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Proteomics: A Tool For Discovering Protein Biomarkers In Animals

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Introduction:

The term “proteome” denotes the set of proteins expressed by the genetic material of an organism under defined environmental conditions (Conrad et al., 2008). It can also be defined as the total complement of proteins expressed in a cell at any given point in time and under a given set of growth conditions. In 1994, Marc Wilkins introduced the term "proteome" to the scientific community. The term proteomics refers to the large-scale study of the proteome, including its structures and functions (James, 1997). The set of proteins varies with the specific requirements of the cell or organism. Proteomics confirms the presence/ absence of a particular protein as well as measures its quantity. Proteomic studies have emerged as a field of research in the last two decades, and with the advent of technologies in this field, it has developed rapidly.

Three major types of techniques have contributed mostly to the development of the field of proteomics: electrophoresis, mass spectrometry, and chip technologies. These approaches have contributed to the development of three major branches of proteomics study, such as descriptive proteomics (protein cataloguing), functional proteomics (study of the dynamic state of the proteome) and interaction proteomics (study of protein-protein interactions). Potential protein biomarkers with diagnostic importance can be explored by detecting up- and down-regulated, uniquely expressed, and post-translationally modified proteins associated with specific patho-physiological conditions by using proteomic methodologies. The advantage of the proteomic approach is that it identifies a panel of proteins, rather than a single protein, to correlate more reliably with specific pathophysiological conditions. Study of proteomics provides the added advantage of studying ultimate functional molecules, compared to transcriptomic analysis.

Requirements of a Proteomic Technique:

A good proteomic technique should be able to satisfy the requirements, such as -

- **Reproducibility:** generate reproducible results that can be performed by different researchers and in different laboratories.
- **Efficiency:** efficient enough to detect the majority of the proteins present in a biological sample.
- **Dynamicity:** efficiently quantify the proteins present in a wide dynamic range of concentrations in a biological sample.

However, meeting these demands by a proteomic technique is quite challenging because of the following facts:

- It is often difficult to reproduce the method of collecting the sample.
- Treatment of protein samples with proteases before proteomic analysis produces smaller fragments and thus increases the variety and confusion.
- The protein concentration varies in biological samples over 6 orders of magnitude.
- No efficient methods have been developed yet to capture the entire proteome in biological samples, *i.e*. very big and very small ones and very acidic and very basic ones, simultaneously.
- Proteins can't be amplified *in vitro* like DNA or RNA. Thus, to get access to the protein present in very low concentration, we have to load a lot.

Techniques in Proteomics:

Conventional approaches for proteomics studies are commonly divided into gel-based and non-gel-based approaches. The gel-based approaches most commonly comprise 2-dimensional gel electrophoresis (2-DE) or 2-dimensional fluorescence difference gel electrophoresis (2-D DIGE) coupled with mass spectrometry (MS). Whereas, for a non-gel-based approach, label-based or label-free LC-MS/MS techniques are employed. The isobaric tag for relative and absolute quantification (iTRAQ) is the most common non-gel label-based approach used in proteomics studies. However, protein identification by LC-MS/MS and quantification by label-free quantitation (LFQ) approach has been proven to be a more efficient non-gel label-free technique for proteomics studies in recent years and also has been preferred by researchers in the identification of potential protein biomarkers.

- **Gel-based proteomic approaches:**

  Among the gel-based proteomic techniques, the two-dimensional gel electrophoresis (2-DE) coupled with matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF) technique is mostly preferred in proteomics research. The technique compares protein profiles between the samples and also detects differences in protein concentrations in a high-throughput manner. The technique was initially introduced by O'Farrell and Klose (Klose, 1975; O'Farrell, 1975) and was widely used for the discovery of protein biomarkers of viral as well as other infectious

diseases. The 2-DE begins with the separation of proteins based on their isoelectric point in the first dimension and molecular mass in the second dimension. The result is that the molecules are spread out across a 2-D gel. The gels are then analyzed using various commercially available software for the identification of unique as well as differentially expressed proteins between two or more biological samples. The selected protein spots are then analyzed by mass spectrometry for their identification.

In mass spectrometry, the MALDI as ionization method and the TOF as a mass analyzer were the most popular in proteomic research. However, with the advancement of science, more efficient ionization techniques such as Electrospray ionization (ESI) or Desorption Electrospray Ionization (DESI) and mass analyzers such as Triple Quadrupole Mass Spectrometer, Quadrupole Time-of-Flight Mass Spectrometer, Fourier Transform Ion Cyclotron Resonance Mass Spectrometry, etc. have been introduced. Although 2-DE is the most preferred technique since the inception of proteomics research, it has certain limitations, such as long, tedious methodology and inability to detect very high and very low abundant proteins, extreme pI proteins, membrane proteins and even hydrophobic proteins. In the 2-D DIGE method, labelling of protein samples with fluorescent molecules (CyDyes), or fluors is carried out before 2-D electrophoresis (Byrne *et al*, 2009). It is possible to separate up to three different samples within the same 2-D gel. Thus, up to two samples and an internal standard can be resolved in every gel. It enables accurate analysis of differences in protein abundance between samples and also post-translational modifications and protein isoform expression. Although 2-D DIGE is more sensitive than the traditional 2-DE, it still has a few drawbacks, such as the inability to resolve hydrophobic proteins, membrane proteins, extreme pI and extreme MW proteins. Moreover, quantification of the detection of very low abundance proteins using 2-D DIGE and their subsequent staining, excision, and identification by MS can be problematic.

- **Non-gel-based proteomic approaches:**

Liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) has emerged as a powerful technique for the proteomic analysis of complex samples and identification of protein biomarkers associated with pathophysiological conditions in animals ((Thomas et al., 2022). It has been increasingly used for the accurate detection of alterations in protein profiles in biological samples and to infer biological function. Consequently, protein identification and quantification by label-based and label-free approaches in combination with LC-MS/MS have been applied by several workers. In label-based approaches, such as isobaric tag for relative and absolute quantitation (iTRAQ), stable isotope labeling of amino acids in cell culture (SILAC), tandem mass tag (TMT), etc., quantification of proteins is done by introducing stable isotopes. In iTRAQ, isobaric tags are used to label peptides via primary amines after protein extraction and digestion. Labelled samples are subsequently mixed (4-plex or 8-plex) and analyzed by LC-MS/MS. This technique enables

simultaneous analysis of multiple samples in a single MS run (multiplexing), resulting in reduced analytical variability. However, iTRAQ method suffers from limitations like variable labelling efficiency, expensive labelling reagents, additional sample processing, difficulty in analyzing LAPs and a finite number of "plexes".

Currently, label-free mass spectrometric proteomic approaches have gained more popularity due to their applicability to any kind of sample, no limit on the number of samples that can be compared and a simple and cost-effective methodology (Anand et al., 2017; Moulder et al., 2016). Label-free approach does not utilize stable isotopes, and the quantification is done based on spectral counting and intensity-based measurements. Though both LFQ and iTRAQ provide high proteome coverage and apparently valid predictions in terms of differential expression, LFQ provides higher sequence coverage, resulting in the detection of a higher number of differentially expressed proteins. Additionally, the added value of LFQ over the iTRAQ is reflected by the more confident protein identification with higher protein sequence coverage. Thus, the LFQ approach gaining popularity and becoming the preferred option when the detection of differential expression is a main objective of the study.

Applications of Proteomics in Biomarker Discovery:

The application of proteomic approaches over the last decade has provided new tools for clarifying the molecular aspects of physiological states and for understanding the etiology and pathogenesis of many diseases. The shotgun LC-MS/MS proteomics method was also used to identify thousands of proteins in human and animal body fluids, including blood, serum/plasma, seminal plasma and tear fluid. Discovery of protein biomarkers in serum related to several physiological and pathological conditions in humans as well as in animals has been extensively carried out by several researchers, and consequently, several serum proteins have been proposed as potential biomarkers related to pathophysiological conditions. These studies revealed serum amyloid A as a biomarker for acute traumatic brain injury in humans, PCK2 and AK2 for Alzheimer's disease, Factor 5, Inter-alpha-trypsin inhibitor heavy chain H4, LRG1, and Vitronectin for total colorectal adenoma, etc. in humans (Wicker et al., 2019; Dey et al., 2019).

Similarly, in animal species, the MX2 protein as a potential candidate for early pregnancy detection in buffaloes, fibroblast growth factor-21 and total hemoglobin for fatty liver in lactating dairy cows, higher expression of haptoglobin (HP), SAA, fibrinogen, interleukins (IL-1α, IL1β, IL-6), TNF-α, and interferon gamma (IFN-γ) for pregnancy toxemia in ewes, ceruloplasmin, fibrinogen, HP and α1-acid glycoprotein in for mastitis caused by S. aureus in goats and sheep, ovotransferrin (OVT) concentration for infection and inflammation in chicken etc (Buragohain et al., 2017; Shen et al., 2018). Proteomics has also been applied in studies on humans, cows, horses, swine, chickens, and some domestic pets to define the protein profiles of tissues and other body fluids during stressful conditions, pathological states, and infections. Proteomic

investigations have been carried out for the identification of biomarkers in milk, whey and serum for bovine mastitis. Generation of proteome maps has also been done for companion animals, which can serve as a reference point in the new studies.

Both gel-based and non-gel-based proteomics approaches were used to identify potential low-abundant protein biomarkers for diseases of canines like babesiosis and leishmaniosis. Similarly, protein biomarkers for animal diseases like bovine respiratory disease in cows, chronic equine laminitis, doping and spontaneous equine recurrent uveitis in horses and azotemia and urinary tract disease in cats have been identified using various proteomics approaches. In case of serum research in pigs, proteomic tools have been used to set up protein map under different pathological conditions such as porcine reproductive and respiratory syndrome (PRRS) infection, diet-induced steatohepatitis and metabolic syndrome, stress, peritonitis-induced sepsis and diagnostic applications (Genini et al., 2012). Also, serum proteomic analysis of pigs infected with CSFV and FMDV was carried out (Liu et al., 2011). Despite the widespread application of protein biomarker discovery, to date, only a few candidate biomarkers have been moved forward to validation, regulatory approval, and clinical use, and to our knowledge, were discovered via immuno-based assays. Effective biomarker discovery has been challenged by many factors, including inherent biological complexity and variability (e.g. intra-individual variation and disease marker promiscuity), stability of analytical platforms, efficiency of data analysis, validation of biomarkers in the general population, and the expense and time needed to bring a test into the clinic.

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