal jugular vein. A blood gas analysis was obtained upon the catheter placement, revealing a 63% $ScVO_2$ value. Aggressive fluid resuscitation was promptly started with a 30 mL/kg crystalloid bolus infusion over 30 min. Norephinephrine infusion was also initiated. The patient was transferred to the ICU with a diagnosis of septic shock, likely originating from a urinary tract infection.

In the ICU, amid microbial cultures collection and empiric antibiotic therapy administration, a whole blood sample was obtained for EA testing. The assay was rapidly performed according to the protocol presented herein.

In order to compute EA results, the chemiluminometer records: the basal luminescence of neutrophils (L1); luminescence of neutrophils activity in response to the LPS in the blood sample (L2); the maximal neutrophils activity in response to massive exposure to LPS (L3). Results are expressed as: Endotoxin Activity (EA) = (L2-L1)/(L3-L1). Therefore, the resulting EA value reflects the degree of patient's neutrophils oxidative burst due to the presence of circulating endotoxin (L2), normalized by the highest level of luminescence which can be measured in the same blood sample in response to a supra-maximal concentration of LPS (L3). Both values are controlled for the basal luminescence of the sample (L1).

After approximately 30 minutes, the clinician acknowledged 0.75 EA units to be the endotoxin activity level of the patient.

An EA value less than 0.40 EA units indicates a low endotoxin activity level, equal to a low circulating LPS concentration, which represents a low risk of progression to a severe disease state. Results between 0.40 EA and 0.59 EA units indicates an intermediate endotoxin activity level, which represents an elevated risk for the development of severe sepsis and septic shock. Results equal or greater than 0.60 EA units indicates a high endotoxin activity level, which represents a high risk for septic shock and poor patient outcomes (**Table 1**).

The diagnosis of septic shock, likely due to GN bacteria, was therefore confirmed. The patient was also categorized as being at extremely high risk, in line with the evidence of concomitant organ failures and lactate level. He was furthermore treated with aggressive volume resuscitation and vasoactive support. Polymixin-B hemopurification therapy was considered, yet not implemented, due to the convincing early response of the patient to fluids and vasopressors. On day 2, *Escherichia Coli* was confirmed as the causative agent through positive blood cultures. With the appropriate antibiotic therapy, the patient recovered, being discharged from the ICU on day 7. A second EA test was performed before ICU discharge. A result of 0.2 EA units indicated complete resolution of the biochemical cascade triggered by septic shock.

Figure 1 shows the distribution of EA values measured within 24 h in a sample population of septic shock patients.

FIGURE AND TABLE LEGENDS:

Figure 1. Distribution of Endotoxin Activity (EA) levels measured within 24 h from septic shock onset in a population of critically ill patients (*n* = 107). Adapted from Bottiroli, et al. with permission[15].

Table 1. Categories of Endotoxin Activity levels. EA = Endotoxin Activity.

DISCUSSION:

Septic shock is still nowadays associated with a mortality as high as 40%, although this rate varies according to the considered reports[16]. The need for novel and better biomarkers is advocated by most experts in the fields in order to aid clinicians in early diagnosis, better management, and prognostication of patients with septic shock[6].

Performing an EA test does not require previous technical knowledge or sophisticated laboratory equipment, and any healthcare provider can easily and quickly learn how to run it. The prompt identification of a high circulating EA might help in stratifying patient's risk, triggering an earlier and more aggressive therapeutic approach. Conversely, an EA result of less than 0.40 EA units might indicate a low risk of progression to multi-organ failure[17]. The use of patient's samples as their own control is simple, makes this test more sensitive and a more accurate representation of the true blood level of endotoxemia.

The use of LPS-antibody complexes and the patient's own neutrophils protects the EA test from being possibly inhibited by other factors (e.g. plasma proteins). The same cannot be said for other test, like the LAL test, which requires the activation of a coagulation cascade. The LAL test performs well when the endotoxin is not bound by a specific receptor, but in plasma and whole blood different proteins bind LPS, interfering with the assay. Moreover, fungal products may trigger the limulus coagulation cascade, making the test less specific for GN bacteria. For these reason, the EA is superior to the still commonly adopted LAL test for the evaluation of endotoxemia[18].

However, for the EA test to be reliable, it is crucial to meticulously follow the aforementioned steps. The endotoxin level of the specimen being tested is calculated by computing chemiluminescence over time, measuring basal (tube #1) and maximal (tube #3) responses for the same blood sample as reference values. Therefore, it is imperative to carefully place the three tubes in the correct order into the chemiluminometer.

The use of EA levels in clinical practice should not replace standard tests (e.g. laboratory tests and blood cultures) in the workup of a potential infectious disease. Although LPS might clearly be associated with the release of GN bacteria membrane products, elevated levels of endotoxemia have also been reported in case of infections due to other agents[19]. It is very well known that endotoxemia might be due to translocation of bacteria through the gut mucosa, particularly whenever tissue hypoperfusion and increased gut barrier permeability are likely to occur[20,21]. Under such conditions, it is possible to expect circulating LPS to be a consequence, rather than the cause, of sepsis and shock. In this scenario, the EA could provide information about the severity of the ongoing tissue injury, regardless of the bacterial etiology[15]. Supporting this principle, in a recent study Grimaldi et al. found the elevation of EA levels to be associated with the severity and duration of shock following out-of-hospital cardiac arrest[13].

Interestingly, Virzí et al. also highlighted the potential role of endotoxin (and its monitoring) in patients with type 5 cardiorenal syndrome, a condition characterized by concomitant cardiac and renal dysfunction in the setting of different systemic disorders, such as sepsis[23] . Endotoxin is

known to induce impairment of cardiac myocytes, although the exact pathophysiological mechanism is largely unclear. On the other hand, endotoxemia has been shown to induce renal dysfunction due to several pathways of local and systemic injury, causing impairments of renal blood flow, glomerular filtration rate, and tubular function[22].

However, a few limitations of the EA must be highlighted. The influence of an ongoing antibiotic therapy on the EA result is currently not well established[24]. Moreover, evidence suggests that in critical ill patients a single-point early EA measurement, while useful, does not reliably predict septic shock mortality, even when greater than 0.6 units[15]. Serial repeated assessments might be needed, although their number and timing are currently unclear. Other authors focused on fluctuations in endotoxemia, hypothesizing that an increased daily variability might be associated with a higher degree of multi-organ dysfunction[25]. Lastly, an additional technical limitation to be considered is the relative scale along which the endotoxin activity is measured (0 to 1 units, with 0.01 minimum detectable increments). This makes extremely high results inevitably plateau around the maximum level, thereby making differentiations in this subgroup of patients (EA > 0.9 units) harder to investigate.

In conclusion, while further studies are needed to evaluate the potential impact of monitoring endotoxin levels on clinical outcome, the EA is currently available as a quick, simple and sensitive test, which might facilitate the decision-making process of ICU clinicians in critical ill septic patients.

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DISCLOSURES:

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Figure

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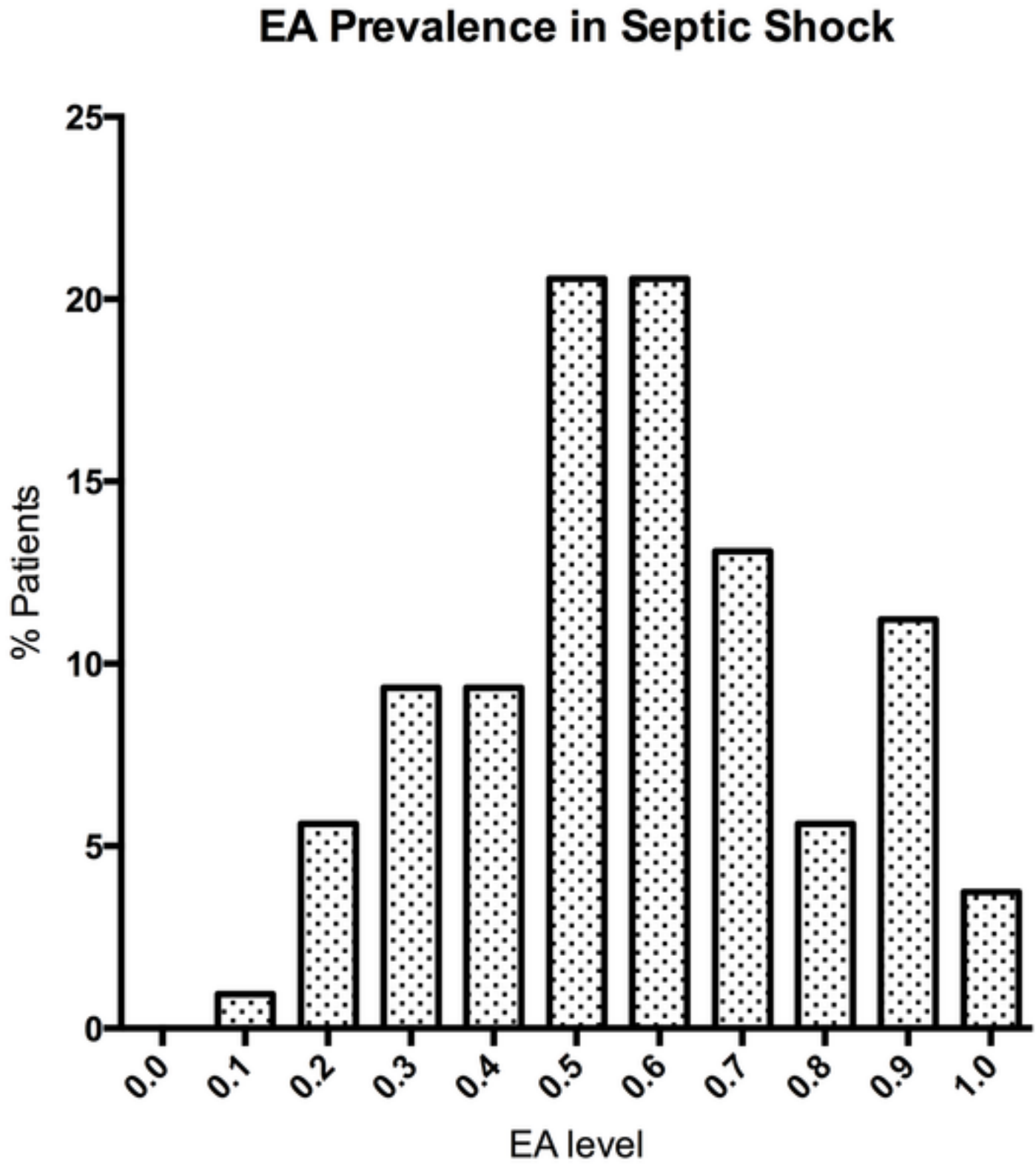

Table 1

EA units	Endotoxin activity level
< 0.40	Low
0.40 – 0.59	Intermediate
≥ 0.60	High

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
EAA kit	Spectral Medical Inc.	EAAST-20	Package with 20 tests + 1 quality control
Smart Line TL	Berthold	EAASL	Luminometer
Incubator shaker	GRANT	ES-20	Mini-incubator shaker
Vortexer	VWR	444-2790	Vortex instrument

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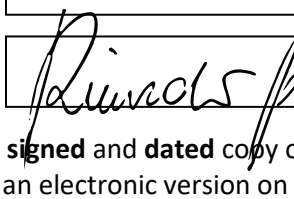
Signature:	
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RE: Resubmission of JoVE58507 ENDOTOXIN ACTIVITY ASSAY FOR THE DETECTION OF WHOLE BLOOD ENDOTOXEMIA IN CRITICALLY ILL PATIENTS,

Response to Review - Cover Letter

Dear Dr. Steindel,

We are pleased to resubmit for publication a new version of our manuscript, modified according to the Editor's recommendations. Particularly, point-by-point:

1. You have indicated in your ALA that you want Open Access but checked Standard in Editorial Manager-which are you choosing? If standard, please sign a new ALA (attached) and include it with your submission.

   A new ALA has been attached to the resubmission. We confirm we want Standard Access.

2. EAA is a commercial term (see http://www.spectraldx.com/eaa.html, e.g.); please use another abbreviation for this assay.

   Throughout the revised version of the manuscript, we referred to the measurement of "Endotoxin Activity (EA)". No brand name has been used.

3. Please include an ethics statement before the numbered protocol steps, indicating that the protocol follows the guidelines of your institution's human research ethics committee.

   Added. See Page 3, lines 102-105.

4.

4. 1: This section should be in the imperative.

The section has been modified as requested (Page 3, from line 110)

5. 2.10: How long do you vortex here?

10 seconds. The text has been changed accordingly.

6. 2.10.2: What does 'for duplicate tubes 1, 2 and 3' mean-repeat the readings for those tubes?

Each EA assay is run in duplicates, meaning the same blood sample is tested through chemiluminometry in two separate sets of tubes (tube #1, tube #2, tube #3 AND tube#1-duplicate, tube#2-duplicate, tube#3-duplicate). The two resulting EAs are averaged. This is repeatedly remarked in the protocol description (1.3, 2.2, 2.5, 2.6, 2.7, 2.10.2, 2.11).

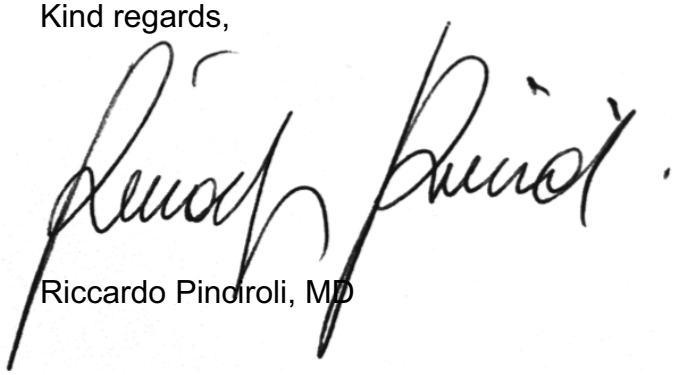
7. 3: This section probably belongs (in paragraph form) in the results section; otherwise it should be in the imperative and entirely numbered steps.

We thank the editor for the useful suggestion. After proper rephrasing, the paragraph was moved to the Representative Results section.

8. Figure 1: It is unclear why this is here; the video will probably convey all essential information about the instrument.

The figure was added during the review process following reviewer #1/comment #3. However, we agree with the editor and removed the figure representing the instrument.

Kind regards,



Riccardo Pinotroli, MD