and 5 μL of each mycoplasma suspension (10^3, 10^4, and 10^5 cfu/mL), which were equal to 2.5×10^2, 2.5×10^3, and 2.5×10^4 cfu/mL. The DNA extraction and purification is unnecessary for this PCR method. PCR was performed in an iCycler PCR System (Biorad, 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 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 transilluminator.

To compare the performance of the simplified PCR with that of standard PCR, we performed a standard PCR assay as previously described [2]. Briefly, DNA from a Mycoplasma suspension was extracted using a DNA extraction kit (Qiagen, Germany) according to the manufacturer's instructions. Standard PCR was performed in a total reaction volume of 20 μL containing 10 × buffer (GE Healthcare, UK), 0.5 U Taq DNA polymerase (GE Healthcare, UK), 4 mM dNTPs, 5 pmol mycoplasma universal primer set, and 5 μL of DNA template which was equal to 2.5×10^2, 2.5×10^3, and 2.5×10^4 cfu/mL of *Mycoplasma*. Conditions of the PCR and electrophoresis were the same as those for the simplified PCR described above.

To evaluate the usefulness of the simplified PCR on commercial dairy farms, we compared the sensitivity and specificity of the simplified PCR to that of a culture method. A total of 1,683 quarter milk samples from lactating cows were randomly collected from 18 commercial dairy farms. A total of 159 milk samples were collected from quarters with clinical symptoms such as swelling, induration, and flare. We confirmed that 202 milk samples were collected from quarters that showed no clinical symptoms but had high somatic cell counts ($> 400 \times 10^3$ cfu/mL). One hundred μL of milk sample were used to inoculate 2.9 mL of mycoplasma broth (Kanto Kagaku, Japan) and incubated at 37°C for 72 h. One hundred μL of the broth culture were then plated on a *Mycoplasma* agar plate (Kanto Kagaku, Japan) and incubated in 5% CO_2 at 37°C for 14 to 28 days to produce typical *Mycoplasma* colonies [7]. Each broth culture was analyzed using the simplified and standard PCR as described above.

In this study, we compared the detection rates for seven major *Mycoplasma* spp. of the simplified and standard PCR assays. All ATCC strains were clearly detected by both methods (Fig. 1). Seven species of two mycoplasma strains isolated from animals with mycoplasma mastitis were also detected by both simplified and standard PCR (data not shown). Gene sequences of PCR-amplified products showed 99% homology with documented sequences in an established gene bank (intergenic spacer region). The minimum detection limit for Mycoplasma by simplified PCR was estimated to be 2.5×10^3 cfu/mL (Fig. 2). The results obtained from the simplified PCR agreed well with those from standard PCR using purified DNA from broth cultures. Our results showed that simplified PCR for detecting *Mycoplasma* spp. permits gene amplification without any DNA preparation and performs as well as standard PCR. In this study, we used Amdirect Plus for the simplified PCR to detect *Mycoplasma* spp. in broth cultures. Ampdirect Plus is a commercially available reagent used for preparing PCR samples without DNA extraction and purification [1]. We have confirmed that the use of Ampdirect Plus reduces labor and time for detecting *Mycoplasma* spp. in samples.

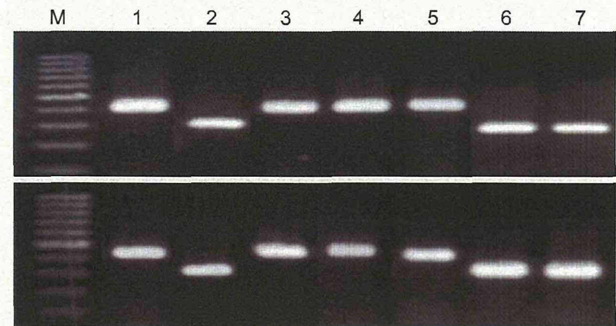

Fig. 1. Detection of major mycoplasma mastitis pathogens by simplified (upper) and standard (lower) PCR. M. marker, 1: *Mycoplasma (M.) bovis* (ATCC 25523), 2: *M. arginini* (ATCC 23838), 3: *M. bovigenitalium* (ATCC 19852), 4: *M. californicum* (ATCC 33461), 5: *M. bovirhinis* (ATCC 27748), 6: *M. alkalescens* (ATCC 29103), 7: *M. canadense* (ATCC 29418).

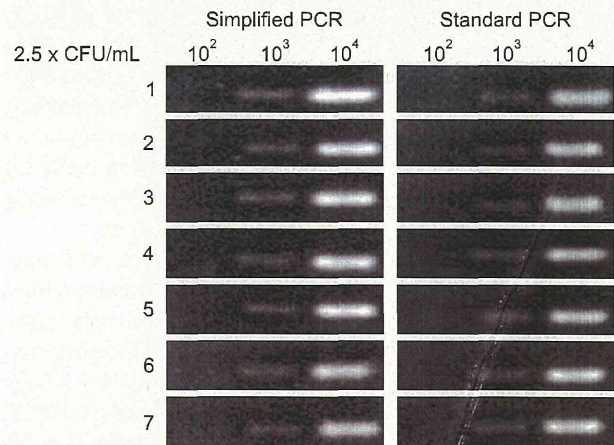

Fig. 2. Minimum limits of detection for major mycoplasma mastitis pathogens by simplified PCR and standard PCR. 1: *M. bovis* (ATCC 25523), 2: *M. arginini* (ATCC 23838), 3: *M. bovigenitalium* (ATCC 19852), 4: *M. californicum* (ATCC 33461), 5: *M. bovirhinis* (ATCC 27748), 6: *M. alkalescens* (ATCC 29103), 7: *M. canadense* (ATCC 29418).

Out of the 1,685 milk samples cultured in mycoplasma broth, simplified PCR detected Mycoplasma DNA in 152 samples that were also positive according to the culture

assay. The concentration of Mycoplasma in these broth cultures was more than 1×10^4 cfu/mL. Four samples were found to be negative by culture and positive by simplified PCR. We speculated that the amplification of DNA from non-viable Mycoplasma in the broth cultures caused the differences between the simplified PCR and culture method results. Two samples were found to be negative by the simplified PCR and positive by culture assay. We confirmed that the concentrations of Mycoplasma in these cultured broths were 2×10^2 and 3×10^2 cfu/mL, which are less than the minimum detection limit of the simplified PCR. Both milk samples were obtained from cows with no clinical symptoms and normal somatic cell counts. Our results suggested that the number of Mycoplasma in the broth cultures of a few milk samples was insufficient for simplified PCR. These samples were not further investigated. The sensitivity and specificity of the simplified PCR method were 98.7% and 99.7%, respectively, of those of the culture assay. Results from the simplified PCR assay completely concurred with those obtained by standard PCR. It has been reported that the sensitivity and specificity of standard PCR for detecting Mycoplasma are 96.2% and 99.1%, respectively, of those of the culture method [2]. In the present study, we succeeded in establishing a simplified PCR method that is able to provide the results identical to those obtained using conventional culture and standard PCR methods.

Standard PCR techniques are labor-intensive and time-consuming, making this method impractical for assaying a large number of milk samples from a commercial dairy farm. This method requires many steps that are quite lengthy; processing about 300 samples requires at least 4 ~ 5 h for DNA template preparation and 3 h for completing the PCR cycle. In contrast, 300 samples to be examined within 3 h using the simplified PCR, which does not require several of these laborious steps including Mycoplasma DNA template isolation and purification. Fast and easy screening for mycoplasma mastitis using a simplified PCR assay would enable the quick isolation of infected cows from herds. Our newly developed simplified PCR assay for detecting *Mycoplasma* spp. is a useful method that can be used to help control and prevent mycoplasma mastitis outbreaks on commercial dairy farms.

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Short Communications

Prevalence of *Mycoplasma* species in bulk tank milk in Japan

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Mycoplasma species are highly contagious pathogens and their ability to cause intramammary infection is a serious problem on dairy farms (Nicholas and Ayling 2003). Since the cure rate of clinical mastitis caused by *Mycoplasma* species is very low due to difficulties in antibiotic therapy, *Mycoplasma*-infected cows on farms must be culled in an emergency to prevent outbreaks of *Mycoplasma* mastitis (Nicholas and Ayling 2003). Bovine *Mycoplasma* mastitis was first reported in 1962 (Hale and others 1962). However, little is known about the prevalence of *Mycoplasma* mastitis on dairy farms in Japan. In this study, the prevalence of *Mycoplasma* species in bulk tank milk from dairy farms in Japan was investigated.

A total of 1241 commercial dairy farms (n=45 to 1125 cows/farm) were randomly selected for bulk tank milk screening. The samples were collected from April to September 2010. Each bulk tank contained milk from two days of production. Milk samples were aseptically collected into 50 ml tubes. One hundred microlitres of milk samples were inoculated into 3.0 ml of *Mycoplasma* broth (Kanto Kagaku; Tokyo) and then incubated at 37°C for 72 hours. One hundred microlitres of *Mycoplasma*-enriched culture broth was plated on a *Mycoplasma* agar plate (Kanto Kagaku; Tokyo) and incubated in 5 per cent CO_2 at 37°C for one month for the development of typical *Mycoplasma* colonies (Nicholas and Ayling 2003). PCR for the detection of *Mycoplasma* species in cultured broth was performed according to the method described by Higuchi and others (2011). Identification of major *Mycoplasma* species including *M bovis*, *M arginini*, *M bovigenitalium*, *M californicum*, *M bovirhinis*, *M alkalescens* and *M canadense* was performed by DNA sequence analysis.

Mycoplasma species were isolated from bulk tank milk obtained from 16 (1.29 per cent) of 1241 farms screened in this study. These results are similar to the results of a previous study carried out in the USA, which showed that the prevalence of *Mycoplasma*-positive samples from bulk tank milk ranged from 1.8 to 5.8 per cent (Kirk and others 1997). Seven species of *Mycoplasma*, *M bovis* (0.56 per cent, n=7), *M californicum* (0.24 per cent, n=3), *M canadense* (0.24 per cent, n=3), *M arginini* (0.16 per cent, n=2), *M bovigenitalium* (0.08 per cent, n=1), *M alkalescens* (0.08 per cent, n=1) and *M bovirhinis* (0.08 per cent, n=1), were isolated from the bulk tank milk samples collected in this study. Intramammary infection with *M bovis* is difficult to treat, and culling of infected cows and loss of milk production can lead to significant economic loss for a dairy farm (Nicholas and Ayling 2003). It is recommended that *Mycoplasma*-infected cows on farms be segregated from healthy animals as soon as they are identified to reduce the risk of spread in an outbreak (Bicknell and others 1983). Mastitis due to *M bovis* has been studied by epidemiological investigations of bulk tank milk in France (Arcangioli and others 2011), New Zealand (McDonald and others 2009), Australia (Jelinek and others 1993), Belgium (Passchyn and others 2011) and the USA (Jasper and others 1979), with the prevalence varying from 0 (New Zealand) to 4 per cent (California, USA). This short communication is the first to report on the prevalence of *M bovis* in bulk tank milk of commercial dairy farms in Japan.

TABLE 1 Prevalence of *Mycoplasma* species in bulk tank milk of 16 commercial dairy farms in Japan

	Herd size	Bulk tank milk			Cows		
Farm Number	(cows/herd)	Culture	PCR	*Mycoplasma* species	Number of infected cows	(%)	*Mycoplasma* species
1	50	+	+	*M bovigenitalium*	1	2.00	*M bovigenitalium*
2	59	+	+	*M arginini*	1	1.69	*M arginini*
3	60	+	+	*M californicum*	5	8.33	*M californicum*
4	64	+	+	*M californicum*	5	7.81	*M californicum*
5	69	+	+	*M bovis*	24	34.78	*M bovis*
6	98	+	+	*M alkalescens*	1	1.02	*M alkalescens*
7	102	+	+	*M canadense*	3	2.94	*M canadense*
8	105	+	+	*M bovirhinis*	2	1.90	*M bovirhinis*
9	112	+	+	*M canadense*	2	1.79	*M canadense*
10	120	+	+	*M bovis*	4	3.33	*M bovis*
11	162	+	+	*M bovis*	4	2.47	*M bovis*
12	166	+	+	*M bovis*	3	1.81	*M bovis*
13	180	+	+	*M bovis*	11	6.11	*M bovis*
14	258	+	+	*M californicum*	18	6.98	*M californicum*
15	358	+	+	*M bovis*	15	4.19	Only *M bovis*
		+	+	*M arginini*	5	1.40	Only *M arginini*
					3	0.84	*M bovis*+*M arginini*
16	420	+	+	*M bovis*	104	24.76	Only *M bovis*
		+	+	*M canadense*	15	3.57	Only *M canadense*
					10	2.38	*M bovis*+*M canadense*

It was confirmed that outbreaks of *Mycoplasma* mastitis on two dairy farms (farm 5, 34.78 per cent; farm 16, 24.76 and 2.38 per cent) were caused by *M bovis*. All of the infected cows showed severe clinical symptoms, including decreased milk production, and swelling and induration of the udder. Similar clinical cases were observed on other *M bovis*-infected farms (farms 10 to 13 and 15) and *M californicum*-infected farms (farms 3, 4 and 14). However, except for a slight increase in somatic cell counts in the milk samples (3.3-4.1x10^5/ml), clear clinical symptoms were not detected in the udders of cows infected with *M arginini*, *M alkalescens*, *M canadense*, *M bovigenitalium* and *M bovirhinis*. These results suggested that periodic inspection of bulk tank milk to detect *Mycoplasma* species, especially *M bovis* and *M californicum*, is

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important to prevent outbreaks of *Mycoplasma* mastitis on dairy farms. It has been reported that *M bovis* could be detected in bulk tank milk on a farm where only one out of 300 cows was excreting organisms (Bicknell and others 1983). In the present study, it was confirmed that 1.8 (farm 12; 3/166) to 6.1 per cent (farm 13; 11/180) of *M bovis*-infected cows and 6.9 (farm 14, 18/258) to 8.3 per cent (farm 3, 5/60) of *M californicum*-infected cows on dairy farms were sufficient to detect *Mycoplasma* mastitis by bulk tank milk screening.

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Prevalence of *Mycoplasma* species in bulk tank milk in Japan

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

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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

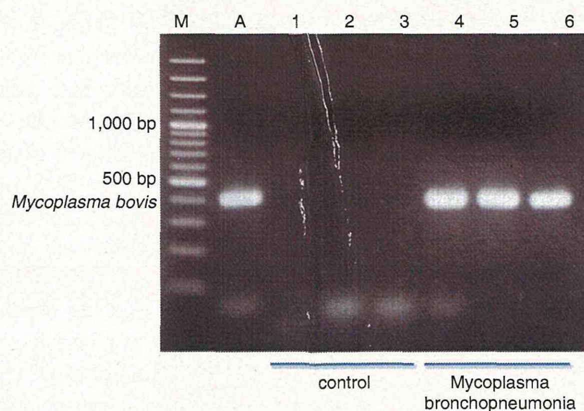

Fig. 1 Detection of *Mycoplasma bovis* in calves by polymerase chain reaction based on 16S rRNA genes. *M* marker, *A* positive standard (ATCC 25523), *lanes 1–3* control, and *lanes 4–6* bronchopneumonia calves

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

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.

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

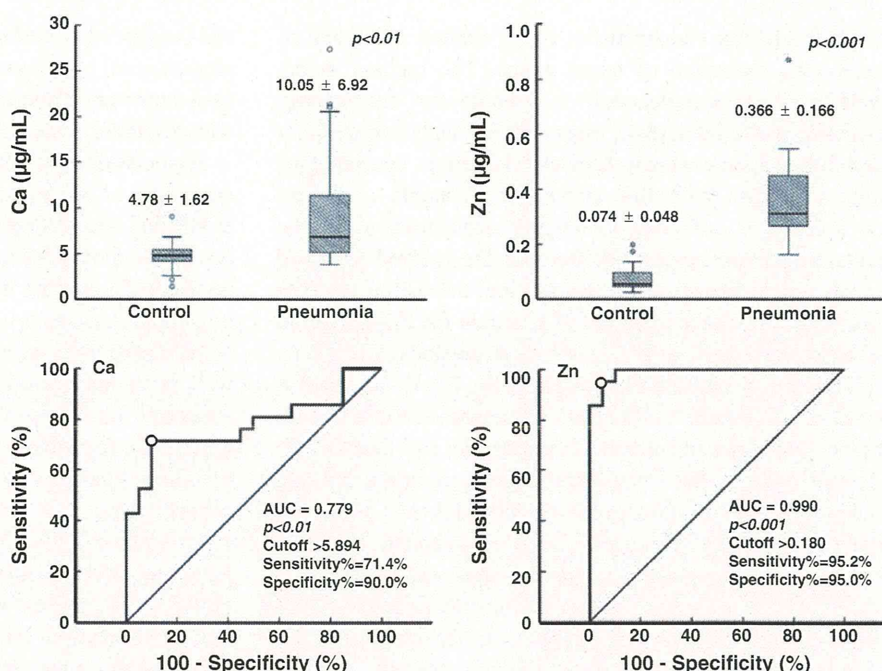

Fig. 2 Receiver operating characteristic (ROC) curves for Ca and Zn concentrations for detection of *Mycoplasma* bronchopneumonia in calves. The mean area under the ROC curve (AUC) is shown for each ROC curve. The optimal cutoff point for test was calculated by the Youden index. *Open circle* cutoff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

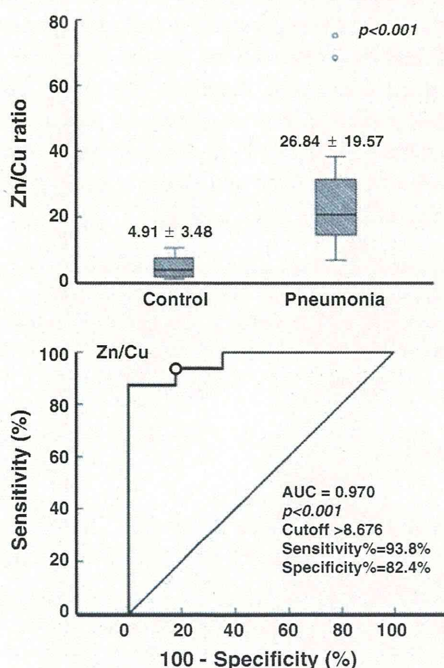

Fig. 3 Receiver operating characteristic (ROC) curves for the Zn/Cu ratio for detection of *Mycoplasma* bronchopneumonia in calves. See Fig. 2 for key

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.

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