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Comparative Proteomic Profile of Canine Uterus with Pyometra

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Abstract : Pyometra, a common disorder in intact bitches, can lead to canine sepsis. Identification of biomarkers for sources of infection in the uterus using two-dimensional electrophoresis (2-DE)-mass spectrometry (MS) analysis may enable the discovery of novel diagnostic markers of sepsis. Toward this end, surgically resected uterus samples from four bitches (three pyometra and one healthy) were randomly selected for 2-DE-MS, which identified 32 differentially expressed proteins, including seven inflammatory proteins, five non-inflammatory proteins, and 20 functionally unknown proteins. Despite the limited information on canine uterus proteomics, we suggest the potential use of differentially expressed uterus proteins as candidate biomarkers to discover targets to attenuate inflammation in pyometra. Further identification of the functionally unknown proteins is warranted.

Key words **:** bitch, inflammatory protein, uterus, 2DE.

Introduction

Canine pyometra is a common disease of older, sexually intact female dogs (4,8). It is characterized by the accumulation of uterine pus with increased risk of opportunistic bacterial infections. Canine pyometra may cause subclinical and unrecognizable conditions but can also rapidly trigger sepsis (3,5). Thus, identification of biomarkers from the sources of inflammation would enable the early detection of canine sepsis and could improve predictions of disease outcome. One of the most precise methods to screen for potential biomarkers is proteomics analysis, which provides information on the entire protein complement of an organism, tissue, or cell (7). Unfortunately, proteomics profile data from dog tissue samples are limited, and there are no proteomics data specifically related to canine pyometra. Thus, the objective of the current study was to identify candidate biomarkers for sources of inflammation in the canine uterus using a proteomics approach, which can reveal proteins and endpoint functions for ultimate identification of novel diagnostic markers of pyometra.

Materials and Methods

We randomly selected uterine tissue samples from pyometra (n = 3; dog #1: 12 years, Poodle; dog #2: 5 years, Maltese; dog #3: 13 years, Maltese) and healthy (n = 13 years, Chihuahua) bitches, which were subjected to two-dimensional electrophoresis (2-DE) combined with matrix-assisted laser desorption/ionization tandem time-of-flight tandem (MALDI-TOF/TOF) mass spectrometry (MS). Confirmation of the diagnosis of pyometra was based on clinical signs, such as vomiting, polyuria, polydipsia, abdominal ultrasound as well as a gross examination of the uterine system (i.e., pus-filled enlarged uterus during ovariohysterectomy). The researcher analysing uterine samples was blinded to the outcome of each dog. This study was conducted with the approval of the Institutional Animal Care and Committees (IACUC) GNU-190218-D0010.

Total proteins were extracted from uterine tissues as described in our previous study (15). Protein concentrations were determined using a Pierce™ BCA protein assay kit (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's protocol, and equal amounts of proteins were loaded and separated using two-dimensional electrophoresis (2-DE). Isoelectric focusing was carried out using GE Healthcare Immobiline™ DryStrip Gels (18 cm, pH 4-7; Amersham Biosciences, Uppsala, Sweden) as previously described (15). Following 1-DE, 2-DE was performed using 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis with Protean-II XI electrophoresis equipment (Bio-Rad, Hercules, CA, USA). After silver nitrate staining (15), the gels were scanned and densitometer image analysis using Progenesis SameSpots software (version 4.0) was performed as described previously (13) to identify significantly differential protein spots (spot intensity fold-change ≥ 1.5 with $P < 0.05$). Selected spots were digested and processed for MS analysis using trypsin digestion and subjected to MALDI-TOF/TOF MS

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using an ABI 4800 Plus TOF-TOF Mass Spectrometer (Applied Biosystems, Woburn, MA, USA). The differentially expressed proteins were identified using the MASCOT program (http://www.matrixscience.com) as described previously (16), and functionally classified using the National Center for Biotechnology Information and Universal Protein Resource Knowledgebase (UniProtKB) databases.

Results

Comparison of the 2-DE patterns of the healthy control (Fig 1A) and pyometra (Figs 1B-D) samples identified a total of 44 differentially expressed protein spots, and 32 proteins were successfully identified by MALDI-TOF/TOF MS. After eliminating duplicate proteins, 12 proteins were finalized according to data available in the UniProt protein database. Table 1 lists the identified proteins with corresponding accession numbers, analytical molecular weight, analytical isoelectric point, sequence coverage, number of peptide matches, MOWSE score, and fold-change between groups (Table 1).

Among the differentially expressed proteins, we further focused on proteins associated with inflammation, which reflects disease conditions. Of the 12 functionally-known proteins, two upregulated proteins—insulin-like growth factor binding protein 1 (IGFBP1) and L-lactate dehydrogenase B chain (LDHB)—and three downregulated proteins—vitamin D-binding protein (VDBP), actin, alpha cardiac muscle 1 (ACTC1), and aldehyde dehydrogenase mitochondrial (ALDH2)—were consistently identified from all three dogs. Of the 20 functionally unknown proteins, only one upregu-

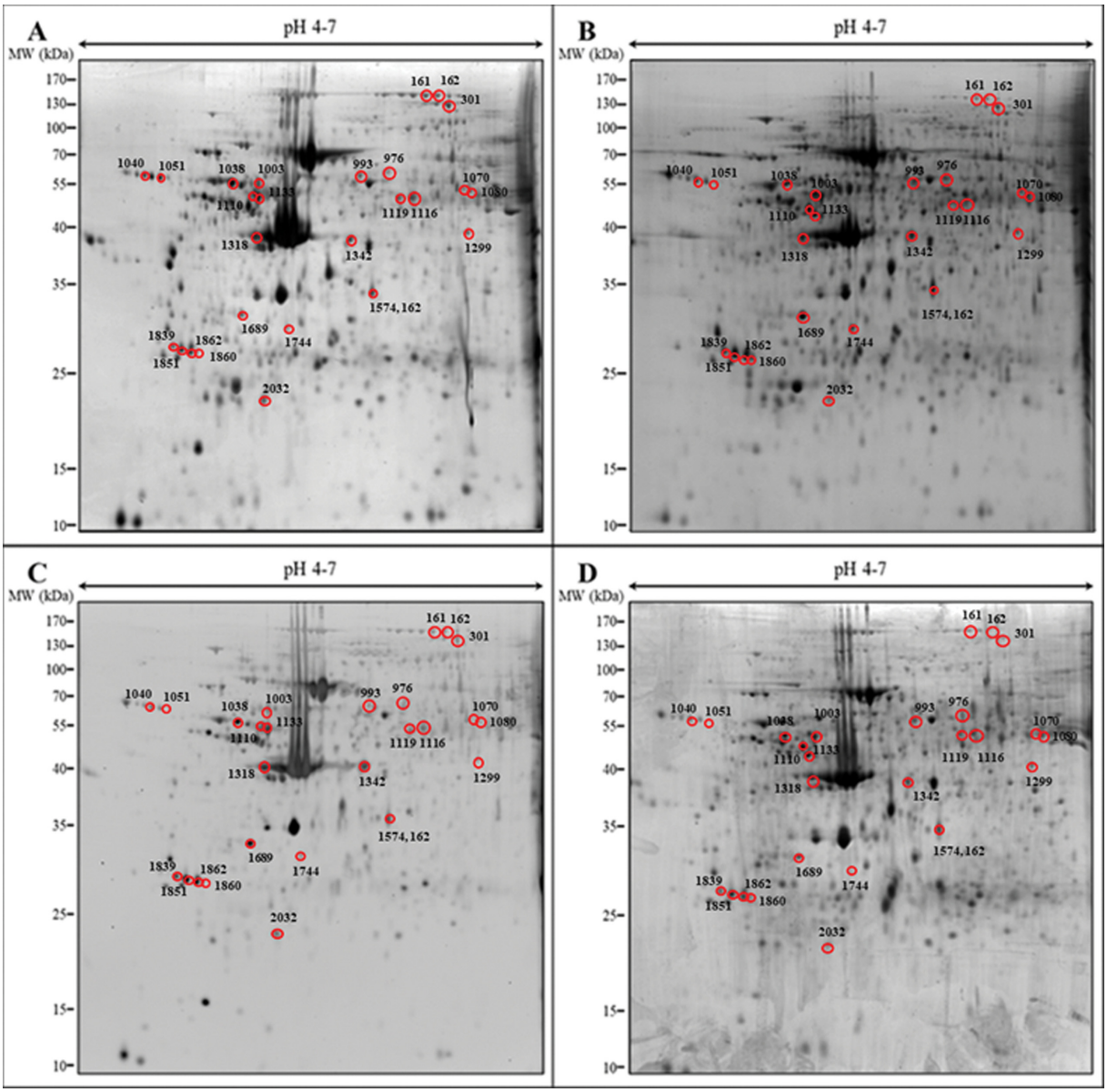

Fig 1. Representative two-dimensional gel electrophoresis (2-DE) maps of uterine tissues from healthy (A) and pyometra dog #1 (B), dog #2 (C), and dog #3 (D). The samples were resolved by 2-DE on pH 4-7 IPG strips followed by separation on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels in the second dimension. Proteins were visualized by silver nitrate staining. The numbers indicate successfully identified protein spots, and the numbers correspond to the spot numbers in Table 1. The experiments were performed in triplicate.

Table 1. List of identified proteins that were differentially expressed between healthy and pyometra dogs

Spot No.	NCBI ID	Protein name	MOWSE score	Sequence coverage (%)	Protein MW (Da)	pI	Fold change	Up & Down pyometra dog #1	Up & Down pyometra dog #2	Up & Down pyometra dog #3	Biological function
161	gi|545560773	Collagen alpha-2(VI) chain-like	181	25	48147	5.13	3.8	↓	↓	↓	N/A
162	gi|1239973556	Vam6/Vps39-like protein isoform X2	40	13	87436	6.59	2.4	↑	↑	↓	N/A
301	gi|345798988	Vinculin	376	24	128023	5.61	3.9	↓	-	↓	Cellular response
976	gi|545485772	T-complex protein 1 subunit alpha isoform X2	87	10	59466	5.7	2.8	↓	-	↓	N/A
993	gi|1239956979	5-azacytidine-induced protein 2 isoform X2	45	20	33262	6.63	2.3	↓	-	↓	N/A
1003	gi|1239898771	Pre-mRNA-splicing factor SLU7	31	13	74000	6.69	3.8	↓	↓	↓	N/A
1038	gi|121583756	Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1 precursor	131	8	46505	5.58	2	↓	↓	↓	N/A
1038	gi|1239973050	Myocardial zonula adherens protein isoform X2	35	10	50621	5.53	2	↓	↓	↓	N/A
1040	gi|545553759	Alpha-2-HS-glycoprotein	78	7	40021	5.12	2.7	↓	-	↓	Inflammation
1051	gi|928186573	Calcium-activated potassium channel subunit alpha-1 (Fragment)	48	7	131922	6.04	2.2	↓	-	↓	Ion homeostasis
1070	gi|545514079	T-complex protein 1 subunit beta isoform X3	99	19	56867	5.69	3.7	↓	-	↓	N/A
1080	gi|73996559	Keratin, type II cuticular Hb6	42	5	55016	5.55	3.3	↓	↓	↓	N/A
1110	gi|73975215	Vitamin D-binding protein isoformX2	342	25	54536	5.2	3.4	↓	↓	↓	Vitamin D metabolism
1116	gi|73995214	Aldehyde dehydrogenase, mitochondrial isoform 2	435	23	57183	6.63	2.2	↓	↓	↓	Inflammation
1119	gi|545528914	Selenium-binding protein 1	260	25	52904	5.79	2.2	-	-	↓	Selenium binding
1133	gi|1150763464	Tubulin alpha-1A chain	364	39	50788	4.94	3.4	↓	↓	↓	N/A
1133	gi|359323129	Tubulin alpha-1C chain	280	39	50562	4.97	3.4	↓	↓	↓	N/A
1299	gi|345794141	Peptidyl-prolyl cis-trans isomerase CWC27 homolog	34	9	53686	6.14	1.9	↓	-	↓	mRNA Splicing
1299	gi|545515462	Calmodulin-lysine N-methyltransferase isoform X5	48	8	30034	6.15	1.9	↓	-	↓	N/A
1318	gi|57108093	Actin, alpha cardiac muscle 1	178	25	42334	5.23	4	↓	↓	↓	Muscle contraction
1318	gi|1239958855	Tumor protein D54 isoform X18	36	13	21636	6.45	4	↑	↑	↑	N/A
1342	gi|359320104	Creatine kinase B-type	419	30	42960	5.47	2.1	↓	-	↓	Cellular homeostasis
1574	gi|356461040	L-lactate dehydrogenase B chain	109	22	36884	5.71	2.4	↑	↑	↑	Carbohydrate metabolism

Table 1. Continued

Spot No.	NCBI ID	Protein name	MOWSE score	Sequence coverage (%)	Protein MW (Da)	pI	Fold change	Up & Down pyometra dog #1	Up & Down pyometra dog #2	Up & Down pyometra dog #3	Biological function
1574	gi|545554868	Polypeptide N-acetylgalactosaminyl-transferase 13 isoform X5	58	15	57857	6.21	2.4	↑	↑	↓	N/A
1689	gi|57100553	Annexin A5 isoform X1	717	37	35978	4.99	3.6	-	-	↓	N/A
1744	gi|1239956606	LOW QUALITY PROTEIN: golgin subfamily A member 4	38	2	263259	5.28	2.9	↑	↑	↓	N/A
1839	gi|545525240	Insulin-like growth factor binding protein 1	141	22	28012	5.89	4.2	↑	↑	↑	Hormone signal
1851	gi|73983524	Mitotic spindle assembly checkpoint protein MAD2A	35	16	23727	5.02	2.1	↑	↑	↓	N/A
1851	gi|928151832	14-3-3 protein zeta/delta	188	44	27899	4.73	2.1	↑	↑	↓	N/A
1860	gi|1239930884	BEN domain-containing protein 3 isoform X2	46	4	86781	5.4	2.3	↑	-	↓	N/A
1862	gi|73992048	14-3-3 protein beta/alpha isoformX2	114	19	27947	4.76	7.2	↑	↑	↓	N/A
2032	gi|359322074	Peroxiredoxin-2 isoform 1	334	31	22112	5.23	2.7	-	↓	↓	Antioxidant

N/A, not available.

lated protein (tumor protein D54 isoform X18) was consistently identified, whereas six downregulated proteins were consistently identified: collagen alpha-2(VI) chain-like; tubulin alpha-1A chain; tubulin alpha-1C chain; serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1 precursor; Myocardial zonula adherens protein isoform X2; keratin, type II cuticular Hb6; Pre-mRNA-splicing factor SLU7.

Discussion

Several previous studies used microarray analyses to determine differences in mRNA levels in canine uterus (1,9,19). However, to the best of our knowledge, this is the first study focused on identifying differentially expressed proteins in the canine uterus following naturally occurring bacterial infections using proteomics analysis. The mRNA expression of IGFBP1 was upregulated (9,19), as was the protein level in this study. However, the upregulation of LDHB has not been reported. Validation of protein by immunoblotting assay, and functional analysis should be confirmed in future studies. Moreover, owing to the limited nature of information available on NCBI databases, only 12 differentially expressed proteins among the 32 spots identified could be individually characterized, seven of which were ultimately determined to be associated with inflammatory pathways, including two upregulated and five downregulated proteins in the pyometra samples. Thus, further identification of the functionally unknown proteins is warranted.

The identified proteins were predominantly related with tumor cell proliferation, cellular homeostasis, autophagy, and inflammation. Changes in the same proteins in different dogs with pyometra could be related to differences in immune status. IGFBP1, which was upregulated in the pyometra uterus, displays normal T cell expression, and was shown to significantly suppress the proliferation and megakaryocytic differentiation of hematopoietic stem cells in vitro (16). Thus, IGFBP1 may play a significant role in the regulation of inflammatory and angiogenic proteins (14). Interestingly, the present 2-DE results are consistent with previous microarray mRNA analyses (9,19). The other upregulated protein, LDH, is considered to regulate efficient anaerobic/glycolytic metabolism in tumor cells, thereby reducing reliance on oxygen to facilitate survival in the hypoxic tumor microenvironment (10,12). Furthermore, LDH was suggested to originate in tumor tissues and in healthy tissues damaged by tumor expansion and invasion, serving as a marker to differentiate lung cancer from benign lung disease and healthy controls (2). Among the three downregulated in pyometra dogs, VDBP is an albumin-like protein that requires cell-surface binding to mediate its functions (21). Its biological roles reportedly include vitamin D metabolite transport, C5a chemotactic factor binding, fatty acid transport, actin sequestration, and inhibition of angiogenesis (11,20). Interestingly, VDBP has drawn attention as a precursor of the potent macrophage activation factor Gc protein-derived macrophage activating factor, which releases pro-inflammatory cytokines in the affected area (6). Thus, VDBP levels are significantly reduced in septic patients and non-septic intensive care patients compared to healthy controls. ALDH2 was also downregulated in all three pyometra dogs, consistent with a previous study (17). ALDH2 is an enzyme that is crucial for the detoxification of reactive

aldehydes (22). ALDH2 exhibits high genetic polymorphism in humans, which severely compromises its enzymatic activity with anti-inflammation effects (18).

Overall, our comparative proteomics analysis is an efficient technique to compare altered protein expression between pyometra and healthy dogs. However, further studies are required to validate the relationship of these proteins and changes in their expression levels with regard to canine pyometra. These findings will be further supported by real-time quantitative polymerase chain reaction and immunohistochemistry. In addition, protein analysis of serum samples would be advantageous in identifying diagnostic biomarkers. Technical challenges, such as depletion of albumin from serum samples, should be considered in order to identify low abundance proteins in the future.

Such precise identification of uterine inflammatory proteins is expected to be beneficial in diagnosing and understanding the pathophysiology of canine pyometra at the proteomic level. As the first study to identify proteomic profiles in response to inflammation within the uterus, these preliminary results can guide further studies through more detailed identification of inflammatory protein biomarkers with clinical application in veterinary medicine.

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Conflict of Interest

No conflicts of interest to declare.

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