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NOTE

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Relationship between bronchoalveolar lavage fluid and plasma endotoxin activity in calves with bronchopneumonia

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ABSTRACT. The aim of this study was to investigate the relationship between the endotoxin activity in plasma and that in bronchoalveolar lavage fluid (BALF) in bronchopneumonia. Thirty-three calves were included in this study (17 healthy calves and 16 calves with respiratory disease). In the calves with bronchopneumonia, the median endotoxin activity in plasma (0.437 EU/ml, $P<0.001$) and BALF (29.45 EU/ml, $P<0.001$) was significantly higher than in the control calves. Plasma endotoxin activity was significantly and positively correlated with that in BALF ($r^2=0.900$, $P<0.001$). Based on the receiver operating characteristics curves, we propose a diagnostic cutoff point for plasma endotoxin activity (0.104 EU/ml, AUC=0.914, $P<0.001$, Se 81.3% and Sp 82.4%) for identification of bronchopneumonia in calves which could die within a week.

KEY WORDS: bronchopneumonia, bronchoalveolar lavage fluid, calf, endotoxin, *Mycoplasma bovis*

The incidence and severity of bovine respiratory diseases complex (BRDC) have been increasing globally, and BRDC is presently considered one of the most important diseases affecting the health of young calves and economics. Bovine *Mycoplasmas* are often detected in pneumonic lungs in combination with other bacteria such as *Pasteurella multocida* and *Mannheimia haemolytica* [4]. Chronic infections with *Mycoplasma bovis* are often associated with lymphocytic "cuffing" pneumonia with marked hyperplasia of peribronchial lymphoid tissue that causes stenosis of the airway lumen, and compression and collapse of the adjacent pulmonary parenchyma [16].

Endotoxin or lipopolysaccharide (LPS), which is known as a component of Gram-negative bacterial cell wall, stimulates the release of pro-inflammatory cytokines from neutrophils and monocytes/macrophages in an infected lung tissue region where bacterial components have accumulated [19]. It is likely that elevated levels of circulating endotoxin and cytokines are associated with poor outcome [9]. The gastrointestinal tract has traditionally been recognized to be the source of systemic endotoxin appearing in the circulation [11]. However, there is a report that the possibility that pulmonary-to-systemic endotoxin translocation could be occurred [14]. We hypothesized that *M. bovis* infects the bronchial region and then breaks the epithelial barrier, letting LPS into the circulation. Also we hypothesized that elevated plasma endotoxin activity correlate with severity of bronchopneumonia.

To the best of our knowledge, comparative studies on the relationship between endotoxin activity in plasma and bronchoalveolar lavage fluid (BALF), and between endotoxin activity and bronchopneumonia have not yet been performed in calves. Therefore, the aim of the present study was to evaluate plasma and/or BALF endotoxin activity in calves with bronchopneumonia. The receiver operating characteristic (ROC) curves were constructed in order to assess the plasma and BALF endotoxin measurement in calves with bronchopneumonia.

All procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals of the School of Veterinary Medicine at Rakuno Gakuen University and the National Research Council (Approved #: VH16C1) [15]. Thirty Holstein (10 female and 20 male) and three Jersey calves (all male) that aged 48.6 ± 33.4 days old were enrolled in this study. The health status of each calf was established based on physical examination, serum biochemical analysis, and thorax radiological examination. Sixteen calves were admitted to the Rakuno Gakuen University Veterinary Teaching Hospital exhibiting clinical signs such as coughing, nasal discharge, fever, and pulmonary adventitious breath sounds. All calves with bronchopneumonia enrolled in this study were classified as severe, as there were shadows in more than 30% of the lung tissue on thorax radiography. These

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calves were culled or died within the first week after hospitalization. As a control, seventeen calves with no abnormal clinical signs and that were negative in *Mycoplasma* and bacterial culture test were also examined. They were purchased at livestock markets in the Ishikari region for educational purpose and were kept at the School of Veterinary Medicine, Rakuno Gakuen University.

Single blood samples were collected via jugular venipuncture into heparinized tubes for 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 (Cryo-TubeTM vials, Nunc, Roskilde, Denmark) at −30°C for later analyses.

The BALF samples were obtained during bronchoscopic examination using a standard protocol described previously [3, 18]. Briefly, bronchoscopy was performed using a flexible video bronchoscope (Olympus VQ Type 6092A, Olympus Co., Tokyo, Japan) under sedation with 0.05 mg/ kg of xylazine solution. The tip of the bronchoscope was wedged into position in the tracheal bronchus. Two hundred milliliters of isotonic, sterile saline solution warmed to 37°C was instilled in 50-m*l* aliquots with a disposable plastic syringe and immediately re-aspirated. The first aliquot was discarded [18]. With this procedure, a recovery rate of at least 60% was required. To ensure endotoxin activity in endoscope were below lower limits of quantification, the bronchoscope was washed 5 times according to our hospital protocol between sampling next calf. The bronchoscope was connected an aspirator and aspirated 2 *l* tap-water, 1 *l* antiseptic solution and 0.5 *l* sterile saline. For antiseptic solution, 5 m*l* of 10 w/v% benzalkonium chloride (Osuban S, Japan Pharmacopoeia) was diluted 200-fold in 1 *l* sterile saline. By repeating this washing more than three times, preliminary study was found that endotoxin activity in bronchoscope falls below the detection of limits.

Sub-samples were cultivated and investigated by polymerase chain reaction (PCR) tests targeting *M. bovis* based on 16S rRNA genes [8]. Sub-samples were cultured in non-selective medium at 35–37°C in aerobic condition for 17–20 hr at commercial laboratory (Daiichi Kishimoto Clinical Laboratory, Sapporo, Japan). *M. bovis* cultured in modified PPLO broth (Kanto Chemical, Tokyo, Japan) at 37°C in 5% CO_2 for 3 days at Rakuno Gakuen University. Briefly, simplified PCR was performed in a total reaction volume of 20 μl containing 10 μl of 2 × AmpdirectPlus (Shimadzu Co., Kyoto, Japan), 0.50 U of Nova taq TM Hot Start DNA polymerase (Merck KGaA, Darmstadt, Germany), 5 *p*mol of a mycoplasma universal primer set (MycoAce; Nihon Dobutsu Tokusyu Shindan Ltd., Eniwa, Japan), and 5 μl of each sample. PCR was performed using an iCycler PCR System (Bio-Rad Laboratories, Hercules, CA, U.S.A.). Conditions for the simplified PCR were as follows: initial denaturation at 95°C for 10 min followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 45 sec, and extension at 72°C for 1 min. The PCR products were separated by electrophoresis on 1.5% (w/v) agarose gels, stained with ethidium bromide, and visualized with a UV trans-illuminator. The *M. bovis* strain (ATCC 25523) was used as a positive standard.

Endotoxin activity in plasma and BALF was measured by conventional limulus amebocyte lysate (LAL)-kinetic turbidimetric (KTA) and chromogenic (KCA) assays, respectively. Immediately prior to testing, plasma and BALF samples were diluted 20- and 100-fold in endotoxin-free water (Otsuka distilled water, Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan), respectively, and the dilutions were vortexed for 10 sec. The plasma dilutions were then heated for 10 min at 80°C in order to inactivate interfering substances, such as protease inhibitors [21]. BALF specimens were used for analysis without heating.

The endotoxin reference standard (CSE, CONTROL STD ENDOTOXIN, Charles River, Charlston, SC, U.S.A.), which contained 10,000 endotoxin units (EU)/vial, was used to establish standard curves. The LAL reagents for LAL KTA (Endosafe® KTA2, Charles River) and KCA (Endosafe® Endochrome-K kit, Charles River) assays were reconstituted with Endotoxin-Specific Buffer Solution (Charles River) to eliminate β-glucan. Both conventional LAL-based assays were performed on 96-well microplates (Endosafe® 96-well, flat bottom microplate M9001, Charles River). Endotoxin activity was measured using a microplate reader (Sunrise™, Tecan Group Ltd., Männedorf, Switzerland) and EndoScan-VTM endotoxin-measuring software (Charles River). The lower limits of detection for this assay in plasma and BALF were 0.042 and 0.140 EU/m*l* in plasma and BALF, respectively. A test result was considered valid when spike recovery and coefficient of variation (CV) met the accepted criteria; spike recovery: 50–200%, CV <25% [5–7, 10]. Plasma endotoxin activity below 0.042 EU/m*l* was statistically analyzed as 0.042 EU/m*l*.

Data were statistically analyzed using the SPSS software program (ver. 21, IBM Japan, Tokyo, Japan). Non-normally distributed data were expressed as the median and range. A difference between two groups was assessed with the Student's *t*-test in the case of normal distribution or Mann-Whitney *U* test in the case of non-normal distribution. Receiver operating characteristic (ROC) curves were used to characterize the sensitivity (Se) and specificity (Sp) of each parameter to severe bronchopneumonia-associated changes. The optimal cut-off point for a test was calculated by the Youden index [2]. Pearson's rank correlation test was also used to evaluate the correlation between endotoxin activity in BALF and plasma. *P*-values lower than 0.05 were considered significant.

As a result of bacterial culture, *P. multocida* and *M. haemolytica* were detected in 1 and 6 of the bronchopneumonia calves, respectively. *Mycoplasma bovis* was detected in the BALF of all calves with bronchopneumonia by a PCR method based on 16S rRNA genes [8] and by a culture-based isolation method using a modified PPLO broth, although the control with no abnormality was not amplified. The culture and PCR results indicated that control calves did not have active infections with *P. multocida, M. haemolytica* and *M. bovis* in this study.

The data were expressed as the median and range because the endotoxin activities of plasma and BALF were non-normally distributed. Differences in endotoxin activity between control and patient groups were analyzed using the Mann-Whitney *U* test setting the significance level at $P<0.05$. The endotoxin activity in plasma and the ROC curve for endotoxin activity in plasma were demonstrated to be useful for detecting severity of bronchopneumonia.

In healthy calves, the plasma endotoxin activity was below the limit of detection in 14 of 17 calves (82.4%), with a median plasma endotoxin activity of 0.042 EU/m*l* (min–max: 0.042–0.802 EU/m*l*). The calves with bronchopneumonia had a significantly higher median endotoxin activity in plasma (0.437 EU/m*l*, min-max: 0.048–2.419 EU/m*l*, $P<0.001$). Based on the ROC analysis,

the proposed optimal cut-off points for plasma endotoxin activity with regard to Se and Sp were 0.104 EU/m*l* (AUC=0.914, *P*<0.001, Se 81.3% and Sp 82.4%).

In the same manner, the endotoxin activity in BALF and the ROC curve for endotoxin activity in BALF were demonstrated to be useful for detecting the severity of bronchopneumonia. In healthy calves, the median endotoxin activity in BALF was 2.43 EU/m*l* (min-max: 0.10–36.33 EU/m*l*). In contrast, the calves with bronchopneumonia had a significantly higher median endotoxin activity in BALF (29.45 EU/m*l*, min- max: 0.50–156.46 EU/m*l*, *P*<0.001). Based on the ROC analysis, the proposed optimal cut-off points for BALF endotoxin activity with regard to Se and Sp were 4.39 EU/m*l* (AUC=0.875, *P*<0.001, Se 81.3% and Sp 88.2%). As described in Fig. 1, plasma endotoxin activity was significantly and positively correlated with that of BALF (r^2=0.900, *P*<0.01).

We investigated the relationship between bronchopneumonia, which is associated with *M. bovis,* and endotoxin activity in plasma and BALF. Calves with bronchopneumonia were found to have higher endotoxin activity in both plasma and BALF than in healthy calves. In addition, the proposed diagnostic cut-off points for endotoxin activity in plasma and BALF based on ROC curve analysis in detecting *Mycoplasma* bronchopneumonia were 0.104 EU/m*l* and 4.39 EU/m*l,* respectively. The clinical and pathological signs for bronchopneumonia caused by *M. bovis* are non-specific; therefore, laboratory diagnosis is necessary to identify this disease. In this study, PCR based on 16S rRNA genes was able to amplify *M. bovis* DNA [8, 13] and was used to confirm *Mycoplasma* bronchopneumonia using BALF samples.

Previous studies reported that *M. bovis* was the common bacterial pathogen of BRDC [17], and *M. bovis* was detected in all calves with severe bronchopneumonia in this study. *M. bovis* likely plays an important role in co-infection. It is believed that *M. bovis* is a predisposing factor in the infectious process leading to invasion by other bacterial pathogens, possibly by compromising host defenses [16]. *M. bovis* induces neutrophils and macrophages in the lumina of terminal airways or small bronchioles on epithelial cells and makes microabscesses. The bronchiolar epithelium is damaged by infiltrating neutrophils and macrophages [1]. Then due to co-infection with Gram-negative bacteria, such as *P. multcida* or *M. haemolytica*, the endotoxin level may increase in bronchoalveolar regions. Infection by *M. bovis* may develop into a severe suppurative bronchopneumonia or necrotizing pneumonia when associated with other organisms or, conversely, into a mild catarrhal broncho-interstitial pneumonia when associated with other microorganisms [17].

Although it is sensitivity and accuracy of a bioassay for detecting LPS has been regarded as problem in recent years, previous

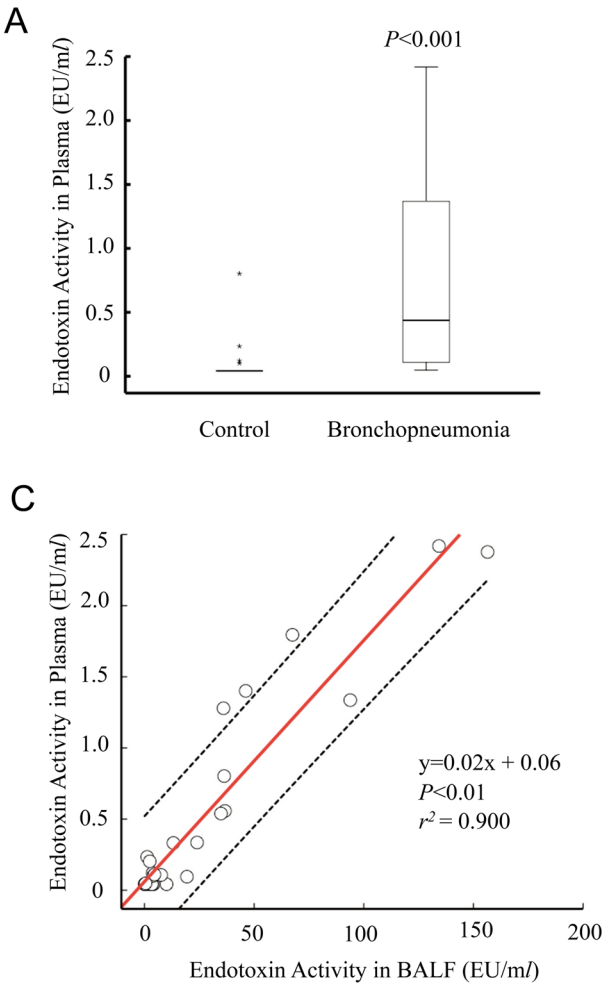

Fig. 1. Relationship between alveolar lavage fluid (BALF) and plasma endotoxin activity in bronchopneumonia calves. The upper left (A) and right graphs (B) show the endotoxin activity levels in the plasma and BALF of bronchopneumonia calves compared with those of healthy calves, respectively. The horizontal line in each box represents the median value. The boxes represent the interquartile range (25 to 75%). Outliers are plotted separately as dots. The lower graph (C) shows the correlation of endotoxin activity in plasma and BALF of bronchopneumonia calves. The endotoxin activity in plasma was positively correlated with that in BALF according to Pearson's product-moment correlation coefficient.

studies in animal experiments have reported translocation of LPS [12]. Murphy *et al*. [14] suggested that it is possible that alveolar capillary stress failure occurred by adverse ventilatory strategy, allowing passage of endotoxin from the alveolus to the pulmonary circulation. Restated from previous paragraph, *Mycoplasma* bronchopneumonia induces severe airway inflammation accompanied by profound and persistent micro-vascular remodeling in tracheobronchial mucosa [20]. The mechanism of endotoxin translocation remains conjectural because we were unable to precisely determine the nature of the epithelial or microvascular disruption responsible for the passage of endotoxin in this study. However, present results that evaluated correlation of plasma and BALF endotoxin activities might support these findings. Our result shows that the calves with high endotoxin activity in BALF due to severe bronchopneumonia associated with *M. bovis* have high plasma endotoxin activity. The status of calves with bronchopneumonia associated with Mycoplasma or bacteria and systemic inflammation seems to be involved in increasing in plasma endotoxin activity. It is possible that plasma endotoxin activity may increase as bronchopneumonia becomes more severe. It was revealed that plasma endotoxin activity is an important prognosticator for BRDC in this study.

In conclusion, we investigated the diagnostic value of endotoxin activity in calves with systemic inflammation caused by bronchopneumonia, and identified plasma endotoxin activity as a sensitive marker of systemic inflammation in calves with bronchopneumonia. Based on the receiver operating characteristics curves, we propose a diagnostic cutoff point for plasma endotoxin activity for identification of severe bronchopneumonia that could be culled or died within a week. Our results demonstrate that measuring endotoxin activity in plasma might help with diagnosis and even predict the prognosis of a calf to BRDC.

CONFLICT OF INTEREST. The authors declare no conflicts of interest associated with this manuscript.

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