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Plasma Proteomics Analysis of Dairy Cows with Milk Fever Using SELDI-TOF-MS

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ABSTRACT

Milk fever is an important metabolic disorder of dairy cows at calving and is characterized by hypocalcemia during the transition period. The aim of this study is to investigate novel changes in the plasma proteomics of cows with milk fever. Surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) was used for many field as a novel proteomics technology. So far, the plasma proteomics of milk fever has not been investigated using SELDI-TOF-MS. Plasma samples were obtained from twenty-one Holstein cows with milk fever (T) and fifty-nine Holstein cows without milk fever (C) at a dairy farm in Heilongjiang, China. Twenty-four differential peptide peaks in the plasma of T and C cows were isolated by SELDI-TOF-MS. Ten of these peaks were identified using the Swissport Protein Database. The peptide peaks represented ten unique proteins and showed significant alterations in their peaks as determined by analysis using the Wilcoxon Rank Sum Test. The four up-regulated proteins were identified as complement c3 frag, hepcidin, amyloid bata a4 protein, serum albumin frag and fibrinogen. Complement c3 frag and hepcidin regulate the inflammatory response. Amyloid beta a4 protein is involved in Alzheimer's disease. Serum albumin frag acts as a transport protein. Fibrinogen beta chain participates in blood coagulation. The two down-regulated proteins were plasma protease c1 inhibitor frag and apolipoprotein a-2 which are associated with, respectively, blood coagulation and cardiovascular disease. The four proteins that were both up-regulated and down-regulated were fibrinogen alpha chain frag, neurosecretory protein vgf frag, serun amyloid a protein and cystatin-c. Based on SELDI-TOF-MS, identify novel plasma proteins that may be closely associates with milk fever in cows. These findings may reveal previously unidentified metabolic changes in cows with milk fever.

Key words: SELDI-TOF-MS, plasma proteomics, milk fever, dairy cows

INTRODUCTION

Milk fever (MF) is a metabolic disease that occurs at calving or during the transition period, especially in high-producing dairy cows (Horst *et al.*, 1997). MF is associated with many important diseases, such as metritis, ketosis, displaced abomasums andretained placenta. The disease is characterized by hypocalcaemia; this is a consequence of fetal development and the formation of colostrum during the transition period which result in considerable loss of blood calcium (Ca). Levels of parathyroid hormone and 1, 25-dihydroxyvitamin D3 (DHVD) increase as a result of the reduction of plasma Ca concentration (DeGaris and Lean, 2008). Previous studies suggest that

many biochemical factors, such as phosphorous, magnesium, alkaline phosphatase, hydroxyproline, osteocalcin and calcitonin, have a close relationship with plasma Ca concentration (Horst *et al.*, 1997; DeGaris and Lean, 2008).

Surface enhanced laser desorption/ionization time of flight mass spectrometry (SELDI-TOF-MS) is a novel technology of proteomic analysis which involves protein-chip bond mass spectrometry. It can be used to search for peptides and/or proteins on the surface of the protein chip that have undergone chemical modification and it allows analysis of complex biological samples (Yuan *et al.*, 2012). SELDI-TOF-MS detects the protein spots on different chromatographic surfaces and maintains their physicochemical properties (Lehmann *et al.*, 2005). Thus, it may eliminate the requirement for pre-purification and the proteins can be purified easily in buffered salt or detergents. For these reasons, SELDI-TOF-MS is easy and simple to perform, rapid and is able to screen smaller peptides than 2-differential in-gel electrophoresis (2D-DIGE) (Grus *et al.*, 2005). In recent years, many fields have research diseases of humans and rats by SELDI-TOF-MS. For example breast cancer (Li *et al.*, 2005), severe acute respiratory syndrome (Yip *et al.*, 2005) and in the discovery of drugs (Ilyin *et al.*, 2005).

The pathogenesis of MF has been reported in relation to physiology, biochemistry and pathology in previous studies. However, there have been no reports about the plasma proteome of cows with MF investigated using SELDI-TOF-MS. Therefore, the aim of this study was to explore the plasma proteomic changes in cows with MF using SELDI-TOF-MS, to provide new information on the pathogenesis of MF.

MATERIALS AND METHODS

Experimental animals: All animals were selected from an intensive dairy farm in accordance with the requirements of the Veterinary Medical Ethical Committee of the Local Agricultural Department (Mishan, Heilongjiang, China). Twenty-one Holstein cows were assigned to the MF group (T, Ca<1.40 mmol L⁻¹ and obviously clinical signs, such as depression, recumbency, unscourseness, etc.,) and fifty -nine Holstein cows to the control group (C, Ca>2.50 mmol L⁻¹ and no clinical signs) (DeGaris and Lean, 2008). Table 1 shows the age, parity and plasma Ca concentration of the two groups of cows. The difference were very significant between two groups for age, parity and plasma Ca concentration (p<0.01). All the cows were fed a total mixed ration (TMR) at parturition which consisted of 8.5 kg concentrated feed, 18.5 kg silage maize, 4 kg hay and 350 g fat. The nutritional analysis was 55.60% DM (dry matter), 16% crude protein, 1.75 mcal DM⁻¹ NEL(net energy for lactation), 5.60% fat, 39.10% NDF(neutral detergent fiber), 20.30% ADF(acid detergent fiber), 180 g Ca and 116 g P.

Blood parameter analysis: All blood samples (10 mL) from MF and C groups were collected by the caudal vein within 6 h after calving. Heparin (150 IU) was added to each sample according to the International Guiding Principles for Biomedical Research Involving Animals. The samples were

Table 1: Age, parity and plasma Ca concentration of tested cows (Mean±SD)

Groups	No.	Age	Parity	Ca (mmol L ⁻¹)
T	21	5.78±1.98**	3.19±1.57**	1.28±0.20**
C	59	3.24±1.69	1.54±1.18	2.56±0.21

T: Milk fever group, C: Control group, **represents very significant difference between two groups (p<0.01)

centrifuged immediately at 3,000 rpm for 10 min, then frozen in liquid nitrogen and stored at -80°C until subsequent analyses. The plasma Ca concentration was detected using an automatic biochemical analyzer (Modular DPP, Roche, Germany) using a commercial kit (651564-01, Roche, Germany).

SELDI-TOF-MS

Sample preparation: After thawing and centrifugation (10,000 rpm for 5 min) at 4°C, 10 µL of each serum sample was added to 10 µL U9 (5 mL 9 M urea, 2% CHAPS(3-[(3-cholesterol aminopropyl) dimethylammonio]-1-propane sulfonic acid), 50 mM Tris-HCl, pH 9.0. Each sample was mixed by gently knocking the bottom of the test tube with a finger. The samples were shaken for 30 min in an ice-bath and gently knocked with the finger to mix them every 5 min. Subsequently, 180 µL NaAC (sodium acetate) (50 mM, pH 4.0) was added to 20 µL of the treated sample, to give a final dilution of the serum sample of 20. The sample was applied to a spot on the chip after mixing, avoiding the formation of bubbles.

Chip processing: Each spot was washed with 10 mM HCL (5 µL) for 5 min. After removal of the acid, 5 µL HPLC (High-performance liquid chromatography) water was added and the sample shaken at 250 rpm for 5 min. This procedure was repeated once. The CM10 (weak cation exchanger array) ProteinChip was put into a Bioprocessor; 100 µL elution buffer (50 mM NaAC, pH 4.0) was put into each well and incubated at room temperature for 5 min with vigorous shaking at 250 rpm. This procedure was repeated once. After rejecting the buffer, 100 µL of sample was added immediately to every spot and incubated for 60 min with vigorous shaking at 250 rpm. After the content had been removed from each spot, the spot was washed with buffer (50 mM NaAC, pH 4.0) for 5min at room temperature with vigorous shaking at 250 rpm. This procedure was repeated once. Each spot was washed with 100 µL HPLC water that was removed after 5 min with vigorous shaking at 250 rpm. After air drying for 10-15 min, 1 µL EAM (energy absorbing molecule) solution was applied to each spot.

Experimental apparatus: SELDI-TOF-MS PBSIIC (BIORAD, USA) and Protein software were used to read the chips and analyze the data. The following settings were used: Laser intensity 240, detector sensitivity 8, voltage 20000 v, vacuum 8.387e-007 Torr, peaks detected from 1000-50000 Da and collection 112 times per spot.

Statistical analysis: In this study many tools were used for statistical analysis, including CIPHERGEN ProteinChip Software (Version 3.1.1), the Swissport protein database, MATLAB 2007b software, Hierarchical cluster using gene cluster 3.0 and tree view cluster software and rweka from bioconductor. One-way analysis of variance was used for analysis the age, parity and plasma Ca concentration of two groups of cows. The value of peaks was shown in table by the CIPHERGEN ProteinChip software (Version 3.1.1). The Wilcoxon rank sum test was used for assess the differences in the peaks between the T and C groups according to P values.

RESULTS

Detection using SELDI-TOF-MS and predicted proteins: The raw data were got from 81 peaks by CIPHERGEN ProteinChip software (Version 3.1.1) between the group of MF and control. The threshold of the p value was 0.01 and the differential peaks were determined with a p value <0.01. Thirty-seven differential peaks were selected (Fig.1a). Figure 1b, c show the most significant difference peak (m/z:3898.65) which have the minimum P value, via Trace view and Gel view.

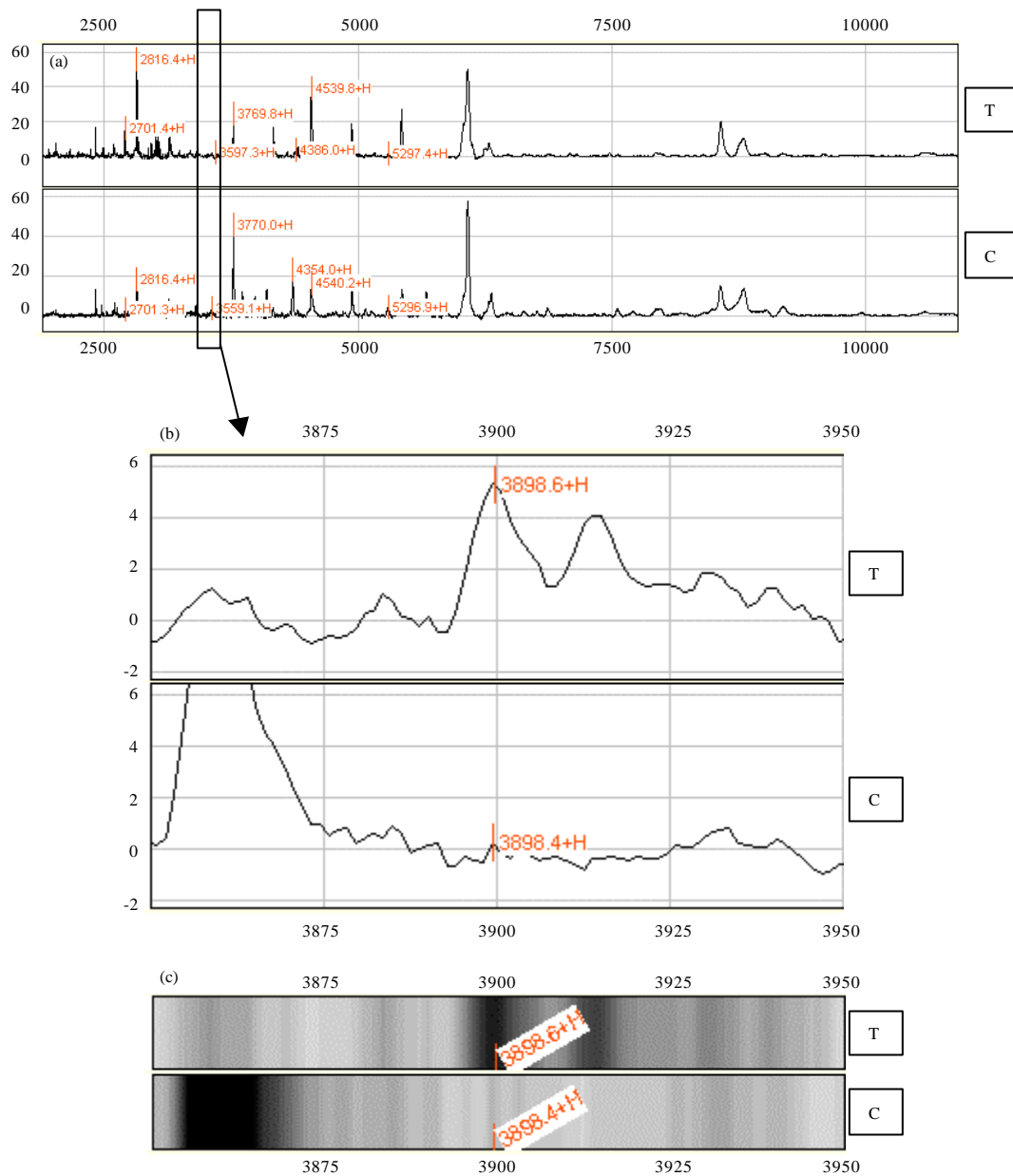


Fig. 1(a-c): Detection using SELDI-TOF-MS and predicted proteins. The differential peaks between milk fever (T) and C control (groups). On the right of the (a) Group I is the test (T) with milk fever, and Group III is control (C). The abscissa is m/z and the ordinate is the peak value (b) The abscissa is m/z and the ordinate is the peak value (b) is enlarged from (a) for the m/z 3868 (amyloid beta a4 protein). The top is the test (T) with milk fever, and the bottom is control (C) and (c) The abscissa is m/z in Gel view. The top is the test (T) with milk fever, and the bottom is control (C). The dark color indicates high content and the light color indicates low content

The true mass to charge ratio (m/z) of the thirty-seven differential peaks and the theoretical m/z of polypeptides from the Swissport protein database, were used to predict the most similar

Table 2: Status of the up-regulated proteins in the T group

Peaks (m/z)	T (Mean±SD)	C (Mean±SD)	p-value (×E = 10)	Name of protein
1859.39	4.55±5.89	0.82±2.02	2.10 E ⁻⁴	Complement c3 frag
1953.27	3.82±3.71	1.78±2.72	1.35 E ⁻⁴	
2001.1	1.98±1.03	1.17±0.75	5.28 E ⁻⁴	Amyloid beta a4 protein
2016.15	8.28±3.86	5.54±3.75	3.38 E ⁻³	
3898.65	5.11±3.73	0.45±0.95	9.80 E ⁻¹⁰	
3912.85	17.15±13.64	3.32±6.76	1.83 E ⁻⁷	
2128.23	1.39±1.21	0.34±0.86	1.77 E ⁻⁶	Serum albumin frag
2156.84	2.41±1.65	1.02±1.15	2.10 E ⁻⁴	
2721.87	4.63±4.87	0.96±1.32	3.25 E ⁻⁵	Hepcidin
2837.35	5.16±3.59	2.57±3.49	1.58 E ⁻³	

T: Milk fever group, C: Control group

Table 3: Status of the down-regulated proteins in the T group

Peaks (m/z)	T (Mean±SD)	C (Mean±SD)	p value (×E = 10)	Name of protein
4039.43	0.46±0.4	2.15±2.2	3.96 E ⁻⁴	Plasma protease c1 inhibitor frag
9189.79	1.1±0.52	2.19±1.4	2.19 E ⁻⁴	Apolipoprotein a-2

T: Milk fever group, C: control group

Table 4: Status of the up-regulated and down-regulated proteins in the T group

Peaks (m/z)	T (Mean±SD)	C (Mean±SD)	p value (×E = 10)	Name of protein
3170.42	3.49±3.03	1.5±1.08	1.69 E ⁻⁴	Fibrinogen alpha chain frag
3381.51	0.27±0.35	0.57±1.4	2.95 E ⁻⁴	
3416.05	11.14±8.93	1.89±4.38	2.16 E ⁻⁰⁸	
5670.34	4.48±2.23	8.76±5.11	2.01 E ⁻⁴	
3687.47	3.94±2.92	1.62±1.3	1.92 E ⁻⁴	Neurosecretory protein vgf frag
3862.41	1.49±1.23	4.43±4.18	5.30 E ⁻³	
4853.84	2.2±1.74	4.24±3.1	6.47 E ⁻³	
11682.7	1.36±0.49	2.44±1.5	8.87 E ⁻⁴	Serum amyloid a protein
12171.1	1.35±0.63	0.66±0.48	1.57 E ⁻⁵	
12789.6	0.67±0.88	1.49±1.32	2.37 E ⁻³	Cystatin-c
12970.9	1.87±1.89	0.78±0.96	4.30 E ⁻³	
13448.9	1.19±2.44	3.75±4.89	3.63 E ⁻³	

T: Milk fever group, C: Control group

protein for each differential peak. From these results, twenty-four differential peaks were predicted, of which ten were identified. In the T group four proteins were up-regulated in Table 2, two proteins were down-regulated in Table 3 and four proteins were both up-regulated and down-regulated in Table 4. The P value of all peaks was under 0.01.

The p values were compared between the two groups and the m/z ratios of the top five peaks were 2126.23, 2959.68, 3416.05, 3898.65 and 3912.85, respectively, as shown by the boxplot in Fig. 2.

PCA analysis: Principal Component Analysis (PCA), used to detect compounds with significant differences, can be used to show the relationships among many samples by compressing the

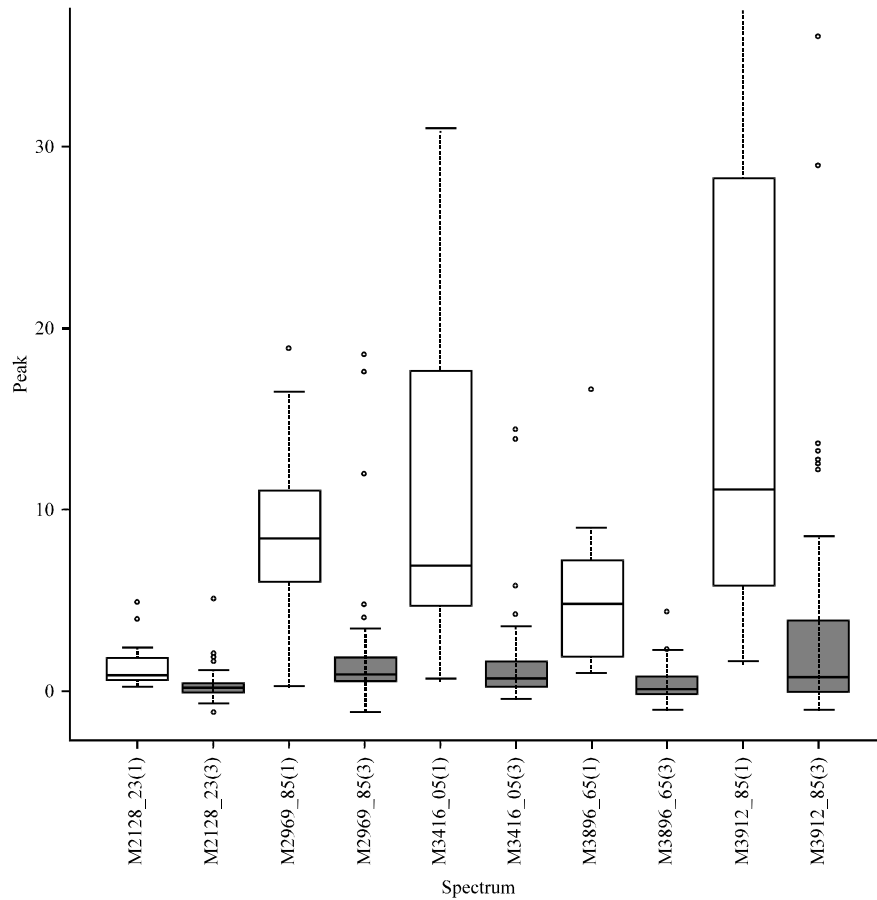


Fig. 2: Detection using SELDI-TOF-MS and predicted proteins. The p-values were compared between the two groups and the m/z ratios of the top five peaks respectively, as shown by the boxplot. Pink box color shows the test (T) group; gray box color shows the control (C). The abscissa is m/z and the ordinate is the peak value. The boxplot displays differences in intensity of 2128.23, 2959.85, 3416.05, 3898.65 and 3912.85 m/z respectively between the T and C groups

characteristics of the samples in low-dimensional space. In this study, the data for twenty-four differential peaks were converted mathematically to PC1 (principal component 1) and PC2 (principal component 2) and then demonstrated by score plot using MATLAB 2007b software. The result indicated that the T and C groups were able to be differentiated clearly in Fig. 3.

Cluster analysis: The relationship of all samples was shown by mode of Hierarchical cluster using Gene cluster 3.0 and tree view cluster software. Figure 4 displayed major of samples in T group lied in left side and of C group lied right. Results suggested that the peaks had significant difference between two groups. However, there were some overlap between two groups and it may be relative to samples difference from same group.

Decision tree analysis: The decision tree analysis was completed using Rweka from Bioconductor. In this study, only one protein peak (2959.85) was used for discriminate cows with

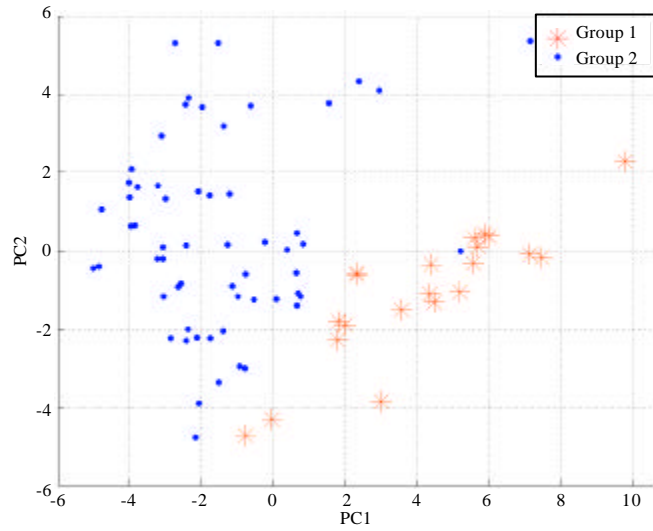


Fig. 3: Principal component analysis (PCA). Group I is the test (T) with milk fever. Group III is control (C). PC1 is principal component 1 and PC2 is principal component 2. The different groups could be distinguished clearly by PC1 and PC2. The result indicated that the T and C groups were able to be differentiated clearly PCA plot

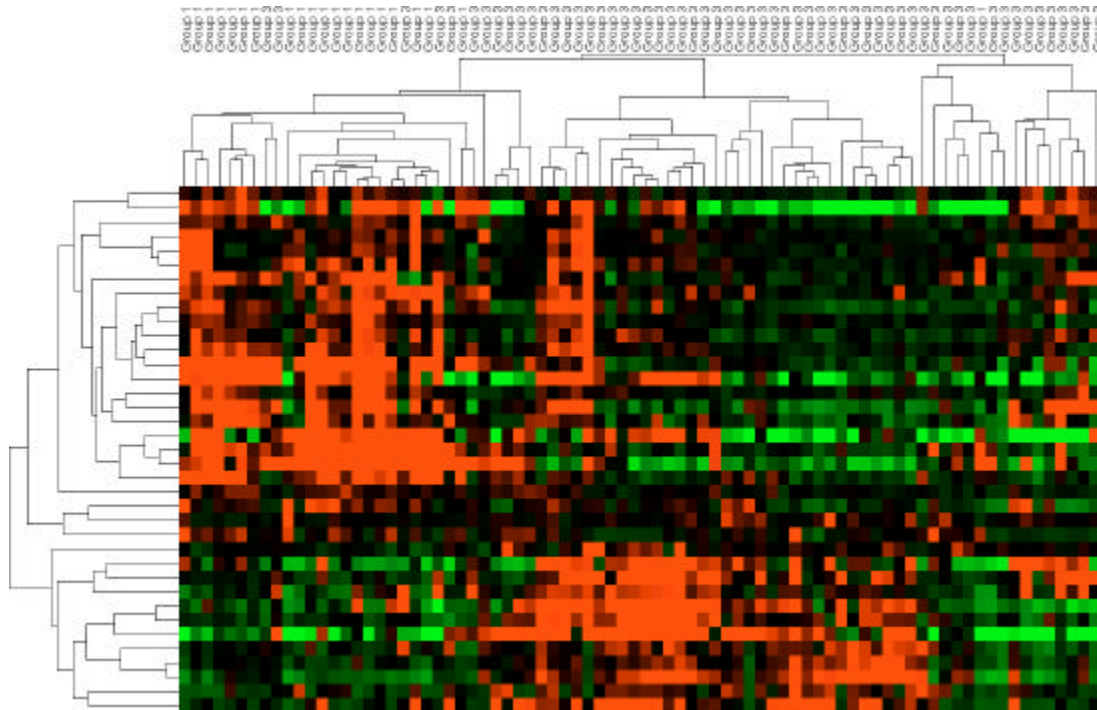


Fig. 4: Cluster analysis of samples. Group I is test (T) with milk fever. Group III is control (C). Each horizontal line is a kind of compound and each vertical line is a sample. Red indicates high content of sample and green indicates low content of sample. Results of Cluster analysis suggested that the peaks had significant difference between two groups

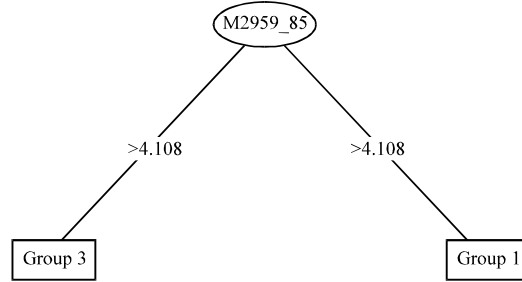


Fig. 5: Decision tree analysis of samples. Results of decision tree modeling allocate the samples to different groups; Group I is test (T) with milk fever. Group III is control (C). The node is labeled sequentially and shows splitting criteria. In this figure $M2959_{85} = 4.108$ would mean that subjects with peak intensities of $= 4.108$ at an m/z ratio of 2959.86 Da would move down the left side and all other subjects would move down the right side

MF from healthy cows (Fig. 5) according to decision tree modeling to allocate the samples to different groups; the model had 90.5% sensitivity, 93.2% specificity and 92.5% accuracy.

DISCUSSION

The result of study was shown for the first time the different proteins that are expressed in the plasma of cows with MF, using SELDI-TOF-MS which combines protein-chip analysis and TOF-MS. A result of high sensitivity and adaptability was obtained quickly and easily (Grus *et al.*, 2005). In this study, four proteins were up-regulated, two proteins were down-regulated and four proteins were both up-regulated and down-regulated in the T group.

The four up-regulated proteins were complement c3 frag (C3), amyloid beta a4 protein (A β), Serum Albumin Frag (SALB) and Hepsidin (HC). C3 is secreted by activated macrophages and adipocytes at sites of inflammation and has a central role in the immune system (Goralski and Sinal, 2007; Zarkadis *et al.*, 2001). In the activated complement system C3 also plays an important role. Activation of complement involves three pathways; each pathway has a distinct mechanism, but all three pathways require activated C3 (Onat *et al.*, 2011; Pangburn *et al.*, 2008). A β is a transmembrane protein that is produced by amyloid precursor protein (APP) on the cell membrane (Zetterberg *et al.*, 2010) and it has the structure of a cell-membrane receptor-like protein (Mattson, 2004). A β is an important risk factor for Alzheimer's disease (AD) since its polymeric product forms a neurotoxin by deposition on the cell matrix which may result in AD (Walsh and Selkoe, 2007; Hampel *et al.*, 2010). HC, an 84-amino acid protein, is an important regulatory protein produced by the liver. It can inhibit erythrocyte function by iron loading and inflammation, leading to anemia, anoxia and increased inflammatory response (Coyne, 2011; Young and Zaritsky, 2009). SALB is a single peptide chain with 580-585 amino acids which is the most common protein produced by the liver in the blood. It has many biological functions, including maintenance of osmotic pressure, transport of molecules such as hormones, fat-soluble vitamins, free fatty acids and drugs, binding of calcium ions (Ca²⁺) and buffering pH (Peters, 1975). The up-regulation of C3 and hepsidin found in this study may be associated with the development of inflammation during MF because the complement system is activated by the C3 pathway and high levels of hepsidin can enhance the inflammatory response. This may explain why affected cows easily succumb to infectious diseases, such as mastitis or metritis. Some studies have reported that the susceptibility

of immunocytes to stimulation may decrease in cows with MF, leading to the inflammatory responses involved in endometritis and mastitis (Kimura *et al.*, 2006). However, the relationship between milk fever and hepcidin still is unclear. In addition, the up-regulated A β which produces a neurotoxin leading to AD in humans, may be involved in the signs of depression and paralysis seen in cows with MF. Likewise, the relationship between A β and milk fever needs further confirmation. Furthermore, there has been no report about the relationship of serum albumin with MF and more evidence is required.

The two down-regulated proteins were plasma protease c1 inhibitor frag (C1INH) and apolipoprotein a-2 (ApoA-II). C1INH is a heavily glycosylated single chain polypeptide with a molecular weight of ~405 kD which is an important inhibitor of the inflammatory response by means of bonding with many proteases (Jackson *et al.*, 1989; Parikh and Riedl, 2011). In the complement system, it plays an important regulatory role and prevents overactivation of the complement cascade (Emonts *et al.*, 2007). It can promote blood coagulation because C1INH can stimulate the synthesis of kallikrein and blood coagulation factor XII which play an important role in blood coagulation (Matsushita *et al.*, 2000; Cai *et al.*, 2005). ApoA-II is the second major protein component of High-density Lipoprotein (HDL), accounting for about 20% of HDL protein. Recent studies have shown that it can protect against Cardiovascular Disease (CVD) by transporting various proteins (Blanco-Vaca *et al.*, 2001; Birjmohun *et al.*, 2007). However, the function of ApoA-II is not totally clear (Winkler *et al.*, 2008). The down-regulation of C1INH may be an important factor in the inflammatory process of cows with MF because of the inhibitory role of this protein in the inflammatory response. The down-regulation of ApoA-II may be related to the tachycardia and arrhythmia seen in cows with MF, because of its role in prevention of CVD. Nevertheless, further confirmation of the relationship of both these proteins to MF is required.

An interesting phenomenon noted in this studies was that four proteins were both up-regulated and down-regulated in the T group. They were fibrinogen alpha chain Frag (FG), neurosecretory protein Vgf Fra (VGF), Serum Amyloid a protein (SAA) and Cystatin-c (CYS-C). FG is a plasma glycoprotein that plays an important role in the inflammatory response and blood coagulation. It is widespread in the tissues during injury and inflammation (O'Donovan *et al.*, 2012; Flick *et al.*, 2004). During Ca²⁺-dependent blood coagulation, it is converted into fibrin. Therefore, in this study the up-regulation of FG was possibly related to the inflammatory response of cows with MF because FG has an inhibitory function in inflammation. Furthermore, the down-regulation of FG may also be related to blood coagulation because the conversion of fibrinogen into fibrin is accompanied by massive consumption of Ca²⁺. This process may be a reason for the decreased Ca²⁺ concentration in cows with MF. VGF, a 617-amino acid protein, has the characteristics of a neuropeptide precursor. It is a response gene for neurotrophic factor and is regulated by Nerve Growth Factor (NGF) in PC12 cells (Canu *et al.*, 1997; Riedl *et al.*, 2009). It is widely distributed in the nervous system and exists selectively in neurons and other nervous tissue (Salton *et al.*, 2000). VGF is up-regulated during injury and inflammation of nerves (Riedl *et al.*, 2009) and down-regulated in patients with AD, Amyotrophic Lateral Sclerosis (ALS) and frontolateral dementia (Selle *et al.*, 2005; Carrette *et al.*, 2003). Therefore, the up-regulation of VGF may play a role in the inflammatory response of cows with MF. In addition, the down-regulation of VGF, like A β , may play a role in the regulation of nerve function related to the signs of depression shown by cows with milk fever. However, this hypothesis requires further research. In humans, SAA, a 12-14 kDa protein, is one of a group of apolipoproteins in HDL that are precursors to the amyloid A protein found in amyloidosis. Its levels can increase 1,000-fold in the 24-36 h after injury or infection and returns

to normal levels in 10-14 days (Bahk *et al.*, 2010). It can promote the catabolism of HDL, reduce the level of esterified serum cholesterol and change the distribution of HDL subpopulations (Salazar *et al.*, 2001). Thus, in this study the up-regulation of SAA may be related to the presence of secondary infections during the development of MF. The down-regulated SAA has the same role as ApoA-II. CYS-C is a 13 kDa protein which is a member of the cysteine inhibitor family, produced by karyocytes. It is widely distributed in body fluids and is an effective inhibitor of cathepsin (Comnick and Ishani, 2011; Lafarge *et al.*, 2010). It can also inhibit antigen processing and presentation, thus reducing the immune response. It can increase filtration in the glomerulus, showing complete reabsorption and catabolism, so that the plasma CYS-C concentration is considered to represent the Glomerular Filtration Rate (GFR) (Taglieri *et al.*, 2009). Thus, the immune function of cows with MF usually decreases which may have a close association with the up-regulation of CYS-C. However, this finding also needs further confirmation. In general, the four proteins which were both up-regulated and down-regulated in affected cows in this study are possibly associated with the different physiological and pathological pathways involved in MF. This suggests that these proteins may play dual roles in the development of MF and are worthy of further research attention in the future.

Finally, the ten differential proteins identified in this study were not able to establish a new model for diagnosis according to the decision tree analysis. However, the compound with m/z of 2959.85 may be considered as a diagnostic biomarker to establish a new model for the diagnosis of MF, due to its high accuracy (92.5%), sensitivity (90.5%) and specificity (93.2%) (Fig. 5). However, it was not identified by the SELDI-TOF-MS analysis. Therefore, it will be necessary to investigate this compound further using other methods.

CONCLUSION

In summary, this study is the first to explore the plasma proteomics of cows with MF, using SELDI-TOF-MS and to identify successfully ten differential proteins. The results suggest that these ten proteins are altered when cows develop MF. This study may contribute to researcher understanding of the relationship between MF and these ten proteins.

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