



# Analysis of differentially expressed proteins in Muscovy duck embryo fibroblasts infected with virulent and attenuated Muscovy duck reovirus by two-dimensional polyacrylamide gel electrophoresis

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**ABSTRACT.** Muscovy duck reovirus (MDRV) belongs to the Orthoreovirus genus of the Reoviridae family, which is a significant poultry pathogen leading to high morbidity and mortality in ducklings. However, the pathogenesis of the virus is not well understood. In the present study, two-dimensional (2D) polyacrylamide gel electrophoresis (PAGE) combined with LC-MS-MS was used to identify differentially expressed proteins between Muscovy duck embryo fibroblasts (MDEF) infected with virulent (MV9710 strain) and attenuated (CA strain) MDRV and non-infected MDEFs. A total of 115 abundant protein spots were identified. Of these, 59 of differentially expressed proteins were detected, with functions in metabolism and utilization of carbohydrates and nucleotides, anti-stress, and regulation of immune and cellular process. GO analysis of the identified proteins showed that they belonged to the classes molecular function (141 proteins), cellular component (62 proteins), and biological process (146 proteins). The results were validated by qRT-PCR, which suggests that the analysis method of 2D PAGE combined with LC-MS-MS used in this study is reliable. This study lays a foundation for further investigation of the biology of MDRV infection in MDEF.

**KEY WORDS:** 2D technology, muscovy duck embryo fibroblasts (MDEF), muscovy duck reovirus (MDRV), qRT-PCR

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Muscovy duck reovirus (MDRV) is the causative agent of a disease characterized by clinical signs of general weakness, viral arthritis/tenosynovitis, diarrhea, growth retardation, fibrinous pericarditis, and even sudden death [4, 18, 19, 25]. The virus is an important cause of morbidity and mortality in Muscovy ducklings between 2 and 4 weeks of age. MDRV was first described in South Africa in 1950 [10], and the virus was isolated in France in 1972 [4].

MDRV belongs to the Orthoreovirus genus of the Reoviridae family, with genomes consisting of 10 segments of double-stranded RNA [13, 29]. With the exception of the S4 gene, most of the genes are mono-cistronic. Reovirus replication occurs in the cytoplasm. However, reovirus infection can perturb host cell nuclear functions, leading to cytopathic effects in infected cells and tissue injury in the infected host. MDRV p10.8 has been confirmed to be functionally related to cellular lesions *in vivo* and *in vitro* [6, 7]. In our previous study, an attenuated MDRV strain was obtained by serial alternate passages on Muscovy duck embryo fibroblasts (MDEF) and chick embryo fibroblasts (CEF) [3]. The attenuated virus did not cause disease in one-day-old ducklings [8]. However, the molecular mechanisms underlying MDRV-host interaction have not been elucidated and little is known about the host cells' response against MDRV strains of differing virulence.

Two-dimensional (2D) PAGE combined with LC-MS-MS is effective for comparing paired protein samples directly on 2D gels, but its previous application in the proteomics of cells infected by MDRV has been very limited, particularly for the comparison of MDEF infected with virulent and attenuated MDRV strains. The aim of this study was therefore to characterize the proteome of MDEFs infected with virulent and attenuated MDRV. The results shed light on the biology and function of MDRV infection in MDEF and contribute to the identification of markers as potential drug targets.

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## MATERIALS AND METHODS

### *Ethics statement*

The animal protocol used in this study was approved by the Research Ethics Committee of the College of Animal Science, Fujian Agriculture and Forestry University (Permit Number PZCASFAFU2014002). All ducks were handled in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals approved by the State Council of China.

### *MDRV strains and cell culture*

The virulent MDRV strain WM9710 was isolated from Muscovy ducks in Fujian Province [8]. The attenuated virus strain CA was obtained by serial alternate passages on MDEF and CEF. After infection with CA strain, ducklings at one day old showed clinical symptoms as identified in a previous study [3]. MDEF were derived from 11-day-old Muscovy duck embryos. Cells were cultured in DMEM supplemented with 10% fetal bovine serum, at 37°C in 5% CO<sub>2</sub>.

### *Collection of virulent and attenuated MDRV*

The WM9710 and CA strains were preserved in liquid nitrogen. Before use, they were rapidly thawed in a 38°C water bath, and two-day-old ducks were infected with the thawed virus intraperitoneally. After three serial passages in ducks, liver samples from ducks infected with virulent and attenuated MDRV were homogenized in PBS (pH 7.2) with 100 units/ml penicillin and 0.1 mg/ml streptomycin to give a 20% suspension (w/v). The suspensions were centrifuged at 12,000 g for 15 min. The supernatant of virulent MDRV (10% v/v) was inoculated into MDEF ( $2 \times 10^5$ ), and 8 days later, the cells were harvested to obtain the WM9710 strain. The supernatant of attenuated MDRV (1% v/v) was inoculated into MDEF ( $2 \times 10^5$ ), and the cells were harvested to obtain the CA strain at 3 days post-infection.

### *Protein extraction*

Cells infected with strains of differing virulence were washed by three rounds of centrifugation and suspension in PBS. Pre-cooled acetone was then added to the sediment and incubated at -20°C for 30 min. After removing the acetone, the cells were lysed in a lysis buffer (L1) containing 8 M urea, 4% CHAPS, 40 mM Tris-HCl (pH 8.5, Amresco, OH, U.S.A.), 1 mM PMSF (Amresco), 2 mM EDTA, and 10 mM DTT, sonicated on ice (180 W, 2 sec duration with 3 sec intervals for 5 min), and centrifuged at 20,000 g for 30 min at 15°C. The supernatant was transferred to a new centrifuge tube and 3 volumes of acetone were added. The mixture was then precipitated at -20°C for 30 min. The precipitate was subjected to a second round of lysis in L1 buffer and then sonicated. Finally, the mixtures were centrifuged at 20,000 g for 30 min at 15°C, and the supernatant was recovered. The concentrations of the samples were determined by 2-D Quant-Kit (GE Healthcare, Pittsburgh, PA, U.S.A.) according to the manufacturer's instructions.

### *Protein separation by 2D PAGE*

A total of 600 µg protein was mixed with 50 mM DTT and 0.8% immobilized pH gradient (IPG) buffer (GE Healthcare) prior to isoelectric focusing electrophoresis (IEF) and subsequent SDS-PAGE. The labeled mixtures were loaded onto Immobiline DryStrip gels IPG, 18 cm, linear pH gradient from 4–7, GE Healthcare). IPG strips were then subjected to IEF. Electrophoresis was performed at 20°C, with the following settings: 500 V for 60 min, 1,000 V for 60 min, 8,000 V for 2 hr, 8,000 V for 3 hr, and finally, 500 V overnight. After IEF, the IPG strips were equilibrated in equilibration solution containing 1% DTT for 15 min and then in the same solution containing 25% iodoacetamide instead of DTT. Proteins were separated by running the gels at 2 W/gel for 45 min and then at 18 W/gel at 10°C until the bromophenol blue reached the bottom of the gels.

### *Spot picking and enzymatic digestion*

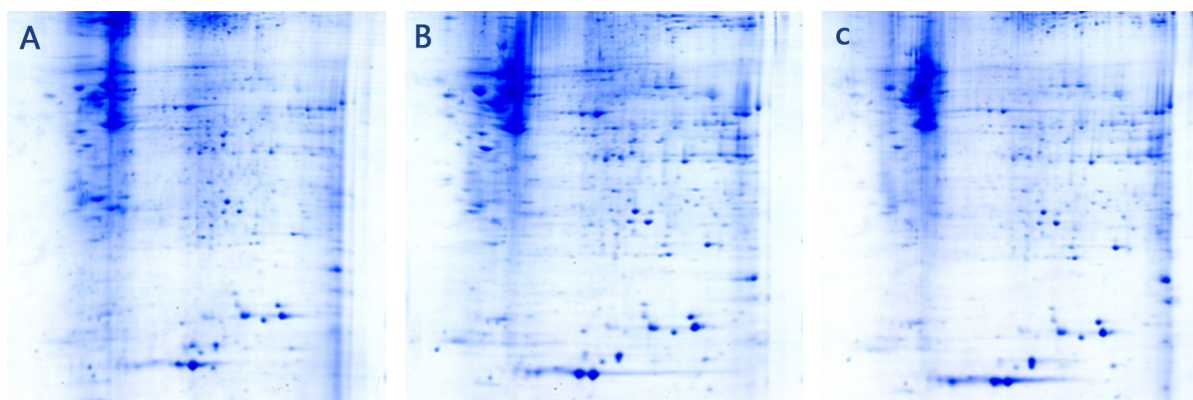
The abundant protein spots were selected, excised manually from the coomassie brilliant blue (CBB)-stained preparative gel and placed into 1.5 ml Eppendorf tubes. The gel pieces were washed twice with 1 ml MilliQ water, destained with 50% acetonitrile (ACN) and 25 mM ammonium bicarbonate (NH<sub>4</sub>HCO<sub>3</sub>), and dried under vacuum. Proteins within the dried gel pieces were then reduced by incubation in 10 mM DTT for 60 min at 56°C and alkylated by incubation in 55 mM iodoacetamide for 45 min in the dark. The gel pieces were then dehydrated in ACN at room temperature for 10 min before rehydration in 25 mM NH<sub>4</sub>HCO<sub>3</sub>. Following a second round of dehydration in ACN, the gel pieces were incubated in 1 µg/µl trypsin (Promega, Beijing, China) at 4°C overnight. The enzyme digestion reaction was terminated in 0.1% formic acid (FA).

### *Identification of protein spots by LC-MS-MS*

A total of 10 µl peptide was loaded onto a 2 cm C18 trap column and then eluted onto a 10 cm analytical C18 column (inner diameter 75 µm) on the Prominence nano 2D system (Shimadzu, Kyoto, Japan). The samples were separated using a gradient of Buffer B (100% ACN and 0.1% FA) from 2 to 80% and returning to 5% in 60 min. The peptides were subjected to nano-electrospray ionization followed by proteomics analyzer mass spectrometry (MS/MS) in MicroTOF-QII (BrukerDaltonics, Billerica, MA, U.S.A.) coupled online to the HPLC. MS/MS was performed as previously reported [17].

### *Database searching and bioinformatics analysis*

The experimental MS data were matched to a corresponding virtual peptide mass database derived from Mascot search engine



**Fig. 1.** Proteomic comparisons among Muscovy duck embryo fibroblasts (MDEF) infected with virulent and attenuated Muscovy duck reovirus (MDRV) and non-infected MDEFs using 2D PAGE. A: MDEF infected with virulent MDRV; B: MDEF infected with attenuated MDRV; C: Control MDEF.

**Table 1.** Primers for verification of mRNA transcriptional levels of genes identified by 2D PAGE with LC-MS-MS analysis from MDEF infected with virulent and attenuated MDRV and non-infected MDEFs

Gene name	Access number	Primer sequence
Annexin A1	Q92108	PF:5'- AAGAGCCTGGAAGAAGACTT-3' PR:5'- TTGAAAGTCTCCAGACG-3'
Alpha-enolase	Q9PVK2	PF:5'- GATCTTGCTGGGAATCCTG-3' PR:5'- ACTGTAGCCAGCCTTGTT-3'
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	O57479	PF:5'-CAACCATGAAAAGTATGACA -3' PR:5'-TCCAGTAGATGCTGGAAT-3'
Alcohol dehydrogenase [NADP (+)]	Q6AZW2	PF:5'- CCAGATGATGTGGAGGAGG-3' PR:5'- GCCAGCCCTTGGTCAACC-3'
Eukaryotic initiation factor 4A	Q2NL22	PF:5'-GATCTTGGCTCCAACAAGAG-3' PR:5'-CAAAGACACGCCAGGTGTG-3'
Guanine nucleotide-binding protein	Q4R7Y4	PF:5'-GGATGAGACCAACTATGGA-3' PR:5'-TCGAGATCCAGAGACAATC-3'
Vimentin	Q4R4X4	PF:5'-ATACCCGCACCGACGAG-3' PR:5'- CCCGCATCTCCTCCTCGT-3'

v2.3.01, the uniprot-fungi database, which consists of 708,539 sequences and 310,953,869 residues. Protein identification was carried out by MALDI-TOF MS using the Mascot software (<http://www.matrixscience.com>). The search parameters were: fixed modification, variable modification, carbamidomethylation; variable modifications, Oxidation (M), peptide tolerance of  $\pm 0.05$  Da, fragment mass tolerance of  $\pm 0.1$  Da. The protein name and functional annotations were analyzed using Blast and the Gene Ontology (GO) database, respectively.

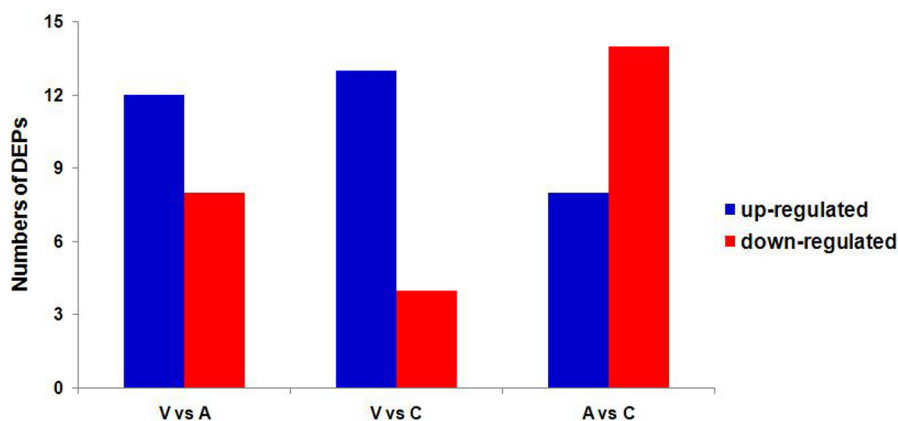
#### Quantitative real-time PCR verification

Total RNA was isolated from cells infected with both virulent and attenuated MDRV using Trizol reagent (Invitrogen, Waltham, MD, U.S.A.), according to the manufacturer's instructions. RNA was then dissolved in RNase-free ddH<sub>2</sub>O (Sangon, Shanghai, China). Ten micrograms of purified mRNA from cells infected by both virulent and attenuated MDRV was used as template in a reverse-transcription reaction performed with random primers and M-MLV reverse transcriptase (Promega) according to standard protocols. The cDNAs were used for quantitative real time PCR (qPCR). The sequences of SYBR Green-labelled primers (listed in Table 1) were synthesized by Sangon. The RT-PCR was performed on an ABI PRISM<sup>®</sup> 7500 Sequence Detection System (Applied Biosystems, Thermo, Shanghai, China).  $\beta$ -actin was used as a housekeeping gene. The differences in qPCR quantitation were compared using one-way ANOVA.

## RESULTS

#### Two-dimensional analysis of abundant proteins

Proteins extracted from cells infected with virulent and attenuated MDRV and non-infected cells were separated using 2D PAGE. Figure 1 shows images of the different samples stained by bromophenol blue. A total of 115 spots were identified across analytical



**Fig. 2.** Numbers of differentially expressed proteins from Muscovy duck embryo fibroblasts (MDEF) infected with virulent and attenuated Muscovy duck reovirus (MDRV) and non-infected MDEFs. V: MDEF infected with virulent MDRV; A: MDEF infected with attenuated MDRV; C: Control MDEF.

gels after the removal of redundant and meaningless peptides. The identified spots were analyzed using pair-wise comparisons (virulent (V) vs. attenuated (A), A vs. control (C), and V vs. C). The number of proteins identified as being differentially expressed between A and C (70 proteins) was slightly higher than that for V vs. A (63 proteins) and V vs. C (61 proteins).

#### Identification of different expressed proteins (DEPs) in MDEF infected with virulent and attenuated MDRV and nothing

Following bioinformatic analysis, 59 spots were identified as showing statistically significant changes in intensity of >1.5-fold among samples. More up-regulated genes were detected in MDEF infected with virulent MDRV than in MDEF infected with attenuated MDRV (Fig. 2). However, after infection with the attenuated MDRV, more genes were down-regulated than up-regulated in MDEF (Fig. 2).

DEPs among the three groups of MDEFs were identified as being involved in diverse functions including metabolism and utilization of carbohydrates and nucleotides (e.g. pyruvate kinase, Guanine nucleotide-binding protein), anti-stress (e.g. thioredoxin-dependent peroxide reductase, stress-induced-phosphoprotein), regulation of immune and cellular process (e.g. annexin, transmembrane emp), and other crucial developmental processes (Table 2).

#### Functional classification of identified proteins

The gene function classification system GO was used to predict the possible functions of the identified proteins. As shown in Fig. 3, three categories were identified: molecular function, cellular component, and biological process. The most common category was biological process (146 proteins), and as the least common was cellular component (62 proteins). Within these categories, the most common sub-categories were enzyme regulator in the molecular function category (22% of proteins identified) and metabolic process in the biological process category (21%) (Fig. 3). EH domain-containing protein 3 was the only protein predicted to belong to the sub-category of membrane-enclosed lumen (0.3%).

#### Quantitative real-time RT-PCR validation of identified proteins

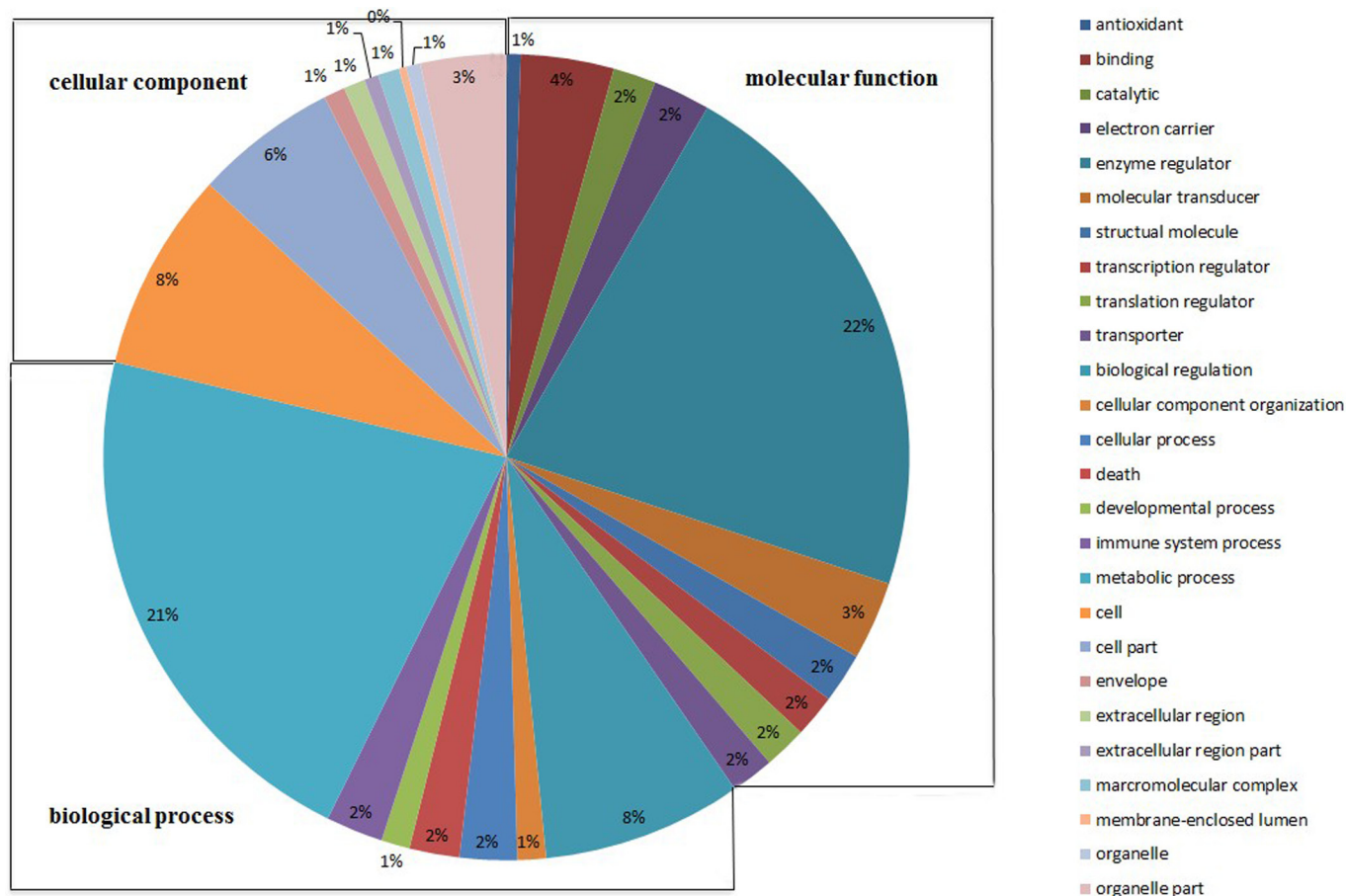
Seven genes identified by 2D PAGE were selected for qRT-PCR analysis to quantify their transcriptional levels: annexin A1 (GenBank access No. Q92108), alpha-enolase (GenBank access No. Q9PVK2), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (GenBank access No. O57479), alcohol dehydrogenase [NADP (+)] (GenBank access No. Q6AZW2), eukaryotic initiation factor 4A (GenBank access No. Q2NL22), guanine nucleotide-binding protein (GenBank access No. Q4R7Y4), and vimentin (GenBank access No. Q4R4X4). The results of qPCR analysis were generally consistent with the results of 2D PAGE with LC-MS-MS listed in Table 2 (Fig. 4), suggesting that the method of 2D PAGE with LC-MS-MS used in this study is reliable.

## DISCUSSION

Quantitative proteomics allows the comparison of distinct proteomes to identify proteins that display changes in abundance or post-translational state. Two-dimensional technology relies on pre-electrophoretic separation on gels, and this technique is widely used [1, 25]. Recently, this technique has been applied for identifying biomarkers, designing novel drug targets, and monitoring therapeutic processes. Here, the 2D technology was used to investigate DEPs from MDEF infected by virulent and attenuated MDRV, and a total of 59 DEPs were identified, providing a foundation for analysis of the pathogenesis of MDRV.

The annexins are a group of cellular proteins that are involved in changes in cell shape, trafficking and organization of vesicles, exocytosis, endocytosis, and other important cellular and physiological processes [5]. Annexins have also been identified



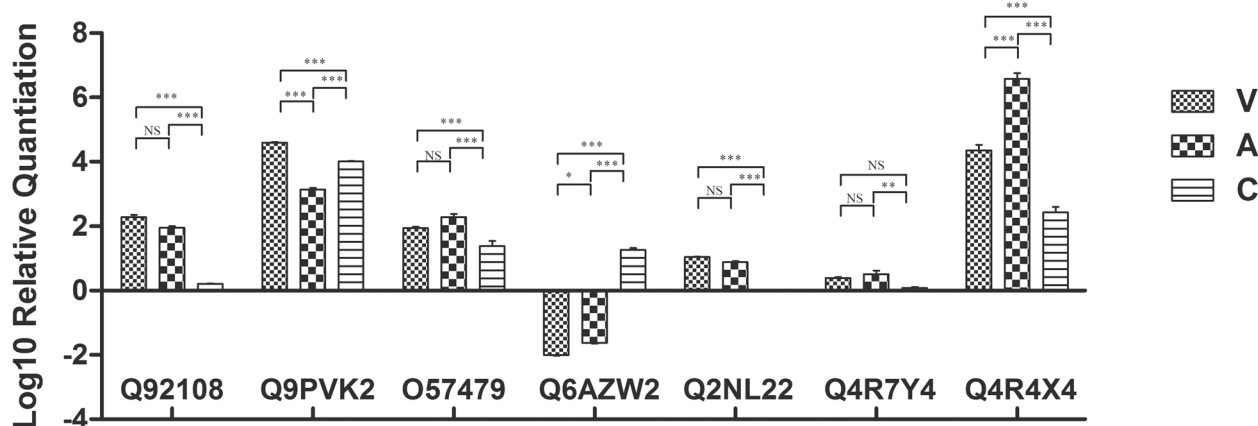


**Fig. 3.** Classification of protein functions by Gene Ontology (GO). Proteins were classified into three ontologies: molecular function, cellular component, and biological process.

**Table 2.** Selected proteins identified from Muscovy duck embryo fibroblasts (MDEF) infected by virulent and attenuated Muscovy duck reovirus (MDRV) and non-infected MDEF cells

Accession	Description	Coverage	Unique peptides	Ratio		P value	
				A/V	V/C	A/V significance	V/C significance
P09525	Annexin A4	19.6	4	0.21	10.78	2.93E-14	5.62E-12
P08132	Annexin A4	9.7	2	0.93	2.19	0.836291	0.0473
P17785	Annexin A2	52.0	15	0.28	6.27	6.98E-10	5.41E-07
Q92108	Annexin A1	13.9	3	0.47	9.18	2.81E-07	1.57E-09
Q9PVK2	Alpha-enolase	5.4	2	0.93	2.39	0.7795	0.048
O57479	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	19.2	3	1.04	1.59	0.9654	0.169
P00340	L-lactate dehydrogenase A	15.3	4	3.81	1.03	0.00420413	0.762
Q6AZW2	Alcohol dehydrogenase [NADP (+)]	5.2	1	0.28	8.57	9.07E-10	6.81E-08
P14950	Annexin A1 isoform p35	10.7	2	0.15	11.38	7.85E-20	5.41E-21
A5A6I5	Fructose-bisphosphate aldolase A	6.9	1	0.64	8.34	0.069	5.73E-08
Q9Z0V6	Thioredoxin-dependent peroxide reductase	20.1	4	0.79	0.91	0.19	0.76
Q5ZLN1	Phosphoglycerate mutase 1	43.6	6	2.83	7.16	0.042	6.50E-08
Q90593	78 kDa glucose-regulated protein	73.2	29	0.29	1.41	9.10E-12	0.27
P00940	Triosephosphate isomerase	62.8	13	1.03	1.19	0.93	0.59
Q90635	Dihydropyrimidinase-related protein 2	49.3	8	0.38	8.16	1.09E-05	7.64E-09
P09117	Fructose-bisphosphate aldolase C	33.9	5	0.28	5.49	2.23E-13	4.35E-07
Q2NL22	Eukaryotic initiation factor 4A	21.3	3	0.94	7.31	0.91	5.17E-09
Q4R7Y4	Guanine nucleotide-binding protein	48.5	7	1.36	1.04	0.18	0.94
Q4R4X4	Vimentin	68.1	14	1.16	6.53	0.4	2.76E-08
P00548	Pyruvate kinase	71.6	14	0.68	1.06	0.25	0.97
P84173	Prohibitin	59.2	12	0.19	11.38	3.06E-17	4.45E-14

Note: V: MDEF infected with virulent MDRV; A: MDEF infected with attenuated MDRV; C: Control MDEF.



**Fig. 4.** Confirmation of differentially expressed genes of from Muscovy duck embryo fibroblasts (MDEF) infected with virulent and attenuated Muscovy duck reovirus (MDRV) and non-infected MDEFs by qRT-PCR. Comparison of the relative quantitation of each gene expressed in V, A and, C in pairs. A: MDEF infected with virulent MDRV; B: MDEF infected with attenuated MDRV; C: Control MDEF. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , NS: not significant.

outside the cell in the extracellular space and have been associated with the processes of fibrinolysis, coagulation, inflammation, and apoptosis [24, 28]. Annexin A-II is the most prominent in mediating fibrinolysis. Annexin A-II serves as a receptor for plasminogen, which functions to produce plasmin [11]. Plasmin initiates fibrinolysis by degrading fibrin, which would otherwise lead to hemorrhage [14]. Here, five annexin isoforms were identified, and four of them had significantly higher expression in MDEF infected with virulent MDRV compared to cells infected with attenuated virus. This result was verified by qRT-PCR. Clinical features of MDRV infection in Muscovy ducks include nephrorrhagia and necrosis of the liver. Further studies are required to elucidate the role of annexins that are highly expressed in ducks infected by virulent MDRV.

MDRV infection in ducks causes apoptosis. The mechanism of MDRV-induced apoptosis is thought to be complex and multifactorial, and has not been well studied to date. The present study suggests that dysregulation of the peroxiredoxin system may be involved in apoptosis in MDRV infection. *In vitro* infection with virulent MDRV resulted in significantly lower expression of TDPR protein compared to infection with attenuated virus. Thioredoxin-dependent peroxidases have previously been shown to protect against apoptosis by scavenging hydrogen peroxide [20]. Thioredoxin 2 is a critical regulator of cytochrome c release and mitochondrial apoptosis [16, 27], and transmembrane thioredoxin-related molecule (TMX) has a protective role in endoplasmic reticulum (ER) stress-induced apoptosis [21]. Further study is needed to identify whether MDRV infection induces dysregulation of the peroxiredoxin system.

Glycolysis is the metabolic pathway that converts glucose into pyruvate, resulting in the release of free energy in the form of the high-energy molecules ATP (adenosine triphosphate) and NADH (reduced nicotinamide adenine dinucleotide). Glycolysis has been reported to be related to infection with viruses, including influenza [2], hepatitis C [9], and Kaposi's Sarcoma-associated Herpesvirus [26]. Here, a series of proteases involved in the glycolysis pathway, including triosephosphate isomerase, pyruvate kinase, phosphoglycerate mutase 1, L-lactate dehydrogenase A, fructose-bisphosphate aldolase A, and glyceraldehyde-3-phosphate dehydrogenase, were identified in cells infected with MDRVs. The relationship between changes in the glycolysis pathway and MDRV infection should be further investigated.

Prohibitin (PHB) is a widely expressed protein that functions as a molecular chaperone and is involved in cell cycle regulation, maintenance of mitochondrial structure, anti-apoptotic processes, senescence, and proliferation [22]. The protein is reported to be selectively up-regulated in infective pathogen forms which could be critical for disease pathogenesis [15, 23]. For example, PHB plays a role in the internalization of Chikungunya virus (CHIKV) as a receptor protein [30], and was shown to have antiviral activity against Theiler's murine encephalomyelitis virus (TMEV) [12]. In the present study, PHB was enriched in all three groups.

GO analysis was used to predict the possible functions of the identified abundant proteins. The analysis revealed that many of these proteins possess critical functions related to viral invasion, duplication, survival, and strain virulence. The functions of these proteins in biological processes should be further validated experimentally.

In conclusion, a total of 115 proteins were identified in this study by 2D PAGE with LC-MS-MS analysis in MDEF infected with virulent and attenuated MDRV and non-infected MDEFs, and 59 proteins were found to be differentially expressed in pairwise comparisons (A versus V, A versus C and V versus C). GO analysis indicated that many proteins are involved in crucial functions regarding enzyme regulation, metabolic processes, and biological regulation. The identification of these proteins has the potential to enable a better understanding of the pathogenesis of MDRV infection in MDEF, and suggests that further studies in other Reoviridae family viruses are warranted.

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