Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Available online at www.sciencedirect.com
ScienceDirect
Agricultural Sciences in China
2009, 8(10): 1263-1269
October 2009

Comparative Proteomic Analysis of Plasma from Clinical Healthy Cows and Mastitic Cows

YANG Yong-xin[1,2], ZHAO Xing-xu[1] and ZHANG Yong[1]

[1] *College of Veterinary Medicine, Gansu Agricultural University, Lanzhou 730070, P.R.China*
[2] *Institute of Animal Husbandry and Veterinary Medicine, Anhui Academy of Agricultural Sciences, Hefei 230031, P.R.China*

Abstract

The current research presents the protein changes in plasma from healthy dairy cows and clinical mastitic cows using two-dimensional gel electrophoresis (2-DE). After staining with silver nitrate and Coomassie Blue, differential expression proteins were detected by PDQuest 7.4 software, and then subjected to ion trap mass spectrometer equipped with a Surveyor HPLC System, differential spots of protein were identified. Three protein spots that originated from preparation gels were identified to be two proteins. Overall, haptoglobin precursor was up-regulated in cows infected with clinical mastitis and could be a mastitis-associated diagnostic marker, whereas SCGB 2A1 (secretoglobin, family 2A, member 1) was down-regulated protein. Plasma protein expression patterns were changed when cows were infected with mammary gland inflammation; it suggests that analysis of differential expression protein might be useful to clarify the mechanisms involved in the pathophysiology, and find new diagnostic markers of mastitis and potential protein targets for treatment.

Key words: dairy cows, clinical mastitis, plasma, proteome

INTRODUCTION

Blood can be regarded as a special form of connective tissue with cells separated by liquid plasma. When the body is infected with the disease, the mediators are derived from the cells and tissues through either active secretion or leakage from blood cells or tissues, and then, enter the circulatory System to cause the changes of the blood composition. In particular, plasma protein is a heterogeneous mixture of a large number of constituents, involved in the organism's defense against infection and regulation of the osmotic pressure as well as small molecule transport, which have been functionally characterized and associated with disease processes.

After the Human Plasma Project, proteomics were widely used to investigate the protein biomarkers and potential protein targets for therapies in plasma for human diseases, including cancer (Ahmed *et al*. 2005; Huang *et al*. 2006). To investigate the mechanisms of mammary gland secreted milk and infected disease, previous research workers studied protein expression patterns using 2-DE and mass spectrometry in plasma from healthy cows, subsequently, the changes of the milk whey proteins in dairy cows affected with mastitic inflammation and from unaffected animals using proteomic approach, and subsequently some proteins were identified to associate with disease (Baeker *et al*. 2002; Hogarth *et al*. 2004; Talamo *et al*. 2003). These results suggested that protein expression patterns during clinical and subclinical milk change could be applied to search for new diagnostic marker of mastitis. Up to the present, the researchers failed to consider the

This paper is translated from its Chinese version in *Scientia Agricultura Sinica.*
YANG Yong-xin, E-mail: yangyongxin66@yahoo.com.cn; Correspondence ZHAO Xing-xu, Professor, Tel: +86-931-7632482, E-mail: zhaoxx@gsau.edu.cn

doi: 10.1016/S1671-2927(08)60337-5

changes of plasma protein when cows were infected with clinical mastitis. This investigation was to detect the expressed proteins in plasma from clinical mastitic and healthy dairy cows by 2-DE providing a platform for parallel analysis, and then, to identify differential proteins by ion trap mass spectrometer equipped with HPLC System, in order to probe the pathogenesis of mastitis and find new biomarkers of mastitis-associated proteins for diagnosis and treatment.

MATERIALS AND METHODS

Samples preparation and quantification

Blood samples were obtained from 12 healthy cows and 12 dairy cows infected with clinical mastitis in a dairy farm by jugular venipuncture and were anticoagulated with 0.5% EDTA. Clinical mastitis cases were identified on the basis of clinical symptoms of heat, pain, redness, and swelling of the udder or clots in the milk. Healthy dairy cows were confirmed with the LMT (Lanzhou Mastitis Test, Lanzhou, China), which is similar to CMT (California Mastitis Test) method. The blood samples were centrifuged at 3 000 × g for 20 min at 4°C, the suspension was collected and pooled, and used as the 2-DE sample that was assayed for protein concentration using 2-D Quant Kit (Amersham Bioscience, Sweden). The remainder of protein samples were stored in aliquots at -75°C until further use.

2-DE

For the first dimension, isoelectric focusing (IEF) was carried out using commercially dedicated apparatuses, Protean IEF cell (Bio-Rad, CA, USA). IPG strips were used according to the manufacturer's instructions. 3-5 μL plasma protein mixed with 10% SDS/2.3% DTT for 5 min at 95°C (Steel *et al*. 2003), containing 200 μg protein for silver nitrate staining gels or up to 1.5 mg for colloidal Coomassie Blue staining gels, were diluted to 300 μL with rehydration solution (7 mol L^{-1} urea, 2 mol L^{-1} thiourea, 4% CHAPS, 65 mmol L^{-1} DTT, 0.5% pH 3-10 IPG buffer, and trace bromophenol blue), and applied by passive rehydration for 12 h with a nonlinear pH 3-10 gradient strips. The IEF steps were carried out at 20°C on the Protean IEF cell as previously described (Yang *et al*. 2007), briefly, for 30 min at 250 V, 1 h at 1 000 V, and then, a gradient was applied from 1 000 to 10 000 V in 5 h, and focusing was continued at 10 000 V for 8 h to give a total of 80 kV h. After the IEF, IPG strips were placed in reducing equilibration buffer that contained 6 mol L^{-1} urea, 2% SDS, 20% glycerol, 50 mmol L^{-1} Tris-HCl (pH 8.8), 0.01% (w/v) bromophenol blue, and 2% DTT for 15 min with shaking followed in alkylating equilibration buffer with 2.5% (w/v) iodoacetamide instead of 2.0% DTT for an additional 15 min. Then, strips were transferred on to 12% T polyacrylamide gel, low melting agarose was overlaid that contained 0.1% SDS, and 37.5 mmol L^{-1} Tris (pH 8.8) was warmed to its melting point and was used to seal the surface of the IPG strips. Separation in the second dimension was carried out using Protean II Xi electrophoresis apparatus (Bio-Rad, CA, USA) and Tris-glycine-SDS (pH 8.3) as the electrode buffer. The gels were started with 50 V and continued with 200 V until the bromophenol blue dye marker had reached the bottom of the gel.

Protein visualization and image analysis

Protein spots were visualized with silver staining as previously described (Mortz *et al*. 2001), and micropreparative gels were stained with Coomassie Blue. Stained gels were matched and detected with the PDQuest software (ver. 7.4, Bio-Rad, USA). Three independent samples were repeated for each pooled sample. Gel images were conducted to remove backgrounds and to automatically detect protein spots. For comparisons of protein levels among gels, normalization was done against the total intensity of all spots present in the gel.

In-gel digestion

Differentially expressed protein spots were excised manually using pipette tips, which were clipped off to form an orifice with an inner diameter of 2 mm and put into 1.5 mL microtubes. The gel particles were washed three times with MilliQ water for 30 min and twice with MilliQ water/acetonitrile (1:1, v/v) for 15 min at room temperature, and then, gel pieces were shrunk in

100 μL acetonitrile. Subsequently, the dried particles were subjected to 50 mmol L^{-1} ammonium bicarbonate containing 0.01% sequence-grade trypsin incubated at 37°C overnight. The peptides were extracted with 50% acetonitrile and 0.2% formic acid, and applied one round of vortexing and sonication (20 min each), and subsequently, the supernatant was transferred to a clean microtube. This step was repeated.

Protein identification and database search

Peptide mass was determined using ion trap mass spectrometer (LCQ Deca XP Plus, Thermo Finnigan) equipped with a Surveyor HPLC System (Thermo, USA). Before injecting the peptide mixes, 150 mm × 0.18 mm BioBasic-18 column (Thermo, USA) was equilibrated at a flow rate of 120 μL min^{-1}. Mobile phase A consisted of water and 0.1% formic acid, and mobile phase B consisted of acetonitrile and 0.1% formic acid. Peptides were eluted with 0.1% formic acid/acetonitrile linear gradient in 120 min. In the full scan mode, MS spectra for all samples were measured with an overall mass/charge (m/z) range of 400-2 000, and MS/MS was performed in data-dependent mode. Peptides were identified using SEQUEST software (Bioworks 2.0, Thermo Finnigan) to search against the publicly available NCBI nonredundant protein database (http://www.ncbi.nlm.nih.gov). The protein identification criteria were based on delta correlation score Delta CN ($\geqslant$0.1) and cross-correlation score Xcorr (one charge$\geqslant$1.9, two charges$\geqslant$2.2, three charges$\geqslant$3.75).

RESULTS

Differential protein analysis in plasma

The experiments were repeated three times under the same experimental conditions. The gel images were automatically matched and analyzed using PDQuest 7.4 software to determine protein spots that differentially expressed. Although protein spots in region A rectangle from healthy cows plasma and region B rectangle from clinical mastitic cows presented significant difference in silver-stained 2-DE maps, they were not different in Coomassie Blue gels; only three spots were detected to be differentially expressed with changes in the stain density of threefold or more in both stained 2-DE maps, that is, spot 1 was a down-regulated protein, whereas spots 2 and 3 were up-regulated in plasma from cows infected with clinical mastitis (Fig.1).

Differential protein spots identification by LC-MS

The differential spots were excised from the gels and tryptic peptides were extracted; then, the peptides were automatically analyzed by HPLC equipped with ion trap mass spectrometer, and protein identification was carried out by peptide sequencing with the search programs of SEQUEST. Three protein spots were identified to be two proteins, listed in Table. A representative tandem spectra of a tryptic peptide obtained from spot 3 was shown in Fig.2. The double-charged peptide ions with a m/z ratio of 726.62 was effectively fragmented to yield sufficient structural information for identification peptide, VETGSEATADSCPK.

Identification proteins analysis

From the search result, we found that spots 2 and 3 were identified as haptoglobin precursor, which have similar molecular weight and different iso-electric point values in 2-DE maps. Moreover, O-linked and N-linked glycosylations are the most common form of post-translational modifications. Then, we used the bioinformatics software tools (http://www.cbs.dtu.dk/services/NetOGlyc/; http://www.cbs.dtu.dk/services/NetNGlyc/) to predict O-linked and N-linked glycosylation sites of haptoglobin precursor, but the research result showed this protein was absent in O-linked and N-linked glycosylation sites. This phenomenon was because haptoglobin precursor occurred in other forms of post-translational modifications as well as mutant.

DISCUSSION

Stained methods analysis

Protein visualized with silver-stained methods is com-

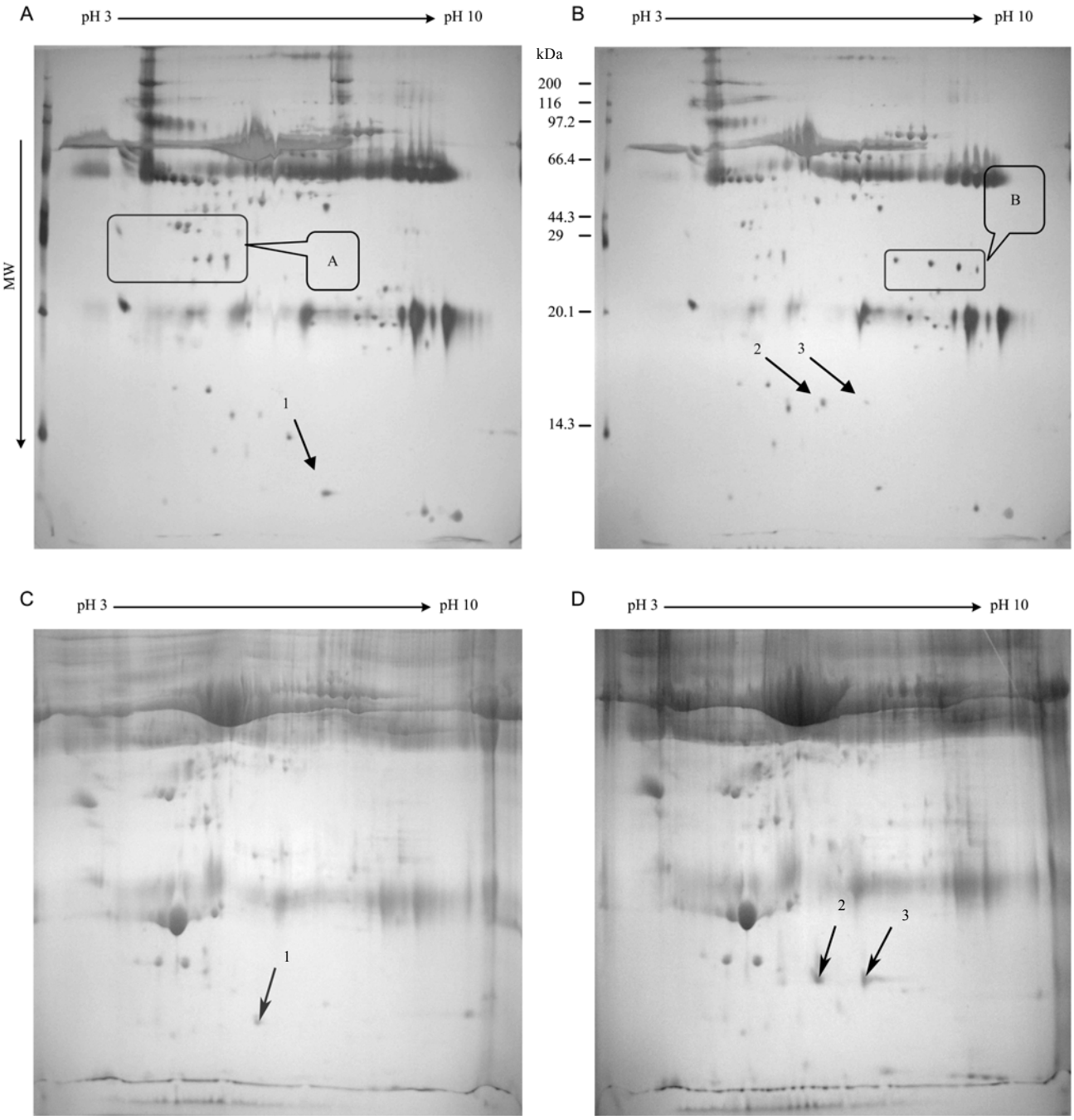

Fig. 1 2-DE maps of plasma protein from healthy dairy cows and clinical mastitic cows. Differential expression of proteins 1, 2, and 3 identified by MS/MS. A, B, proteins were stained by silver nitrate; C, D, proteins were stained by colloidal Coomassie Blue. A, C, 2-DE maps of healthy dairy cows; B, D, 2-DE maps of clinical mastitic cows.

Table Identification of differential expression plasma proteins by LC-MS/MS

Spot	Protein name	Accession	*p*I; MW	Sequence	Coverage (%)
Down-regulated proteins in cows infected with CM					
1	Secretoglobin, family 2A, member 1	AAH62218	5.48; 10 884.8	TINSDISIPEYK	31.58
Differentially expressed proteins only in cows infected with CM					
2	Haptoglobin precursor	HPBO	4.64; 4 681.2	VETGSEATADSCPK	31.11
3	Haptoglobin precursor	HPBO	4.64; 4 681.2	VETGSEATADSCPK	31.11

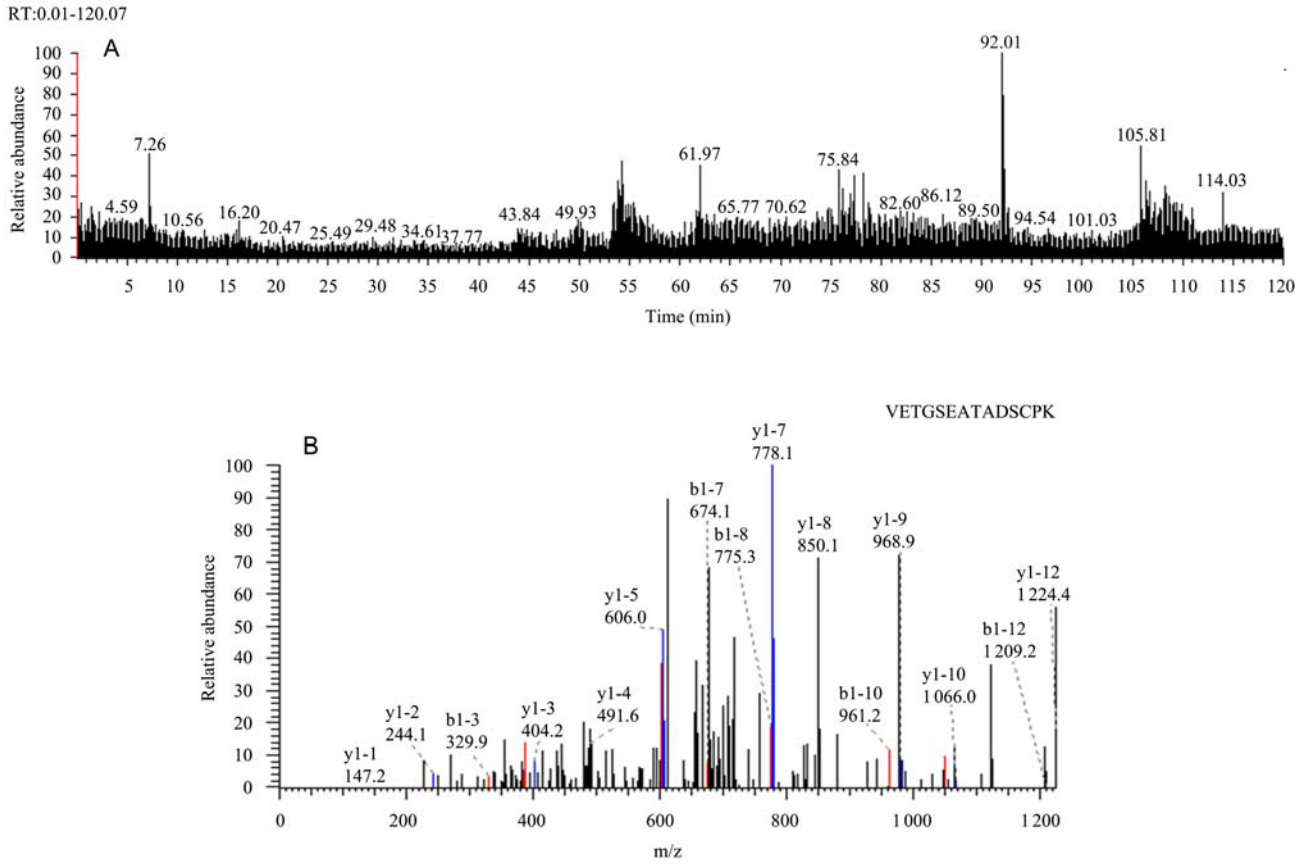

Fig. 2 LC-MS/MS profile analysis of protein spot 3. A, the total ion current of spot 3 using mass spectrum analysis. The *x* axis represents the retention time of the analysis, and the *y* axis represents ion current detected; B, MS/MS analysis of the double-charged peptide ions with a m/z ratio of 726.62. b and y designate the N- and C-terminal fragments of the peptide produced by breakage at the peptide bond in LCQ, respectively, corresponding to the peptide VETGSEATADSCPK.

monly used in analytical gels of comparative proteomics approach, because of their highly sensitive range of detection, typically 1-10 ng of protein per spot. However, silver-staining methods are hardly sensitive to visualization for some proteins, which have not provided compatible identification using mass spectrometry. Colloidal Coomassie is commonly used with preparative gels that is greatly compatible with mass spectrometry and has a detection of 8-50 ng (Simpson 2003). Although spots in regions A and B rectangle between healthy cows and clinical mastitic cows plasma presented significant difference in silver-stained 2-DE maps, in this study, they were not different in Coomassie Blue gels, because protein samples in micropreparative gels had not effectively separated or as a result of the protein visualized with silver-stained and Coomassie Blue G-250 methods inherently had significant difference in sensitivity.

Haptoglobin precursor

Haptoglobin was a major acute phase protein, which has host defense and antibacterial activities. It connects to free hemoglobin released from erythrocytes to form haptoglobin-haemoglobin complex, and thereby, inhibits its oxidative activity and sequesters the iron within hemoglobin, and furthermore preventing iron-using bacteria benefitting from hemolysis. Recently, haptoglobin was an up-regulated one in hepatocellular carcinoma, severe acute respiratory syndrome, and ovarian cancer using proteomic approach, and regarded as a useful diagnostic marker (Ahmed *et al*. 2005; Ang *et al*. 2006; Wan *et al*. 2006). However, comparative analysis of breast cancer serum using 2-D differential gel electrophoresis combination with MALDI-TOF/TOF found that haptoglobin was a down-regulated protein (Huang *et al*. 2006). Interestingly, haptoglobin con-

centrations were increased in milk and serum from cows with experimentally induced acute and chronic *Staphylococcus aureus* matitis and from cows with clinical mastitis, indicating that it is an acute phase protein (Eckersall *et al*. 2001; Eckersall *et al*. 2006; Gronlund *et al*. 2003). Our data showed that the haptoglobin precursor also increased in plasma of cows infected with clinical mastitis, and it might play a significant role in the early response to invasion of mammary by pathogenic bacteria.

SCGB 2A1

Recently, SCGB 2A1 (also know as mammaglobin) is predicted to be a secreted protein and when mRNA as lymph node micrometastases in breast cancer is expressed, there is an increase in the expression of mammaglobin in mammary gland of breast cancer patients, and it is also identified as a novel serum marker for detection of breast cancer (Bernstein *et al*. 2005; Ooka *et al*. 2000; Watson *et al*. 1999). However, mammaglobin was found in human normal prostate tissue and absent in tumor samples without significant differences (Tucker *et al*. 2005). Also, its mRNA levels did not vary significantly between wild-type and estramustine-resistant cells in prostate and ovarian cancer cell lines. In our data, although mammaglobin is identified in serum and is a down-regulated protein when cows are infected with clinical mastitis, the function of the mammaglobin protein associated with intramammary inflammation in cows remains unknown. It might provide valuable information to clarify the mechanisms involved in the pathogenesis of mammary.

CONCLUSION

To probe protein expression pattern changes in plasma from healthy dairy cows and clinical mastitic cows, this subject analyzed and identified differential protein spots using 2-DE and ion trap mass spectrometer equipped with HPLC System. Three protein spots originated from preparation gels were identified to be two proteins, haptoglobin precursor was an up-regulated protein, while SCGB 2A1 was a down-regulated protein in cows infected with clinical mastitis. Proteomic approach analysis of protein changes in plasma might be useful to clarify the mechanisms involved in the pathophysiology of cows infected with clinical mastitis, to find new diagnostic markers of mastitis, and to find potential protein targets for treatment.

Acknowledgements

The project was supported by the China International Sci & Tech Cooperation Project (2005 DFA30720).

References

Ahmed N, Oliva K T, Barker G, Hoffmann P, Reeve S, Smith I A, Quinn M A, Rice G E. 2005. Proteomic tracking of serum protein isoforms as screening biomarkers of ovarian cancer. *Proteomics*, **5**, 4625-4636.

Ang I L, Poon T C, Lai P B, Chan A T, Ngai S M, Hui A Y, Johnson P J, Sung J J. 2006. Study of serum haptoglobin and its glycoforms in the diagnosis of hepatocellular carcinoma: a glycoproteomic approach. *Journal of Proteome Research*, **5**, 2691-2700.

Baeker R, Haebel S, Schlatterer K, Schlatterer B. 2002. Lipocalin-type prostaglandin D synthase in milk: a new biomarker for bovine mastitis. *Prostaglandins and Other Lipid Mediators*, **67**, 75-88.

Bernstein J L, Godbold J H, Raptis G, Watson M A, Levinson B, Aaronson S A, Fleming T P. 2005. Identification of mammaglobin as a novel serum marker for breast cancer. *Clinical Cancer Research*, **11**, 6528-6535.

Eckersall P D, Young F J, McComb C, Hogarth C J, Safi S, Weber A, McDonald T, Nolan A M, Fitzpatrick J L. 2001. Acute phase proteins in serum and milk from dairy cows with clinical mastitis. *Veterinary Record*, **148**, 35-41.

Eckersall P D, Young F J, Nolan A M, Knight C H, McComb C, Waterston M M, Hogarth C J, Scott E M, Fitzpatrick J L. 2006. Acute phase proteins in bovine milk in an experimental model of *Staphylococcus aureus* subclinical mastitis. *Journal of Dairy Science*, **89**, 1488-1501.

Gronlund U, Hulten C, Eckersall P D, Hogarth C, Persson W K. 2003. Haptoglobin and serum amyloid A in milk and serum during acute and chronic experimentally induced Staphylococcus aureus mastitis. *Journal of Dairy Research*, **70**, 379-386.

Hogarth C J, Fitzpatrick J L, Nolan A M, Young F J, Pitt A, Eckersall P D. 2004. Differential protein composition of bovine whey: a comparison of whey from healthy animals and from those with clinical mastitis. *Proteomics*, **4**, 2094-2100.

Huang H L, Stasyk T, Morandell S, Dieplinger H, Falkensammer

G, Griesmacher A, Mogg M, Schreiber M, Feuerstein I, Huck C W, Stecher G, Bonn G K, Huber L A. 2006. Biomarker discovery in breast cancer serum using 2-D differential gel electrophoresis/MALDI-TOF/TOF and data validation by routine clinical assays. *Electrophoresis*, **27**, 1641-1650.

Mortz E, Krogh T N, Vorum H, Görg A. 2001. Improved silver staining protocols for high sensitivity protein identification using matrix-assisted laser desorption/ionization-time of flight analysis. *Proteomics*, **1**, 1359-1363.

Ooka M, Sakita I, Fujiwara Y, Tamaki Y, Yamamoto H, Aihara T, Miyazaki M, Kadota M, Masuda N, Sugita Y, Iwao K, Monden M. 2000. Selection of mRNA markers for detection of lymph node micrometastases in breast cancer patients. *Oncology Reports*, **7**, 561-566.

Simpson R J. 2003. *Proteins and Proteomics: A Laboratory Manual*. Cold Spring Harbor, New York.

Talamo F, D'Ambrosio C, Arena S, Del Vecchio P, Ledda L, Zehender G, Ferrara L, Scaloni A. 2003. Proteins from bovine tissues and biological fluids: defining a reference electrophoresis map for liver, kidney, muscle, plasma and red blood cells. *Proteomics*, **3**, 440-460.

Tucker J M, Lipatova Z, Beljanski V, Townsend D M, Tew K D. 2005. Evaluation of lipophilins as determinants of tumor cell response to estramustine. *Journal of Pharmacology and Experimental Therapeutics Fast Forward*, **315**, 1158-1162.

Wan J, Sun W, Li X, Ying W, Dai J, Kuai X, Wei H, Gao X, Zhu Y, Jiang Y, Qian X, He F. 2006. Inflammation inhibitors were remarkably up-regulated in plasma of severe acute respiratory syndrome patients at progressive phase. *Proteomics*, **6**, 2886-2894.

Watson M A, Dintzis S, Darrow C M, Voss L E, DiPersio J, Jensen R, Fleming T P. 1999. Mammaglobin expression in primary, metastatic, and occult breast cancer. *Cancer Research*, **59**, 3028-3031.

(Edited by ZHANG Juan)