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REVIEW

Application of proteomics to investigation of viral diseases in livestock and poultry

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Summary. – Proteomics is widely used to investigate and understand viral infections in livestock and poultry. The effects of infections on abundance, post-translational modifications, and interactions of host cell proteins have been systematically studied using proteomic methods, such as two-dimensional electrophoresis and mass spectrometry. Such research increases our understanding of the pathogenesis of viral infections and contributes to the development of novel anti-viral drugs for the livestock and poultry industries.

Keywords: proteomics; infectious diseases; poultry and livestock; application

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Abbreviations: AIV = avian influenza virus; CSFV = classical swine fever virus; ESI-MS = electrospray ionization mass spectrometry; Hp = haptoglobin; hpi = hours post infection; IBDV = infectious bursal disease virus; IFN = interferon; iTRAQ = isobaric tag for relative and absolute quantitation; LC-MS/MS = liquid chromatography tandem mass spectrometry/mass spectrometry; MALDI-TOF-MS = matrix-assisted laser analytical desorption/ionization time-of-flight mass spectrometry; MS = mass spectrometry; MDV = Marek's disease virus; NF-κB = nuclear factor kappa B; PBMCs = peripheral blood mononuclear cells; PCV2 = porcine circovirus type 2; PEDV = porcine epidemic diarrhea virus; PK-15 = porcine kidney cells 15; PRRSV = porcine reproductive and respiratory syndrome virus; PTMs = post-translational modifications; SILAC = stable isotope in labeling by amino acids in cell culture; TGEV = transmissible gastroenteritis virus

1. Advances in proteomic technologies

The proteome encompasses all proteins expressed in cells, tissues, or whole organisms (Fields, 2001). The term "proteomics" is derived from a combination of "protein" and "genomics". Proteomics mainly involves studying the types and abundance of all proteins expressed in cells, tissues, or entire organisms, followed by exploration of protein functions and the molecular basis of biological activities through bioinformatics. Traditional quantitative proteomic techniques include two-dimensional polyacrylamide gel electrophoresis (2-DE) combined with mass spectrometry (MS), label-free quantitative proteomic technology, isotope labeling of relative and absolute quantitative technology (isobaric tag for relative and absolute quantitation, iTRAQ), and cell-subculture isotope labeling technology (stable isotope labeling by amino acids in cell culture, SILAC) (Fig. 1). These quantitative proteomic methods, combined with the continuous development of MS, are leading to a new era of proteomic research.

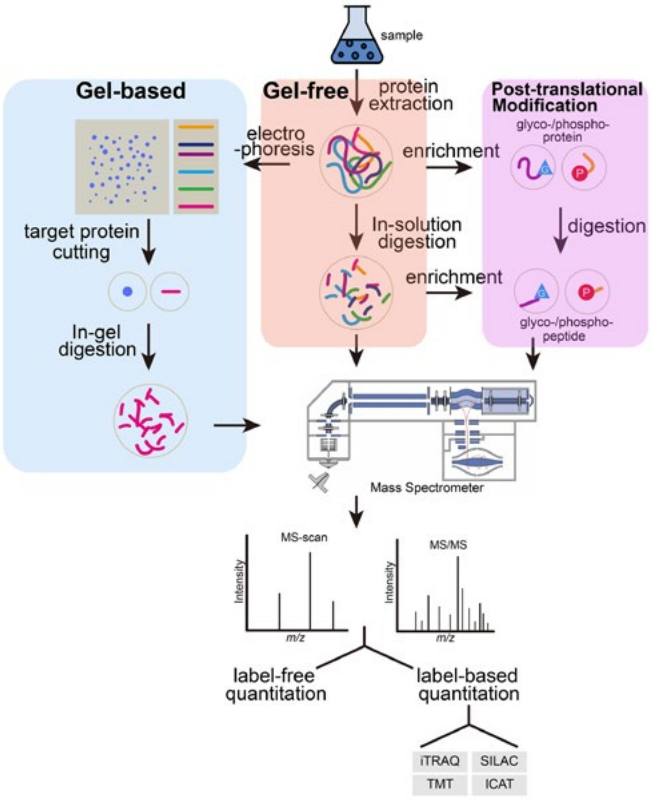

Fig. 1

Overview of the approaches of proteomics

1.2 Gel-based proteomics

Proteomic techniques can be divided into gel-based and gel-free proteomics (Zargar *et al.*, 2016). The first stage of 2-DE is isoelectric focusing which uses different pH gradients according to chemical characteristics of the proteins and causes them to accumulate at different pH values, achieving separation. The second stage is sodium dodecyl sulfate polyacrylamide gel electrophoresis, which transfers the isoelectric focusing electrophoresed proteins to the top of sodium dodecyl sulfate polyacrylamide gel electrophoresis gel for separation according to their different molecular masses. Following electrophoresis, a two-dimensional electrophoretogram can be obtained by staining with Coomassie blue. The protein of interest can be identified by analyzing the diagram with a relevant software and searching against a relative protein database (Gorg *et al.*, 2000). Improvements in the sensitivity of 2-DE have been continuous, and currently up to 10,000 proteins can be separated in a single diagram, making it possible to study proteins on a large scale. However, 2-DE also has limitations, namely that low-abundance proteins cannot be detected, insoluble proteins are not detected by electrophoresis, and repeatability is poor (Wu *et al.*, 2006).

1.3 Gel-free approaches

MS is one of the main techniques used for protein identification. The basic principles of MS are the separation and constituent determination of substances according to differences in molecular weight to weight ratios. Various protein parameters obtained by MS are important for protein identification. The most important component of a mass spectrometer is the ion mass analyzer. Electrospray ionization mass spectrometry (ESI-MS) and matrix-assisted laser analytical desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) are widely used in proteomics at present (Smith *et al.*, 1990). MALDI-TOF-MS is simple and rapid to operate, there is a one-to-one correspondence between peak and sample composition quality, the spectrum is easily analyzed, the ionization efficiency is high, there are few fragment ions and the sensitivity is high (Pieles *et al.*, 1993). The main advantage of ESI-MS is that it can be used in combination with high performance liquid chromatography separation

to achieve the integrated separation and identification of complex compounds and therefore improve detection efficiency (Smith *et al.*, 1990).

Developed by Applied Biosystems in the United States, iTRAQ is an *in vitro* isotope-labeling technique based on the iTRAQ reagent. In recent years, iTRAQ has become one of the main methods utilized in proteomic quantitative research. Compared with traditional proteomic techniques and the unlabeled proteomic quantitative technique, iTRAQ has advantages of high sensitivity, high accuracy and, most importantly, high-throughput functions, which can analyze eight samples simultaneously (Wu *et al.*, 2006). The key to iTRAQ is the tag reagent, which consists of a peptide reaction group and an isobaric tag comprising a reporting ion/group and equilibrium or balancing group. Taking the four-standard kit as an example, the main steps are protein extraction, denaturation/enzyme hydrolysis, peptide labeling, sample mixing, liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS), and the qualitative and quantitative analysis of peptides. The mass number of the ion and the molecular weight of the equilibrium group are reported to constitute a label reagent of equal mass. The reactive group reacts with the amino terminus or lysine side chain of the peptide to label the digested peptide. After the sample is labeled, it enters the first round of MS. Because the isobaric tag has the same molecular weight, the same peptides from different samples show the same mass/charge ratios and mass/charge peaks. When entering the second round of MS, the neutrality of the equilibrium group is lost and the reporting group is separated from the entire label. Different peaks appear at the low mass/charge ratios of the secondary MS, which reflect the relative abundance of the labeled peptide and provide quantitative information for the protein containing the peptide (Smith *et al.*, 1990).

SILAC technology is an isotope metabolic labeling method used to label proteins during cell culture by introducing stable isotopes via a medium containing ^{13}C, ^{13}C-glucose, or ^{15}N-labeled amino acids. Most of the amino acids used for SILAC stable isotope labeling are lysine and arginine. During *in vitro* cell growth and proliferation, isotope-labeled amino acids are used in the synthesis of proteins, which then become isotope-labeled after several cell passages. After MS, the area sizes of the three isotope-labeled peptides in the first round of MS are quantitatively analyzed, and proteins can be also identified by their peptide sequences using secondary MS. SILAC technology is only applicable for *in vitro* subcultured cells; it is difficult to use with samples such as tissues and body fluids, and has a high labeling cost for animal models. However, because of its high labeling efficiency, tiny margin of error, high sensitivity, and accurate quantification, it is one of the most commonly used methods in current comparative proteomic research (Graumann *et al.*, 2008).

Label-free protein quantification differs from isotope-labeling because it does not use expensive isotope labels but uses proteolytic enzymes to decompose peptides during MS analysis. This method overcomes the shortcomings of traditional isotope methods and can obtain quantitative results for complex protein samples quickly and easily.

2. Progress of post-translational modification proteomic research

Protein post-translational modifications (PTMs) are an important regulator of protein function and play a key role in proteomic research into complex regulatory mechanisms of signal transduction in biological activities. It is also the main research focus of dynamic epigenetics. PTMs currently includes 20 common protein modifications, such as SUMOylation (Cuijpers *et al.*, 2017), phosphorylation (Taracha *et al.*, 2017), acetylation (Diallo *et al.*, 2019), glycosylation (Bagdonaite *et al.*, 2018), methylation (Wang *et al.*, 2017), hydroxylation (Elenewski *et al.*, 2015), ubiquitination (Faktor *et al.*, 2019), and ADP-ribosylation (Cohen *et al.*, 2018).

Phosphorylation and dephosphorylation, catalyzed by protein kinases and phosphatases, can alter protein functions such as increasing or decreasing their biological activities, activating or inhibiting signaling pathways, or initiating interactions (Cohen, 2002). At least 30% of proteins in eukaryotes undergo phosphorylation, with the most common type being serine phosphorylation followed by threonine and tyrosine phosphorylation at a ratio of 1800:200:1 (Alonso *et al.*, 2004). However, phosphorylated peptides account for approximately 1%–2% of the total enzymatic peptides (Zarei *et al.*, 2011). Phosphate groups are very unstable and often lost in the process of sample pretreatment, leading to the omission of peptides. Currently, more effective phosphopeptide-enrichment methods, such as TiO_2, immobilized metal affinity chromatography, and affinity chromatography, in combination with high sensitivity and high resolution MS (Chen *et al.*, 2018), are widely used to study protein phosphorylation in the fields of human and animal diseases.

Protein glycosylation is one of the most important and common PTMs and plays an important role in biological processes, such as the immune response (Lowe, 2001) and signal transduction (Daniels *et al.*, 2002). Abnormalities in glycosylation sites and glycosylation levels are closely related to a variety of diseases, such as the occurrence and metastases of tumors, and neurodegenerative and metabolic diseases (Conserva *et al.*, 2016). Many glycoproteins

have been used as biomarkers or drug targets for early warning diagnoses and treatment (Palmigiano *et al.*, 2016). An important branch of proteomics, glycoproteomics, has become a hot topic in proteomic research (Rodriguez *et al.*, 2018).

Glycoproteins are formed by carbohydrate chains covalently linked to specific amino acid residues. In general, protein glycosylation can be divided into N-, O-, and C-glycosylation and glycosylated phosphatidylinositol anchors (Lowe, 2001), according to the amino acid residues linked to the carbohydrate chains and the different connection modes. N-glycosylation is formed when the hemiacetal hydroxyl group at the reduction end of the carbohydrate chain attached to the specific amino acid sequence, resulting in the side chain amide group of an asparagine residue linked through an N-glycosidic bond (Skeene *et al.*, 2017). The amino acid sequence mentioned above refers to N-X-S/T/C (X is any amino acid except proline, S is serine, T is threonine, and C is cysteine). The N-carbohydrate chain can be efficiently and specifically excised by peptide N-glycosidase F. Because N-glycosylation occurs at a specific amino acid sequence, N-glycosylation can achieve large-scale site identification via MS and database retrieval, and it has become the most widely studied form of glycosylation in recent years. Many large-scale N-glycosylation databases have been published, but only 5%–10% of the theoretical predictions have been identified (Ahn *et al.*, 2015). The proportion of glycoproteins in mammals is as high as 50%, but the abundance is usually low. Only 2%–5% of the peptides of glycosylated proteins obtained after enzymatic digestion are glycosylated peptides, and the ionization efficiency of glycosylated peptides in MS is not high. As a result, glycosylated proteins are not easily identified using this technique (Skeene *et al.*, 2017). Therefore, selective enrichment of glycosylated proteins is highly beneficial for glycoproteomics. Currently, the enrichment methods of N-glycosylated proteome samples are mainly divided into lectin-affinity hydrophilic chromatography and boron-affinity chromatography according to the different enrichment mechanisms (Ahn *et al.*, 2015).

3. Application of proteomics to research on livestock and poultry infectious diseases

At present, the frontier of proteomic research includes three main aspects: constitutive, comparative, and interaction proteomics. Due to its strong practicability and wide-scale potential application, comparative proteomics has become the preferred method for studying important life processes and major infectious diseases. The properties of virus-infected cells are not only the result of viral genome replication and expression but also a comprehensive picture of the interactions between viruses and host cells. By comparing the different protein profiles before and after viral infection, we can systematically study the protein abundance, PTMs, localization, and interactions of viral and related host proteins in combination with traditional virology methods. The research can lead to an in-depth understanding of the interactions between viruses and host cells and viral pathogenesis. The following is a review of the effects of several common viral infections of livestock and poultry on host protein expression, PTMs of host proteins, and the interactions between virus and host proteins. Identified proteins with significantly differential expression and interacting proteins are shown in Table 1.

3. 1 Avian influenza virus

Avian influenza is an acute infectious disease caused by avian influenza virus (AIV) and results in huge economic losses to the global poultry industry (Beato *et al.*, 2009). To further understand the molecular mechanisms, by which the AIV subtype H5N1 causes neurological symptoms, 2-DE combined with MS was used to identify differentially expressed proteins in the brain tissues of chickens after H5N1 infection. A total of 31 significantly changed proteins were identified, mainly including cytoskeletal, ubiquitin proteasome-related and nerve signal transduction proteins. Some identified proteins, such as collapsin response mediator protein and septin 5 were found to be involved in Parkinson's and Huntington's diseases, causing neurological dysfunction and even encephalitis. The functional identification of these proteins would provide a strong basis to further understand the mechanisms of AIV-induced neurological dysfunction (Zou *et al.*, 2010).

3.2 Infectious bursal disease virus

Infectious bursal disease, caused by infectious bursal disease virus (IBDV), is an acute, highly contagious disease of chickens (Qi *et al.*, 2016), which induces immunosuppression and leads to co-infection with other infectious agents. A combination of 2-DE and MALDI-TOF/TOF-MS was used to analyze differentially expressed proteins in chicken embryo fibroblasts at 12, 48, and 96 h post IBDV infection, finding that 51 proteins were significantly changed. IBDV had a significant inhibitory effect on the abundance of ubiquitin-regulating proteins, energy metabolism-related proteins, and intermediate filaments, while the virus significantly up-regulated proteins related to signal transduction, poly-ubiquitin protein, heat shock protein (HSP) 27, and eukaryotic translation initiation factor 4A (Zheng *et al.*, 2008). Dynamic changes in the proteomics of chicken fasciae after IBDV infection were

Table 1. Proteomics-based studies for identification proteins after viral infections

Viruses	Samples	Methods	Significantly changed proteins identified	References
AIV	brain tissues of chickens	2-DE combined with LC-MS/MS	31	Zou *et al.*, 2010
IBDV	CEF cells	2-DE combined with MALDI-TOF/TOF-MS	51	Zheng *et al.*, 2008
	chicken fasciae	2-DE combined with MALDI-TOF/TOF-MS	54	Wu *et al.*, 2012
	DF-1 cells	iTRAQ combined with LC-MS/MS	23 cytoplasmic proteins and 34 nuclear proteins	Sun *et al.*, 2015
	chicken bone marrow-derived DCs	iTRAQ combined with LC-MS/MS	134	Yasmin *et al.*, 2019
MDV	chicken spleen	ESI-MS/MS	48	Thanthrige-Don *et al.*, 2009
	chicken bursa	2-DE combined with MS	24	Lu *et al.*, 2010
	chicken tumor tissues	Imaging MS and noncontact laser capture microdissection	IFN-γ induced protein 30 and HSP70	Pauker *et al.*, 2019
PRRSV	PAMs	LC-MS/MS	95	Li *et al.*, 2017
	HEK 293T, MARC-145 and PK-15 cells	Pull-down-coupled MS	14-3-3 and CD2AP	Xiao *et al.*, 2016
	MARC-145, HEK293T cells	Pull-down-coupled MS	PARP-1	Liu *et al.*, 2015
	PAMs	LC-MS/MS	242 and 249 phosphorylated proteins at 12 and 36 hpi	Luo *et al.*, 2014
	PAMs	LC-MS/MS	273 lysine acetylated sites on 218 up-regulated proteins, and 109 lysine acetylated sites on 90 down-regulated proteins at 6 hpi	Fang *et al.*, 2021
	PAMs	LC-MS/MS	983 lysine ubiquitination sites on 717 proteins were altered at 36 hpi	Zhang *et al.*, 2018
PEDV	Vero cells	iTRAQ combined with LC-MS/MS	661 (virulent strain) and 474 (vaccine strain CV777) differentially expressed proteins	Guo *et al.*, 2016
	jejunum tissues of piglets	iTRAQ combined with LC-MS/MS	269 (YN13) and 301 (YN144) differentially expressed proteins	Li *et al.*, 2016
TGEV	PK-15 cells	iTRAQ combined with LC-MS/MS	60 up-regulated proteins and 102 down-regulated proteins	An *et al.*, 2014
	PK-15 cells	iTRAQ combined with LC-MS/MS	69 differentially expressed proteins at 24 hpi	Zhao *et al.*, 2018
CSFV	pig serum	2-DE combined with MALDI-TOF/TOF-MS	10	Sun *et al.*, 2011
	PBMCs	2-DE combined with MALDI-TOF/TOF-MS	34	Sun *et al.*, 2010
CSFV-PCV2	PK-15 cells	iTRAQ-coupled LC-MS/MS	304 up-regulated proteins and 198 down-regulated proteins	Zhou *et al.*, 2017
PCV2	the major pig plasma	2-DE combined with MALDI-TOF/TO F-MS and Nano-LC-ESI MS/MS	Hp was O-glycosylated, the glycosylation level of IgG and IgA were reduced	Marco-Ramell *et al.*, 2014
	PK-15 cells	2-DE combined with MALDI-TOF/TOF-MS	21	Zhang *et al.*, 2009

also studied by 2-DE combined with MALDI-TOF/TOF-MS, and the abundance of most proteins was seen to change between 24 and 96 h post infection (hpi). Among the 54 significantly changed proteins identified by MS, 12 were significantly up-regulated, while 42 proteins were significantly down-regulated, including the proteins involved in protein degradation, energy metabolism, stress responses, macromolecule biosynthesis, and material transport (Wu *et al.*, 2012). Researchers used iTRAQ combined with LC-MS/MS to quantitatively analyze the dynamic proteomic changes after IBDV infection in chicken embryo fibroblast cell line DF-1, finding that 23 cytoplasmic proteins and 34 nuclear proteins changed significantly. These significantly altered proteins were identified as being involved in the immune response, signal transduction, biosynthetic energy metabolism, viral adhesion, and cell apoptosis. Ingenuity pathways analysis software was used to enrich the signaling pathways of these significantly differentially expressed proteins and showed them to be significantly enriched in the mTOR, phosphatidylinositol 3-kinase / Akt, and interferon (IFN)-dependent-signaling pathways (Sun *et al.*, 2015).

Chicken dendritic cells (DCs) have been proven to be sensitive to IBDV infection. To better understand the effect of virulent IBDV infection on chicken bone marrow-derived DCs, the membrane proteins of DCs were extracted and further identified by proteomics. A total of 134 differentially expressed proteins were identified. It was found that IBDV altered RNA/DNA/protein synthesis at 3 hpi by destroying several important proteins, but did not activate proteins related to signal transduction. At the late stage of infection, IBDV induced the expression of CD200 receptor1-A, integrin α-5, HSP 90, cathepsins, lysosome-associated membrane proteins and RAS-associated proteins. These proteins play important roles in signal transduction, apoptosis, stress response and secretion of risk-associated proteins (Yasmin *et al.*, 2019).

3.3 Marek's disease virus

Marek's disease is a type of proliferative infectious disease of lymphoid tissues in chickens caused by Marek's disease virus (MDV). Marek's disease is not only an important pathogen in the chicken industry but also a natural model for herpesvirus-induced tumor formation (Mcpherson *et al.*, 2016). Understanding the mechanisms of MDV-host interactions and tumor-induction will provide new avenues of research on the prevention and control of the disease. Changes in the spleen protein expression profile on days 7, 14, and 21 after MDV infection of 5-day-old specific pathogen-free chickens were studied by ESI-MS/MS; a total of 48 host proteins were identified, including cytoskeletal, ubiquitin-proteasome-degrading, signal transduction, and translation regulatory proteins (Thanthrige-Don *et al.*, 2009). Using 2-DE and MS, the differentially expressed protein profiles of bursa in specific pathogen-free chickens with different stages of MDV-infection were analyzed, and 24 differentially expressed proteins were identified to be mainly involved in cell differentiation, cell apoptosis, immune defense and tumor-related life processes, providing the basis for further studies of MDV pathogenesis and virus tumorigenesis (Lu *et al.*, 2010). Imaging MS and noncontact laser capture microdissection were first used to visualize the proteomics of MDV-induced tumors. Potential biomarkers of MDV-induced tumorigenesis, namely IFN-γ-induced protein 30 and HSP70, were found and verified to be differentially expressed in MDV-induced tumor tissues, which provided evidence for the pathogenesis and diagnosis of this malignant tumor (Pauker *et al.*, 2019).

3.4 Porcine reproductive and respiratory syndrome virus

Porcine reproductive and respiratory syndrome is a disease caused by porcine reproductive and respiratory syndrome virus (PRRSV) that results in reproductive dysfunction in pregnant sows and respiratory symptoms in all-aged pigs (Prieto *et al.*, 2005). Label-free quantitative proteomics was used to quantitatively detect secreted proteins in supernatants that were differentially expressed after PRRSV infection of porcine alveolar macrophages (PAMs) compared with mock-infected cells. A total of 95 differentially expressed secretory proteins were found in this screening. Among them, 49 proteins were up-regulated and 46 proteins were down-regulated. These differentially expressed secretory proteins were involved in immune and inflammatory related pathways, such as nuclear factor kappa B (NF-κB) and toll-like receptor signaling pathway (Li *et al.*, 2017). Host proteins named 14-3-3 and CD2-associated protein were determined to interact with nonstructural protein 2 using green fluorescent protein pull-down-coupled tandem MS technology (Xiao *et al.*, 2016). Host proteins interacting with the viral nucleocapsid protein were analyzed by MS, which showed that Poly [ADP-ribose] polymerase 1 can interact with nucleocapsid protein. After treating PRRSV-infected cells with Poly [ADP-ribose] polymerase 1 inhibitor 3-AB, the viral replication was significantly inhibited (Liu *et al.*, 2015). Furthermore, 242 and 249 significantly differentially expressed phosphorylated proteins were identified by quantifying the proteome after PRRSV infection of PAMs for 12 and 36 h, respectively. Bioinformatic analysis found that these phosphorylated proteins were mainly enriched in the mitogen-activated protein kinase and NF-κB pathways, indicating that PRRSV induced the production of

inflammatory factors by activating these two pathways (Luo *et al.*, 2014a). Acetylation of lysine is an important PTM after viral infection; it plays an important role in the host cell antiviral response. Using acetylation-based antibody enrichment technology and tandem mass tag labeled LC-MS/MS to draw a broad-spectrum acetylation map of PRRSV-infected PAMs, 3,731 lysine acetylation modification sites on 1,421 cell proteins were identified. These modification sites were identified as being involved in many biological processes such as the host immune response and energy metabolism (Fang *et al.*, 2021). Ubiquitination of lysine is a dynamic and variable PTM that plays an important role in the regulation of cell processes. On the basis of the ubiquitination antibody enrichment and MS identification technology, the broad-spectrum ubiquitination modification map of PRRSV-infected PAMs was studied. A total of 4,044 modification sites on 1,580 proteins were identified, of which 983 sites on 717 proteins were significantly changed at 36 hpi. Through in-depth bioinformatic analysis, PRRSV infection was found to inhibit the innate immune response of host cells by regulating the ubiquitination of important molecules, including TNF receptor associated factor 6, signal transducer and activator of transcription 1, Janus kinase 1 and IFN-stimulated genes. Ubiquitination was also observed on 15 PRRSV proteins, including important viral proteases and structural proteins that play a role in viral infection and neutralizing antibody induction. The results indicated that a highly organized system of ubiquitin proteasome system was needed for PRRSV efficient replication (Zhang *et al.*, 2018) .

3.5 Porcine epidemic diarrhea virus

When an iTRAQ-based quantitative proteomic was used after Vero cells infected with either a virulent strain of porcine epidemic diarrhea virus (PEDV) or a vaccine strain CV777, 661 and 474 differentially expressed proteins were identified, respectively. It was found that the PEDV virulent strain inhibited the protein synthesis of Vero cells by downregulating the activities of mTOR and its downstream targets 4EBP1 and p70S6K. The results also showed that the virulent strain activated the NF-κB signaling pathway more strongly than the vaccine strain did, causing a more robust inflammatory response (Guo *et al.*, 2016). In addition, an iTRAQ combined with LC–MS/MS was used in a study of the differential protein abundance in intestinal tissues after infection with a virulent PEDV strain YN13 and attenuated strain YN144. As a result, 269 and 301 differentially expressed proteins were identified in the jejunum tissues of piglets, respectively. Bioinformatic analysis showed that these proteins were involved in the stress response, signal transduction, and the immune system. IFN-stimulated genes can be up-regulated after infection with both virulent and attenuated PEDV strains. YN13 and YN144 caused different changes in heteronuclear protein A1, eukaryotic initiation factor 4G1, and members of the HSP family post infection, which may have led to the differences in pathogenicity (Li *et al.*, 2016).

3.6 Transmissible gastroenteritis virus

Transmissible gastroenteritis virus (TGEV) is a porcine enteropathogenic coronavirus, which causes fatal watery diarrhea and severe dehydration in piglets (Schwegmann-Wessels *et al.*, 2006). Proteins differentially expressed following infection of porcine kidney cells (PK-15) with TGEV were identified using an iTRAQ-based comparative proteomic method. Sixty significantly up-regulated proteins and 102 significantly down-regulated proteins were identified. Bioinformatic analysis revealed that the up-regulated proteins were significantly enriched in IFN-related signaling pathways. Western blotting and immunofluorescence assay further confirmed that TGEV infection caused the phosphorylation and nuclear translocation of signal transducer and activator of transcription 1(An *et al.*, 2014).

PK-15 cells that were transfected with microRNA (miR)-4331 and infected with TGEV were studied by quantitative proteomics. 69 significantly differentially expressed proteins were identified, and the target of miR-4331 was found to be RB1. The results showed that miR-4331 increased the level of calcium ions in the mitochondria, decreased the mitochondrial membrane potential, up-regulated interleukin-1 receptor, and activated the p38 mitogen-activated protein kinase signaling pathway (Zhao *et al.*, 2018) .

3.7 Classical swine fever virus

Classical swine fever is a highly infectious disease. Its clinical symptoms are characterized by hemorrhagic fever and immunosuppression, causing great losses to the pig industry (Luo *et al.*, 2014b). To reveal the proteomic changes in pig serum, five out of 10 pigs were inoculated with the highly pathogenic classical swine fever virus (CSFV) strain, and the remainders were used as uninfected controls. Serum samples were taken from each pig 3 days after infection. After removing serum albumin and immunoglobulin, the samples were subjected to 2-DE. The results showed that the abundance of 17 proteins had changed more than 1.5-fold. 10 of these proteins were successfully identified by MALDI-TOF-MS, of which 4 proteins were up-regulated and 6 proteins were down-regulated, including those with coagulation, anti-inflammatory, and antigenic functions (Sun *et al.*, 2011). The co-infection or re-infection of a host by a virus is one

of the key factors in viral pathogenesis. On an established model, the response of host cells to a co-infection with porcine circovirus type 2 (PCV2)-CSFV was investigated by iTRAQ-coupled LC-MS/MS proteomic analysis. Compared with uninfected cells, 304 proteins were up-regulated and 198 proteins were down-regulated. Bioinformatic analysis confirmed the dominant role of PCV2. It also suggested that mitochondrial dysfunction, Nrf 2-mediated oxidative stress response, and apoptotic signaling pathways may be specific targets following PCV2-CSFV co-infection (Zhou *et al.*, 2017). Leukopenia and immunosuppression are the typical clinical manifestations of CSFV, and peripheral blood mononuclear cells (PBMCs) are the main targets of CSFV. To investigate changes in PBMC protein abundance after CSFV infection, proteins from PBMCs infected with CSFV were analyzed by 2-DE and MS. The results showed that 66 proteins were significantly altered after CSFV infection, of which 34 were identified by MALDI-TOF-MS/MS, including those involved in cytoskeleton, energy metabolism, protein translation and processing, antioxidant stress, and HSPs-related proteins. Western blot analysis confirmed that annexin A1 abundance was up-regulated while abundance of cofilin was down-regulated after CSFV infection. This provides a theoretical basis for understanding the in vivo response of target cells to CSFV infection at the molecular level and for exploring the pathogenesis of leukopenia and immunosuppression caused by CSFV (Sun *et al.*, 2010).

3.8 Porcine circovirus 2

Porcine circovirus 2 (PCV2), a single-stranded DNA virus, is associated with post-weaning multiple system failure syndrome, which is a disease of great economic significance for pig production (Chae, 2005). Haptoglobin (Hp) and immunoglobulin are plasma glycoproteins involved in the immune response after PCV2 infection. Through the identification of Hp protein types in healthy and PCV2-infected pigs as well as the protein skeleton and glycan chain composition of porcine Hp, the total Hp level in PCV2-infected pigs was found to be increased, but the interspecific ratio of Hp was unaffected. Glycoproteomic analysis of the Hp β subunit confirmed that porcine Hp was O-glycosylated. Glycosylation of the heavy chain of pig IgG and IgA showed that the levels of both proteins were reduced in PCV2-infected pigs but there were no significant changes in the N- and O-glycosylation patterns of the major pig plasma glycoproteins (Marco-Ramell *et al.*, 2014). To study the interactions between target cells and PCV2, 2-DE was used to analyze the differentially expressed proteins between PK-15 cells infected with PCV2 and mock-infected cells. As a result, 21 host proteins with significantly altered abundances were identified by MALDI-TOF/TOF, including proteins related to the component of cytoskeleton maintenance, macromolecule biosynthesis, stress response, signal transduction, energy metabolism, and the ubiquitin-proteasome pathway. Analysis of the subcellular distribution of cytoskeletal proteins demonstrated the interaction between PCV2 cap protein and α-tubulin (Zhang *et al.*, 2009).

4. Potential for application of proteomics to research on infectious diseases of livestock and poultry

As a frontier of modern research, comparative proteomics provides a powerful tool for in-depth study of viral infections in animals and the development of new antiviral drugs. However, because some species' databases are incomplete, many differentially expressed protein functions remain to be described. On the basis of the large amount of information obtained, it will be possible to analyze the specific roles of many differentially abundant proteins during viral infections and pathogeneses, combined with the power of modern bioinformatic techniques. At present, the cells used for comparative proteomic studies of viral infections are mostly cultured *in vitro*, and thus cannot fully represent the mechanisms of interactions between viruses and host cells under the influence of the whole host immune system and other physiological conditions. In addition, after a virus infects cells or tissues, some proteins change before the emergence of detectable signs. An appropriate time point must be selected when studying these altered proteins for the early diagnosis of a viral infection. With the increasing depth of research and the emergence of new technologies, comparative proteomics will lead to further breakthroughs in the development of new vaccines and early diagnosis, prevention, and treatment of major viral infections in livestock and poultry. Therefore, it is likely that proteomics will significantly contribute to prevention and control of livestock and poultry viral diseases.

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