Proteomics in Veterinary Medicine and Animal Science: Neglected Scientific Opportunities with Immediate Impact

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Animal/veterinary proteomics is an evolving field which holds a great promise not only for fundamental and applied discoveries regarding biology and pathology of domestic species, but can also be implemented in comparative applications of human diseases research. Experimental proteomics in domestic animals have advantages over use of rodents, such as multiple sampling in time series and availability of biological samples in sufficient volume for multiple analyses, such that both experimental and natural disease processes can be investigated. While there are certain technical limitations in the expansion of the field, they can currently be circumvented and in the future mastered with a greater participation of proteomic experts, which will in turn drive the accessibility of species-specific reagents, data volume expansion in bioinformatic databases, and increased funding. This Viewpoint highlights some comparative proteomics studies addressing important issues and encourages readers to expand their horizons of domestic animal proteomics research. It will hopefully inspire new fruitful collaborations between veterinary and animal scientists and proteomic specialists for research in these areas that can have immediate and direct impact on health, society, and the economy.

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1. Introduction

Proteomic investigations in veterinary medicine and animal health have increased recently but are still a minor component of the large body of reports in the proteomics canon. Investigation of the proteomes of tissue and biological fluids from animals may be for health and diseases in veterinary medicine, in which case there is a parallel with the investigation of human disease. However, there are additional features of comparative proteomics that give added scientific value to proteomics in species such as cattle, dogs, poultry, cats, pigs, horses, and fish and they are:

1) Animal proteomics is a field of study in its own right with application to the biology and pathology of domestic species providing valuable insight in fundamental aspects of each species.
2) Comparative proteomics allows differences and similarities between proteome in health and disease to be compared between species, yielding fascinating insight into how species proteomes have evolved.
3) Experimental proteomics in domestic animals has inherent advantages over use of rodents, for instance with greater use of multiple sampling in time series studies and with non-invasive (milk, saliva, urine) or minimally invasive (serum, plasma) samples being available in sufficient volume for multiple analyses.
4) Both experimental and natural disease processes can be investigated by proteomics in the same species, unlike disease studies in humans that can only be undertaken in patient samples from natural disease.
5) Species other than rodents are often more suitable as models for human physiology with, for example, pig or dog required for drug safety testing as novel pharmaceuticals approach regulatory approval.
6) Interaction between the proteomic phenotype and genetics in domestic animals can be assisted by the knowledge of the population genetics of species where decades of recorded breeding are an invaluable resource on proteome–genome interactions.

There have recently been several excellent reviews in animal proteomics, devoted to advances in the area over the last decade[1–6] and has been the subject of books.[7,8] Rather than repeat the information contained therein, this Viewpoint is to encourage greater participation in this neglected niche of proteomics, where fascinating questions and hypothesis-driven investigations can be generated. The article will first focus on technical issues that may be limiting further expansion and second on where comparative proteomics has been utilized in addressing important issues in areas that may be unfamiliar to many readers.

2. Technical Aspects of Animal Proteomics

2.1. Sample Selection and Preparation

Animal studies have been an essential component of every field of medical and basic biological research at least for the last 150 years. Various animals like rats, mice, dogs, cats, rabbits, fishes, birds (mainly chicken), pigs, cows, and primates are being used in research. Domestic animals offer advantages for research in proteomics in experimental design, sample selection, and sample preparation due to the possibility to adapt to the environment and the broad availability of diverse biological samples including biofluids (urine, serum/plasma, milk, saliva, sperm), cells, and tissues.

In many proteomics-based approaches, most abundant proteins (albumin, IgG, α1-antitrypsin, IgA, transferrin, uromodulin) must be depleted in order to reduce the dynamic range of the samples and gain access to low abundance proteome. A general limitation for animal proteomics research till recently was that most of the commercially available depletion procedures were based on immunoaffinity and were specific for human, rat, or mouse high-abundance proteins. The increased availability of species-specific antibodies, commercially available combinatorial peptide libraries, and library display technologies, essential for the low-abundance proteome enrichment[9] and the isolation of specific high-affinity binding molecules[10] is facilitating the depletion of abundant proteins and validation of candidate protein biomarkers from any domestic animal species. The development of species-specific binding molecules will also contribute to the expansion of animal research in biomarker discovery to extracellular vesicle (EV) research. EVs are rapidly emerging as mediators of communication between cells and it is known that in many pathologies, EV amount and/or composition can vary, representing a potential source of biomarkers or "liquid biopsies."[11]

Sample types used for animal proteomics have required development of specific preparation protocols. Investigations of milk in relation to dairy cow production has led to separation protocols for milk fractionation into whey proteins, normal milk exosomes, and milk fat globule membranes within which neutrophil extracellular traps have been examined.[12] A sample type that may be thought to be resistant to proteomic analysis is wool from sheep farming given the predominance of keratin in its protein structure especially with keratin contamination being the bugbear of careful sample preparation in conventional proteomics. However, protocols have been devised using extensive urea extraction in order to examine and compare wool composition and morphogenesis.[13]

2.2. The Recent Trend to Mass Spectrometry

During the last two decades, the utilization of MS-based methods for protein identification and quantification in veterinary science has evolved tremendously.[9] Whole genome sequencing of the most important animal species in veterinary sciences, such as chicken, pig, cow, sheep, horse, dog, and cat[14] has been a prerequisite for application of MS in animal proteomics. Although commercially available MS instrumentation has improved tremendously in terms of high sensitivity, resolution, molecular specificity, and wide dynamic range, the major barriers in the field of veterinary proteomics still concern incompletely characterized animal genome sequences, as well as incomplete GO annotations and mapped pathways, which present challenges for study of non-model organisms.[5] This can be circumvented by using a homology-driven approach in both database search and bioinformatic analyses, or performing de novo sequencing for protein identification.[9]

There are three MS-based proteomics strategies that are employed in veterinary medicine: a top-down approach (measurement of the intact proteins),[15] a middle-down approach (analysis of incomplete protein digests and protein fragments) on the protein level,[16] and a bottom-up approach (analysis of digested proteins)[17] on the peptide level. Lack of expertise, knowledge, and limited availability of instrumentation has made top- and middle-down approaches extremely rare in veterinary medicine.[15] The bottom-up approach has been the method of choice for protein identification and quantification used in both, gel-based and LC-based proteomic approaches. Although the majority of the proteomic investigations reported in veterinary medicine has been gel-based (2DE/DIGE), there is an increasing number of shotgun (gel-free) proteomic studies in farm animals, small animals, and aquatic animals, especially those of economic value.[1] In most of the shotgun proteomic studies in veterinary medicine, mass spectrometers have been operated in data-dependent acquisition mode addressing new biomarker discovery in body fluids where thousands of proteins have been identified and relatively quantified. Reliable workflows have been established using label-free or label-based (iTRAQ and tandem mass tags) proteomic approaches for discovery proteomics. The new imperatives in global research push the trends in veterinary medicine toward the novel acquisition strategies—data-independent acquisition (DIA),[18] and one step further—to targeted proteomics used for absolute protein quantification.[6] Sequential window acquisition of all theoretical fragment ion mass spectra (SWATH-MS) is a technique which combines DIA with reference peptide MS/MS spectral library[19] match for label-free quantification on proteome scale.[20] The SWATH technique has been used in veterinary medicine through the study of porcine seminal plasma proteome in order to identify biomarkers for sperm quality and fertility.[21] The use of SWATH with animal species datasets in public repositories such as SWATHAtlas has the potential to expand into different animal (sub)proteomes and pathophysiology. Targeted proteomics is used to validate the quantitative results obtained by discovery proteomics

experiments and it is based on SRM/MRM.[6] Targeted analyses based on absolute quantification peptide standards such as Absolute Quantification peptides[22] or quantification concatamer coupled to SRM have great potential in clinical diagnostics, especially in the development and application of quantitative assays.[23]

Emerging MALDI MS-based methodology in veterinary medicine includes MALDI fingerprinting, as well as MALDI imaging mass spectrometry (IMS), which are currently underrepresented in the field. MALDI-TOF MS fingerprinting has been already used for identification of food-borne pathogens in raw milk,[24] and in aquaculture after using a reference database library. MALDI IMS is a relatively new label-free analytical technique for the analysis of biological and clinical tissue sections.[25] Most of the samples for IMS originate from humans, rats and mice,[26] but increasingly IMS proteomic studies are occurring in veterinary medicine, for example, to monitor honeybee venom distribution within a transverse section of the pig ear skin and organism response to sting.[27] This can reveal detailed tissue morphology and enabling more analysis of veterinary disease diagnosis and prognosis.

2.3. Gel-Based Proteomics

Gel-based proteomics have an important place in the proteomic armory and have recently been reviewed both for method[28] and applications in animal proteomics.[29] These approaches are particularly important in assessment of post-translation modifications which can be more easily observed in 2DE. For instance these are particularly relevant to changes in the glycosylation in plasma protein observed in responses to disease.[30]

2.4. Bioinformatics in Animal Proteomics

Bioinformatic analysis of data recently emerged as a key step in research. In proteomics, bioinformatics is notably used to decipher a list of proteins into usable knowledge to better understand cellular pathways. Furthermore, it is also used to compare results generated on different species and to complete knowledge using in silico rather in vitro/in vivo experiments.

The genes/proteins which are inherited from a common ancestor, or orthologs, conserve similar sequences and functions.[31] For example, orthologs can be requested for one gene/protein on the Ensembl database (www.ensembl.org). A common point in different domestic animal species is that the volume of data generated for a specific species is very low compared with more productive research such as in humans, mouse, or yeast. As a consequence, domestic animals databases are well behind the information volume of human/mouse databases (**Table 1**). Thus for a given protein or gene, one request performed on human

database will produce more results than in domestic animal databases leading to potentially missed data. A pragmatic solution exists: comparing proteomics/genomics databases between different species, to fill the gap in one species using information from another species. Therefore, thanks to application of phylogenetics and orthologs properties, it is possible to exploit results generated on one domestic animal species with knowledge from another domestic species. It is important to use closely related species wherever possible for instance investigation of buffalo proteomes can gain from comparison to the bovine proteome and genome. Using the same orthologs approach, it is then possible to gather information from human/mouse databases and apply them to results generated from domestic animal experiments.[32,33]

An application of animal proteomics is in utilization of domestic animal research as a model to decipher pathways in humans. Moreover, application of animal proteomics to answer human research issues is a promising prospect with the horizontal transfer of knowledge. Such data exploitation in bioinformatics involves tools, databases, and the skills to use them. A lack of trained bioinformaticians in animal sciences exists, so there is need of investment (training, knowledge, and experience sharing) in the area. The most efficient way forward is to organize specialized services of people, software, and hardware for bioinformatics to be able to manage horizontal activities for different research projects in animal proteomics.

The level of data repository and sharing in animal sciences is increasing. For example, the ProteomeXchange consortium (www.proteomexchange.org) aims to standardized data submission from mass spectrometry and supports data dissemination. PeptideAtlas (www.peptideatlas.org) is a repository of different organism peptides and proteins data. It offers data from different domestic animals species like cows, horses, or pigs.[34] The recently updated PigPeptideAtlas is a unique resource for use in animal proteome research, which includes data from 25 tissues and three body fluid types.

Bioinformatics will help the ongoing development of high-throughput proteomics applied to animal sciences, ensuring a significant and positive impact not only on animal sciences but also on human research.

2.5. Validation of Proteomics Investigation

An ongoing issue in investigations and especially with the rapid development of quantitative proteomics is the need for validation of results prior to full acceptance. A variety of methods can be used to confirm that proteins measured by either relative or absolute proteomics, the main approach being use of antibody-based Western blot or enzyme linked immune assay. Animal proteomics has a limitation in these validation procedures due to

Table 1. Status of reviewed proteins and GO annotations in different species (data from www.ebi.ac.uk/GOA, accessed: March 9, 2018).

	Human	Mouse	Rat	Cow	Chicken	Dog	Cat
Swiss-Prot reviewed proteins	20 316	16 966	8022	6002	2289	817	224
Total GO annotations	556 623	496 404	435 391	187 683	136 694	171 324	—

the lack of species specific antibody; however, where an antibody is available results should be validated to demonstrate the veracity of results. As an example, the change in milk proteins haptoglobin and mammary-associated serum amyloid A detected by LC-MS/MS were comparable to change in the proteins' concentrations in milk during mastitis with immunoassays known to measure the bovine protein.[35,36] A further example using cross species antibodies was the validation of Atlantic salmon serum proteome changes in response to α-salmonid virus, when the appearance of the glycolytic enzyme enolase in serum was validated using antibody raised against this protein in zebrafish.[37]

3. Comparative Applications

For comparative experimentation, rodents generally represent the most-economical models; they are readily available and easily managed in the laboratory. However, large animals such as dog, sheep, goat, and pig are physiologically more similar to humans than rodents, though there are some limitations when studying diseases in companion and farm animals, such as time-consuming collection of samples, high cost of upkeep while the annotation of the animal genomes is still ongoing. A recent development is the recognition of the importance of study on related diseases between animals and humans exemplified by the One Health Initiative (www.onehealthinitiative.com). This brings together human, animal, and environmental health, and is increasingly recognized as a critical framework for addressing a range of health problems, particularly antimicrobial resistance.[38] Proteomic approaches will have significant potential impact in this area for instance by development of more accurate diagnostic tools for animal diseases leading to limiting unnecessary antibiotics use.

3.1. Companion Animals

Most proteomic studies in domestic animals have been on farm animals, due to their economic impact but recently companion animal proteomics has been developing a momentum.[39] Here, the focus will be the dog, which is a common companion animal and the most represented in the scientific literature (340 990 entries in PubMed, https://www.ncbi.nlm.nih.gov/pubmed/).

Companion animals and humans frequently suffer from similar diseases, such as cancer, heart disease, kidney disease, and obesity,[40] which makes them excellent models for comparative proteomics research. Dogs or cats share anatomical and physiological similarities with humans and live in the same environment with their owners. Their compressed survival times, the high level of veterinary health care, and limited genetic variation with purebred dogs often prone to inherited diseases matched with different human genetic disorders, make them important models of some genetic diseases.[41]

An interesting example of genetic disease model is Golden Retriever muscular dystrophy (GRMD), comparable to Duchenne muscular dystrophy (DMD). GRMD shows a much more severe muscle pathology than the *mdx* mouse model and better reflects the clinical course of human DMD. A recent proteomic study of muscle samples from GRMD dogs and healthy controls by ICAT profiling revealed that proteins involved in metabolic pathways were decreased in abundance in GRMD muscle, with defective energy metabolism as a hallmark of the disease.[42] This animal model of DMD can provide new insights into the nature and time course of molecular derangements of dystrophic muscle.

It is recognized that dogs often develop tumors of similar biology, histology, molecular mutations, and response to therapies, to those in humans, as they are exposed to the same mutagenic and carcinogenic threats.[43] For example, immunoproteomic approach led to the discovery of four disease-associated autoantigens in dogs with breast cancer, which have been reported to elicit autoantibody response also in the human breast cancer.[44]

Due to the similarities in anatomy and physiology, as well as the high degree of human heart proteome conservation, dogs (along with pigs) are the most commonly used animals in human cardiovascular disease research. Kooij et al.[45] showed that 6575 from 44 272 proteins identified in human myocardium, based on data available from Uniprot database, shared the minimum 90% of the amino acid sequence both with the dog and the pig. Heart diseases are one of the most common diseases of pet dogs and cats, affecting 10–15% of all dogs and cats. A recent study on serum of dogs with canine idiopathic dilated cardiomyopathy (iDCM) using label-based quantitative proteomics approach revealed potential heart tissue remodeling markers which could also be of relevance in human iDCM.[32]

Proteomic analyses of different biological fluids in healthy companion animals have been performed, allowing the creation of proteome catalogues which can serve as a reference point in the new studies. Proteomes of canine serum,[46] urine,[47] saliva,[48] seminal plasma,[49] tears,[50] cerebrospinal fluid,[51] bronchoalveolar lavage fluid,[52] and follicular fluid[53] were characterized using proteomic methods such as 2DE or gel-free LC-MS. In 2016, Fernandes et al.[39] presented CanisOme, a database of all proteomics studies with identified proteins of various tissues in healthy or diseased dogs. This will be valuable in advancement of dog health and their use as models in studies of human diseases.

The identification of biomarkers related to diseases provides diagnostic tools as well as potential targets for drug development and vaccine candidates. For example, a gel-based proteomic approach with enrichment of low abundance plasma proteins was employed for finding biomarkers associated with canine babesiosis[54]; while gel-free, isobaric-based quantitative proteomic approach enabled the identification of new potential biomarkers for the treatment monitoring of canine leishmaniosis.[55] The translation of these results into technology for use in routine veterinary practice is keenly anticipated.

While canine models of human diseases have been used in certain extent in proteomic studies, feline diseases are underutilized which is a missed opportunity as they can serve as good models of type 2 diabetes mellitus or inherited muscular dystrophies disorders, being clinically similar to those in humans.[41] One of the examples where cats were used in a proteomic study is research on familial hypertrophic cardiomyopathy (HCM).[56] Cats with HCM represent a good intermediary model between the mouse models and humans to study disease progression, development of the congestive heart failure, and different therapeutic strategies.[40]

3.2. Farm Animals

Farm animal proteomics have been dominated by studies on understanding the traits that are important for production of meat, milk, and other animal products. These studies were focused on identifying biomarkers that can help optimize a sustainable balance between productivity, product quality, and animal welfare.[1,4] Recently, farm animal proteomic studies have also focused on the application of proteomics to animal disease pathogenesis and diagnostics, cancer biomarker research, and identification of stress markers in order to monitor animal welfare in increasingly intensified farm management practices.[3,8] There have been more proteomic investigations of cattle than other domestic species often with a focus on milk for use in dairy products and in mastitis, the infection of the mammary gland, which is the most important disease encountered in dairy farming.[57]

A recent example of the potential extent of an omic study in mastitis encompassed peptidomics, proteomics, and metabolomics.[35,36,58] The investigation showed that there were more than 500 upregulated proteins in the milk from cows with mastitis, with cathelicidins and peptidoglycan recognition protein 1, and acute phase proteins such as haptoglobin being the most upregulated.[36,59] This is clear example of research not feasible in rodents due to low volume of milk that could be collected. A further example is the examination of bovine nasal secretion by proteomic analysis.[60] Proteomic investigation of the bovine nasal secretion identified serum proteins such as albumin and IgG but also secretory proteins such as IgA, lactoferrin, and also odorant-binding protein. It would be interesting to determine changes in nasal secretion during pulmonary disease in this species where milliliter volume can be collected.

Proteomic investigation has been applied to many aspects of cattle production, including adipose tissue[61] and liver metabolism,[62] and also the several conditions that constitute the periparturient diseases. While including mastitis, these transition period conditions also include endometritis, lameness, fat cow syndrome, milk fever, and ketosis which together make the period around calving of special research interest. The applications of proteomics and other omic technologies in a systems biology approach to bring a fresh view on these dairy farming problems have been recently reviewed[63] and may contribute to a wholesale alteration to our understanding of the aetiology and pathology of these diseases.

Domestic pig is of immediate relevance for food production, animal husbandry, and welfare. The pig shows similarity in size, shape, and physiology to human and has been used as a major mammalian model for many human diseases, due to the close similarity on the gene and proteome level between pig and humans.[64,65] Domestic pigs are being substituted by miniature pigs in some studies when advantages are achieved in terms of space requirements and reduced housing and food costs.[64] A miniature pig model was used by Wang and co-workers,[66] utilizing multistage proteomics techniques, which unveiled underlying functional changes related to the development of acute liver failure (ALF) and facilitated the discovery of novel ALF markers, suggesting a significant enrichment of ALF-related proteins involved in energy metabolism. In clinical validation study, prognostic values of these markers were tested in human patients,

among which some were highlighted as promising biomarkers for early detection and short-term prognosis of ALF.

Other farm species have also been used as models of human disease, which is reviewed in several papers[67] such as the one by Ceciliani et al. (2014),[68] describing in detail the use of pigs, cows, and horses as well-established models, especially in infectious diseases with emphasis on the host–pathogen interactions. Also, Tholey et al. mention the potential of farm animals as models in research of nutrition influences to human health.[69]

Discovery of stress markers is an important objective of proteomics in animals, as it is known that stress will adversely affect growth of animals but can also have deleterious effects on meat for human consumption. Both 2DE and iTRAQ methodologies have been used to identify the serum proteins altering in response to stress induced by housing the pigs in individual rather than mixed stalls.[3]

Quantitative proteomics has demonstrated that responses of pig intestine to infection by *Salmonella typhimurium* includes eukaryotic initiation factor 2 signaling, free radical scavengers or antimicrobial peptides expression, and the impairment of bile acid and lipid metabolism by means of underregulation of fatty acid–binding protein 6, as well as farnesoid X receptor/retinoid X receptor, and liver X receptor signaling pathways.[70] This is not only of value in understanding the response to intestinal infection in the pig but provides a valid model for investigation of infection of humans with the same bacteria.

Proteomics research in less studied species, such as sheep, has examined mastitis, respiratory and reproductive infections, paratuberculosis, and scrapie,[71] as well as milk production, including the characterization of the proteome of ewes' colostrum[72] and milk.[73] The buffalo is a very important farmed animal in many parts of the world such as the Indian subcontinent and Southeast Asia. Recently, plasma proteome of buffaloes was characterized[74] while a heterologous fibrin sealant has been developed from the buffalo blood fibrinogen and applied as biological glue in the treatment of chronic venous ulcers.[75]

4. Conclusions

Animal proteomics despite being a neglected area of research has made disproportionate impact and contribution to our knowledge. This has encompassed the health and welfare of farm animals and pet animals being beneficial in the pursuit of happiness of their owners. There are further potential benefits from experimental or clinical studies especially in the investigation on zoonotic infections that interact with the One Health Initiative.

The objective of this Viewpoint article will be achieved if it is able to expand the concepts and potential impacts of domestic animal proteomics with three segments of readers such that:

1) Veterinary and animal scientists consider proteomics as a valuable component of their investigations and to understand the potential impact that these advanced technologies can have.

2) Proteomic specialists in mass spectrometry and bioinformatics to recognize that engagement with veterinary and animal sciences can lead to fruitful collaborations.

3) Perhaps of most importance, the evaluators of research project applications to understand that research in these areas can have immediate and direct impact on society and the economy.

In conclusion, there is a wealth of virtually unlimited unknown unknowns to be revealed by proteomics in animal research going from fundamental understanding to directly translatable research impact.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

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