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Profiles of serum amino acids to screen for catabolic and inflammation status in calves with *Mycoplasma* bronchopneumonia

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ABSTRACT. The aim of the present study was to investigate the relationships between serum amino acid profiles in normal and calves with *Mycoplasma* bronchopneumonia. Serum free amino acid concentrations in serum obtained from 34 calves with or without *Mycoplasma* bronchopneumonia were determined by high-performance liquid chromatography. The calves with *Mycoplasma* were characterized by significantly lower total amino acid and total essential amino acid concentrations and molar ratios of branched-chain amino acid (BCAA) to aromatic amino acid (BCAA/AAA) and BCAA to tyrosine (BTR), and by a significantly higher molar ratio of serine phosphorylation (SPR). The proposed diagnostic cutoffs for BCAA/AAA, BTR and SPR in serum based on ROC analysis for detection of catabolic states associated with *Mycoplasma* bronchopneumonia were set at <1.75, <2.86 and >0.85, respectively. Our results suggest that determining the profiles of amino acids, especially BTR and SPR, could provide useful diagnostic information in terms of predicting protein catabolism in *Mycoplasma* bronchopneumonia.

KEY WORDS: amino acid, branched-chain amino acid, bronchopneumonia, calf, *Mycoplasma*

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The frequency and severity of bovine respiratory disease (BRD) have increased globally, and respiratory disease is currently regarded as the principal health problem and the most economically important disease of young calves [7, 23, 27]. *Mannheimia haemolytica*, *Pasteurella multocida*, *Arcanobacterium pyogenes* and *Histophilus somni* are all frequently implicated in BRD [27]. Bovine *Mycoplasmas* are often isolated from pneumonic lungs in combination with other pathogens, such as *P. multocida* and/or *H somni* [4]. *Mycoplasma bovis* infection often progresses to severe necrosuppurative bronchopneumonia, fibrinonecrotizing pneumonia with a large number of organisms or mild catarrhal bronchointerstitial pneumonia when associated with low numbers of organisms [2]. Pulmonary lesions in naturally infected calves comprise an exudative bronchopneumonia and extensive foci of coagulation necrosis surrounded by neutrophils, monocytes and lymphocytes. In calves with *Mycoplasma* bronchopneumonia, the inflammatory process involves endothelial cells, intravascular macrophages, alveolar macrophages and alveolar epithelial cells.

Pro-inflammatory cytokines, such as interleukin (IL)-1, IL-6 and tumor necrosis factor (TNF), induce marked metabolic changes leading to hyperthermia, anorexia and muscle protein catabolism as well as increased protein synthesis by the liver [12, 20]. As a consequence, nutrients are diverted from growth processes toward tissues and cells involved in inflammatory and immune responses. Metabolic changes associated with inflammatory processes and immune response can modify protein and amino acids requirements. During immune challenge, decreases in total amino acid (TAA) concentrations in plasma can be explained by an increase in amino acid utilization for energy, cell proliferation or serving as substrates for molecules involved in inflammation, host defense or be funneled into metabolic pathways specifically related to host defense [4].

Systemic effects of chronic obstructive pulmonary disease (COPD) in humans are correlated with altered plasma levels of hormones including cortisol, leptin, ghrelin and insulin-like growth factor 1 (IGF-1) during inflammation and catabolism [16, 18]. Clinically, patients with COPD develop progressive weight loss and generalized skeletal muscle wasting known as pulmonary cachexia syndrome associated with metabolic changes induced by the inflammatory and immune responses in pneumonia. These afflictions modify the animal requirements for protein and amino acids. Amino

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acids play a pivotal role in intermediary metabolism both as the building blocks of proteins carbohydrates, and as precursors for other biomolecules [16]. Changes in insulin concentrations also influence amino acid metabolism, particularly that of the branched-chain amino acids (BCAAs). BCAAs promote protein transcription and translation and inhibit protein degradation. Many studies have confirmed that plasma levels of BCAAs, particularly leucine, are reduced in patients with COPD [8, 14, 32].

Amino acid metabolism in cancer cells is known to be significantly altered compared with that of normal cells, and these changes are also reflected in the plasma amino acid profiles of patients with various types of cancer [19, 21]. In addition, severe sepsis is associated with changes in serum amino acid profiles associated with net proteolysis and negative nitrogen balance [6, 31]. The serum amino acid profiles observed in sepsis is characterized by elevated concentrations of the aromatic amino acids (AAA) phenylalanine and subnormal BCAA concentrations [6, 31]. Therefore, serum amino acid profiles, especially alterations in BCAAs, are utilized as a screening tool for identification of the severity of disease and nutritional management of patients with COPD [8, 14, 32], various types of cancer [19, 21] and sepsis [6, 31]. Alterations in BCAAs have been assessed by determining the molar ratio of BCAAs to AAAs (BCAA/AAA) [10] or by the molar ratio of BCAAs to tyrosine (BTR) [24]. Inflammation has also been observed to precede serine phosphorylation of insulin receptor substrate-1 (IRS-1) [3]. Increased serine phosphorylation of IRS reduces the ability of this messenger to undergo tyrosine phosphorylation and may accelerate the degradation of IRS-1. Profiles of serine phosphorylation as assessed by the molar ratio of phosphoserine to serine (SPR) may be useful as indicators of serine phosphorylation caused by inflammation.

The aim of this study was to investigate the relationships, if any, of the serum concentrations of amino acids in calves with lung inflammation associated with *Mycoplasma* bronchopneumonia. Our hypothesis that the serum amino acid profiles of calves with *Mycoplasma* bronchopneumonia would be similar to those in human COPD, neoplasia and sepsis due to the inflammation and generalized catabolic state of these animals. The serum amino acid profiles, TAA, BCAA/AAA, BTR and SPR were examined. Receiver operating characteristic (ROC) curves were constructed to describe the performance of amino acid profiles in calves with *Mycoplasma* bronchopneumonia. These data may be useful diagnostically and prognostically in calves with bronchopneumonia.

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 [22]. Thirty-four Holstein calves that were 70.1 ± 37.5 days old were enrolled in this study. The health status of each animal was established on the basis of physical examination screening, biochemical analysis, thoracic ultrasound and radiological examinations. Eighteen calves were patients at the Rakuno Gakuen University Veterinary Teaching Hospital showing clinical signs, such as coughing, nasal discharge, fever and pulmonary adventitious breath sounds. None of the calves showed serious anorexia. All calves with bronchopneumonia enrolled in this study were classified as severe cases, as there was a shadow in more than one-third of the images of the thoracic area in X-ray image of the thorax. *M. bovis* was detected in bronchoalveolar lavage fluid of all 18 calves by a PCR method based on the 16S rRNA gene [13]. As controls, sixteen Mycoplasma-free calves with no abnormal clinical signs were purchased at livestock markets in the Ishikari region for educational purposes and were kept at the School of Veterinary Medicine, Rakuno Gakuen University. All calves consumed concentrated pellets (Calf-starter, Satsuraku Agriculture Cooperative, Hokkaido, Japan) in accordance with the manufacturer's guidelines and had *ad libitum* access to hay and water throughout the study.

Single blood samples were collected by jugular venipuncture from all calves within one week after hospitalization. All blood samples were allowed to sit for 30 min and centrifuged for 10 min at 3,000 g with a standardized procedure to harvest serum.

Free amino acid concentrations in serum were determined using an automated amino acid analysis system (Shimadzu Prominence LC-20AD amino acid analysis system, Shimadzu, Kyoto, Japan). Serum was diluted 1:1 with 5% trichloroacetic acid, swirled for 30 sec and centrifuged for 3 min at 10,000 g at 4°C. The supernatant was diluted 10 times with 0.2 N lithium citrate buffer (pH=2.2) and was filtered through a 0.45 μm membrane filter (GHP Acrodisc® Syringe filter GF (4559), Shimadzu GLC, Tokyo, Japan). A portion of each sample (20 μl) of the filtrate was injected onto a HPLC column (Shim-pack AMINO-Li type, 6.0 mm in diameter × 100 mm in length, Shimadzu). The mobile phase was a linear gradient from pH 2.2 with 0.15 N-lithium citrate and 7% methyl cellosolve solution to pH 10.0 with 0.3 N lithium citrate produced using a commercial mobile reagent kit (flow rate, 0.6 m*l*/min: column temperature, 39°C: Amino Acid Mobile Phase Kit (Li Type), Shimadzu). The post-column fluorescence derivatization was conducted using an *o*-phthalaldehyde/N-acetylcysteine method and a commercial reagent kit (flow rate, 0.2 m*l*/min: reaction temperature, 39°C: Amino Acid Reaction Reagent Kit, Shimadzu) as described by Gnanou *et al*. [11]. Individual amino acids were detected by a fluorescence detector (run time, 160 min: excitation on wavelength, 350 nm: and emission wavelength, 450 nm: RF-10Axs, Shimadzu). A mixed solution of amino acids was prepared as above and used as a standard (Type AN-II (011-14463) and Type B (123-02505), Wako Pure Chemical Industries, Osaka, Japan).

Statistical analysis: All amino acids detectable using this method and their abbreviations are listed in Table 1. We calculated the essential amino acid (EAA: Thr + Val + Met + Ile + Leu + Phe + His + Lys+ Arg), nonessential amino acid (NEAA: Ser + Glu + Gly + Ala + CysCys + Tyr), TAA (EAA + NEAA), BCAA (Val + Ile + Leu) and AAA (Tyr

Table 1. Mean ± SD of free amino acid concentrations in blood serum (μmol/m*l*) in the calves with *Mycoplasma* bronchopneumonia

No*	Amino acids	Abbreviations	Control (n=16)	Bronchopneumonia (n=18)	*P* value
1	Phosphoserine	P-Ser	2.42 ± 0.92	6.25 ± 2.40	*P*<0.001
2	Taurine	Tau	7.86 ± 3.53	9.96 ± 4.73	NS
3	*o*-Phosphoethanolamine	P-ET-Amine	1.53 ± 2.00	3.40 ± 1.89	*P*<0.01
4	Threonine	Thr	1.99 ± 0.94	1.79 ± 0.76	NS
5	Serine	Ser	6.60 ± 1.85	2.39 ± 2.88	*P*<0.001
6	L-Glutamic Acid	Glu	4.32 ± 3.06	4.23 ± 2.62	NS
7	Alpha-amino adipic acid	α-AAA	7.37 ± 9.22	12.83 ± 4.07	NS
8	Glycine	Gly	11.43 ± 7.81	7.50 ± 6.96	NS
9	Alanine	Ala	9.62 ± 7.97	1.36 ± 2.71	*P*<0.001
10	Citrulline	Cit	1.97 ± 2.21	5.55 ± 3.60	*P*<0.01
11	Alpha-amino-N-butyric acid	α-ABA	0.23 ± 0.28	1.05 ± 1.31	NS
12	Valine	Val	9.82 ± 9.20	0.05 ± 0.15	*P*<0.001
13	Cystine	CysCys	1.07 ± 1.07	3.94 ± 2.68	*P*<0.001
14	Methionine	Met	1.23 ± 0.56	1.64 ± 0.47	NS
15	Isoleucine	Ilu	3.49 ± 0.99	3.89 ± 1.20	NS
16	Cystathionine	Cystio	0.014 ± 0.046	2.064 ± 2.936	NS
17	Leucine	Leu	7.02 ± 3.89	3.16 ± 2.20	*P*<0.001
18	Tyrosine	Tyr	1.98 ± 1.37	4.76 ± 2.70	*P*<0.001
19	Phenylalanine	Phe	1.22 ± 0.71	0.37 ± 0.34	*P*<0.001
20	Beta-alanine	β-Ala	0.040 ± 0.067	0.095 ± 0.189	NS
21	Beta-amino isobutyric acid	β-AIBA	0.006 ± 0.006	0.001 ± 0.002	NS
22	Gamma-aminobutyric acid	γ-ABA	1.03 ± 1.35	1.33 ± 0.98	NS
23	Histidine	His	2.15 ± 2.25	3.37 ± 1.37	NS
24	3-Methylhistidine	3-ME-His	0.377 ± 0.637	0.569 ± 0.316	NS
25	Carnosine	Carno	0.141 ± 0.218	0.498 ± 0.267	*P*<0.01
26	OH-lysine	OH-Lys	0.262 ± 0.084	0.506 ± 0.244	*P*<0.01
27	Ornithine	Orn	0.861 ± 0.549	0.476 ± 0.398	*P*<0.05
28	Lysine	Lys	1.50 ± 0.72	1.65 ± 0.96	NS
29	Ammonia	NH_4	0.301 ± 0.157	0.495 ± 0.418	NS
30	Arginine	Arg	5.14 ± 3.14	7.19 ± 3.97	NS

UNIT, μmol/m*l*: *ID, Detection order: NS, not significant.

+ Phe) concentrations, BCAA/AAA, BTR and SPR (P-Ser/Ser), respectively. Data were tableted and described as the mean ± standard deviation (SD). Statistical analyses were performed using a software package (IBM SPSS Statistics, Ver. 21, IBM Corp, Armonk, NY, U.S.A.). The mean values for each dependent variable were compared with the normal values, using the Student's *t*-test after ANOVA as the *F* test. Receiver operating characteristic (ROC) curves were used to characterize the sensitivity and specificity of each parameter with respect to changes associated with *Mycoplasma* bronchopneumonia. The optimal cutoff 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 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 [1]. The significance level was $P<0.05$.

RESULTS

The mean concentrations of serum amino acids in calves with *Mycoplasma* bronchopneumonia are summarized in Table 1. The average concentrations of serine, alanine, valine, leucine, phenylalanine and ornithine were significantly lower in the calves with bronchopneumonia than those of normal animals ($P<0.001$). In contrast, the calves with *Mycoplasma* bronchopneumonia were found to have large amounts of phosphoserine ($P<0.001$), *o*-phosphoethanolamine ($P<0.01$), citrulline ($P<0.01$), cysteine ($P<0.001$), tyrosine ($P<0.001$), carnosine ($P<0.01$) and OH-lysine ($P<0.01$) compared with those without respiratory disease. There were no significant differences in the levels of the remaining 16 amino acids and NH_4.

Serum TAA, EAA and NEAA levels and EAA/NEAA also varied between calves with bronchopneumonia and normal calves (Fig. 1). Amino acid analysis demonstrated significant decreases in TAA in calves with bronchopneumonia (46.25 ± 8.77 μmol/m*l*) compared with normal animals (67.09 ± 24.99 μmol/m*l*, $P<0.01$). The essential amino acids (EAA) were significantly lower in calves with bronchopneumonia (15.41 ± 12.02 μmol/m*l*), compared with control calves (28.28 ± 12.02 μmol/m*l*, $P<0.001$), but there was no

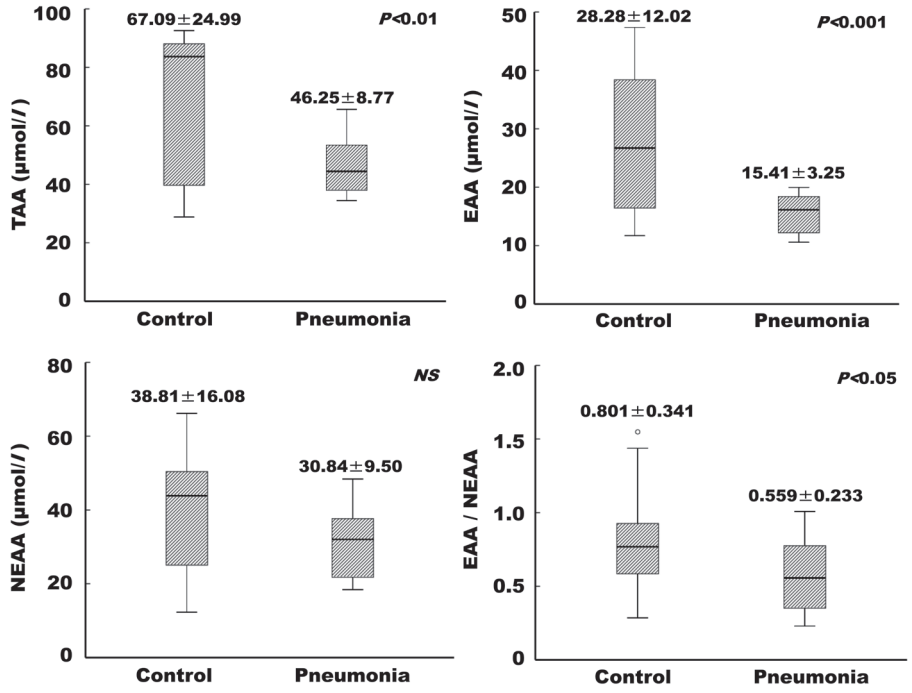

Fig. 1. Amino acid profiles of total amino acids (TAAs), essential amino acids (EAAs), nonessential amino acids (NEAAs) and EAA/NEAA in the calves with *Mycoplasma* bronchopneumonia. TAA=EAA+NEAA, EAA=Thr + Val + Met + Ile + Leu + Phe + His + Lys+ Arg; NEAA=Ser + Glu + Gly + Ala + CysCys + Tyr; NS, not significant.

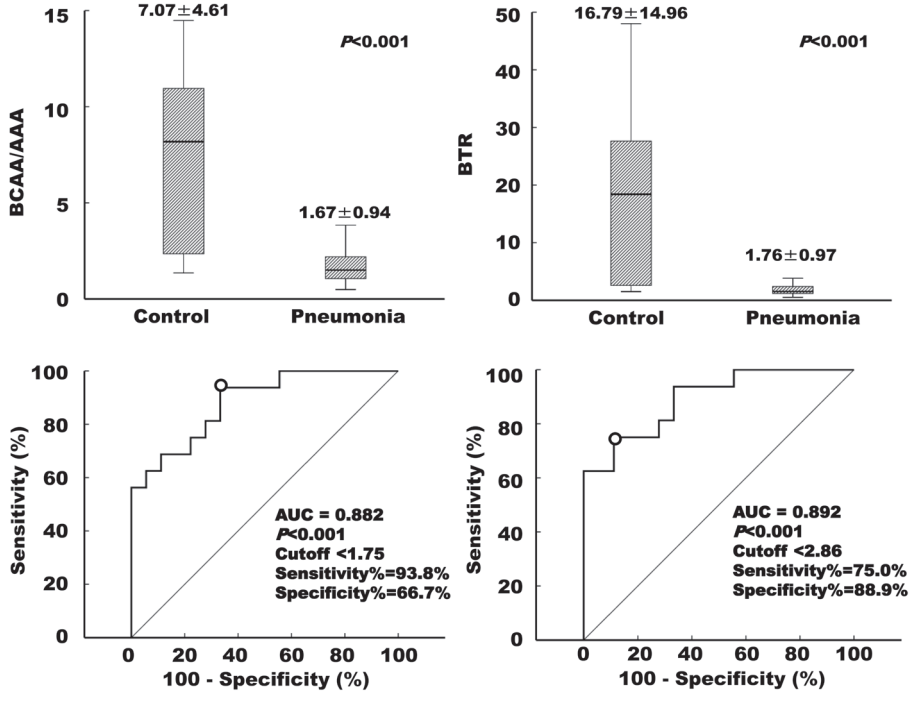

Fig. 2. Receiver operating characteristic (ROC) curves for BCAA/AAA and the molar ratio of branched-chin amino acids to tyrosine (BTR) in detecting *Mycoplasma* bronchopneumonia in calves. The mean area under the ROC curve (AUC) is shown for each ROC curve. The optimal cutoff point for the test was calculated by the Youden index. Open circle, cutoff point.

significant difference in NEAA between groups. Therefore, the EAA to NEAA ration (EAA/NEAA) of the calves with bronchopneumonia (0.559 ± 0.233) was significantly lower than that of the control calves (0.801 ± 0.341, $P<0.05$).

ROC curves for BACC/AAA and BTR (Fig. 2) demonstrate their utility in documenting catabolism in *Mycoplasma* bronchopneumonia of calves. Amino acid analysis showed significant decreases in the BCAAs in calves with bronchopneumonia compared with normal calves (6.74 ± 2.49 vs. 20.3 ± 12.9 μmol/m*l*, $P<0.001$); however, there was also a significantly greater serum concentration of AAAs in the calves with bronchopneumonia compared with normal calves (5.07 ± 2.91 vs. 3.05 ± 1.06 μmol/m*l*, $P<0.05$). Therefore, the BACC/AAA was significantly lower in the calves with bronchopneumonia (1.67 ± 0.94) than in the control calves (7.07 ± 4.61, $P<0.001$). In the same manner, the serum BTR in the calves with *Mycoplasma* bronchopneumonia (1.76 ± 0.97) was significantly lower than that of the healthy control (16.79 ± 14.96, $P<0.001$). The area under the ROC curves (AUC) for BCAA/AAA and the BTR were 0.882 ($P<0.001$) and 0.892 ($P<0.001$), respectively. The proposed cutoff points for BCAA/AAA and the BTR in serum for identifying catabolism in calves with *Mycoplasma* bronchopneumonia based upon analysis of the ROC curves were set at <1.75 and <2.86, respectively. The sensitivities and specificities of proposed diagnostic cutoff for BCAA/AAA in serum were 93.8% and 66.7%, respectively. In the same manner, this sensitivities and specificities of the proposed diagnostic cutoff for the BTR in serum were 75.0% and 88.9%, respectively.

Figure 3 shows the ROC curves for SPR in detecting phosphoserine in the serum of calves with *Mycoplasma* bronchopneumonia. Serum phosphoserine was significantly higher in the calves with bronchopneumonia (6.25 ± 2.40) than in the control calves (2.42 ± 0.92 μmol/m*l*, $P<0.001$); however, there was a significant decrease in serum serine in the calves with bronchopneumonia (2.39 ± 2.88 vs. 6.60 ± 1.85 μmol/m*l*, $P<0.001$). Therefore, SPR was significantly higher in the calves with *Mycoplasma* bronchopneumonia (8.112 ± 8.253) than in the control calves (0.393 ± 0.244, $P<0.001$). The proposed cutoff point for the serum SPR of calves with *Mycoplasma* bronchopneumonia based analysis of the ROC curves (AUC=0.967, $P<0.001$) was set at >0.850. The sensitivities and specificities of proposed diagnostic cutoff for the ratio of phosphoserine in serum were 94.4% and 93.3%, respectively.

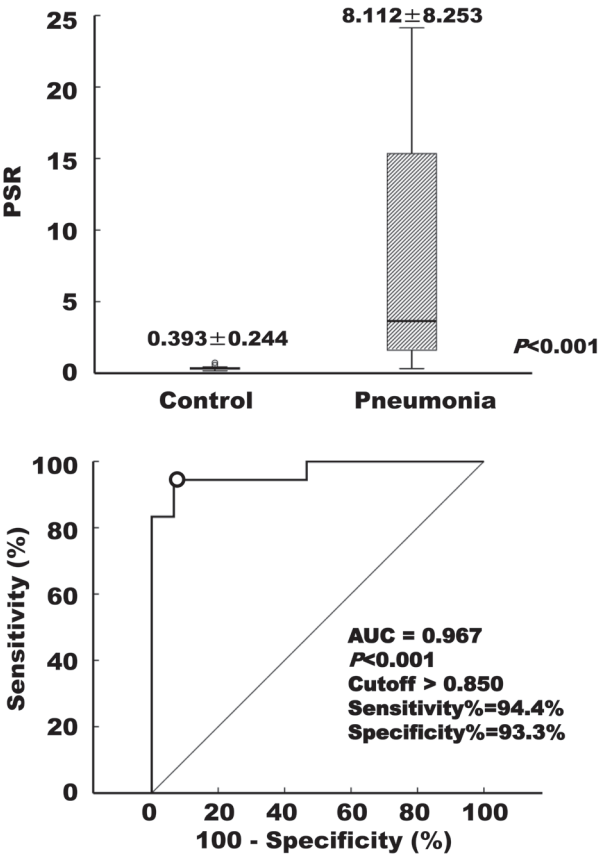

Fig. 3. Receiver operating characteristic (ROC) curves for the molar ratio of phosphoserine to serine (SPR) in detecting *Mycoplasma* bronchopneumonia in calves. SPR=phosphoserine/serine. See Fig. 2 for key.

DISCUSSION

The calves with *Mycoplasma* bronchopneumonia were found to have significant decreases in TAA, EAA, BCAA/AAA and BTR in serum compared with healthy animals; however, there was a significant increase in the SPR of the calves with bronchopneumonia compared with the normal calves. In addition, proposed diagnostic cutoffs of <1.75,<2.86 and >0.85 were set for BCAA/AAA, BTR and SPR in serum, respectively, based on the ROC curves analysis in calves with *Mycoplasma* bronchopneumonia. These values may be useful when adjusting nutritional factors and inflammation status during therapy.

Routine clinical and pathological changes observed in calves with bronchopneumonia are not characteristic for *M. bovis*. The diagnosis of *M. bovis* bronchopneumonia requires additional diagnostic methods for identification. *M. bovis* specific PCR has been used to detect *M. bovis* directly in milk, nasal and lung tissue samples [15]. Several studies have demonstrated that sampling BALF is more predictive of lower respiratory airway pathogens, including *M. bovis*, than nasal swabs, although it is clearly not as convenient [23]. Therefore, PCR-based evaluation using the 16S rRNA genes specific for *M. bovis* DNA was conducted to confirm *Mycoplasma* bronchopneumonia, using BALF samples from all calves in this study [13].

M. bovis infection compromises host defense mechanisms, leading to invasion by other respiratory pathogens [4, 27]. Buchvarova and Vesselinova [4] demonstrated that up to 1/3 of the lungs of calves that die of pneumonia are infected only with *M. bovis*, while the remaining 2/3 of calves contain combinations of *M. bovis* with *P. multocida* and/or *H. somnus*. Coinfections with bovine viral diarrhea virus (BVDV)

are also commonly reported [28]. Other factors play a role in bovine respiratory disease, such as concurrent viral and bacterial diseases, as well as environmental factors; however, 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 [23].

In this study, we demonstrated that serum TAAs, EAAs and BCAAs (Val, Leu) were significantly reduced in calves with *Mycoplasma* bronchopneumonia, compared with healthy animals. BCAAs may undergo net degradation, primarily in skeletal muscle and may be utilized as a noncarbohydrate energy substrate; increased clearance of BCAAs may contribute to decreases in BCAAs in serum. Significant respiratory muscle weakness is also observed due to reduced negative inspiratory force in severe bronchopneumonia. Engelen *et al*. [8] demonstrated total body protein synthesis and protein breakdown are elevated in patients with stable, severe COPD in the post absorptive state, indicating increased whole-body protein turnover. Plasma free amino acid concentrations represent the balance between exogenous uptake and intercurrent metabolites in protein synthesis and breakdown. Several investigators have reported that the amino acid profile is altered in the plasma and skeletal muscles of patients with COPD [14, 32]. Therefore, decreased levels of serum EAA may be associated with increased protein catabolism and turnover associated with bronchial inflammation and moderate anorexia. One other potential association between decreasing BCAA and inflammation is the development of insulin resistance. Systemic inflammation accompanying with bronchopneumonia is associated with serum BCAA concentrations [9]. BCAAs are important not only as essential substrates for protein synthesis but also as regulators of protein synthesis in skeletal muscle [5]. Furthermore, BCAAs are oxidized principally by the skeletal muscle [32]. *In vivo* studies have demonstrated roles of IL-1 and TNFα in the stimulation of muscle BCAA catabolism and amino acid uptake by hepatocytes. Additionally, an increase in the activity of muscle branched-chain keto acid dehydrogenase has been reported [9].

Decreased serum levels of BCAAs in hyper metabolic states may be associated with lung dysfunction and respiratory muscle weakness [8, 32]. Various disturbances in serum amino-acid levels have been demonstrated in underweight COPD patients [32]. The serum concentration of alanine was also decreased in calves with *Mycoplasma* bronchopneumonia in this study. This confirms the findings of Pouw *et al*. [26] and Yoneda *et al*. [32]. Alanine is the main gluconeogenic amino acid in states of malnutrition. This suggests that anorexia and inflammation in *M. bovis* bronchopneumonia may alter gluconeogenesis and muscle anabolic or catabolic processes. The BCAA–alanine–glucose pathway could be activated in calves with *Mycoplasma* bronchopneumonia, as indicated in the findings of Odessey [25], because the rates of uptake and utilization of gluconeogenesis exceed the rate of supply.

Our results demonstrate that the serum phosphoserine concentrations in calves with *Mycoplasma* bronchopneumonia are higher than those in normal animals. This suggests increased utilization of phosphoproteins via protein catabolism. Therefore, the SPR may indicate that serine phosphorylation is significantly higher in calves with lung inflammation than in normal calves. Phosphorylation commonly occurs on serine residues in eukaryotic proteins. Serine phosphorylation is increased by bacterial cells, flagellin and binding of flagellin to mucin gene products [17]. These things may induce phosphorylation of cellular extracellular signal-regulated kinase among other critical kinase modules [17]. Increased concentrations of reactive molecules trigger the activation of serine/threonine kinase cascades, such as c-Jun, EPK and p38 N-terminal kinases that in turn phosphorylate multiple targets, including the insulin receptor and the IRS proteins [30]. This offers a plausible explanation for the molecular basis of oxidative stress-induced insulin resistance [9, 29]. Our results demonstrated that SPR might be an effective index in evaluation of the severity of bronchopneumonia associated with inflammation.

For *IRS-1 and −2*, an increase in serine phosphorylation decreases the extent of tyrosine phosphorylation and is consistent with the attenuation of insulin action [21, 29]. The present study describes inflammation resulting in an increase in the serum concentration of tyrosine, one component of the serum AAAs. This increase was also associated with decreases in serum BCAAs. Therefore, the amino acid profile in this study demonstrated that BCAA/AAA and BTR were significantly lower in calves with bronchopneumonia than in the normal calves. In our study, the BTR (AUC=0.892) was similar to BCAA/AAA (AUC=0.882). Thus, these ratios characterize the metabolic state.

The present study investigated the changes in the amino acid profiles of *Mycoplasma* bronchopneumonia. The BCAA–alanine–glucose pathway could be activated in calves with *Mycoplasma* bronchopneumonia, because the rates of uptake and utilization of gluconeogenesis exceed the rate of supply. Therefore, *Mycoplasma* bronchopneumonia may alter gluconeogenesis and muscle anabolic or catabolic processes associated with severe inflammation. Therefore, *Mycoplasma* infection plays a central role in the development of the BRDC, indicating systemic inflammation symptoms. However, it is unclear whether the same result is obtained in other bronchopneumonia cases, such as Pasteurellosis. Therefore, further studies on the relationship between the amino acid profiles and other bronchopneumonia cases are required. In addition, future studies need to focus on dissecting those changes associated with inflammation and anorexia and those specific to *Mycoplasma* bronchopneumonia.

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REFERENCES

1. Akobeng, A. K. 2007. Understanding diagnostic tests 3: receive operating characteristic curves. *Acta Paediatr.* **96**: 644–647.

[Medline] [CrossRef]
2. Arcangioli, M. A., Duet, A., Meyer, G., Dernburg, A., Bézille, P., Poumarat, F. and Le Grand, D. 2008. The role of Mycoplasma bovis in bovine respiratory disease outbreaks in veal calf feedlots. *Vet. J.* **177**: 89–93. [Medline] [CrossRef]
3. Barreiro, G. C., Prattali, R. R., Caliseo, C. T., Fugiwara, F. Y., Ueno, M., Prada, P. O., Velloso, L. A., Saad, M. J. and Carvalheira, J. B. 2004. Aspirin inhibits serine phosphorylation of IRS-1 in muscle and adipose tissue of septic rats. *Biochem. Biophys. Res. Commun.* **320**: 992–997. [Medline] [CrossRef]
4. Buchvarova, Y. and Vesselinova, A. 1989. On the aetiopathogenesis of mycoplasma pneumonia in calves. *Arch. Exp. Veterinarmed.* **43**: 685–689. [Medline]
5. Buse, M. G. and Reid, M. 1975. Leucine a possible regulator of protein turnover in muscle. *J. Clin. Invest.* **56**: 1250–1261. [Medline] [CrossRef]
6. Chiarla, C., Giovannini, I. and Siegel, J. H. 2004. The relationship between plasma cholesterol, amino acids and acute phase proteins in sepsis. *Amino Acids* **27**: 97–100. [Medline] [CrossRef]
7. Ellis, J. A. 2001. The immunology of the bovine respiratory disease complex. *Vet. Clin. North Am. Food Anim. Pract.* **17**: 535–550. [Medline]
8. Engelen, M. P., Deutz, N. E., Wouters, E. F. and Schols, A. M. 2000. Enhanced levels of whole-body protein turnover in patients with chronic obstructive pulmonary disease. *Am. J. Respir. Crit. Care Med.* **162**: 1488–1492. [Medline] [CrossRef]
9. Evans, J. L., Maddux, B. A. and Goldfine, I. D. 2005. The molecular basis for oxidative stress-induced insulin resistance. *Antioxid. Redox Signal.* **7**: 1040–1052. [Medline] [CrossRef]
10. Fischer, J. E., Yoshimura, N., Aguirre, A., James, J. H., Cummings, M. G., Abel, R. M. and Deindoerfer, F. 1974. Plasma amino acids in patients with hepatic encephalopathy. Effects of amino acid infusions. *Am. J. Surg.* **127**: 40–47. [Medline] [CrossRef]
11. Gnanou, J. V., Srinivas, S. K. and Kurpad, A. V. 2004. Automated derivatization with *o*-phthalaldehyde for estimation of amino acids in plasma using reversed-phase high performance liquid chromatography. *Indian J. Biochem. Biophys.* **41**: 322–325. [Medline]
12. Grimble, R. F., Jackson, A. A., Persaud, C., Wride, M. J., Delers, F. and Engler, R. 1992. Cysteine and glycine supplementation modulate the metabolic response to tumor necrosis factor-α in rats fed a low protein diet. *J. Nutr.* **122**: 2066–2073. [Medline]
13. Higuchi, H., Iwano, H., Kawai, K., Ohta, T., Obayashi, T., Hirose, K., Ito, N., Yokota, H., Tamura, Y. and Nagahata, H. 2011. A simplified PCR assay for fast and easy mycoplasma mastitis screening in dairy cattle. *J. Vet. Sci.* **12**: 191–193. [Medline] [CrossRef]
14. Hofford, J. M., Milakofsky, L., Vogel, W. H., Sacher, R. S., Savage, G. J. and Pell, S. 1990. The nutritional status in advanced emphysema associated with chronic bronchitis. A study of amino acid and catecholamine levels. *Am. Rev. Respir. Dis.* **141**: 902–908. [Medline] [CrossRef]
15. Hotzel, H., Sachse, K. and Pfützner, H. 1996. Rapid detection of Mycoplasma bovis in milk samples and nasal swabs using the polymerase chain reaction. *J. Appl. Bacteriol.* **80**: 505–510. [Medline] [CrossRef]
16. Jagoe, R. T. and Engelen, M. P. 2003. Muscle wasting and changes in muscle protein metabolism in chronic obstructive pulmonary disease. *Eur. Respir. J. Suppl.* **46**: 52s–63s. [Medline] [CrossRef]
17. Lillehoj, E. P., Kim, H., Chun, E. Y. and Kim, K. C. 2004. Pseudomonas aeruginosa stimulates phosphorylation of the airway epithelial membrane glycoprotein Muc1 and activates MAP kinase. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **287**: L809–L815. [Medline] [CrossRef]
18. Luo, F. M., Liu, X. J., Li, S. Q., Wang, Z. L., Liu, C. T. and Yuan, Y. M. 2005. Circulating ghrelin in patients with chronic obstructive pulmonary disease. *Nutrition* **21**: 793–798. [Medline] [CrossRef]
19. Maeda, J., Higashiyama, M., Imaizumi, A., Nakayama, T., Yamamoto, H., Daimon, T., Yamakado, M., Imamura, F. and Kodama, K. 2010. Possibility of multivariate function composed of plasma amino acid profiles as a novel screening index for non-small cell lung cancer: a case control study. *BMC Cancer* **10**: 690. [Medline] [CrossRef]
20. Melchior, D., Sève, B. and Le Floc'h, N. 2004. Chronic lung inflammation affects plasma amino acid concentrations in pigs. *J. Anim. Sci.* **82**: 1091–1099. [Medline]
21. Mustafa, A., Gupta, S., Hudes, G. R., Egleston, B. L., Uzzo, R. G. and Kruger, W. D. 2011. Serum amino Acid levels as a biomarker for renal cell carcinoma. *J. Urol.* **186**: 1206–1212. [Medline] [CrossRef]
22. National Research Council. Guide for the Care and Use of Laboratory Animals. 1996. pp. 1–70. National Academy Press, Washington, D.C.
23. Nicholas, R. A. and Ayling, R. D. 2003. Mycoplasma bovis: disease, diagnosis, and control. *Res. Vet. Sci.* **74**: 105–112. [Medline] [CrossRef]
24. Ninomiya, T., Yoon, S., Sugano, M., Kumon, Y., Seo, Y., Shimizu, K., Nagano, H., Kasuga, M. and Hayashi, Y. 1999. Improvement of molar ratio of branched-chain amino acids to tyrosine (BTR) associated with liver fibrosis in chronic hepatitis C patients treated with interferon-alpha. *Dig. Dis. Sci.* **44**: 1027–1033. [Medline] [CrossRef]
25. Odessey, R. 1979. Amino acid and protein metabolism in the diaphragm. *Am. Rev. Respir. Dis.* **119**: 107–112. [Medline]
26. Pouw, E. M., Schols, A. M., Deutz, N. E. and Wouters, E. F. 1998. Plasma and muscle amino acid levels in relation to resting energy expenditure and inflammation in stable chronic obstructive pulmonary disease. *Am. J. Respir. Crit. Care Med.* **158**: 797–801. [Medline] [CrossRef]
27. Radaelli, E., Luini, M., Loria, G. R., Nicholas, R. A. and Scanziani, E. 2008. Bacteriological, serological, pathological and immunohistochemical studies of Mycoplasma bovis respiratory infection in veal calves and adult cattle at slaughter. *Res. Vet. Sci.* **85**: 282–290. [Medline] [CrossRef]
28. Shahriar, F. M., Clark, E. G., Janzen, E., West, K. and Wobeser, G. 2002. Coinfection with bovine viral diarrhea virus and Mycoplasma bovis in feedlot cattle with chronic pneumonia. *Can. Vet. J.* **43**: 863–868. [Medline]
29. Sun, X. J. and Liu, F. 2009. Phosphorylation of IRS proteins Yin-Yang regulation of insulin signaling. *Vitam. Horm.* **80**: 351–387. [Medline] [CrossRef]
30. Tanti, J. F. and Jager, J. 2009. Cellular mechanisms of insulin resistance: role of stress-regulated serine kinases and insulin receptor substrates (IRS) serine phosphorylation. *Curr. Opin. Pharmacol.* **9**: 753–762. [Medline] [CrossRef]
31. Vente, J. P., von Meyenfeldt, M. F., van Eijk, H. M., van Berlo, C. L., Gouma, D. J., van der Linden, C. J. and Soeters, P. B. 1989. Plasma-amino acid profiles in sepsis and stress. *Ann. Surg.* **209**: 57–62. [Medline] [CrossRef]
32. Yoneda, T., Yoshikawa, M., Fu, A., Tsukaguchi, K., Okamoto, Y. and Takenaka, H. 2001. Plasma levels of amino acids and hypermetabolism in patients with chronic obstructive pulmonary disease. *Nutrition* **17**: 95–99. [Medline] [CrossRef]