Spectral Diagnostics Incorporated
Endotoxin Activity Assay (EAA™)

For In Vitro Diagnostic Use Only

For the measurement of endotoxin activity in human whole blood samples

Catalogue Numbers: EAA20-1, EAQC-5

Intended Use: The EAA™ is a rapid in vitro diagnostic test that utilizes a specific monoclonal antibody to measure the endotoxin activity in EDTA whole blood specimens. When used in conjunction with microbial cultures and other relevant diagnostic tests (i.e. ultrasound, bronchoscopy or CAT scan) the test is indicated for use in A) ruling out the presence of Gram negative bacterial infections. The EAA™ is also intended to be used B) in conjunction with other clinical information such as clinical signs, other laboratory and/or radiographic test results to aid in the risk assessment of patients on their first day of admission to the ICU for progression to severe sepsis.

Summary and Explanation: Endotoxin or lipopolysaccharide (LPS) is a major cell wall constituent of Gram negative bacteria and the primary Gram negative bacterial product responsible for severe sepsis[1-2]. Elevated endotoxin levels cause changes in the expression of more than 300 genes and activated macrophages, neutrophils, endothelial cells and the coagulation cascade producing the sepsis cascade[3].

This assay uses the biological response of the neutrophils in a patient's blood to an immunological complex of endotoxin and exogenous antibody as a measure of the endotoxin activity in the patient[4]. The assay reacts specifically with LPS of Gram negative bacteria and does not cross-react with cell wall constituents of Gram positive bacteria and other microorganisms.

The assay is to be used in conjunction with:
A) Conventional microbiological cultures and other relevant diagnostic tests such as ultrasound, chest X-ray, bronchoscopy or CAT scan to facilitate the "rule-out" of Gram negative infection by providing additional rapid diagnostic information.
B) All available clinical and laboratory findings including physician judgment to determine the risk for severe sepsis.

Principle: The EAA™ is a rapid testing device, which measures the endotoxin activity in whole blood. The test is based on the reaction of endotoxin with a specific anti-endotoxin antibody. Complement proteins opsonize the endotoxin-antibody complex. The opsonized immune complex primes neutrophils in the blood to enhance their respiratory burst in response to zymosan. The respiratory burst of the neutrophils yields oxidants that react with luminol in the reaction mixture to emit chemiluminescence. The chemiluminescence can then be detected in a photon counting luminometer.

A basal activity measurement (Tube 1) in the absence of the specific anti-endotoxin antibody measures the non-specific oxidative burst of the patient's neutrophils. An additional control measurement including the specific anti-endotoxin antibody and an excess of exogenous endotoxin (Tube 3) measures the maximum oxidative burst of the patient's neutrophils. The test measurement (Tube 2) includes the specific antibody to measure the neat level of endotoxin activity. The EAA™ level is calculated by normalizing the chemiluminescence in the test sample (Tube 2) against the maximum chemiluminescence (Tube 3), correcting both measurements for the basal activity chemiluminescence (Tube 1).

Specimen Collection and Preparation: EDTA anti-coagulated whole blood samples are required for this procedure. Guidelines recommended by the NCCLS[6] should be followed when collecting, transporting and processing patient samples. Samples must be collected in blood collection tubes containing EDTA anti-coagulant by venipuncture or through an indwelling catheter. If sampling through an indwelling arterial or venous cannula or catheter, please flush the lines per your facility's policy prior to collecting blood in the appropriate tube. Blood samples may be stored for up to 180 minutes at an ambient temperature range of 18°C to 25°C prior to analysis. Blood must be thoroughly mixed for at least 20-30 seconds by gentle inversion immediately prior to analysis. A minimum of 2.5 mL of whole blood is required to run the patient assay (1.0 mL patients blood) and QC assay (1.0 mL) which may be obtained from independent samples.

Materials Provided:

Cat. No.	EAA™ Trays*	EAA™ Quality Control Tests*	EAA™ Reagent Bottle**	Instructions for Use
EAA20-1	4	1	2	Yes
EAQC-5***	0	5	1	Yes

* EAA™ Trays and QC Tests are contained within a sealed foil pouch. See below for a description of the contents of each test type. ** See below for a description of the contents of each bottle. ***EAQC-5 configuration supplies for additional QC tests.

***Each EAA™ tray contains the following reagents sufficient for 5 duplicate tests**:

Content	Label
10 tubes containing stabilizers and luminol-zymosan	(Tube 1)
10 tubes containing 1.0-2.0 μg of murine monoclonal anti-endotoxin antibody, stabilizers and luminol-zymosan	(Tube 2)
10 tubes containing 1.0-2.0 μg of murine monoclonal anti-endotoxin antibody, stabilizers and luminol-zymosan	(Tube 3)
5 tubes containing 2.3 ng of endotoxin (*E coli* 055:B5) with stabilizers	(LPS MAX Tube)
5 empty tubes	(Aliquot Tube)

*** Each EAA™ Quality Control single test contains the following reagents**:

Content	Label
2 tubes containing stabilizers and luminol-zymosan	(Tube 1)
2 tubes containing 1.0-2.0 μg of murine monoclonal anti-endotoxin antibody, stabilizers and luminol-zymosan	(Tube 3)
1 tube containing 1.0-2.0 μg of murine monoclonal anti-endotoxin antibody, stabilizers and luminol-zymosan	(High Control Tube)
1 tube containing 1.0-2.0 μg murine monoclonal antibody, stabilizers and luminol-zymosan	(Low Control Tube)
1 tube containing 2.3 ng of endotoxin (*E coli* 055:B5) with stabilizers	(QC LPS MAX Tube)
1 empty tube	(QC Aliquot Tube)

**** Each EAA™ Reagent bottle contains about 70 mL of EAA™ Reagent containing HBSS and Heparin in sufficient amount for one kit.**

Reagent Storage and Stability: Do not use the EAA™ kit beyond the expiration date. Do not mix components from different kits with different lot numbers**.** Store the EAA™ kit at 2°- 8° C or remove the EAA™ Reagent bottles to store at 2°- 8° C with the unopened foil pouches at room temperature.

EAA™ Test Components	Temperature	Stability
EAA™ Assay Trays		
Unopened	Room temperature(18°-25° C)	Until expiration
Opened, resealed	2°- 8° C	30 days Reseal after opening
EAA™ QC Single Test	Room temperature(18°-25° C)	Until expiration
EAA™ Reagent		
Unopened	2°- 8° C	Until expiration Do not freeze
Opened bottle	2°- 8° C	30 days Reseal after opening Do not freeze

Additional Materials Required:

1. Combipipettor – capable of delivering 40µl, and 1000µl volumes and a micropipette capable of delivering a 500 µl volume.
2. Sterile combipipette and micropipette tips
3. Timer
4. EDTA blood collection tubes

Warnings and General Precautions:

CAUTION:
Device Materials:

The device contains materials that are not classified as hazardous due to the physical and chemical nature and/or concentration in solution, in accordance with California and Federal OSHA regulations (United States), Workplace Hazardous Materials Information System (WHMIS, Canada) and the Directive 2008/98/EC of the European Parliament and of the Council of 19 November 2008 on waste and repealing certain Directives.

1. For the reagents in the kit, there are no known direct risks to health.
2. Use general laboratory precautions when handling and storing the unused device materials.
3. For in-vitro diagnostic use only.
4. The EAA™ contains lyophilized beaded reagents in test tubes. Under certain conditions (damaged packaging, improper sealing of the pouches or improper storage) these reagents may shrink in the presence of excess humidity. Do not use tubes that contain shrunken beads as test results may be inaccurate.
5. For manual pipetting of samples and controls, use individual pipette tips to eliminate carryover.

Used Device Materials:

6. Use recommended universal precautions[5] for handling used reagents and human specimens.
7. Handle or dispose used EAA reagents and all human blood products as though capable of transmitting infectious agents.
8. Do not pipette solutions by mouth.
9. Clean up spills immediately with a 0.5% sodium hypochlorite solution.

EAA™ Test Procedure – See Appendix A for instructions for use
EAA™ QC Procedure – See Appendix B for instructions for use

Interpretation of EAA™ Test Results:
A) Rule –out of Gram Negative Infection
The EAA™ is an endotoxin activity assay with a numeric cut-off to rule-out Gram negative infection set at 0.40. Results less than 0.40 (0.00-0.39) support the absence of Gram negative infection for ICU patients with suspicion of infection.

As with all diagnostic tests, each laboratory should establish its own diagnostic cut-off to assure proper representation of specific populations and to reflect current practice and criteria for Gram negative infections at their institution.

B) Risk Assessment for Severe Sepsis
Results $\geq$ 0.4 is indicative of endotoxemia. Several factors can cause elevated levels of circulating endotoxin. These include invasive Gram negative infection and the translocation of endotoxin from the lumen of the intestine. Endotoxin is the primary Gram negative bacterial product responsible for septic shock.

Precise diagnosis of primary and secondary causes of Endotoxemia requires further clinical and diagnostic approaches to determine its exact causes and appropriate therapies.

The EAA™ is intended to help assess the risk that a patient will develop severe sepsis during the first critical hours following admission in the ICU and test results are most useful when considered in conjunction with other sepsis risk factors. The EAA™ is not intended for the differential diagnosis of a current sepsis condition. In a prospective, multi-center, controlled clinical study (see Clinical Performance below) the risk of developing severe sepsis among ICU patients was investigated and study results supported the following interpretative risk assessment criteria:

- Patients tested on their first day of admission to the ICU where the EAA™ value is ≥ 0.60, are three times more likely to develop severe sepsis within the next 24 hours than subjects whose EAA™ values are < 0.40 (0.00-0.39).

Results less than 0.40 EAA™ units indicate a low endotoxin activity level. Results in this range represent a low risk for progression to severe sepsis.

- Severe sepsis is a syndrome with more than one cause. Low endotoxin activity levels do not rule out the possibility of the development of severe sepsis from non-endotoxemic triggers. Therefore in this context, clinicians would be encouraged to focus on non-endotoxemic causes of sepsis. The EAA™ is to be used in conjunction with other clinical information.

Results of between 0.40 - 0.59 EAA™ units indicates an intermediate endotoxin activity level and represent an elevated risk for severe sepsis.

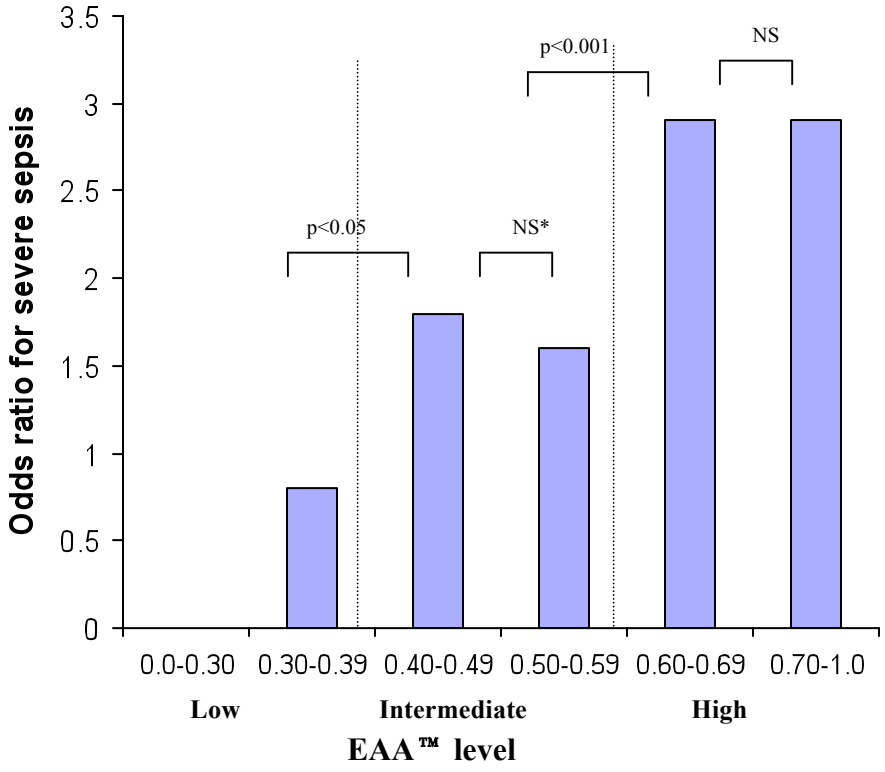
- Patients in the intermediate range have an odds ratio for severe sepsis of 2.0 versus patients with low EAA™ results.

A result greater than or equal to 0.60 EAA™ units indicates a high endotoxin activity level.

- Patients with EAA™ levels in the high range have an odds ratio for severe sepsis of 3.0 versus patients with low EAA™ results.
- Increased levels of endotoxin can be associated with other conditions in critically ill patients such as shock (9) and hypoxemia (10). Endotoxemia has pathogenic properties in patients with liver disease (11,12), in patients post-operative (13,14,15) and in patients undergoing cardiopulmonary bypass (16).

While increased levels of endotoxin may not be the only risk factor for severe sepsis, its presence in graded levels, low (0.00-0.39 EAA™ units), intermediate (0.40-0.59 EAA™ units) and high (≥0.60 EAA™ units) has a strong association with the presence of the disease. The figure below demonstrates the intervals chosen for EAA™ results, and the odds ratio for development of severe sepsis as well as the statistical evaluation for differences between all three levels. In addition, the trend across all three levels of endotoxemia is consistent and highly statistically significant (p< 0.0001).

Figure 1. Odds Ratio for Severe Sepsis and Level of Endotoxemia



*NS= non statistically significant differences between odds ratios for deciles of EAA™ levels within the intermediate EAA™ level and within the high EAA™ level. Note, however that the differences between the levels, low versus intermediate and intermediate versus high EAA™ levels are statistically significant.

Limitations:

Analytical

1. In common with all immunoassays or immunochemical reactions, careful technique is required.
2. The user should be alert to the possible effects on results of potential interferences from medications or unknown endogenous substances. See Interfering Substances below.

Clinical

1. The EAA™ should not be used outside the critical care setting as the test's performance characteristics have not been established outside this setting.
2. The EAA™ should not be used at times other than the first day of ICU admission as the test's performance characteristics has not been established beyond the first ICU day.
3. The findings of positive endotoxin results in patients without severe sepsis have been reported previously in published studies.
4. The results of the EAA™ should be evaluated in context of all laboratory findings and the total clinical status of the patient. In cases where the laboratory results do not agree with the clinical picture or history, additional tests should be performed.

Expected Results:

A) Rule –out of Gram Negative Infection

The EAA™ is an endotoxin activity assay with a numeric cut-off to rule-out Gram negative infection set at 0.40. Results less than 0.40 (0.00-0.39) support the absence of Gram negative infection for ICU patients with suspicion of infection.

The Multi-Center Endotoxin Detection in Critical illness (MEDIC) trial was a multi-center, prospective observational study, performed in 10 Intensive Care Units (ICUs) of academic hospital settings in North America and Europe. The MEDIC trial was conducted to evaluate the performance characteristics of the EAA™ compared to culture determination of invasive Gram negative infection. Eligible patients were those with clinical suspicion of infection plus and order for a diagnostic culture.

Invasive Gram negative infection was identified by culture in 36 of 465 (8%) of evaluable patients. Using a cut-off value for the EAA™ of < 0.40, the test performed as follows:

	No Gram Negative Infection	Gram Negative Infection Meeting *CEC Criteria	Total
< 0.40 (0.00-0.39) EAA™	134	8	142
≥ 0.40 (0.40-1.00) EAA™	295	28	323
Total	429	36	465

NPV (95%CI)	94.4 (88.4 – 97.8)
Sensitivity (95%CI)	77.8 (56.3 – 92.1)
Specificity (95%CI)	31.2 (25.3 – 37.7)
PPV (95%CI)	8.7 (5.8 – 12.3)

*Clinical Evaluation Committee: All cultures showing bacterial growth were further reviewed by a committee (CEC) using explicit adjudication rules to achieve agreement regarding the presence or absence of infection.

As with all diagnostic tests, each laboratory should establish its own diagnostic cut-off to assure proper representation of specific populations and to reflect current practice and criteria for Gram negative infections at their institution.

Results ≥ 0.40 are indicative of endotoxemia. Several factors can cause elevated levels of circulating endotoxin. These include invasive Gram negative infection and the translocation of endotoxin from the lumen of the intestine. Endotoxin is the

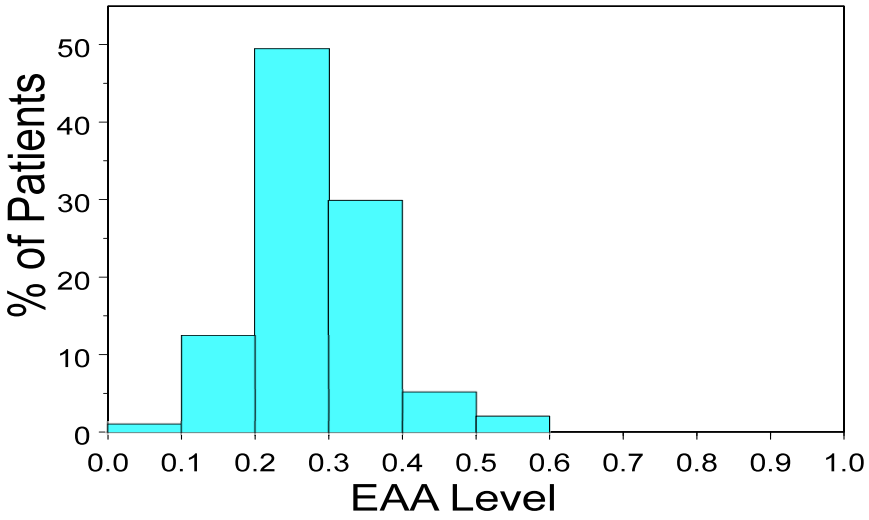
primary Gram negative bacterial product responsible for septic shock. In the MEDIC study, patients with an EAA™ level ≥ 0.60 had an Odds Ratio for sepsis of 3.0 (See Risk Assessment for Severe Sepsis below).

Precise diagnosis of primary and secondary causes of Endotoxemia requires further clinical and diagnostic approaches to determine its exact causes and appropriate therapies.

B) Risk Assessment for Severe Sepsis

The EAA™ is an endotoxin activity assessment assay with three intervals of endotoxemia: low < 0.40 (0.00-0.39) EAA™ units, intermediate 0.40-0.59 EAA™ units and high ≥ 0.60 EAA™ units.

Figure: Histogram of EAA™ values in Healthy Subjects



An EAA™ level of 0.40 represents a value that is +2 standard deviations above the mean. In a population of healthy subjects 93% had an EAA™ level below this value. As such it is reasonable to assume that a level of 0.40 represents a conservative cutoff below which most individuals should be "healthy". In addition, an EAA™ level of 0.60 represents a value of +4 standard deviations above the mean. Individual baseline variations may occur. An unexpected finding of slight elevations of endotoxin have been reported in ambulatory conditions such as during periodontitis[7], or cigarette smoking[8]. No volunteers had a measured EAA™ level of ≥0.60. As such this represents a significant level above which an EAA™ level may be indicative of an underlying adverse process.

Clinical Performance: The Multi-Center Endotoxin Detection in Critical illness (MEDIC) trial was a multi-center, prospective observational study, performed in 10 Intensive Care Units (ICUs) of academic hospital settings in North America and Europe. The presence of endotoxemia was evaluated on the first day of the patients ICU stay to determine the odds of developing severe sepsis within 24 hours of ICU admission. The target population for the Risk Assessment Study included all eligible patients enrolled in the MEDIC trial on first day of ICU admission who had evaluable samples, N=857. Patient demographics are shown in the following Table

Demographics and Baseline Features of Analyzed Patients

Characteristic	
Age: mean (SD) median [IQR]* years	60 (±17) 62 [49, 74]
Gender (% male)	58.9%
Race (% Non-Caucasian)	15.8%
APACHEII: mean (SD) median [IQR]*	15.2 (±9.5) 14 [8, 21]
Days in ICU: mean (SD) median [IQR]*	5.4 (± 10.9) 2.0 [1, 5]
Days in Hospital: mean (SD) median [IQR]*	23.3 (± 27.0) 14.0 [7,29]
ICU Mortality	13.3%
Hospital Mortality	19.9%

*IQR = inter-quartile range

The critical care patient population is the most appropriate clinical environment for EAA™ testing. The utility of the EAA™ test has only been established for the critical care population. The relationship between endotoxin levels and the risk for developing severe sepsis in critically ill patients is intended as a supplement to currently available clinical information to establish risk for severe sepsis on the first day of ICU stay.

EAA™ Results and the Risk for Severe Sepsis Within the first 24 hours in the ICU

EAA™ results	Incidence of Severe Sepsis within first 24 hours	Odds Ratio	95% CI	Chi-square, p value
Low < 0.40 (0.00-0.39)	4.9 %			
Intermediate 0.40-0.59	9.2%	2.0	1.02-3.78	4.3, <0.05
High ≥ 0.60 (0.60-1.00)	13.4%	3.0	1.65-5.41	14.2, <0.001

Of the 857 patients evaluated, 214 patients had high (≥ 0.60) EAA™ values on Day 1 but did not progress to severe sepsis on Day 1. Pertinent clinical conditions that may have contributed to endotoxemia in these patients included: shock (n=30), hypoxemia (n=127), chronic renal failure (n=25), and post-operative (n=54). Some patients exhibited more than one of these conditions.

Performance Characteristics:
Interfering Substances:

- Triglyceride levels up to 1000 mg/dL do not interfere with EAA™ measurements. At triglyceride concentrations greater than 1500 mg/dL a decrease in EA values has been demonstrated. Grossly lipemic samples should be avoided for use in the EAA™.

- Hemoglobin at 100 mg/dL does not interfere with EAA™ measurements while levels of 500 mg/dL attenuated low EAA™ measurements by less than 0.03 units and attenuated elevated EAA™ readings by an average of 15%. Grossly hemolyzed samples should be avoided for use in the EAA™.

- Exogenous infusion of endotoxin-free albumin into patients at levels which increase plasma albumin by more than 10 g/L above the upper limit of normal may lower EAA™ values. Samples from patients with hyper-protein anomalies or receiving albumin therapies should have protein determinations performed to ensure that elevated albumin levels are not a source of interference with the EAA™.

Precision: Whole blood samples, neat and with added exogenous endotoxin, were analyzed in eight replicates on each of three instruments. Both the within-run and total precision was calculated according to NCCLS procedures in EP5-A.

Sample	N	Mean EAA	Within 1SD	Run % CV	Total 1SD	Precision % CV
1	24	0.11	0.015	14	0.023	22
2	24	0.20	0.030	15	0.029	14
3	24	0.30	0.043	15	0.042	14
4	24	0.50	0.059	12	0.064	13
5	24	0.52	0.036	7	0.034	6
6	24	0.59	0.050	8	0.046	8

Analytical Sensitivity: The total precision on a low patient sample was used to estimate sensitivity of the EAA™ method. Twenty-four EAA™ determinations were made on a single patient sample with a low EAA™ level; eight measurements on each of three instruments. The mean EAA™ level of the patient was 0.11 with a SD of 0.023. Sensitivity, by the two standard deviation method, is thus estimated at 0.046 EAA™ units.

Analytical Specificity: Endotoxin from the Gram negative bacteria, *E. coli* 055:B5, *P. aeruginosa, K. pneumoniae, S. enterides, E. coli* 0127:B8 and *S. marcescens*, and *S. flexnevi* induced an increase in EAA™ levels of 0.10 to 0.52 when 100 pg/mL of the endotoxin was added to a blood sample with a neat mean level of 0.33 EAA units. In addition, preparations of *V. cholerae* LPS produced statistically significant increases in EA values. No reactivity is seen with up to 2000 pg/mL lipoteichoic acid extracts from the following strains of Gram positive bacteria: *S. mutans, S. pyogenes, S. sanguis, S. faecalis, S. aureus* and *B. subtilis*. Similarly, there was no reactivity of yeast mannan up to 2000 pg/mL and cell wall extracts of *C. albicans* and *A. fumagatis*.

Linearity of Results: The assay has been optimized to be highly sensitive to low levels of endotoxin and to resist a high dose hook effect induced by grossly high endotoxin levels. Therefore the curve is curvilinear in shape. When patient samples contain low levels of endotoxin, small increases in endotoxin will result in significant increases in EAA™ values. Dilutions are not required for samples that have generated high EAA™ values.

References:

1. Van Deventer SJH, et al. Endotoxemia: an early predictor of septicemia in febrile patients. The Lancet (March 19) 1988; 605-609.
2. Parrillo JE, Parker MM, Natanson C, et al. Septic shock in humans: advances in the understanding of pathogenesis, cardiovascular dysfunction, and therapy. Ann Int Med 1990; 113: 227-242.
3. Zhao B, Bowden RAS, Stavchansky SA, et al. Human endothelial cell response to gram-negative lipopolysaccharide assessed with cDNA microarrays. Am J Cell Physiol 2001; 281: C1587-C1595.
4. Romaschin AD, et al. A rapid assay of endotoxin in whole blood using autologous neutrophil dependent chemiluminescence. J. of Immunology. Methods 1998; 212: 169-185.
5. NCCLS (National Committee for Clinical Laboratory Standards, 711 East Lancaster Avenue, Villanova, PA 19085) Document # M29-A Protection of Laboratory Worker from Instrument Biohazards and Infectious Disease Transmitted by Blood, Body Fluids and Tissues; Approved Guideline, 1997.
6. NCCLS (National Committee for Clinical Laboratory Standards, 711 East Lancaster Avenue, Villanova, PA 19085) Document # H3-#a Procedures for the Collection of Diagnostic Blood Specimens by Venipuncture – Third Edition; Approved Standard, 1991.
7. Geerts SO, Nys M, De MP, et al. Systemic release of endotoxins induced by gentle mastication: association with periodontitis severity. J Periodontal 2002; 73(1): 73-78.
8. Hasday JD, Bascom R, Costa JJ, et al. Bacterial endotoxin is an active component of cigarette smoke. Chest 1999; 115(3): 829-835.
9. Van Langevelde, P, Joop, K, van Loon, et al. Endotoxin, cytokines, and procalcitonin in febrile patients admitted to the hospital: identification of subjects at high risk of mortality. CID 2000; 31(December): 1343-1348.
10. Murphy DB, Cregg N, Tremblay L, et al. Adverse ventilatory strategy causes pulmonary-to-systemic translocation of endotoxin. Am J Respir Crit Care Med 2000; Jul 162(1): 27-33.
11. Bion JF, Badger I, Crosby HA, et al. Selective decontamination of the digestive tract reduces Gram-negative pulmonary colonization but not systemic endotoxemia in patients undergoing elective liver transplantation. Crit Care Med 1994; 22: 40-49.
12. Gaeta GB, Perna P, Adinolfi LE et al. Endotoxemia in a series of 104 patients with chronic liver diseases: prevalence and significance. Digestion 1982; 23: 239-244.
13. Soong CV, Blair PHB, Halliday ML et al. Endotoxemia, the generation of the cytokines and their relationship to intramucosal acidosis of the sigmoid colon in elective abdominal aortic aneurysm repair. Eur J Vasc Surg 1993; 7: 534-539.
14. Roumen RMH, Frieling JTM, van Tits HWHJ et al. Endotoxemia after major vascular operations. J Vasc Surg 1993; 18:853-857.
15. Schlag G, Redl H, Dinges HP, Davies J. Sources of endotoxin in the posttraumatic setting. Bacterial Endotoxins: Cytokine Mediators and New Therapies for Sepsis: 121-134.
16. Jansen PGM, Te Velthuis H, Oudemans-Van Straaten HM, et al: Perfusion-related factors of endotoxin release during cardiopulmonary bypass. Eur J Cardio-thorac Surg 1994; 8:125-129.

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