NOTE *Internal Medicine*

Detecting endotoxin activity in bovine serum using an automated testing system

Kazuyuki SUZUKI[1,2)]*, Toshio SHIMAMORI[1)], Ayano SATO[1)], Kenji TSUKANO[1)], Masakazu TSUCHIYA[3)] and Jeffrey LAKRITZ[2)]

[1)]*School of Veterinary Medicine, Rakuno Gakuen University, 582 Midorimati, Bunnkyoudai, Ebetsu, Hokkaido 069–8501, Japan*
[2)]*College of Veterinary Medical Science, Ohio State University, 601 Vernon L. Tharp St., Columbus, OH 43210–1089, U.S.A.*
[3)]*Endotoxin and Microbial Detection, Charles River, 1023 Wappoo Road, Suite 43B, Charleston, SC 29407, U.S.A.*

(Received 17 October 2014/Accepted 3 March 2015/Published online in J-STAGE 15 March 2015)

ABSTRACT. The aim of the present study was to compare the ability of the commercially available portable test system (PTS^{TM}) to detect endotoxin activity in bovine serum, with that of the traditional LAL-kinetic turbidimetric (KT) and chromogenic (KC) assays. Prior to testing, serum samples, which were obtained from endotoxin-challenged cattle, were diluted 1:20 in endotoxin-free water and heated to 80°C for 10 min. The performance of the PTS^{TM} was not significantly different from that of the traditional LAL-based assays. The results using PTS^{TM} correlated with those using KT (r^2=0.963, P<0.001) or KC assays (r^2=0.982, P<0.001). Based on these findings, the PTS^{TM} could be applied as a simplified system to assess endotoxin activity in bovine serum.
KEY WORDS: cattle, endotoxin, limulus amebocyte lysate, portable test system, serum

doi: 10.1292/jvms.14-0545; *J. Vet. Med. Sci.* 77(8): 977–979, 2015

Since Levin and Bang [7] discovered the role of endotoxin in the coagulation of horseshoe crab blood in 1964, numerous methods incorporating limulus amebocyte lysate (LAL) have been developed for the detection of endotoxin and endotoxin testing of parenteral drugs [2, 3]. In 1977, the United States Food and Drug Administration (FDA) approved the more widely-use LAL assays. However, these assays are very complex and thus inadequate for field use [8, 10]. The downfall of each of the traditional assays is their failure to yield timely results, which is essential when therapy must be immediately instituted.

Charles River (Charleston, SC, U.S.A.) recently introduced a portable test system (PTS^{TM}) for the detection of endotoxin. This automated miniaturized kinetic chromogenic LAL-based assay (the Endosafe® PTS^{TM}) delivers results in 15 min. The concentration of endotoxin in each sample is determined by the degree of color intensity [1, 4–6]. Unlike the PTS^{TM}, the traditional toxinometer [9], LAL-kinetic turbidimetric (KT) and chromogenic (KC) assays [5] require 75% to 85% more processing time. The PTS^{TM} is also advantageous when time-sensitive treatments are needed, because it is a hand-held portable machine that can be applied as a simple test. The aim of the present study was to compare the ability of the PTS^{TM} to detect endotoxin activity in bovine serum, with that of the KT and KC assays.

Animals and sample collection: All procedures were reviewed and approved by the Institutional of Animal Care and Use Committee (IACUC) of the College of Veterinary Medicine, Ohio State University (U.S.A.) and School of Veterinary Medicine, Rakuno Gakuen University (Japan). To determine the reference range for endotoxin activity in normal bovine serum, blood was collected by jugular venipuncture from 3 Jersey and 47 Holstein dairy cattle that were kept at the School of Veterinary Medicine, Rakuno Gakuen University, aged 3.9 ± 1.5 years old, and centrifuged for 10 min at 3,000 × g at 4°C. Approximately 1.8 m*l* of serum were harvested and stored in sampling tubes (CryoTubeTM vials, Nunc, Roskilde, Denmark) at −20°C until analyzed. Immediately prior to testing, serum samples were diluted 20-fold in endotoxin-free water (R5005-01 Sterile Water for Irrigation USP, B. Braun Medical Inc., Bethlehem, PA, U.S.A.) and agitated in a vortex for 10 sec. Specimens were then heated for 10 min at 80°C to inactivate interfering substances, such as protease.

Six 2-month–old Jersey calves (140.9 ± 36.3 kg of BW) kept at the College of Veterinary Medicine, Ohio State University were enrolled in this study to assess the correlation between the PTS^{TM} and traditional LAL methods. All calves received an intravenous bolus of 2.5 μg/kg body weight of O111:B4 LPS (L4391, Sigma-Aldrich, St. Louis, MO, U.S.A.) in 10 m*l* of autologous serum via the jugular vein (median: 152,641 endotoxin units (EU)/head, min-max; 117,968–229,296 EU/head). Ten milliliter (10 m*l*) of blood samples was collected at 0.5, 1.0, 12 and 24 hr after LPS challenge (24 samples). The PTS^{TM}, KT and KC assays were used to quantify serum endotoxin activity.

Traditional LAL assay, KT and KC assays: Endotoxin-free water was used as the blank in all tests. USP endotoxin reference standard (RSE, USP Endotoxin Reference Standard Lot G, the United States Pharmacopeial Convention, Inc., Rockville, MD, U.S.A.), which contained 10,000 EU/vial, was used as the positive control. The LAL reagents for LAL KT (Endosafe® KTA^2, Charles River) and KC (Endosafe® Endochrome-K kit, Charles River) assays were reconstituted

*CORRESPONDENCE TO: SUZUKI, K., School of Veterinary Medicine, Rakuno Gakuen University, 582 Midorimati, Bunnkyoudai, Ebetsu, Hokkaido, 069–8501, Japan. e-mail: kazuyuki@rakuno.ac.jp

with Endotoxin-Specific Buffer Solution (Charles River) to eliminate any interference from β-glucans. Both traditional LAL-based assays were performed on a 96-well microplate (Endosafe® 96-well, flat bottom microplate M9001, Charles River), and endotoxin activity was determined using a microplate reader (Sunrise™, Tecan Group Ltd., Männedorf, Switzerland) and EndoScan-V™ endotoxin-measuring software (Charles River). The range covered by the standard curve (0.003 to 3.0 EU/m*l*) was established according to the package insert of the LAL product. The lower limit of quantitation for this assay was 0.028 EU/m*l*.

Measurement of endotoxin activity using a PTS™: The Endosafe PTS™, which was used in the present study, is comprised of spectrophotometer, reader and LAL reagent cartridge (Fig. 1). Precise amounts of LAL reagents, buffer components, oligosaccharides as a β-glucan blocker, chromogenic substrates and control standard endotoxin were dried on the channels of the commercially available LAL reagent cartridges (Charles River). The cartridges contain 2 sample and 2 spiked channels. The analyst loaded 25-μl samples into the cartridge sample reservoirs, and the reader drew, mixed and incubated the samples at different time intervals after the assay was started. In this study, 20-fold diluted serum samples, which were heated to 80°C for 10 min, were evaluated for endotoxin activity. Results were automatically multiplied by the dilution factor entered into the system. A detailed description of PTS™ is provided elsewhere [1, 6].

Statistical analysis: A test result was considered valid when spike recovery and coefficient of variation (CV) parameters met the accepted criteria (<25%) established by the Endosafe® PTS™ and traditional LAL methods. Spike recovery values were considered valid, if the results were between 50% and 200% according to the Bacterial Endotoxin Test in the US pharmacopeia [1, 4–6].

Non-normally distributed data were expressed as the median and ranges. Sample endotoxin activities were statistically analyzed by using the SPSS software program (ver 21. IBM Japan, Tokyo, Japan). Serum endotoxin activity below 0.028 EU/m*l* was statistically analyzed as 0.028 EU/m*l*. The results of the PTS™ and each of the traditional assays were compared using the Friedman test. The Pearson product-moment correlation coefficient was used to measure the strength and direction of association between any two assays measured on an interval scale. A linear regression model analysis was also performed. The significance level was set at $P<0.05$.

Determining the reference range for bovine serum endotoxin activity: The KT assay effectively recovered endotoxin from serum samples over the range of concentrations tested. The coefficient of determination (r^2) for the KT assay was 0.98441. Endotoxin was detected in 20% (10/50) of the serum samples obtained from healthy cattle. The median range of endotoxin activity detected in the serum of healthy cattle was 0.028 EU/m*l* (0.028−0.123 EU/m*l*).

Determination of serum-endotoxin activity using the PTS™: Each of the three assays (the PTS™, KT and KC) effectively recovered endotoxin from the serum of endotoxin-challenged calves at the each of the designated time points

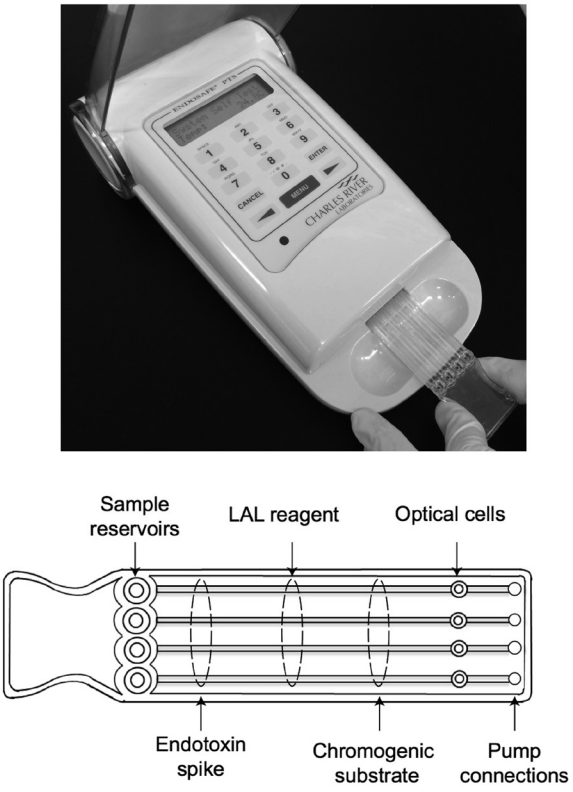

Fig. 1. The Endosafe® PTS™. The limulus amebocyte lysate (LAL) spectrophotometer (upper) and reagent cartridge (bottom) for PTS™.

(0.5, 1.0, 12 and 24 hr). The rate of recovery by each of these tests was 100% (24/24), 95.8% (23/24) and 100% (24/24), respectively. The median range of endotoxin activity detected by each of the tests, PTS™, KT and KC, was 0.395 (0.113–2.130), 0.255 (0.044–2.772) and 0.397 (0.074–2.009) EU/m*l*, respectively. As depicted in Fig. 2, results obtained from the PTS™ correlated well with those from the KT (r^2=0.963, $P<0.001$) and KC (r^2=0.982, $P<0.001$), respectively. Based on the results of the Friedman Test, the ability of the PTS™ to recover endotoxin from serum is not significantly different from that of either the KT or KC assay ($P>0.05$).

The PTS™ is able to effectively detect serum endotoxin activity. It offers several advantages over the microplate kinetic LAL assays currently in use by diagnostic laboratories. It is small and portable, requires only small quantities of specimen and provides results rapidly [1, 6]. However, the results obtained from simplified test will often dictate the course of clinical care and hygiene management.

In conclusion, the photometric PTS™ represents a rapid, simple and accurate technique, which uses a quantitative kinetic chromogenic LAL method for the assessment of endotoxin activity in serum. Results of the current study therefore confirm that the PTS™ is appropriate for use as a practical simplistic assay for the detection of endotoxin activity in bovine serum.

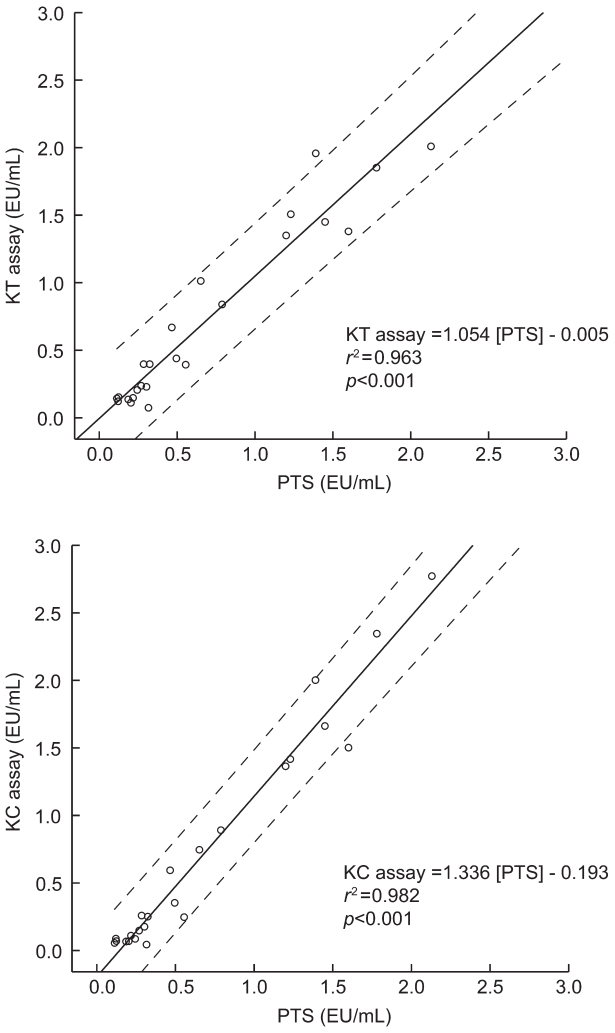

Fig.2. Correlation of endotoxin activity in serum between the traditional kinetic limulus amebocyte lysate (LAL) analysis and portable test system (PTS™). The endotoxin activities detected in serum samples PTS™ positively corrected with those using the kinetic turbidimetric (KT: upper) and kinetic chromogenic (KC: bottom) assays by Person's product-moment correlation coefficient.

ACKNOWLEDGMENTS. This study was supported by a Grant-in-Aid for Science Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (no. 21580393 and 26450431), and by a Grant-in-Aid from Rakuno Gakuen University foundation awarded to K. Suzuki.

REFERENCES

1. Cooper, J. F., Latta, K. S. and Smith, D. 2010. Automated endotoxin testing program for high-risk-level compounded sterile preparations at an institutional compounding pharmacy. *Am. J. Health Syst. Pharm.* **67**: 280–286. [Medline] [CrossRef]
2. Cooper, J. F., Levin, J. and Wagner, H. N. Jr. 1971. Quantitative comparison of in vitro and in vivo methods for the detection of endotoxin. *J. Lab. Clin. Med.* **78**: 138–148. [Medline]
3. Elin, R. J. and Hosseini, J. 1985. Clinical utility of the Limulus amebocyte lysate (LAL) test. *Prog. Clin. Biol. Res.* **189**: 307–327. [Medline]
4. Fukumori, N. T., de Campos, D. G., Massicano, A. V., de Pereira, N. P., da Silva, C. P. and Matsuda, M. M. 2011. A portable test system for determination of bacterial endotoxins in 18F-FDG, 99mTc, and lyophilized reagents for labeling with 99mTc. *J. Nucl. Med. Technol.* **39**: 121–124. [Medline] [CrossRef]
5. Gee, A. P., Sumstad, D., Stanson, J., Watson, P., Proctor, J., Kadidlo, D., Koch, E., Sprague, J., Wood, D., Styers, D., McKenna, D., Gallelli, J., Griffin, D., Read, E. J., Parish, B. and Lindblad, R. 2008. A multicenter comparison study between the Endosafe PTS rapid-release testing system and traditional methods for detecting endotoxin in cell-therapy products. *Cytotherapy* **10**: 427–435. [Medline] [CrossRef]
6. Jimenez, L., Rana, N., Travers, K., Tolomanoska, V. and Walker, K. 2010. Evaluation of the Endosafe® Portable Testing SystemTM for the Rapid Analysis of Biopharmaceutical Samples. *PDA J. Pharm. Sci. Technol.* **64**: 211–221. [Medline]
7. Levin, J. and Bang, F. B. 1964. The roll of endotoxin in the extracellular coagulation of Limulus blood. *Bull. Johns Hopkins Hosp.* **115**: 265–274. [Medline]
8. Svensson, A. and Hahn-Hägerdal, B. 1987. Comparison of a gelation and a chromogenic Limulus (LAL) assay for the detection of gram-negative bacteria, and the application of the latter assay to milk. *J. Dairy Res.* **54**: 267–273. [Medline] [CrossRef]
9. Trump, D., Danckwart, H., Jacobson, M. and Hung, J. 2006. Evaluation of Toxinometer ET-Mini and Endosafe-PTS endotoxin testing systems. *J. Nucl. Med.* **47**: 527P.
10. Waage, S., Jonsson, P. and Franklin, A. 1994. Evaluation of a cow-side test for detection of gram-negative bacteria in milk from cows with mastitis. *Acta Vet. Scand.* **35**: 207–212. [Medline]