

Evaluation of a portable test system for assessing endotoxin activity in raw milk

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ABSTRACT. The aim of the present study was to compare endotoxin activities detected in raw milk samples obtained from cattle by a commercially available portable test system (PTS) and traditional microplate limulus amoebocyte lysate (LAL)-based assay, which determined activities using a kinetic turbidimetric (KT) assay. Raw milk samples were obtained from 53 and 12 dairy cattle without and with clinical mastitis, respectively. Comparison between the KT and PTS was performed by the Friedman test. The Pearson product moment correlation coefficients were calculated to evaluate associations between any two continuous variables. Linear regression model analysis was also performed to obtain the equation describing the relationship between PTS and KT assay. The endotoxin activities detected in 200- or 400-fold diluted milk samples were similar between PTS and KT assay, whereas a significant difference was observed in 100-fold diluted milk ($P < 0.001$). The results obtained from 200- ($r^2 = 0.778$, $P < 0.001$) and 400-fold diluted milk samples ($r^2 = 0.945$, $P < 0.001$) using PTS correlated with those using KT assay. The median milk endotoxin activities in Gram-positive and Gram-negative clinical mastitis cows were 0.655 and 11,523.5 EU/ml, respectively. The results of the present study suggest that PTS as a simple and easy test to assess endotoxin activity in raw milk is efficient, simple and reproducible.

KEY WORDS: cattle, endotoxin, limulus amoebocyte lysate, milk, portable test system

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Mastitis is one of the most frequent diseases reported in dairy cows and is a most common cause of economic losses [17, 21]. Gram-negative bacteria are among the most common environmental pathogens to cause mastitis and are responsible for approximately one third of all cows affected with clinical mastitis in United States [1, 14]. The systemic complications and deleterious outcomes associated with Gram-negative infections have been attributed to the exaggerated inflammatory responses largely elicited by a highly pro-inflammatory component of the Gram-negative bacterial envelope known as endotoxin or bacterial lipopolysaccharide [6]. Endotoxin plays a major role in the pathophysiology of Gram-negative bacterial sepsis; therefore, attempts have been made to detect and quantify it, with conflicting findings, in various states of infection.

Since Levin and Bang [18] discovered the role of endotoxin in the coagulation of horseshoe crab blood in 1964, numerous methods incorporating limulus amoebocyte lysate (LAL) have been developed for the detection of endotoxin [5]. In 1971, Cooper *et al.* [4] recognized the potential of the LAL test to detect endotoxin in parenteral drugs and demonstrated the application of this test as an alternative method to the rabbit pyrogen test. In 1977, the United States Food

and Drug Administration (FDA) approved two methods for endotoxin testing; the rabbit pyrogen test [13] and the more widely used LAL assay. There are currently several accepted methodologies for performing the LAL test. Previous studies indicated the applicability of the LAL test for determining endotoxin activity in pasteurized [10] and raw milk [2, 12, 14, 16, 19, 20, 22, 25]. However, these traditional LAL tests are very complex and, thus, inadequate for field tests. Therefore, the detection of endotoxin activity in milk in the field may represent an alternative and effective method for the early diagnosis and prognosis of coliform mastitis and assessment of hygiene conditions in dairy farms.

Charles River recently introduced a portable test system (PTS) that can assay endotoxin in approximately 15 min using an automated miniaturized LAL-based assay (The Endo-safe® PTS system, Charles River, Charleston, SC, U.S.A.). PTS cartridges have been licensed and approved by the FDA for endotoxin testing. PTS is based upon the kinetic chromogenic detection of endotoxin by measuring color intensities related to endotoxin concentrations in samples [3, 8, 9, 15]. This system is composed of two parts. Figures 1 and 2 show the hand-held spectrophotometer reader and test cartridges, respectively. Therefore, PTS needs to be compared to the traditional LAL test in order to confirm its variability regarding percentage spike recovery and the percentage coefficient of variation (CV) before it can be recommended as a portable and easy LAL test to assess endotoxin activity in raw milk.

The aim of the present study was to compare endotoxin activities detected in raw milk samples obtained from healthy dairy cattle without mastitis by a commercially available PTS™ and traditional microplate LAL-based assay, which

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Fig. 1. The Endosafe[®] PTS system. Spectrophotometer and reader.

determined activities using a kinetic turbidimetric (KT) assay. In addition, a present study was verified whether PTS could measure the milk endotoxin activity of mastitis cattle caused by Gram-positive or negative bacteria.

MATERIALS AND METHODS

Animals and sample collection: Experiments were conducted at the School of Veterinary Medicine, Rakuno Gakuen University and College of Veterinary Medicine, Ohio State University. All procedures were reviewed and approved by the Institutional of Animal Care and Use Committee (IACUC) at the College of Veterinary Medicine, Ohio State University.

Twenty-four Holstein dairy cattle, aged 3.5 ± 1.6 years old, were enrolled in the study to clarify the reference values of healthy animals. These Holstein dairy cattle did not exhibit any clinical symptoms, had linear somatic cell count (SCC) scores less than 3 and were kept at the School of Veterinary Medicine, Rakuno Gakuen University. The medians (min to max) of the linear SCC score and days in milk were 1.7 (0.1–2.9) and 175 days (32–316 days), respectively.

Ten Holstein and nineteen Jersey dairy cattle with linear SCC scores of 3 or more, aged 3.6 ± 0.7 years old, were used in the study to determine the relationship between PTS and traditional microplate LAL methods. Holstein and Jersey dairy cattle were kept at the School of Veterinary Medicine, Rakuno Gakuen University and College of Veterinary Medicine, Ohio State University, respectively. These cattle did not have systemic inflammation including severe mastitis and did not receive any medication. The medians (min to max) of the linear SCC score and days in milk were 4.5 (3.0–9.1) and 108 days (50–188 days), respectively. In 12 of these

Jersey cows, endotoxin activities using the LAL-based KC assay were measured twice at 4-month intervals in order to evaluate the effects of freezing (Oct., 2011 vs. Feb., 2012).

Twelve dairy cattle with clinical mastitis were patients at the Rakuno Gakuen University Veterinary Teaching Hospital and also used in the study. Those dairy cattle indicated swelling, redness and/or edema of one or more quarter with or without systemic signs, and the production of abnormal milk. The pathogens isolated from clinical mastitis milk were *Staphylococcus aureus* (n=6) and *Escherichia coli* (n=6).

Approximately 4 ml of raw milk was collected and stored in sampling tubes (CryoTube[™] vials, Nunc, Roskilde, Denmark) at -20°C until analyzed them within three months. Results greater than 5.0 endotoxin unit (EU)/ml can be run using dilutions in order to obtain results that are within the linearity of the assay. Immediately before being used in the assays, raw milk samples were diluted 100-, 200- or 400-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.

Traditional microplate LAL-based KT assay: Endotoxin-free water was used as a blank in all tests. The reference standard endotoxin (RSE, USP Endotoxin Reference Standard Lot G, the United States Pharmacopeial Convention, Inc., Rockville, MD, U.S.A.) contained 10,000 EU (endotoxin units)/vial was used for a positive control. The LAL reagent (Lot# C1152E; Endosafe[®] KTA², Charles River) was reconstituted with endotoxin-free specific buffer solution (Endotoxin-Specific Buffer Solution, Charles River) to eliminate the interference of β -glucan in the samples. KT assay was performed on a 96-well microplate (Endosafe[®] 96-well, flat bottom microplate M9001, Charles River) using a microplate reader (Sunrise[™], Tecan Group Ltd., Männedorf, Switzerland) at 340 nm and endotoxin-measuring software (EndoScan-V[™] endotoxin-measuring software, Charles River). A standard curve was established for each assay in the range between 0.002 EU/ml and 2.0 EU/ml, according to the package insert of the LAL product. One hundred microliters of the blank was used according to standard endotoxin concentrations (ie, 0.002, 0.02, 0.2 and 2 EU/ml), and 100 μl of the 200-fold diluted samples was added in a 96-well microplate with a respective positive product control.

The spike procedure of all samples obtained from cattle without clinical mastitis was performed according to the manufacturer's instructions by the addition of a known concentration value of endotoxin for KT assay in order to detect any possible inhibition or enhancement from the samples in relation to the LAL substrate. To verify the lack of product inhibition, an aliquot of a dilution of test sample was spiked with a known amount of endotoxin (0.20 EU/ml). The spiked solution was assayed along with the unspiked samples, and their respective endotoxin concentrations were determined. The difference between these two calculated endotoxin values was defined a "spike recovery". All reactions were achieved in duplicate. The lower limit of quantitation for this assay using 200-fold diluted sample was 0.28 EU/ml.

Measurement of endotoxin activity using PTS: All samples tested with the PTS system used 1–0.001EU/ml sensitivity

cartridges. The PTS, which comprised a spectrophotometer, reader (Fig. 1) and LAL reagent cartridge (Fig. 2), was used in the present study. The reagent cartridges (Lot# 3183249) were prototype that does not react in the β -glucan and were provided from Charles River Laboratories. Precise amounts of LAL reagents, buffer components and oligosaccharides as a β -glucan blocker, chromogenic substrates and control standard endotoxin were dried on the channels of the cartridges. The reagent cartridges were potency tested, spike recovery was performed, and then, the calibration code was determined. The calibration code (Cal# 419065608093) contains the reagent cartridge test parameters that were determined during potency testing, as well as the archived curve for that batch of cartridges. The cartridges contained 2 sample channels and 2 spiked channels (Fig. 2). The analyst loaded 25- μ l samples into the cartridge sample reservoirs, and the reader drew, mixed and incubated the samples at different time intervals after the assay was started. Product endotoxin concentration (endotoxin activity), product positive control with a known endotoxin concentration, percentage sample coefficient of variation, percentage endotoxin spike coefficient of variation and percentage recovery of product positive control were automatically calculated using a software for research use involving extrapolation function. In this study, 100-, 200- and 400-fold diluted raw milk samples obtained from cattle without mastitis were used to measure endotoxin activity. To obtain results that are within the linearity of the assay cartridge, serial dilution of raw milk sample obtained from cattle with mastitis were performed, if necessary. Results are automatically multiplied by the dilution factor entered into the PTS system. A detail description of PTS was provided elsewhere [3, 15].

Statistical analysis: A test result was considered valid based on the percentage spike recovery and percentage CV parameters falling within the acceptance criteria (25%) established by the PTS and KT assay. Spike recovery values were considered valid, if the results were between 50% and 200%, according to the Bacterial Endotoxin Test in the US pharmacopeia [24]. The absolute value of the correlation coefficient of the standard curve generated using reference standard endotoxin is greater than or equal to 0.980, for the range of endotoxin concentrations set up, according to the Bacterial Endotoxin Test in the US pharmacopeia [24].

Normally distributed data were reported as the mean \pm standard deviation (SD), and non-normally distributed data were expressed as the median and ranges. Endotoxin activities were statistically analyzed using a software program (SPSS ver. 21 for Macintosh, Japanese edition. IBM Japan, Tokyo, Japan). Comparisons between the KT assay and PTS were performed by the Friedman test. Friedman test is a non-parametric statistical test and used to detect differences in across multiple test attempts [7]. The Pearson product moment correlation coefficients were calculated to evaluate associations between any two continuous variables. Linear regression model analysis was also performed to obtain the equation. Statistical analysis of effects of freezing was also performed in the same way. The significance level was set at $P < 0.05$.

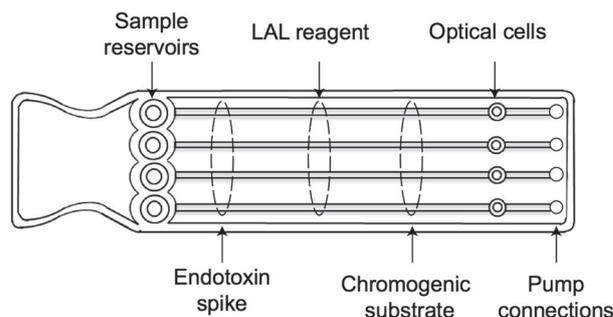


Fig. 2. The Endosafe[®] PTS System. The *limulus amoebocyte lysate* (LAL) reagent cartridge for PTS. Kinetic chromogenic reagents and endotoxin controls are dried in the channels of a polystyrene cartridge. The specimen is pumped into the channels in which it is mixed with reagents and monitored for changes in optical density. Results are interpolated against an archived endotoxin standard curve.

RESULTS

Accuracy and precision of the LAL-kinetic turbidimetric (KT) assay: The KT assay test effectively recovered endotoxin from raw milk samples (84.0%, range 52.1–152.0%) over the range of concentrations tested. The linearity of the standard curve was also good for the KT assay (r^2 , range 0.988–0.999) over the range of concentrations tested. The coefficient of variation, a parameter in the KT assay for endotoxin activity, was 1.55% (range, 0–21.9%). In the KT assay, endotoxin activity was detected in 50% (12/24) and 100% (29/29) of the raw milk samples obtained from dairy cattle with low (<3) and high linear SCC scores (≥ 3), respectively. The medians (range) of the KT assay for cattle with low and high linear SCC scores were 0.28 EU/ml (min to max, 0.27–2.16 EU/ml) and 2.70 EU/ml (0.28–42.0 EU/ml), respectively. Endotoxin activity values were not different even when frozen for 4 months ($P=1.000$ by the Friedman test and $r^2=0.989$, $P < 0.001$ by Pearson product moment correlation coefficients).

Determination of milk-endotoxin activity using the PTS: Regardless of PTS, endotoxin activity was detected in 100% of the raw milk samples obtained from healthy Holstein and Jersey cattle with linear SCC scores of 3 or more. As depicted in Fig. 3, the endotoxin activities detected in 200- or 400-fold diluted milk samples using PTS were similar to those using the KT assay ($P=0.705$ and $P=1.000$ by the Friedman test, respectively), whereas a significant difference was observed in endotoxin activity detected in 100-fold diluted milk ($P < 0.001$). The results obtained for 200- ($r^2=0.778$, $P < 0.001$) and 400-fold diluted milk samples ($r^2=0.945$, $P < 0.001$) using PTS correlated with those using the KT assay, respectively. The medians (range) of endotoxin activities in 100-, 200- and 400-fold diluted raw milk samples were 15.0 EU/ml (range: 0.89–83.1 EU/ml), 2.99 EU/ml (range: 0.10–43.1 EU/ml) and 2.69 EU/ml (range: 0.10–40.8 EU/ml), respectively. PTS for endotoxin activity effectively recovered reference endotoxin from 100-, 200- and 400-fold diluted raw milk samples.

The median milk endotoxin activity in Gram-positive clinical mastitis cows was 0.66 EU/ml (range: 0.28–450.0 EU/ml). Therefore, the dilution factor was adequate for 200 or 400-fold. On the other hand, a sample dilution was required of more than 160,000-fold to measure of endotoxin activity by PTS, because the median of milk in coliform mastitis cow was a significant high (median; 11,523.5 EU/ml, range: 4,707.4–49,035.2 EU/ml).

DISCUSSION

The simple and easy PTS for endotoxin activity offers several advantages over the traditional microplate kinetic LAL-based used by diagnostic laboratories. It is small and portable, requires only small quantities of raw milk and provides results rapidly [3, 15]. The ready availability of this cartridge system adds to its attractiveness. However, since the results obtained using this portable test will often dictate the course of clinical care and hygiene management, it is important that the cartridge system provides accurate information.

In the present study, we showed that PTS could accurately detect endotoxin activities in both 200- or 400-fold diluted milk samples, and the results obtained correlated with the traditional KT assay. However, a significant difference was observed between the PTS and KT assay for endotoxin activity in 100-fold diluted milk samples. We observed the reaction courses of PTS and found significant initial noise with 100-fold diluted milk. Therefore, the high values obtained in 100-fold diluted milk using PTS were caused by the initial noise, which may result from turbidity of the samples by the interference of milk fat and proteins. The raw milk dilutions verified in this study were intended to avoid bacterial endotoxin test interference conditions without sacrificing sensitivity for the detection of harmful levels of endotoxin. Therefore, the 200- or 400-fold dilution may be applied to overcome interference problems without exceeding the limit for endotoxin concentrations in raw milk obtained from healthy cows without mastitis. The results of the present study suggest that PTS to assess endotoxin activity in raw milk is efficient, simple and reproducible.

The present study indicated that coliform mastitis showed a significantly higher endotoxin activity in milk than that in mastitis caused by Gram-positive bacteria. Therefore, PTS might be useful diagnostic tools to differentiation of Gram-negative or positive mastitis. Previous studies reported that, in cases of acute coliform mastitis associated with Gram-negative bacteria, the concentrations of endotoxin were very high in both milk and plasma samples, whereas those in both milk and plasma samples obtained from similar cases due to Gram-positive bacteria were normal [11, 14, 25]. Therefore, the LAL test could be a valuable portable test for the veterinary practitioner, aiding the selection of the antibacterial drugs of choice for the initial treatment of clinical mastitis [1, 2, 25]. Traditional methods including kinetic chromogenic [10, 11, 22] and gel-clot [2, 19, 20] LAL tests for assessing endotoxin activity in raw and pasteurized milk have routinely been performed in the laboratory and are accepted by the FDA as meeting regulatory requirements. However, these methods

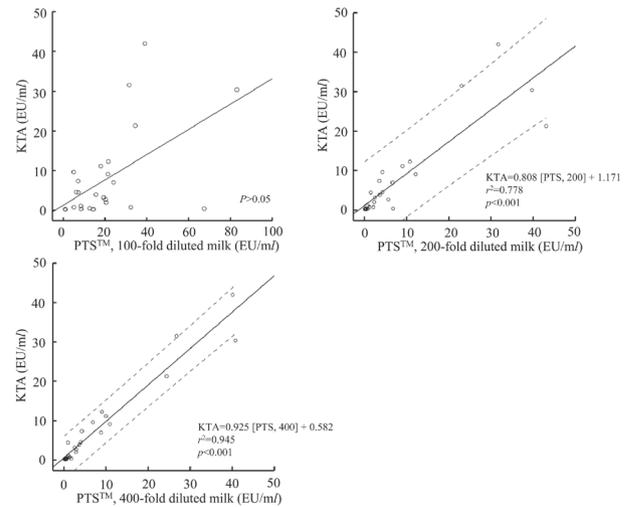


Fig. 3. Correlation of endotoxin activity in milk between the traditional LAL-base kinetic turbidimetric (KT) assay and portable test system (PTS). The endotoxin activities detected in 100-, 200- and 400-fold diluted milk samples using the PTS method positively correlated with those using the KT assay by Pearson's product-moment correlation coefficient. The central line represents the regression line; the dashed lines represent the 95% confidence interval.

take several hours to complete and become problematic, if time-sensitive treatments are needed. However, PTS delivers results within 15 min, whereas the KT assay takes several hours [9], representing 75% to 85% reductions in the time needed to assess endotoxin activity in raw milk obtained from cows. PTS is also advantageous when time-sensitive treatments are needed, because it is a hand-held portable machine that can be applied as a simple and easy portable test.

USP chapter 85 [24], which addresses photometric bacterial endotoxin test methods, allows for a wide recovery range for the positive control, between 50% and 200%, because small discrepancies in test conditions and cartridge flaws contribute to variable recovery values for the positive control [3, 8, 9, 15]. An out-of-specification percentage recovery for the positive control was previously associated with a calculated product endotoxin concentration that expressed any interference, such as inhibition and enhancement [8]. When any criteria, mainly percentage recovery of the positive control, were not within the acceptable range, the test was not considered to be valid [8]. In the present study, positive control recoveries in the traditional KT assay and PTS were rarely outside the acceptable range.

The distribution of the standard plate counts in raw milk obtained from cattle with mastitis was in the range of 10^4 to 5×10^5 /ml [23]. In more than 30% of milk samples from subclinical mastitis, bacteria fail to grow even after 48 hr of conventional culture. In addition, low concentration of bacteria in milk has been proposed as one reason for no growth in bacterial culturing. For example, the detection limit for bacterial growth in culture using 0.01 ml of milk is 10^2 /ml [23]. May *et al.* [19] suggested that the microplate method, which uses half the lysate reagent, was a good indicator

of the bacterial quality of raw milk, consistently detecting bacterial levels greater than 10^3 to 10^4 /ml [23]. In this study reported here, endotoxin activity was detected in 50% and 100% of the raw milk samples obtained from dairy cattle with low and high linear SCC scores using both KT assay. The PTS was possible to calculate the endotoxin activity of all milk samples from healthy Holstein and Jersey cattle with linear SCC scores of 3 or more. Therefore, the endotoxin activity detected by KT assay or the PTS is a useful tool to assess Gram-negative bacterial status of milk samples with no growth in conventional culturing.

In conclusion, photometric PTS represents a rapid, simple and accurate technique using the quantitative kinetic chromogenic LAL method for assessing endotoxin activity in raw milk and meets all the requirements for endotoxin activity including the percentage of CV and recovery of the positive control. In addition, the results of PTS using 200- and 400-fold diluted milk samples correlated with those obtained by the traditional KT assay. Therefore, the results of the present study confirmed that PTS is practical for simple and easy use to assess endotoxin activity in raw milk.

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