for genomic DNA quantification with PicoGreen fluorescent dye (Invitrogen, Carlsbad, CA, USA). A prepared spike mRNA cocktail solution containing known quantity of five mRNAs of bacillus subtilis was added to the tissue lysate in proportion to the DNA quantity. Total RNA was purified from the lysate using the RNeasy kit (Qiagen). One microgram of total RNA was reverse-transcribed with SuperScript II (Invitrogen). Quantitative real time PCR was performed with an ABI PRISM 7900 HT sequence detection system (Applied Biosystems) using SYBR Green PCR Master Mix (Applied Biosystems), with initial denaturation at 95°C for 10 min followed by 40 cycles of 30 sec at 95°C and 30 sec at 60°C and 30 sec at 72°C, and Ct values were obtained. Primers for Cyp3a11 were Cyp3a11 FW and Cyp3a11 RV. Primers for Ces6 were Ces6 FW and Ces6 RV. Primers for mouse SXR selective quantification were mouse SXR FW and mouse SXR RV. Primers for hSXRki selective quantification were human SXR FW and human SXR RV. Primers for both mouse SXR and hSXRki quantification were mouse-human SXR FW and mouse-human SXR RV that amplify the DBD region of the chimera.

In Situ Hybridization analysis

Digoxigein-labeled cRNA probe for Cyp3a11 was synthesized according to Suzuki *et al.* (2005) by RT-PCR using mouse liver cDNA as a template. The primers used were as follows: forward 5'-GATTGGTTTTGATGCCTGGT-3' and reverse 5'-CAAGAGCTCACATTTTTCATCA-3'. The amplified product was sequence confirmed and ligated with Block-iT T7-TOPO (Invitrogen) Linker, which contains the T7 promoter site. A secondary PCR was performed to generate the sense and antisense DNA templates. For antisense template, Block-iT T7 Primer and Cyp3a11 forward primer (or reverse primer for generation of sense DNA template), the same primer as for the first PCR amplification, were used. With these DNA templates, both sense and antisense digoxigenin-labeled riboprobes were synthesized using a DIG RNA labeling kit (Roche Diagnostics, Germany) according to the manufacturer's protocol.

ISH on paraffin sections was carried out according to Suzuki *et al.* with a modification; permeabilization condition 98°C for 15 min in HistoVT One (Nacalai tesque, Japan).

Animals experiments

Male hSXRki and WT mice were maintained under a 12 hr light/12 hr dark cycle with water and chow (CRF-1, Oriental Yeast Co. Ltd., Tokyo, Japan) provided *ad libitum*. The animal studies were conducted in accordance with the Guidance for Animal Studies of the National Institute of Health Sciences under Institutional approval. The expression level of the hSXRki and WT SXR mRNA of ten organs (brain, thymus, heart, lung, liver, stomach, spleen, kidney, small intestine and testis) were analyzed on 15 weeks old male mice (n = 2) by the Percellome quantitative RT-PCR.

For the demonstration of selective gene induction by RIF and PCN in hSXRki and WT male mice on 13 weeks

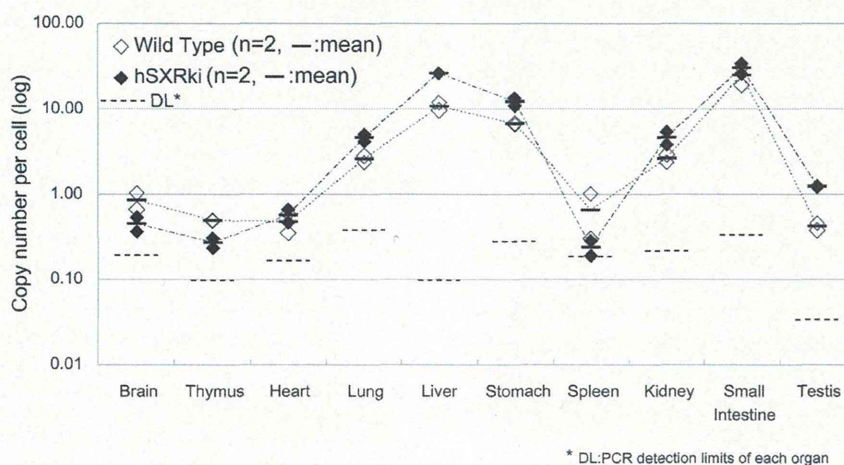

Fig. 2. Conservation of tissue expression patterns of hSXRki mRNA in the knock-in mouse. Percellome quantitative RT-PCR analysis was performed to measure the absolute expression levels of WT SXR mRNA and hSXRki mRNA in ten organs of WT and hSXRki mice. The expression levels of hSXRki mRNA among organs were comparable to WT.

old, three mice per group were singly dosed orally with vehicle (corn oil+0.1% DMSO), 10, 30, or 100 mg/kg of RIF, or 20, 70, or 200 mg/kg PCN (approximately equivalent in molar dose). Eight hours later, mice were sacrificed by exsanguination under ether anesthesia and the liver and the small intestine mucosa were sampled. Liver samples in small pieces were stored in RNA later (Applied Biosystems, Foster City, CA, USA) for further analysis. The small intestine under ice-cooled condition was longitudinally opened, gently rinsed with RNase-free saline and the epithelium was scraped with a glass slide and immersed in RNAlater. For *in situ* hybridization (ISH) of Cyp3a11 in the liver, 15 weeks old male hSXRki and WT mice were dosed orally with vehicle (corn oil), RIF (10 mg/kg), or PCN (40 mg/kg) daily for 3 days and liver sampled 24 hr later. All mice were sacrificed by exsanguination under ether anesthesia.

Statistical analysis

All values are expressed as the means ± S.D. and group differences analyzed by unpaired Student's *t* test or one-way ANOVA followed by Dunnett's post hoc comparison. Level of significance was set at $p < 0.05$.

RESULTS

Generation of hSXRki knock-In mice

Among 144 neomycin resistant TT2 ES clones, six PCR positive clones were further submitted to Southern blotting for the confirmation of homologous recombination. As shown in Fig. 1C, five clones were confirmed, and two (#4 and #25) were used to generate chimeric mice. The resulting mice were backcrossed to ICR strain to confirm germline transmission. One clone (#4) was crossed to a mouse constitutively expressing Cre recombinase to remove the neomycin resistance gene (Fig. 1D) and backcrossed to C57BL/6 CrSlc for at least 6 generations before further analysis.

Tissue distribution of hSXRki mRNA

Ten tissues, i.e., brain, thymus, heart, lung, liver, stomach, spleen, kidney, small intestine and testis from both hSXRki and WT mice were measured for hSXRki or WT SXR mRNA expression by the Percellome quantitative RT-PCR. As shown in Fig. 2, the levels of hSXRki mRNA are comparable to that of SXR in WT mouse and expressed in all tissues analyzed.

Humanized responses in hSXRki mouse

Humanized response of hSXRki was demonstrated by administration of the mouse-specific ligand PCN and the

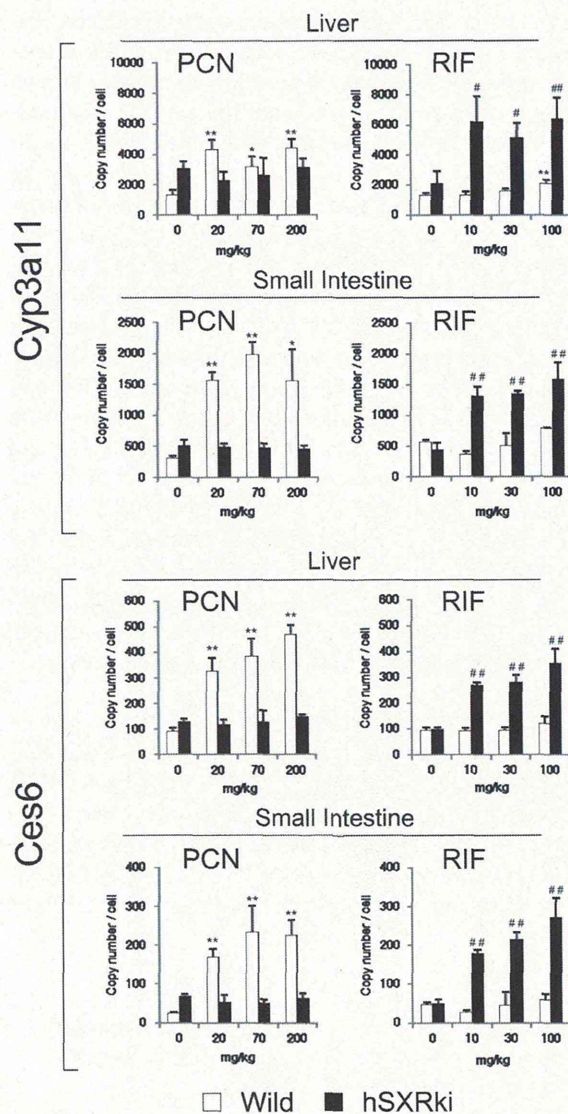

Fig. 3. Humanized response of hSXRki mice to RIF and PCN; Percellome quantitative RT-PCR. WT mice and hSXRki mice (n = 3 each) were singly dosed orally with vehicle (corn oil+0.1% DMSO), 20, 70, or 200 mg/kg PCN, or 10, 30, or 100 mg/kg of RIF (approximately equivalent in molar dose each other). Percellome quantitative RT-PCR data of Cyp3a11 and Ces6, both known as SXR target genes, in liver and small intestinal mucosa showed humanized responses in hSXRki. Bars = S.D., *; $p < 0.05$, **; $p < 0.01$ compared with vehicle group of WT, #; $p < 0.05$, ##; $p < 0.01$ compared with vehicle group of hSXRki. Analyzed by one-way ANOVA followed by Dunnett's post hoc comparison. Level of significance was set at $p < 0.05$.

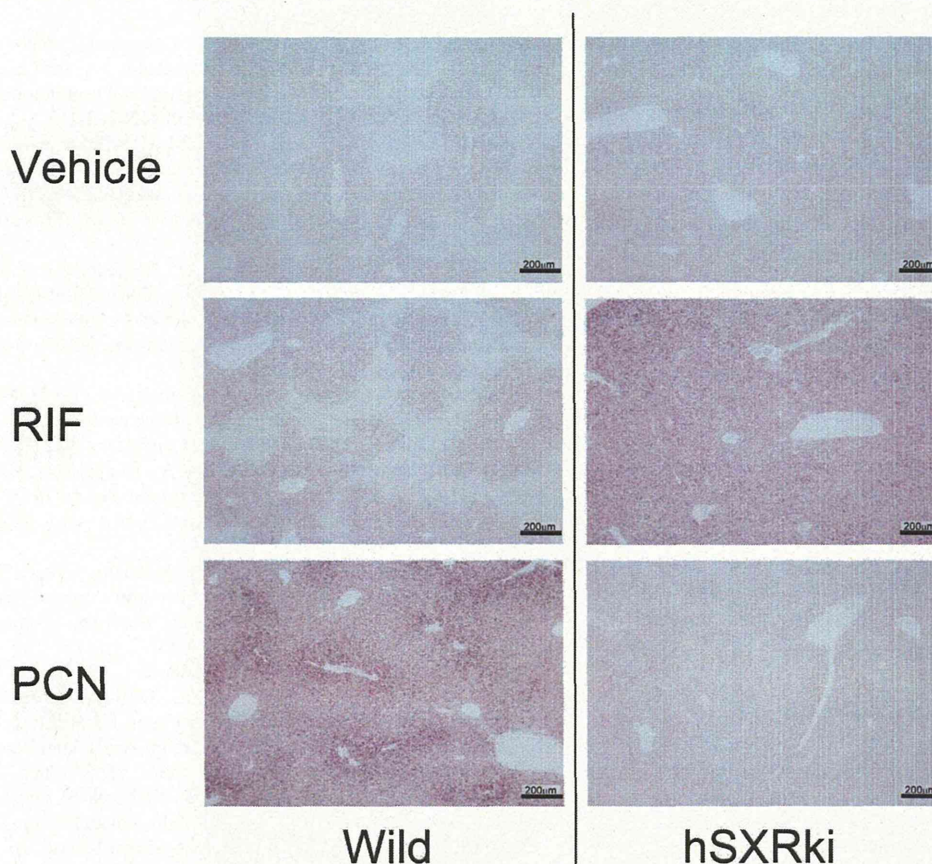

Fig. 4. Humanized response of hSXRki mice to RIF and PCN; *In situ* hybridization for Cyp3a11 mRNA in liver. A DIG-labeled cRNA probe for Cyp3a11 was hybridized and developed for purplish blue chromogenic reaction. Histologically, Cyp3a11 induction was localized around the central veins in both mice with species-specific ligands, respectively.

human-specific ligand RIF to the mice. Induction of the well-known SXR-regulated genes, Cyp3a11 and Ces6 was monitored by Percellome quantitative RT-PCR. As shown in Fig. 3, in the liver and small intestinal mucosa, RIF, but not PCN, induced Cyp3a11 and Ces6 in hSXRki mice (closed column), whereas PCN exclusively induced these genes in WT mice (open column). ISH of Cyp3a11 of the liver also showed humanized responses in hSXRki mice (Fig. 4).

DISCUSSION

We generated a new humanized mouse model in which the ligand binding domain (LBD) of human SXR was homologously knocked-into the murine SXR gene so that systemic response induced by human-selective SXR ligands can be monitored in mice. Firstly, we showed that mRNA from this chimeric gene was expressed at appropriate levels in the same tissues as the endogenous mouse SXR gene in WT mice. Then the humanized response of the mouse was confirmed by monitoring its response to the human-selective activator RIF, and the lack of response to the rodent-selective activator PCN.

There are relatively few reports about the regulation of SXR expression to date. Aouabdi *et al.* (2006) reported the presence of a PPAR alpha binding site 2.2 kb upstream of the transcription start site in human SXR. This site corresponded to the induction site with clofibrate in the rat and they further confirmed its importance using human liver cancer cell line (Huh7). Jung *et al.* (2006) reported the presence of four FXR binding sites in intron 2 of the mouse SXR gene that were required for FXR regulation of SXR expression. This intron 2 region is completely intact in our hSXRki mouse. Therefore, the regulation by FXR should be preserved in our mice.

Compared to the previously generated humanized Alb-SXR, SXR BAC, and hSXR genome mice, we contend that our hSXRki mouse has an advantage because the human-mouse chimeric gene is expressed in the same tissues and at similar levels to endogenous SXR in WT mice under control of the mouse promoter. This feature would make this model suitable not only for systemic toxicity but also toxicity at various stages of development of the embryo and fetus, maturation of infant, and of senescence, where the *cis* and *trans* regulations might be critical in its regulation (Sarsero *et al.*, 2004) (Konopka *et al.*, 2009). Thus, we believe that our system has a broader application range for toxicological studies.

ACKNOWLEDGMENTS

The authors thank Ms. Yuko Matsushima, Mr. Masaki Tsuji, Ms. Maki Otsuka, Mr. Yusuke Furukawa, Mr. Kouichi Morita, Ms. Maki Abe, and Ms. Shinobu Watanabe for technical support. This study was supported in part by the Health Sciences Research Grants H19-Toxico-Shitei-001 from the Ministry of Health, Labour and Welfare, Japan.

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Biol Trace Elem Res (2012) 145:166–171
DOI 10.1007/s12011-011-9180-0

Analysis of Trace and Major Elements in Bronchoalveolar Lavage Fluid of Mycoplasma Bronchopneumonia in Calves

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Received: 30 May 2011 / Accepted: 12 August 2011 / Published online: 26 August 2011
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Abstract The aim of this study was to evaluate the reliability and effectiveness of direct determination of trace and major element concentrations in bronchoalveolar lavage fluid samples from Holstein calves with *Mycoplasma* bronchopneumonia ($n=21$) and healthy controls ($n=20$). The samples were obtained during bronchoscopy using a standard examination method. A total of 18 elements (aluminum, bromine, calcium, chlorine, chromium, copper, iron, potassium, magnesium, manganese, molybdenum, nickel, phosphorous, sulfur, silicon, strontium, titanium, and zinc) were detected by particle-induced X-ray emission. The average bromine, iron, potassium, magnesium, and phosphorous concentrations were higher in calves with bronchopneumonia than in controls ($p<0.05$). They were found to have higher amounts of calcium and zinc, and a higher zinc–copper ratio than that in healthy calves ($p<0.001$). Based on the receiver operating characteristics curves, we propose a diagnostic cutoff point for zinc–copper ratio for identification of *Mycoplasma* pneumonia of 8.676. Our results indicate that assessment of the elemental composition of broncholaveolar lavage fluid is a promising diagnostic tool for *Mycoplasma* bronchopneumonia.

Keywords Bronchoalveolar lavage fluid · Calf · Trace elements · *Mycoplasma* bronchopneumonia · PIXE

Abbreviations

BALF	Bronchoalveolar lavage fluid
MMP	Matrix metalloproteinase
PCR	Polymerase chain reaction
PIMs	Pulmonary intravascular macrophages
PIXE	Particle-induced X-ray emission
ROC	Receiver operating characteristic

Introduction

Mycoplasma bovis is an important cause of calf pneumonia worldwide. Because immune prophylaxis and treatment with antibiotics are not very effective, control measures must include the introduction of strict hygiene standards, confinement of infected herds, and culling of clinically diseased animals [1]. 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 [2]. Pulmonary lesions in naturally infected calves comprise an exudative bronchopneumonia and extensive foci of coagulative necrosis surrounded by inflammatory cells [2]. Chronic infections are often associated with a lymphocytic "cuffing" pneumonia with marked hyperplasia of peribronchial lymphoid tissue that causes stenosis of the airway lumen and compression and collapse of adjacent pulmonary parenchyma [1].

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Pulmonary intravascular macrophages (PIMs) are present in ruminants and horses [3]. These species are highly sensitive to acute lung inflammation compared with non-PIM-containing species such as rats and humans. As the source of TNF-alpha, PIMs promote recruitment of inflammatory cells including IL-8-containing platelets to stimulate acute inflammation in lungs [3]. Lung injury in human and animals are associated with modifications of the extracellular matrix metabolism that lead to an accumulation of several elements and the development of organ fibrosis [3]. In inflamed lungs, matrix metalloproteinase (MMP)-9 is a key contributor to degradation of lung tissue and it potentiates activation of neutrophil chemotactic chemokines. MMP-9 is overexpressed in inflammatory pulmonary disorders of lung in human with adult respiratory distress syndrome [4]. Elevated levels of both serine proteinases and MMPs have been reported in bronchoalveolar lavage fluid (BALF) taken from humans with adult respiratory distress syndrome [4, 5], dogs with pulmonary eosinophilia [6] and horses with chronic obstructive pulmonary disease [7]. Lakritz et al. [8] indicated that gelatinases MMP-2 and MMP-9 were detected in BALF of healthy calves and that lipopolysaccharide-stimulated alveolar macrophages express MMP-9. In addition, an association between pneumonias attributable to *Pasteurella multocida* or *Mycoplasma bovirhinis* in calves and accumulation of MMP-9 in tracheobronchial lavage fluid has been reported [9]. MMPs are a family of zinc and calcium-dependent endopeptidases involved in remodeling and physiological homeostasis of extracellular matrix [10]. Therefore, it is important to investigate the relevance of bronchopneumonia and trace and major element status for food animal health care. However, no comparative studies are available on the trace and major elements status in BALF form calves with *Mycoplasma* bronchopneumonia.

Thus, the aim of this study was to investigate the concentrations and relationships between trace and major elements in BALF from calves with *Mycoplasma* bronchopneumonia. The receivers operating characteristic (ROC) curves were used to describe the performance of BALF in screening for *Mycoplasma* bronchopneumonia and to propose diagnostic cutoffs for calves.

Materials and Methods

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 [11].

Forty-one Holstein calves, 31 males and 10 females, aged 85.3±46.1 days old, were enrolled in this study. The health status of the animals was established on the basis of physical, biochemical, thoracic ultrasound, and radiological examinations. Twenty-one calves were isolated at the Rakuno Gakuen University Veterinary Teaching Hospital showing clinical signs such as coughing, nasal discharge, fever, and pulmonary wheezing sounds. As controls, 20 healthy calves with none of these clinical symptoms were kept at the School of Veterinary Medicine, Rakuno Gakuen University.

The BALF samples were obtained during bronchoscopic examination using a standard protocol described elsewhere [12–14]. 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 2% xylazine solution. The tip of the bronchoscope was wedged into a position in a tracheal bronchus. Two hundred milliliters isotonic, sterile saline solution warmed to 37°C was instilled in 50 mL portions with a disposable plastic syringe and immediately re-aspirated. The first aliquot was discarded [14]. In this procedure, a recovery rate of at least 60% is required.

Sub-samples were cultivated and investigated by polymerase chain reaction (PCR) tests targeting the *M. bovis*, based on 16S rRNA genes [15]. Briefly, simplified PCR was performed in a total reaction volume of 20 μL containing 10 μL of 2× AmpdirectPlus (Shimadzu Co., Kyoto, Japan), 0.5 U of Nova taq TM Hot Start DNA polymerase (Novagen, UK), 5 pmol of a mycoplasma universal primer set (MycoAce; Nihon Dobutsu Tokusyu Shindan Ltd., Hokkaido, Japan), and 5 μL of each samples. PCR was performed in an iCycler PCR System (Bio-Rad Laboratories, USA). 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 s, annealing at 60°C for 45 s, 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 positive standard.

The BALF was then centrifuged at 180×*g* for 10 min at 4°C to remove cell debris and the supernatant was stored at −80°C until assay. The mean concentrations of trace and major elements in BALF were detected by the particle-induced X-ray emission (PIXE) method. A detailed description of the experimental arrangement is shown elsewhere [13, 16]. Briefly, 100 μL BALF supernatants were placed on a subtlety Myler membrane and desiccated. The supernatants were directly irradiated with proton beams. A small (baby) cyclotron used for positron nuclear medicine at the Nishina Memorial Cyclotron Center (Iwate, Japan) provides a 2.9-MeV proton beam on a target after passing through a graphite beam collimator. A Si (Li) detector (0.0254 mm Be window) with 300 and 1,000-μm thick Mylar absorbers was used to select X-rays with energy higher than that of K–K alpha. For lower-energy X-rays, another Si (Li) detector (0.008 mm Be) was used without absorber.

The data are shown as means±standard deviation (SD). Statistical analyses were performed using a commercial software package from IBM SPSS Statistics, v.19 (IBM Co, Somers, NY, USA). The mean values for each dependent variable were compared to the control values using the unpaired Student's t test after analysis of ANOVA as F test. The ROC curves were used to characterize the sensitivity and specificity of a parameter to *Mycoplasma* bronchopneumonia. The optimal cutoff point for a test was calculated by the Youden index [17]. The Youden index (J) is defined as the maximum vertical distance between the ROC curve and the diagonal or chance line and is calculated as J=maximum [sensitivity+specificity−1]. The cutoff point on the ROC curves that corresponds to J is taken to be the optimal cutoff point [17]. The significance level was set at $p<0.05$.

Results

Figure 1 shows the detection of *M. bovis* by simplified PCR based on 16S rRNA genes. The PCR for *Mollicutes* detected *M. bovis* in only one sample (5%) from a healthy calves (controls, n=20) and in all samples (n=21, 100%) from calves with bronchopneumonia. Therefore, the statistical analysis enrolled 19 healthy controls that had not detected *M. bovis* and 21 bronchopneumonia calves.

The mean concentrations of trace and major elements in BALF from calves with *Mycoplasma* bronchopneumonia are summarized in Table 1. The PIXE method allowed detection of 18 elements: Al, Br, Ca, Cl, Cr, Cu, Fe, K, Mg, Mn, Mo, Ni, P, S, Si, Sr, Ti, and Zn. The average concentrations of Br, Fe, K, Mg, and P were higher in the calves with bronchopneumonia than those of the controls ($p<0.05$). Additionally, the calves with *Mycoplasma* bronchopneumonia were found to have larger amounts of Ca and Zn compared to those without respiratory disease ($p<0.01$ and $p<0.001$, respectively). There are no significant differences in the levels of the remaining 11 elements.

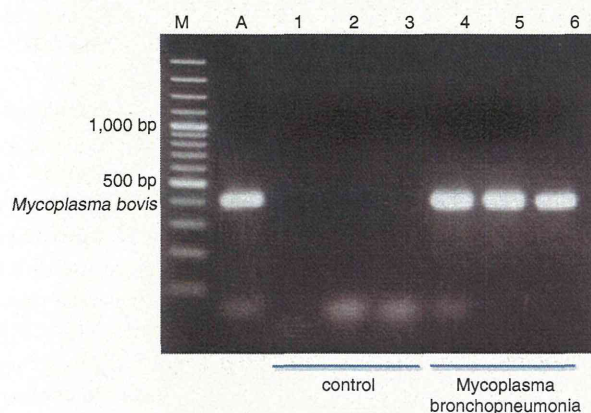

Fig. 1 Detection of *Mycoplasma bovis* in calves by polymerase chain reaction based on 16S rRNA genes. *M* marker, *A* positive standard (ATCC 25523), *lanes 1–3* control, and *lanes 4–6* bronchopneumonia calves

Table 1 Comparison of 18 trace and major elements status measured in broncoalveolar lavage fluid of the calves with or without *Mycoplasma* bronchopneumonia

(μg/mL)	Control (n=19)	*Mycoplasma* pnuemonia (n=21)	p value
Al	0.365[a]±0.238	0.942±0.924	NS[b]
Br	0.409±0.203	1.010±0.814	$p<0.05$
Ca	4.78±1.62	10.05±6.92	$p<0.01$
Cl	704.3±176.4	1,110.4±874.0	NS
Cr	0.028±0.016	0.038±0.021	NS
Cu	0.026±0.036	0.034±0.040	NS
Fe	0.099±0.070	0.201±0.190	$p<0.05$
K	34.4±12.5	65.3±32.9	$p<0.05$
Mg	1.13±0.75	3.11±2.39	$p<0.05$
Mn	0.012±0.008	0.014±0.016	NS
Mo	0.052±0.036	0.029±0.025	NS
Ni	0.009±0.005	0.007±0.005	NS
P	3.21±1.89	15.33±10.45	$p<0.05$
S	8.62±2.26	27.35±21.23	NS
Si	1.19±0.68	1.93±1.09	NS
Sr	0.017±0.015	0.016±0.016	NS
Ti	0.094±0.085	0.124±0.058	NS
Zn	0.074±0.048	0.366±0.166	$p<0.001$
Ca/P	2.01±1.63	1.01±0.66	NS
Zn/Cu	5.93±5.48	26.84±19.57	$p<0.001$

[a] micrograms per liter (μg/mL)

[b] Not significant

The areas under the ROC curves for Ca and Zn concentrations were 0.779 ($p<0.01$) and 0.990 ($p<0.001$), respectively (Fig. 2). The proposed diagnostic cutoff points for Ca and Zn concentrations in BALF for identifying *Mycoplasma* bronchopneumonia based on the analysis of the ROC curves were set at 5.894 and 0.180 μg/mL, respectively. Sensitivities and specificities of proposed diagnostic cutoffs for Ca concentration in BALF were 71.4% and 90.0%, respectively. In the same manner, sensitivities and specificities of proposed diagnostic cutoffs for Zn concentration in BALF were 95.2% and 95.0%, respectively.

Figure 3 shows a ROC curves for Zn/Cu ration in detecting *Mycoplasma* bronchopneumonia in calves. In the body, Ca and P, and Zn and Cu are regulated and restricted by each other, so variations in the Ca/P and the Zn/Cu ratios reflect the effects of these two microelements, respectively [13, 16]. However, in the calves with *Mycoplasma* bronchopneumonia, no characteristic difference of the Ca/P ratio was found in BALF. In contrast, the Zn/Cu ratios of

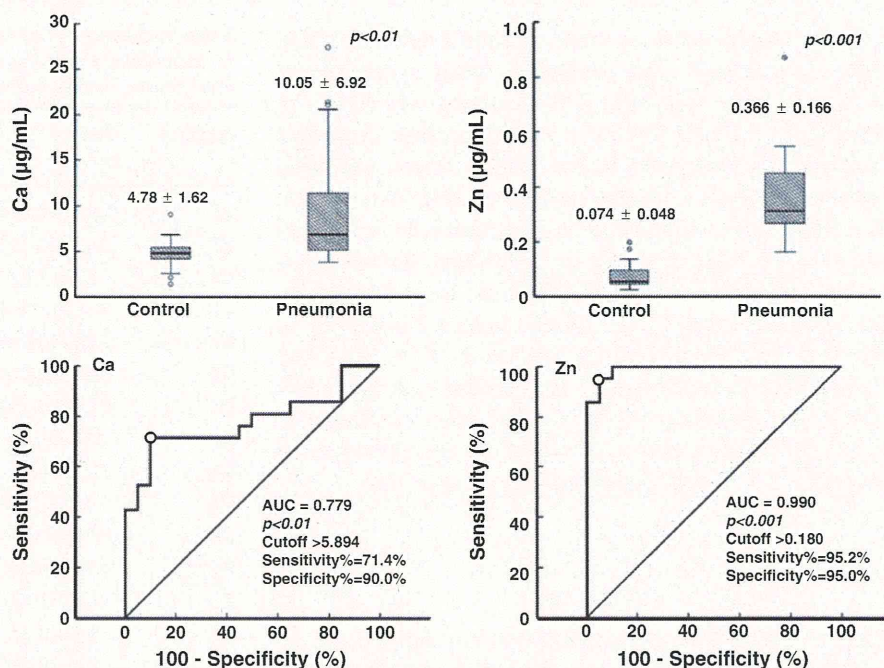

Fig. 2 Receiver operating characteristic (ROC) curves for Ca and Zn concentrations for detection of *Mycoplasma* bronchopneumonia in calves. The mean area under the ROC curve (AUC) is shown for each ROC curve. The optimal cutoff point for test was calculated by the Youden index. *Open circle* cutoff point

the BALF in the calves with *Mycoplasma* bronchopneumonia (26.84±19.57) were significantly higher than that of the healthy control (4.91±3.48, $p<0.001$). Proposed diagnostic cutoff points for Zn/Cu ratios in BALF for identifying *Mycoplasma* pneumonia based on the analysis of the ROC curves were set at 8.676. Sensitivities and specificities of proposed diagnostic cutoffs for Zn/Cu ratio in BALF were 93.8% and 82.4%, respectively.

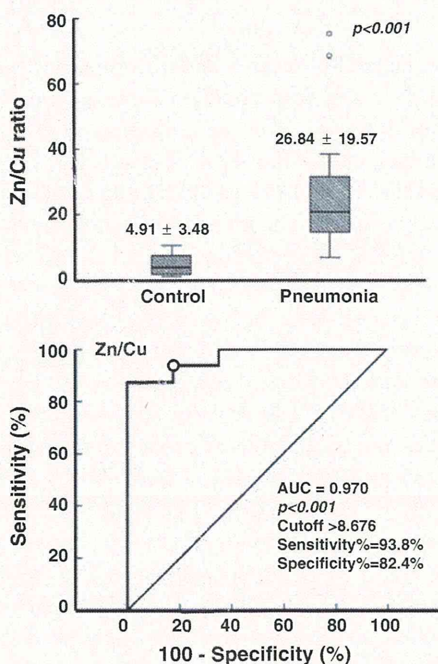

Fig. 3 Receiver operating characteristic (ROC) curves for the Zn/Cu ratio for detection of *Mycoplasma* bronchopneumonia in calves. See Fig. 2 for key

Discussion

We found how *Mycoplasma* bronchopneumonia in calves is associated with the concentrations of some trace and major elements in BALF. The calves with *Mycoplasma* bronchopneumonia were found to have larger concentrations of Br, Ca, Fe, K, Mg, P and Zn, and a high Zn/Cu ratio in BALF compared to those without bronchopneumonia. In addition, the proposed diagnostic cutoffs for Ca and Zn concentrations and Zn/Cu ratio in BALF based on ROC curves analysis in detecting a *Mycoplasma* bronchopneumonia were set at 5.894 and 0.180 μg/mL, and 8.676, respectively.

The clinical and pathological signs for bronchopneumonia caused by *M. bovis* are non-specific, so laboratory diagnosis is necessary for identification of the disease. To that effect, PCRs have been used to detect *M. bovis* directly in milk and nasal samples [18]. Several researches demonstrated that sampling by BALF was more useful for prediction of lower respiratory airway pathogens than nasal swabs although clearly not as convenient [19, 20]. Therefore, in this study, PCRs based on 16S rRNA genes amplified *M. bovis* DNA [15, 21] and were used to confirm *Mycoplasma* bronchopneumonia, using BALF samples.

The PIXE method used in the present study is a fast and reliable multi-element qualitative and quantitative analytical tool that is easily accomplished [22]. In this technique, a

detector analyzes characteristic X-rays emitted as a result of inner-shell ionization of target atoms. The method works well in small samples and is suitable for determining elements in a solid surface, especially for analyzing medium and higher atomic weight elements in a matrix consisting of light elements. With this technique, a sample of a few micrograms is sufficient to analyze concentrations in the parts-per-million range [22]. Because the method does not involve complicated sample preparation, the risk of contamination during the preparation of a sample for PIXE method is remarkably lower than that for other methods [13, 16].

Our results show that the average Br, Fe, K, Mg, and P concentrations in BALF from bronchopneumonia calves were higher than those in controls. A structurally and functionally distinct enzyme from neutrophil myeloperoxidase has the unique ability to use halides or pseudohalides (X^-) and H_2O_2 derived from the respiratory burst to generate cytotoxic hypohalous acids, especially hypobromous acid (HOBr) [23, 24]. The eosinophil peroxidase (EPO), such as EPO–H_2O_2–Br^- system, is also an effective cytotoxin for multiple targets such as multicellular worms or parasites, bacteria, viruses, and host cells [23]. Both HOBr and the EPO–H_2O_2–Br^- system are involved in many of the pathophysiological features of inflamed respiratory disease [24].

Iron is involved in many enzymatic activities. Significant changes in Fe concentration have been reported in BALF of patients with acute respiratory distress syndrome [25]. These changes have been interpreted as indicating that lungs require basal levels of extracellular redox-active Fe [26].

Potassium, magnesium, and phosphorus leak out to the extracellular fluid from tracheal epithelial cell injury because these elements are mostly contained in the intracellular fluid. Majeschak et al. [27] suggested that the Mg^{2+}/ATP-dependent 26S proteasome complex exists outside the cell and is released into the lung epithelial lining fluid after lung injury and contributes to the proteolysis of the bulk of protein in the alveolar space. BAL phospholipid content in lung injury rats correlated with the severity of alveolar-capillary leak [3]. Therefore, increased levels of Br, K, Fe, Mg, and P in BAL might be highly correlated with bronchial inflammation caused by *M. bovis*.

It was also found that BALF from calves with *Mycoplasma* bronchopneumonia were found to have larger concentrations of Ca and Zn and a high Zn/Cu ratio compared to those without respiratory disease. It is known that a calcium ionophore induces airway hyper-responsiveness to intravenous histamine and substance P possibly by reducing the nitric oxide levels in the airway tissues. This may be due to damaged airway epithelium and/or NO breakdown by activated inflammatory cells in the airway [28]. It is speculated that there is a correlation between Ca levels in BALF and the damage of the airway epithelium in calves with *Mycoplasma* bronchopneumonia. MMPs are a family of Zn and Ca-dependent endopeptidases involved in remodeling and physiological homeostasis of the extracellular matrix, shown to be important in the early stages of inflammation associated with respiratory disease in cattle [8, 29].

Associations between pneumonias attributable to *P. multocida* or *M. bovirhinis* in calves and accumulation of MMP-9 in tracheobronchial lavage fluid have been reported [9]. These molecules have high Zn-binding ability, containing three Zn-binding histidines and a glutamate that acts as a general base/acid during catalysis [30]. Furthermore, MMPs have three α-helices and a five-stranded β-sheet, as well as at least two Ca sites and a second Zn site with structural functions. Consequently, MMPs depend upon ionized Zn for activity and on Ca for stability. The changes of these elements are not specific with *Mycoplasma* bronchopneumonia because they result from reactions to inflammation of the bronchus and the tracheal branches. However, *Mycoplasma* bronchopneumonia induces severe airway inflammation accompanied by profound and persistent micro-vascular remodeling in tracheobronchial mucosa. The present results support these findings.

The pathogenesis of *Mycoplasma* bronchopneumonia is usually studied by genetic, proteomic, or molecular biology approaches. This study suggests that direct determination of trace and major elements concentrations in BALF could be a useful approach to the study of the pathogenesis of *Mycoplasma* bronchopneumonia. Infected calves were found to have higher amounts of Ca and Zn and a high Zn/Cu ratio in BALF compared to those without respiratory disease.

In conclusion, it is suggested that measuring the Br, Ca, Fe, K, Mg, P, and Zn concentrations and the Zn/Cu ratio status in BALF might help with diagnosis and even predict the susceptibility of a calf to *Mycoplasma* bronchoneumonia. Future studies need to focus in determining whether there is a correlation between zinc and calcium levels in BALF and the severity of bronchopneumonia.

Acknowledgments This study was supported by a grant-in-aid for Science Research from the Ministry of Education, Culture and Sciences of Japan (no.21580393) awarded to K. Suzuki and by a grant from Rakuno Gakuen University Research to K. Suzuki.

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