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Invited review

Clinical veterinary proteomics: Techniques and approaches to decipher the animal plasma proteome

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ABSTRACT

Over the last two decades, technological advancements in the field of proteomics have advanced our understanding of the complex biological systems of living organisms. Techniques based on mass spectrometry (MS) have emerged as powerful tools to contextualise existing genomic information and to create quantitative protein profiles from plasma, tissues or cell lines of various species. Proteomic approaches have been used increasingly in veterinary science to investigate biological processes responsible for growth, reproduction and pathological events. However, the adoption of proteomic approaches by veterinary investigators lags behind that of researchers in the human medical field. Furthermore, in contrast to human proteomics studies, interpretation of veterinary proteomic data is difficult due to the limited protein databases available for many animal species. This review article examines the current use of advanced proteomics techniques for evaluation of animal health and welfare and covers the current status of clinical veterinary proteomics research, including successful protein identification and data interpretation studies. It includes a description of an emerging tool, sequential window acquisition of all theoretical fragment ion mass spectra (SWATH-MS), available on selected mass spectrometry instruments. This newly developed data acquisition technique combines advantages of discovery and targeted proteomics approaches, and thus has the potential to advance the veterinary proteomics field by enhancing identification and reproducibility of proteomics data.

Introduction

Collection and analysis of blood has long been used to diagnose and monitor animal disease. The non-cellular component of blood contains a wide range of proteins and peptide molecules, many of which can be utilised as biomarkers for physiological and pathological conditions. Routine biochemical analysis can quantify high abundance proteins, such as albumin and globulin, for diagnosis of diseases. In addition, electrophoretic techniques are widely available to provide a higher resolution that quantifies major groups of proteins. However, there are numerous lower abundance proteins that are likely to positively or negatively correlate with several disease states, such as stress, inflammation and subclinical infection. In addition, they also have significance in monitoring drug and vaccine responses. These less abundant proteins include positive and negative acute phase proteins (APPs), oxidative stress markers and cytokines.

Specialised immunoassays, such as ELISAs and radioimmunoassays (RIAs), have been employed to identify and quantify proteins that are not routinely assayed in diagnostic biochemistry panels (Celi, 2011; Islam et al., 2013; Thomas et al., 2015). However, there are several disadvantages associated with these assays, particularly the limitation that separate kits are often required for each protein, the increasing costs and the required time. These assay kits also often rely upon species-specific antibodies, which must be validated for each species. In addition, they also frequently suffer from lack of concordance due to variations in epitope structure between individuals and even within individuals that lead to differences in immunoreactivity in different assays. Other issues, such as Hook's effect, whereby antibody saturation prevents sandwich formation and results in a false low concentration of analytes of interest, are further potential frustrations for the investigator (Hoofnagle and Wener, 2009).

An increasingly popular approach to protein measurement in human medicine is the use of mass spectrometry-based proteomics. Proteomics is the large-scale study of the protein component of a cell, tissue or an organism at a given time under given conditions (Marc et al., 1996). It complements the study of

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genomes and transcriptomes by facilitating interpretation of the true biochemical consequences of the gene activity. Proteomics provides highly sensitive and specific identification of hundreds to thousands of proteins and therefore has been used to understand and diagnose important human diseases, such as cancer (Chae and Gonzalez-Angulo, 2014), neurological disorders (Kroksveen et al., 2011) and several cardiovascular conditions (Edwards et al., 2008).

In veterinary science, the use of mass spectrometry-based proteomics is still in its infancy due to the lack of completely characterised genome sequences and incomplete annotation of gene functions in most domestic species. However, the publication of the genome sequences of several important species, such as cattle (Bovine Genome Sequencing Analysis Consortium Elsik et al., 2009), dogs (Lindblad-Toh et al., 2005), horses (Wade et al., 2009) chickens (International Chicken Genome Sequencing Consortium, 2004), pigs (Martien et al., 2012) and sheep (Jiang et al., 2014), has facilitated the application of mass spectrometry for protein analysis in these important veterinary species. The major advantage in using mass spectrometry techniques to identify biomarkers is that they are highly specific and sensitive approaches to measuring proteins that are crucial to the regulation of physiology and energy metabolism, reflecting changes in homeostasis.

Since proteins are the mediators of cell function, the nature of the proteome is dynamic. The differences in protein expression or degrees of change in post-translational modifications may represent changes in physiological homeostasis; thus, proteins are ideal biomarker targets for drug/or therapeutic agents. For example, APPs are already recognised as biomarkers of pathological changes and perturbations in the innate immune response. Therefore, they are used as sensitive and reliable indicators for diagnosis of several diseases (Eckersall and Bell, 2010). Protein mass spectrometry can also be used to detect and monitor subclinical infections (Seth et al., 2009) and response to vaccines (Rodrigues et al., 2015). The method can be applied in a range of biological samples, including urine (Zhou et al., 2006), cerebrospinal fluid (CSF) (Noben et al., 2006), saliva (Zhang et al., 2013; Gutiérrez et al., 2014; Jacobsen et al., 2014; Sousa-Pereira et al., 2015), seminal fluid (De Canio et al., 2014), tissue biopsies (John, 2009), milk (Gagnaire et al., 2009; Roncada et al., 2012; Hernández-Castellano et al., 2016; Verma and Ambatipudi, 2016), eggs (Qiu et al., 2013), meat products (Bendixen, 2005) and serum or plasma (Henning et al., 2014).

More recent reviews of proteomics in veterinary science have focussed on the application of proteomics to animal disease pathogenesis and diagnostics (Ceciliani et al., 2014) and cancer biomarker research (Kycko and Reichert, 2014; Klopfleisch, 2015; Ceciliani et al., 2016). Some reviews have described the range of proteomic techniques that are presently used (Almeida et al., 2015; Campos and Almeida, 2016). This review will focus on animal proteomics and the workflows involved in traditional and modern proteomics strategies, including results from several recent studies. It will explain techniques now widely available to the veterinary investigator, including liquid chromatography tandem mass spectrometry (LC–MS/MS) and multiple reaction monitoring (MRM), and will also discuss the inevitable challenges that accompany MS-based protein identification and data interpretation. There will also be an explanation of the sequential window acquisition of all theoretical fragment ion mass spectra (SWATH-MS) technique, which is currently making an impact in human medicine.

Modern proteomics approaches used in veterinary science

Proteomic techniques permit the identification of a large set of proteins in complex biological samples. This can be accomplished with or without the use of mass spectrometry. Without mass spectrometry, protein identification relies on biochemical interactions and therefore antibody availability or characteristic enzymatic reactions (Fig. 1).

Gel-based proteomics

Gel-based proteomics is the most popular method to separate a complex mixture of proteins, and it is also the most economical approach to detect protein expression changes on a large scale. Gel electrophoresis and, in particular, two-dimensional gel electrophoresis, has accompanied proteomics since its beginning. Each dimension consecutively evaluates two distinct characteristics of proteins, the first is the *pI* value during isoelectric focusing (IEF) and the second characteristic is the molecular mass during sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

The usual workflow for quantitative proteomics combines conventional protein separation using high-resolution two dimensional (2D) gel electrophoresis, followed by mass spectrometric identification for protein spots of interest. Thus, it facilitates comprehensive quantification of proteins in a given sample. However, poor reproducibility of the technique, considerable time imposition and under-representation of several classes of proteins, such as highly hydrophobic proteins and those with extreme *pI* values, remain challenging issues.

While some of these problems (e.g. reproducibility) have been addressed by the introduction of difference gel electrophoresis (DIGE) technology (Wu and MacCoss, 2002), new issues have arisen. These include inability to fully separate proteins in complex samples into individual spots on a gel; this has led to the advent of so called shotgun approaches (LC–MS/MS) that do not rely on separating proteins inside gels. Despite the limitations associated with gel-based techniques, the majority of studies examining farm animal species have used these techniques for protein analysis (Puerto et al., 2011; Kim et al., 2013; Ferlizza et al., 2015; Hernández-Castellano et al., 2016). This is primarily because of their relatively low representation in protein databases, which presents a technical limitation in selecting other advanced MS-based approaches.

Gel-free proteomics

The introduction of mass spectrometry has not only substantially increased the number of proteins which can be measured, but also improved the specificity of the assay. Liquid chromatography coupled with mass spectrometry presents a breakthrough in terms of specificity, sensitivity and robustness. Mass spectrometry-based proteomic analysis can be broadly divided into two main domains: discovery proteomics and targeted proteomics. The former enables the unbiased identification and quantification of hundreds to thousands of proteins from complex mixtures using shotgun proteomic techniques. Albeit fast and sensitive, this approach relies on stochastic sampling of peptides by a mass spectrometer, often resulting in the problem of missing values. It is also abundance biased and the presence of single highly abundant proteins in a sample (e.g. albumin in the case of blood) can skew the results.

Targeted proteomics focuses only on a subset of selected protein targets relevant to the biological process under study, with quantification either relative to a control sample or on an absolute scale. Targeted proteomics is therefore most commonly employed to validate shotgun proteomics findings.

Discovery (shotgun) proteomics

Shotgun proteomics eliminates the need for extracting proteins from gels and was introduced to identify and/or quantify the