FULL PAPER *Wildlife Science*

Plasma endotoxin activity in kangaroos with oral necrobacillosis (lumpy jaw disease) using an automated handheld testing system

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ABSTRACT. The aim of the present study was to evaluate the reliability and effectiveness of directly determining endotoxin activity in plasma samples from kangaroos with lumpy jaw disease (LJD, n=15) and healthy controls (n=12). Prior to the present study, the ability of the commercially available automated handheld portable test system (PTS^{TM}) to detect endotoxin activity in kangaroo plasma was compared with that of the traditional LAL-kinetic turbidimetric (KT) assay. Plasma 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 assay. The data obtained using PTS^{TM} correlated with those using KT (r^2=0.963, P<0.001). These findings indicated that the PTS^{TM} is applicable as a simplified system to assess endotoxin activity in macropods. In the present study, we demonstrated the diagnostic value of plasma endotoxin activity in kangaroos with systemic inflammation caused by oral necrobacillosis and identified plasma endotoxin activity as a sensitive marker of systemic inflammation in kangaroos with LJD. Based on ROC curves, we proposed a diagnostic cut-off point for endotoxin activity of >0.22 EU/m*l* for the identification of LJD. Our results indicate that the assessment of plasma endotoxin activity is a promising diagnostic tool for determining the outcome of LJD in captive macropods.
KEY WORDS : captive, endotoxin test, lumpy jaw, macropod, portable test system

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Oral necrobacillosis, commonly referred to as "lumpy jaw disease (LJD)", is a term used to describe progressive pyogranulomatous osteomyelitis involving the mandible or maxilla of human beings, wild sheep [15] and captive macropods [3, 10, 14, 17, 21]. LJD is one of the most significant causes of illness and death in these macropods [17]. As shown in Fig. 1, LJD commences as periodontitis with invasion of the mucosa by saprophytic bacteria, such as *Fusobacterium necrophorum*, *Corynebacterium pyogenes* and *Dichelobacter nodosus*, and infection frequently extends into adjacent bones, resulting in osteomyelitis [17]. The primary cause of LJD in the kangaroo is *F. necrophorum* [2, 4], whereas the agents commonly associated with this condition in cattle and humans are members of the genus *Actinomyces* [22]. Numerous species of predominantly Gram-negative anaerobic bacteria have been isolated from lesions, with most members of the normal oral bacterial flora being present; however, the disease has only been reproduced experimentally by injecting *F. necrophorum* into the gingival mucosa [3].

F. necrophorum, a Gram-negative, non-spore-forming anaerobe, is a normal inhabitant of the alimentary tracts of animals and humans. The pathogenic mechanism of *F. necrophorum* is complex and has not yet been elucidated in detail. Several toxins or secreted products, including leukotoxin, endotoxin, hemolysin, hemagglutinin, proteases and adhesin, have been implicated as virulence factors [20]. Periapical lesions that begin as *F. necrophorum* infection in the dental pulp subsequently lead to inflammatory bone resorption [16]. 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 [9]. The accumulation of bacterial components, such as endotoxin in an infected area, may stimulate the release of pro-inflammatory cytokines from neutrophils and monocytes/macrophages. Endotoxin is the primary virulence factor of Gram-negative bacteria, is responsible for damage to animals and is released from bacteria at the time of cell death, thereby initiating an inflammatory response [5]. Endotoxin released from an infected root canal has been shown to trigger the synthesis of interleukin-1 alpha and TNF-alpha from macrophages [16]. These pro-inflammatory cytokines up-regulate the production of matrix metalloproteinase by macrophages in order to promote periapical bone resorption. 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.

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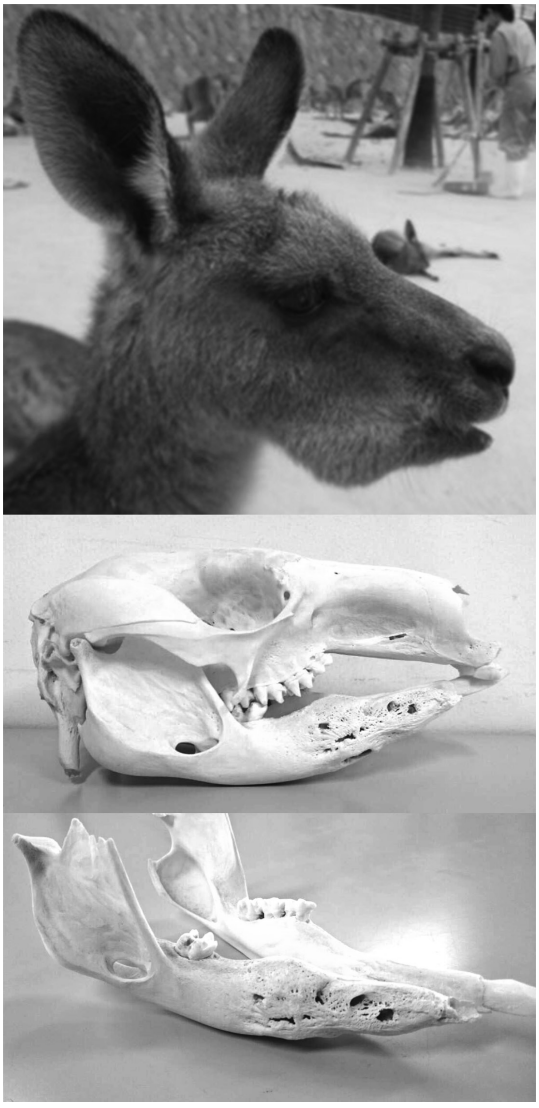

Fig.1. Lumpy jaw disease (LJD) commences as periodontitis with invasion of the mucosa by saprophytic bacteria, and infection frequently extends into adjacent bones, resulting in osteomyelitis.

Since Levin and Bang [19] 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 [7, 8]. However, these assays are very complex and, thus, inadequate for field use [23]. Charles River (Charleston, SC, U.S.A.) recently introduced an automated handheld testing system, named the Endosafe® portable test system (PTS^{TM}), to detect endotoxin. This automated miniaturized kinetic chromogenic LAL-based assay delivers results in 15 min [6, 12, 13]. Unlike the PTS^{TM}, the traditional toxinometer [24], LAL-kinetic turbidimetric KT assays [13] require 75% to 85% longer processing times. The PTS^{TM} is also advantageous when time-sensitive treatments are needed, because it is an automated handheld portable machine that is applied as a simple test.

To the best of our knowledge, comparative studies on the relationship between plasma endotoxin activity and LJD have not yet been performed in macropods. Therefore, the aim of the present study was to determine plasma endotoxin activity in kangaroos with LJD using a commercially available PTS^{TM}, such as an automated handled testing system. Receiver operating characteristic (ROC) curve was constructed in order to describe the performance of plasma endotoxin activity in kangaroos with LJD. Furthermore, endotoxin activities detected in plasma samples obtained from kangaroos with or without LJD by a commercially available PTS™ and traditional microplate LAL-based assay, which determined activities using a kinetic turbidimetric (KT) assay, were compared.

MATERIALS AND METHODS

All procedures were reviewed and approved by the Institutional Animal Care and Use Committee of the School of Veterinary Medicine, Rakuno Gakuen University (Japan). Fifteen Eastern grey kangaroos (*Macropus giganteus*) with LJD aged (mean ± SD) 3.5 ± 2.2 years old and with a body weight of 15.5 ± 6.3 kg were examined in this study. The definitive diagnosis of LJD was made based on clinical findings, such as facial swelling, weight loss, excessive salivation and flicking of the tongue [17]. Twelve Eastern grey kangaroos aged 3.6 ± 2.3 years old and with a body weight of 20.5 ± 11.7 kg were used as the control group. The health status of the control animals was determined on the basis of a physical examination and serum biochemical analysis by zoo veterinarians. All animals were kept at Hibiki Animal World (Fukuoka, Japan) and consumed concentrated pellets (ZC Pellets, Oriental Yeast Co., Ltd., Tokyo, Japan) for herbivores in accordance with the manufacturer's guidelines and had *ad libitum* access to hay (timothy and alfalfa), vegetables (including carrots, cabbage and potatoes), apples and water.

Four milliliters of whole blood was collected via jugular venipuncture into heparinized tubes for the endotoxin analysis and then centrifuged for 10 min at 3,000 g at room temperature within 1 hr of collection. Approximately 1.8 m*l* of plasma was harvested and stored in sampling tubes (CryoTubeTM vials, Nunc, Roskilde, Denmark) at –30°C for later analyses. Immediately prior to testing, plasma samples were diluted 20-fold in endotoxin-free water (Otsuka distilled water, Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan) and agitated in a vortex for 10 sec. Specimens were then heated for 10 min at 80°C in order to inactivate interfering substances, such as protease.

Endotoxin-free water was used as the blank in all tests. The 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 endotoxin units (EU) per vial, was used as the positive control. The LAL reagent for the LAL KT (En-

dosafe® KTA^2, Charles River) assay was reconstituted with Endotoxin-Specific Buffer Solution (Charles River) in order to eliminate any interference from β-glucans. The traditional LAL-based assay was 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 0.027 EU/m*l*.

All samples tested with the PTS system used 1–0.001 EU/m*l* sensitivity cartridges (Fig. 2). The PTS system, which comprised a spectrophotometer, reader and LAL reagent cartridge, was used in the present study. The reagent cartridges (Lot# 3183249) were prototypes that did not react with β-glucan, and were provided by 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 the calibration code was then determined. The calibration code (Cal# 419065608093) contained 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. 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. The 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 the product positive control were automatically calculated using software for research use involving an extrapolation function. In the present study, 20-fold diluted plasma, which was heated for 10 min at 80°C, was used to measure endotoxin activity. Results were automatically multiplied by the dilution factor entered into the system. The lower limit of quantitation for this assay was 0.050 EU/m*l*. A detailed description of PTS™ is provided elsewhere [6, 18].

Statistical analysis: A test result was considered valid based on the percentage spike recovery and percentage coefficient of variation (CV) parameters falling within the acceptance criteria (25%) established by the PTS system 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 [25]. The absolute value of the correlation coefficient of the standard curve generated using reference standard endotoxin was greater than or equal to 0.980 for the range of endotoxin concentrations established, according to the Bacterial Endotoxin Test in the US Pharmacopeia [25].

Sample endotoxin activities were statistically analyzed using the SPSS software program (ver 21. IBM Japan, Tokyo, Japan). The results of the PTS™ and KT assay were compared using the Friedman test, which is a non-parametric statistical test used to detect differences across multiple test attempts [11]. Pearson's product moment correlation coefficients were calculated to evaluate relationships between any two continuous variables. A linear regression model analysis was also performed in order to obtain the equation.

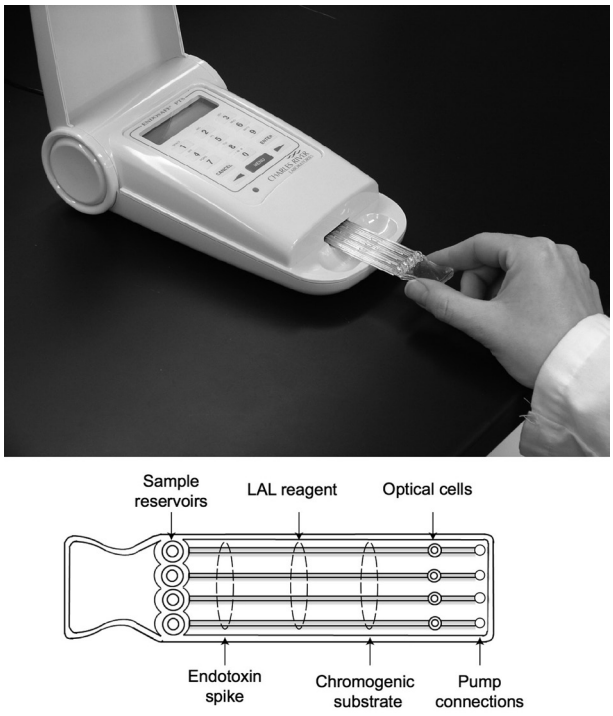

Fig.2. The limulus amebocyte lysate (LAL) spectrophotometer (upper) and reagent cartridge (bottom) for the Endosafe® PTS™ system.

The median values for endotoxin activity obtained from the PTS™ method were compared with the healthy controls, and the Mann-Whitney U test was employed for comparisons between groups. ROC curves were used to characterize the sensitivity and specificity of each parameter with respect to changes associated with LJD. The optimal cut-off point for a test was calculated by the Youden index [1]. The Youden index (J) is defined as the maximum vertical distance between the ROC curve and diagonal or chance line and is calculated as J=maximum [sensitivity+specificity−1]. The cut-off point on the ROC curve that corresponds to J was regarded as the optimal cut-off point [1]. The significance level was $P<0.05$.

RESULTS

The KT test effectively recovered endotoxin from plasma (82.4%, range 73.2% – 88.5%) over the range of concentrations tested. The linearity of the standard curve was also satisfactory for the KT assay (r^2=0.984) over the range of concentrations tested. CV, a parameter in the KT assay test for endotoxin activity, was 10.9% (range, 0.2% – 18.5%). The PTS™ effectively recovered endotoxin from plasma

(98.3%, range 50% – 180%) over the range of concentrations tested. CV, a parameter in the PTS™ for endotoxin activity, was 11.1% (range, 2.4% – 24.4%). Each of the assays (KT and the PTS™) effectively recovered endotoxin from the plasma of kangaroos with or without LJD.

The median ranges of endotoxin activity detected by each of the tests, KT and the PTS™, were 0.206 (min to max, 0.027–1.06) and 0.222 (min to max, 0.050–1.43) EU/m*l*, respectively. As shown in Fig. 3, the results obtained from the PTS™ correlated well with those from the KT assay (r^2=0.915, P<0.001). Based on the results of the Friedman Test, the ability of the PTS™ to recover endotoxin from plasma was not significantly different from that of the KT assay (P>0.05).

Figure 4 shows the relationships between plasma endotoxin activity in kangaroo with LJD. Plasma endotoxin activities were higher in kangaroos with LJD (0.326 EU/m*l*; min to max, 0.05 to 1.56 EU/m*l*) than in those without LJD (0.100 EU/m*l*; min to max, 0.05 to 0.74 EU/m*l*, P<0.05). The area under the ROC curve for plasma endotoxin activity was 0.793 (Fig. 5, P<0.05). The proposed diagnostic cut-off point for plasma endotoxin activity in order to identify kangaroos with LJD based on analyses of ROC curves was set at >0.22 EU/m*l*. The sensitivity and specificity of the proposed diagnostic cut-offs for plasma endotoxin activity were 80.0% and 80.0%, respectively.

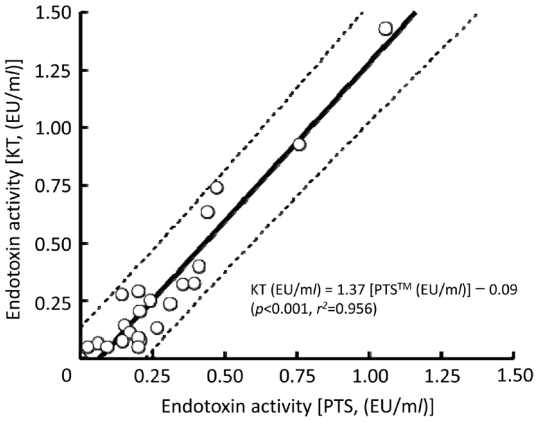

Fig. 3. Relationship between endotoxin activity in plasma between the portable test system (PTS™) and traditional limulus amebocyte lysate kinetic turbidimetric (KT) analysis. Endotoxin activities detected in plasma samples using the PTS™ positively correlated with those using the KT assay by Pearson's product-moment correlation coefficient.

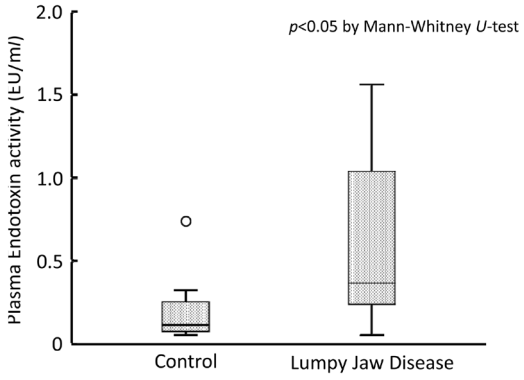

Fig. 4. Medians of plasma endotoxin activity in kangaroos with lumpy jaw disease (LJD). The horizontal line in each box represents the median value. The boxes represent the interquartile range (25 to 75 percentiles). Outliers are plotted separately as dots.

DISCUSSION

We investigated the relationship between oral necrobacillosis, commonly referred to as LJD, in captive kangaroos and plasma endotoxin activity using an automated handheld endotoxin testing system named the PTS™. In the present study, positive control recoveries in the traditional KT assay and PTS™ were rarely outside the acceptable range. The PTS™ effectively detects plasma endotoxin activity in the kangaroo and is practical for simple and easy use to assess endotoxin activity in plasma. It offers several advantages over the microplate kinetic LAL assays currently in use by diagnostic laboratories, namely, it is small and portable, requires only small quantities of specimens, and rapidly provides results [6, 18]. The activity of endotoxin in the plasma was higher in kangaroos with than in those without LJD. Furthermore, plasma endotoxin activity was significantly higher in kangaroos with LJD than in the healthy control group. Therefore, the proposed diagnostic cut-off for plasma endotoxin activity based on the ROC curve analysis to detect LJD was set at >0.22 EU/m*l*. The sensitivity and specificity of the proposed diagnostic cut-off for plasma endotoxin activity were 80.0% and 80.0%, respectively.

USP chapter 85 [25], 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 [6, 12, 13, 18]. 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 [12]. When any criteria, mainly percentage recovery of the positive control, were not within the acceptable range, the test was not considered to be valid [12]. In the present study, positive control recoveries in the traditional KT assay and PTS™ were rarely outside the acceptable range. The photometric PTS™ represents a rapid, simple and accurate technique using the quantitative kinetic chromogenic LAL method to assess plasma endotoxin activity in kangaroos and meets all the requirements for endotoxin activity including the percentage of CV and recovery of the positive control. Furthermore, the results of the PTS™, which used plasma diluted 1:20 in endotoxin-free water and heated to 80°C for 10 min, correlated with those obtained by the traditional KT assay. Therefore, the results of the present study confirmed that the PTS™ is practical for simple and easy use in order

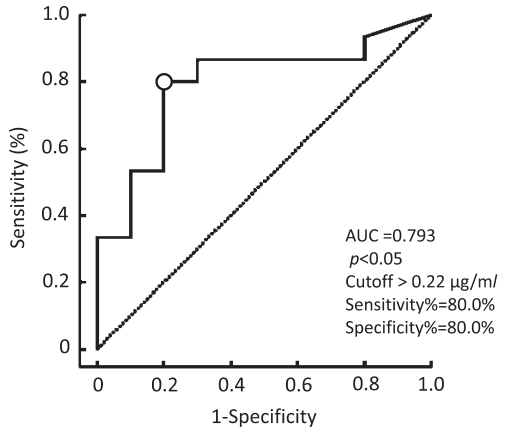

Fig. 5. ROC curve for plasma endotoxin activity in order to detect kangaroos with lumpy jaw disease (LJD). The optimal cut-off point for the test was calculated by the Youden index. Open Circle: Cut-off point.

to assess endotoxin activity in plasma.

Our results showed that average plasma endotoxin activity was higher in kangaroos with LJD than in controls. Based on ROC curves, we proposed a diagnostic cut-off point for endotoxin activity of >0.22 EU/m*l* for the identification of LJD. Endotoxin, which is released from bacteria including *F. necrophorum* at the time of cell death and initiates an inflammatory response [20], refers to the lipopolysaccharide protein of the Gram-negative bacterial wall and is the primary virulence factor of Gram-negative bacteria responsible for damage to the kangaroo. Endotoxin is known to be responsible for many of the pathophysiological signs observed during Gram-negative bacterial infections in mammalians, such as fever, leukopenia, complement activation, the activation of macrophages and changes in the plasma levels of metabolites, minerals, acute phase reactants and hormones.

In conclusion, we herein investigated the diagnostic value of plasma endotoxin activity in kangaroos with systemic inflammation caused by oral necrobacillosis and identified plasma endotoxin activity as a sensitive marker of systemic inflammation in kangaroos with LJD. Based on ROC curves, we proposed a diagnostic cut-off point for endotoxin activity of >0.22 EU/m*l* for the identification of LJD. Our results indicate that the assessment of plasma endotoxin activity is a promising diagnostic tool for the outcome of LJD in captive macropods. In addition, the photometric PTS^{TM} represents a rapid, simple and accurate technique, which uses a quantitative kinetic chromogenic LAL method for the assessment of plasma endotoxin activity in kangaroos. Therefore, the results of the present study confirmed that the PTS^{TM} is appropriate for assessing endotoxin activity in plasma.

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REFERENCES

1. Akobeng, A. K. 2007. Understanding diagnostic tests 3: Receiver operating characteristic curves. *Acta Paediatr.* **96**: 644–647. [Medline] [CrossRef]
2. Barber, D., Campbell, J., Davey, J., Luke, T., Agren, E. and Beveridge, I. 2008. Unilateral failure of development of mandibular premolars and molars in an Eastern Grey kangaroo (Macropus giganteus) and its effects on molar progression. *Aust. Vet. J.* **86**: 64–66. [Medline] [CrossRef]
3. Borland, D., Coulson, G. and Beveridge, I. 2012. Oral necrobacillosis ('Lumpy Jaw') in a free-ranging population of eastern grey kangaroos (Macropus giganteus) in Victoria. *Aust. Mammal.* **34**: 29–35. [CrossRef]
4. Brookins, M. D., Rajeev, S., Thornhill, T. D., Kreinheder, K. and Miller, D. L. 2008. Mandibular and maxillary osteomyelitis and myositis in a captive herd of red kangaroos (Macropus rufus). *J. Vet. Diagn. Invest.* **20**: 846–849. [Medline] [CrossRef]
5. Burvenich, C., Bannerman, D. D., Lippolis, J. D., Peelman, L., Nonnecke, B. J., Kehrli, M. E. Jr. and Paape, M. J. 2007. Cumulative physiological events influence the inflammatory response of the bovine udder to Escherichia coli infections during the transition period. *J. Dairy Sci.* **90** Suppl 1: E39–E54. [Medline] [CrossRef]
6. 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]
7. 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]
8. Elin, R. J. and Hosseini, J. 1985. Clinical utility of the Limulus amebocyte lysate (LAL) test. *Prog. Clin. Biol. Res.* **189**: 307–327. [Medline]
9. Erskine, R. J., Tyler, J. W., Riddell, M. G. Jr. and Wilson, R. C. 1991. Theory, use, and realities of efficacy and food safety of antimicrobial treatment of acute coliform mastitis. *J. Am. Vet. Med. Assoc.* **198**: 980–984. [Medline]
10. Finnie, E. P. 1976. Husbandry and diseases of orphaned pouch-young marsupials. *Aust. Vet. Practitioner* **6**: 19–28.
11. Friedman, M. 1937. The use of ranks to avoid the assumption of normality implicit in the analysis of variance. *J. Am. Stat. Assoc.* **32**: 675–701. [CrossRef]
12. 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]
13. 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]
14. Hartley, M. P. and Sanderson, S. 2003. Use of antibiotic impregnated polymethylmethacrylate beads for the treatment of chronic mandibular osteomyelitis in a Bennett's wallaby (Macropus rufogriseus rufogriseus). *Aust. Vet. J.* **81**: 742–744. [Medline] [CrossRef]
15. Hoefs, M. and Bunch, T. D. 2001. Lumpy jaw in wild sheep and

its evolutionary implications. *J. Wildl. Dis.* **37**: 39–48. [Medline] [CrossRef]
16. Hong, C. Y., Lin, S. K., Kok, S. H., Cheng, S. J., Lee, M. S., Wang, T. M., Chen, C. S., Lin, L. D. and Wang, J. S. 2004. The role of lipopolysaccharide in infectious bone resorption of periapical lesion. *J. Oral Pathol. Med.* **33**: 162–169. [Medline] [CrossRef]
17. Jakson, S. 2003. Chapter 9. Macropods. pp. 245–295. *In*: Australian Mammals: Biology and Captive Management. CSIRO Publishing, Melbourne.
18. Jimenez, L., Rana, N., Travers, K., Tolomanoska, V. and Walker, K. 2010. Evaluation of the Endosafe(R) Portable Testing SystemTM for the Rapid Analysis of Biopharmaceutical Samples. *PDA J. Pharm. Sci. Technol.* **64**: 211–221. [Medline]
19. 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]
20. Nagaraja, T. G., Narayanan, S. K., Stewart, G. C. and Chengappa, M. M. 2005. Fusobacterium necrophorum infections in animals: pathogenesis and pathogenic mechanisms. *Anaerobe* **11**: 239–246. [Medline] [CrossRef]
21. Samuel, J. L. 1983. Jaw disease in macropod marsupials: bacterial flora isolated from lesions and from the mouths of affected animals. *Vet. Microbiol.* **8**: 373–387. [Medline] [CrossRef]
22. Soto, E., Arauz, M., Gallagher, C. A. and Illanes, O. 2014. Nocardia cyriacigeorgica as the causative agent of mandibular osteomyelitis (lumpy jaw) in a cat. *J. Vet. Diagn. Invest.* **26**: 580–584. [Medline] [CrossRef]
23. 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]
24. 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.
25. The United States Pharmacopeial. 2014. Chapter 85 Bacterial endotoxin test. pp. 92–96. *In*: The United States Pharmacopeia, 37th ed., The National Formulary, Convention, Rockville.