Proteomic Research in Urine and Other Fluids

Gloria Isani, Enea Ferlizza, and Jarlath E. Nally

Abstract Urine is an ideal biological sample. It is an important source of clinical biomarkers of systemic and renal diseases and can be obtained repeatedly without causing stress. Different analytical protocols for the study of the urine proteome have been used, from the simplest electrophoresis on agarose gels to the most sensitive and complex 2DE. As with urine, a range of bodily fluids (saliva, cerebrospinal, bronchoalveolar, amniotic fluids) are amenable to the tools of proteomics. The identification and characterization of the protein content of such fluids are being used to provide novel insights into the evolutionary adaptations of farm and domestic animal species and to characterize their normal physiological state. A comprehensive review on the application of proteomics to these biological samples is presented.

Keywords Saliva • Cerebrospinal fluid • Bronchoalveolar fluid • Amniotic fluid • Electrophoresis • Veterinary medicine • Farm animals

Abbreviations

2DE	Two-dimensional electrophoresis
Ig	Immunoglobulins
MS	Mass spectrometry
AKI	Acute kidney injury
DIGE	Difference gel electrophoresis
BSE	Bovine spongiform encephalopathy

G. Isani (✉) • E. Ferlizza
Department of Veterinary Medical Sciences (DIMEVET), Alma Mater Studiorum-University of Bologna, Bologna, Italy
e-mail: gloria.isani@unibo.it

J.E. Nally
Bacterial Diseases of Livestock Research Unit, National Animal Disease Center, Agricultural Research Service (ARS), United States Department of Agriculture (USDA), Ames, IA, USA
e-mail: Jarlath.Nally@ars.usda.gov

A.M. de Almeida et al. (eds.), *Proteomics in Domestic Animals: from Farm to Systems Biology*, https://doi.org/10.1007/978-3-319-69682-9_7

AGE	Agarose gel electrophoresis
CAM	Cellulose acetate membrane
HRE	High-resolution electrophoresis
PrPC	Physiological prion protein
GO	Gene ontology
CSF	Cerebrospinal fluid
BALF	Bronchoalveolar fluid

1 Urine

Urine, produced by healthy kidneys under normal physiological conditions, contains a relatively small amount of protein. In general, molecules with an effective radius exceeding ~4 nm are not able to pass via the glomerular filter, and most proteins and protein fragments are reabsorbed along the tubule (Geckle 2007). A few proteins are secreted by the kidney, e.g., uromodulin. The abnormal presence of protein in urine is defined as *proteinuria*. Proteinuria provides insights into the systemic (presence of proteins derived from plasma) and local (proteins of kidney origin) pathophysiology from a clinical perspective. As a consequence, urine is considered an ideal source for biomarkers of clinical significance. Moreover, urine samples can be obtained repeatedly, in large quantities and noninvasively.

As the study of the urinary proteome originated and developed in human medicine, a short introduction summarizing the fundamental stages of urinary proteome characterization in humans is presented. Subsequently, different analytical protocols for the study of the urinary proteome will be discussed, from the simplest assays used during clinical assessments to the most sophisticated used only for research, with some reference to their use in veterinary medicine, and a possible standard protocol for the analysis of urine applicable to normal and proteinuric samples.

1.1 Urinary Proteomics: A Long-Standing Tradition

The first studies on the protein composition of normal urine were performed in 1979, applying two-dimensional electrophoresis (2DE) at a time when this proteomic technique was still in its infancy (Anderson et al. 1979). In 2004 Oh et al. (2004) defined a preliminary urine proteome map and annotated 113 different proteins on a 2D gel by peptide mass fingerprinting. Many other studies followed in subsequent years, and recently, a number of large-scale proteomic studies have been carried out to improve our knowledge on the urinary proteome from healthy individuals. By combining data from different studies published to date, at least 3000 different proteins and peptides have been identified in urine (Molina et al. 2011; Di Meo et al. 2017). Marimuthu et al. (2011) compared the proteins identified in their study with two other large-scale studies previously performed by Adachi

et al. (2006) and by Li et al. (2010). Six hundred and fifty-eight proteins were commonly identified in all three studies and thus represent a universal panel of proteins physiologically present in human urine. Out of these, albumin, uromodulin, heavy and light chains of immunoglobulins (Ig), and transferrin are the most represented. These proteins are generally classified following nephronal localization and functional criteria. Kidney secretory and structural proteins, serum and transport proteins, coagulation and complement factors, immunoglobulins, and other immune proteins, enzymes, metal-binding proteins, and lipoproteins are some of the possible functional categories reported by Candiano et al. (2010). The most abundant proteins in human healthy urine are reported in Table 1.

Wide variability of the urine proteome from healthy individuals has been related to gender, age, diet, physical exercise, drug consumption, and environmental conditions. The extent and sources of this intra- and interindividual variability have been investigated by several studies (Oh et al. 2004; Thongboonkerd et al. 2006; Nagaraj and Mann 2011; Molina et al. 2011; Liu et al. 2012; He et al. 2012; Guo et al. 2015; Pastushkova et al. 2016).

In addition, the urinary proteome is characterized by a highly dynamic range of almost five orders of magnitude (Nagaraj and Mann 2011). Therefore, the most challenging proteins to identify are low abundance proteins, and many of them are yet to be characterized. One innovative technique that has been developed to overcome this limitation is the use of beads coated with hexameric peptide ligand libraries; this allows for the enrichment of low abundant proteins while removing the most abundant ones (Decramer et al. 2008; Candiano et al. 2010; Filip et al. 2015).

Many of the proteins identified in large-scale proteomic analysis on normal human urine are now under investigation in clinical trials to validate their use as sensitive and specific biomarkers of disease. Chronic kidney disease, acute kidney injury (AKI), and diabetic nephropathy are only three of the diseases studied extensively in human medicine using proteomic techniques on urine (Andersen et al. 2010; Lhotta 2010; Devarajan 2011). Such diseases are also highly prevalent in animal medicine, which could benefit from the discovery of novel biomarkers of health.

1.2 Urinary Proteomics in Veterinary Medicine: An Unexplored but Promising Field

Despite the importance of proteomics techniques and their potential for biomarker discovery and diagnostics, high costs and limited interest by animal researchers and clinicians have resulted in few applications of proteomics to urine samples in veterinary medicine, and, as a consequence, fragmentary data are present in the literature. Pyo et al. (2003) studied the urinary proteome in cows by 2DE to search for potential biomarkers of early pregnancy. Simon et al. (2008) applied difference

Table 1 Most abundant proteins found in urine from healthy humans (Molina et al. 2011; He et al. 2012)

He et al. (2012)		Molina et al. (2011)	
A1BG	Alpha-1B-glycoprotein	A1BG	Alpha-1B-glycoprotein
AHSG	Alpha-2-HS-glycoprotein	ACP2	Lysosomal acid phosphatase
AMBP	α1-Microglobulin/bikunin precursor	ALB	Serum albumin
AZGP1	Zinc alpha-2-glycoprotein 1	AMBP	α1-Microglobulin/bikunin precursor
CD14	Monocyte differentiation antigen CD14	AZGP1	Zinc-alpha-2-glycoprotein
COL6A1	Collagen alpha-1(VI) chain	CADM4	Cell adhesion molecule 4
CTSD	Cathepsin D	CD14	Monocyte differentiation antigen CD14
DNASE1	Deoxyribonuclease-1	CDH1	Epithelial cadherin
GAA	Lysosomal alpha-glucosidase	GAA	Lysosomal alpha-glucosidase
GC	Vitamin D-binding protein	GSN	Gelsolin
HPX	Hemopexin	HSPG2	Basement membrane-heparan sulfate proteoglycan core protein
KNG1	Kininogen 1	KLK3	Prostate-specific antigen
ORM1	Orosomucoid 1	KNG1	Kininogen-1
PIGR	Polymeric immunoglobulin receptor	MASP2	Mannan-binding lectin serine protease 2
PTGDS	Prostaglandin-H2 D-isomerase	PEBP4	Phosphatidylethanolamine-binding protein
RBP4	Plasma retinol-binding protein	PRSS3	Trypsinogen 4
SERPING1	Plasma protease C1 inhibitor	PTGDS	Prostaglandin -H2 D-isomerase
UMOD	Uromodulin	QPCT	Glutaminyl-peptide cyclotransferase
		SDR9C7	Orphan short-chain dehydrogenase/reductase
		SECTM1	Secreted and transmembrane protein 1
		SERPINA1	Alpha-1-antitrypsin
		TF	Serotransferrin
		UMOD	Uromodulin

gel electrophoresis (DIGE) to discover biomarkers of bovine spongiform encephalopathy (BSE) in bovine urine. Nabity et al. (2011) applied DIGE and SELDI-TOF to urine samples from dogs affected by hereditary nephropathy.

More recent studies have applied proteomic techniques to explore the urinary proteome in healthy dogs, cows, and cats (Brandt et al. 2014; Bathla et al. 2015; Ferlizza et al. 2015), while the urinary proteome of pigs and other farm animals remains mostly unexplored. These preliminary studies are a starting point for future

research that can focus on the proteome of urine under physiological conditions compared to that during specific clinical pathologies.

1.3 Methods and Protocols for Sample Preparation

The prerequisite for each analytical technique to obtain reliable results is the appropriate treatment of samples in order to preserve their integrity and, at the same time, avoid/remove any interference. Therefore, one of the priorities in the field of urinary proteomics is to optimize the protocol for sample preparation, due to the presence of interfering molecules and ions, of a wide dynamic range and of the differences in protein concentration between healthy/non-proteinuric and diseased/proteinuric animals.

1.3.1 Sample Collection and Stability

The discussion on sample collection procedures (free catch, cystocentesis, catheterization) is out of the scope of the present chapter, but it is important to bear in mind that they can modify the urine protein composition, particularly in animals, because it is not possible to collect 24-h urine as routinely done in humans. Although in humans it has been reported that the variation of intraday samples is limited (Khan and Packer 2006; Thongboonkerd et al. 2006), where possible, we suggest collecting a morning urine sample by free catch in order to limit variables and stress for animals.

The literature reports that urine is stable for hours at room temperature, a few days in the fridge (+4 °C), and for years in the freezer (−20 °C) (Candiano et al. 2010). Molina et al. (2011) found differences in the number of protein spots in urine samples frozen at −20 °C (without protease inhibitors) compared to fresh urine samples and to samples stored at −80 °C; therefore, we recommend storing samples at −80 °C. The use of protease inhibitors is not recommended because they may change the urinary proteome, in particular in healthy/non-proteinuric samples which contain few proteases.

1.3.2 Sample Treatment and Protein Quantification

Different protocols have been proposed to concentrate and desalt urine from healthy individuals before 2DE or SDS-PAGE, including ultrafiltration, precipitation, lyophilization, and ultracentrifugation (Thongboonkerd et al. 2006; Martin-Lorenzo et al. 2014). We recommend precipitation with trichloroacetic acid and washing the pellet with ethanol or 80% acetone as the method of choice for 2D urine proteome analysis, because this protocol provides high protein recovery yield and a large number of protein spots that can be visualized in 2D gels. An interesting alternative can be offered by centrifugal filtration with 3 kDa cutoff filters; it is less time-consuming and

allows the elimination of interfering ions and metabolites and is easy to perform without chemical treatment. Different methods are available for protein quantification, from automated analysis used in the clinical laboratory to protocols applied mainly in research. In the clinical routine, urine proteins are determined by a spectrophotometric automated method based on pyrogallol red, while other methods are the preferred choice in the research field (e.g., Bradford or Lowry).

1.4 Gel-Based Proteomics: From Zone Electrophoresis to DIGE

Different electrophoretic approaches are available, characterized by increasing analytical sensitivity and protein separation performances from semiautomated agarose gel electrophoresis to 2D-DIGE. All these techniques have advantages and disadvantages when applied to urine samples obtained from farm animals.

1.4.1 Agarose Gel Electrophoresis

Agarose gel electrophoresis (AGE) and, less frequently, cellulose acetate membrane (CAM) electrophoresis are used to separate serum proteins in routine clinical analysis, but they are rarely applied to urine. Zone electrophoresis on CAM or AGE allows the separation of proteins in native conditions on the basis of the mass/charge ratio yielding the identification of four to six zones (albumin, alpha 1, alpha 2, beta 1, beta 2, and gamma globulins) (Eckersall 2008). CAM is currently seldom applied to urine samples because separated zones are relatively wide, while the resolution and limit of detection are low; as a consequence, in many laboratories, it has been replaced by AGE. AGE, generally performed on semiautomated systems, is characterized by good reproducibility for both qualitative and quantitative analysis and can be considered to be the easiest, cheapest, and fastest approach to separate, visualize, and quantify urine albumin and globulins. Ferlizza et al. (2017) applied AGE on high-resolution gels (HRE) to urine samples collected from healthy and diseased cats. The same analytical approach was previously used by Giori et al. (2011) for canine urine. These results suggest the use of HRE-AGE in clinical practice to obtain additional information useful for the evaluation of kidney function. Nevertheless, this technique presents a few disadvantages; the first relates to the low sensitivity of staining based on acid violet that limits the analysis of urine samples with protein concentrations lower than 40 mg/dL (Fig. 1). The second limitation relates to the difficulty in defining the limit of detection and thus quantification. Despite these disadvantages, AGE can be considered to be a useful diagnostic tool offering additional clinical information to urinalysis.

A method complementary to AGE, which can provide additional information to improve characterization of the urinary proteome, is SDS-AGE, also performed by semiautomated systems. This method separates proteins according to their

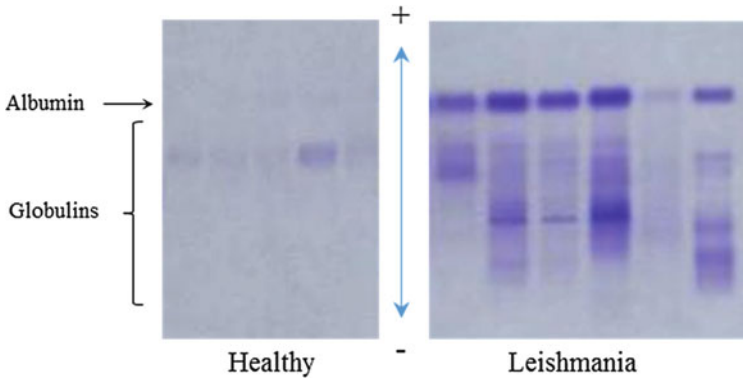

Fig. 1 Examples of urine samples analyzed by HRE-AGE. Samples were collected from healthy dogs and from dogs affected by leishmaniasis

molecular mass and allows the visualization of different protein profiles related to their nephronal origin: glomerular, tubular, or both. The glomerular pattern is characterized by high molecular mass bands (>70 kDa) that contain proteins derived from plasma due to damage of the glomerular filtration barrier. On the other hand, the tubular pattern is characterized by low molecular mass bands (<70 kDa) that contain proteins derived from impaired tubular reabsorption; a mixed pattern is characterized by the presence of both high and low molecular mass proteins. SDS-AGE has been applied by different authors to evaluate kidney function in dogs and cats affected by several pathologic conditions (Bonfanti et al. 2004; Abate et al. 2005; Schellenberg et al. 2008; Gerber et al. 2009; Giori et al. 2011; Paltrinieri et al. 2015; Lavoué et al. 2015). The predominant limitations of analysis are time-consuming protocols, the inability to cut bands for the subsequent identification by mass spectrometry (MS), and the relatively high cost of the analysis.

1.4.2 1D SDS-PAGE

The classical approach for protein separation is based on 1D SDS-PAGE; in this case, proteins are separated on the basis of their molecular mass under denaturing conditions. This method provides for veterinary medicine the best quality/price ratio, with a good resolution for protein separation, high reproducibility and high or very high sensitivity, depending on the staining used (Coomassie, silver nitrate or fluorescent), and relatively low cost (Fig. 2, Isani et al. unpublished results). This technique was used to investigate urine protein composition particularly in companion animals (Raila et al. 2007; Miyazaki et al. 2007; Schaefer et al. 2011; Lemberger et al. 2011; Brandt et al. 2014; Ferlizza et al. 2015) and to a lesser extent in farm animals and nonconventional species (Halbmayr and Schusser 2002; McLean et al. 2007; Alhaider et al. 2012). The higher sensitivity of the staining

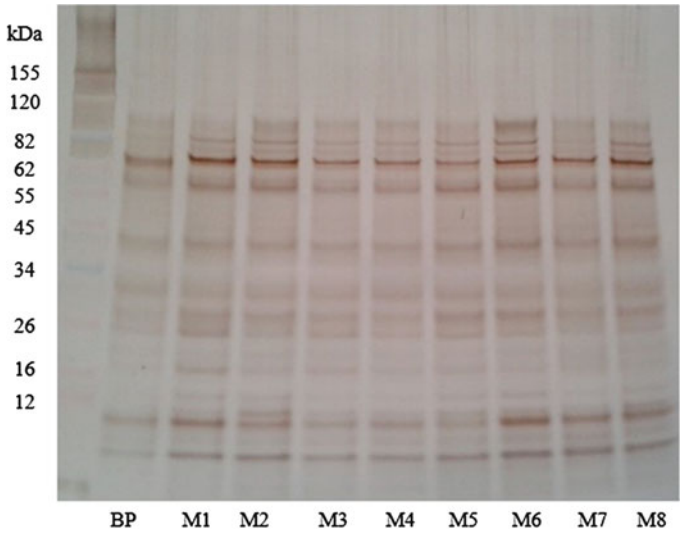

Fig. 2 Example of urine samples collected from cows analyzed by 1D SDS-PAGE. Urine samples collected from one cow before (BP) and during pregnancy (months [M] 1-2-3-4-5-6-7-8). Two μg of proteins were loaded, separated on 12% gel in MOPS buffer, and stained with silver staining

protocols allows relatively easy characterization of a healthy urine profile. SDS-PAGE allows for the visualization of different patterns (tubular, glomerular, or mixed) to identify the nephronal origin of proteins in urine. Moreover, since this technique can be performed also in nonreducing conditions, it is possible to separate proteins according to their original size allowing a better visualization of the native proteome, and nonreducing SDS-PAGE can be performed to better discriminate the different kinds of gammopathies. Furthermore, SDS-PAGE can be performed as a preparative step for subsequent protein identification by MS allowing an accurate characterization of the protein profile and the respective identification of putative biomarkers of nephropathy.

The main disadvantage of SDS-PAGE is that the electrophoretic profile of proteinuric samples can contain many protein bands, indicating a proteome too complex to allow complete separation and characterization.

1.4.3 2DE in Combination with MS, Including DIGE

The best method to separate and visualize the complexity of the urine proteome by means of gel-based proteomics is 2DE. However, in veterinary medicine, its application to urine samples is still limited and restricted to a few species (Pyo et al. 2003; Miller et al. 2004, 2014; Bathla et al. 2015; Ferlizza et al. 2015). Low reproducibility is one of the major disadvantages affecting 2DE, but this has been

significantly improved in recent years. 2D-DIGE overcomes this limitation but has rarely been applied in veterinary medicine research. 2D-DIGE has been used to identify markers of BSE in bovine urine samples (Simon et al. 2008; Plews et al. 2011). In 2011, Nabity et al. applied 2D-DIGE on urine samples of dogs affected by X-linked hereditary nephropathy identifying putative markers of nephropathy.

Figure 3 presents a simplified scheme of a standard protocol used in our laboratory (Isani and Ferlizza) for urine analysis from sample collection to protein identification by mass spectrometry.

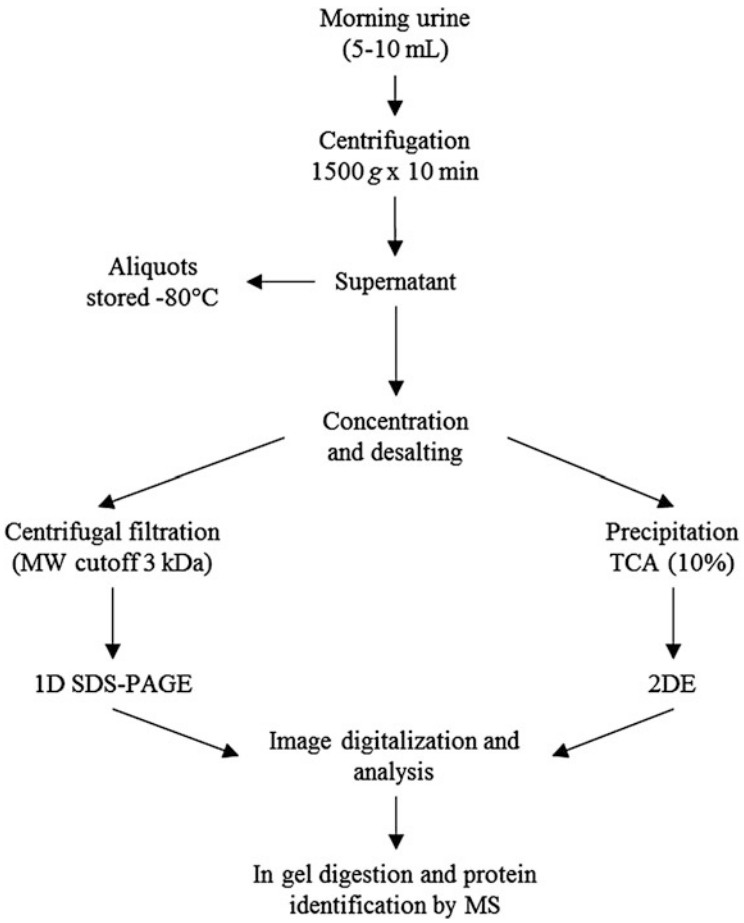

Fig. 3 Standard protocol for urine proteome separation. 1D SDS-PAGE: optimal protein amount of 5–10 μg for minigel (8 × 8 cm) and Coomassie staining. Depending on urine protein concentration, centrifugal filtration (3 kDa cutoff) in case of healthy urine, or dilution with ultrapure water for proteinuric samples. 2DE: optimal protein amount of 250–300 μg for 17 cm strip and Coomassie staining. After 10%TCA precipitation, wash pellet with ethanol or 80% acetone, air-dry, and store at −20 °C

1.5 Urine Proteome and Clinical Application in Farm Animals

Despite the economic importance and the worldwide distribution of farm animals, limited data is available on the characterization of the urinary proteome of healthy animals. The authors are not aware of a complete map of the urinary proteome for any species of veterinary interest, including farm animals (cow, sheep, goat, horse). On the other hand, proteomic techniques have been applied to the characterization of other biological samples from farm animals, e.g., milk and meat, as recently reviewed (Almeida et al. 2015).

1.5.1 The Urinary Proteome of Bovine and Other Ruminants

Under normal physiological conditions, ruminant urine contains a low amount of proteins and has a basic pH that can interfere not only with the semiquantitative dipstick test used in a clinical setting but also with other analytical methods. As a consequence, attention should be paid when determining total proteins in urine of ruminants, and the use of pyrogallol red is suggested. In healthy dairy cows and heifers, mean values of 13.4 mg/dL and 19.4 mg/dL total urine proteins were, respectively, determined by the authors.

Typical 1DE profiles of urines from Friesian heifers and cows (*Bos taurus*) are reported in Fig. 4 (Isani et al., unpublished results). There are common bands with apparent molecular mass of 97, 86, 78, 70, 59, 38, 27, and <12 kDa, the most abundant of which probably corresponds to albumin (70 kDa).

The first characterization of bovine urine was reported by Pyo et al. (2003) applying 2DE on urine samples collected from 30 pregnant and 20 nonpregnant cows. However, of the over 200 spots detected and selected for MS identification, less than 5% could be identified due to the incomplete database annotation for the bovine species, whose genome was not sequenced at that time. Nevertheless, one of the protein spots was identified as bovine pregnancy-associated protein which showed a high correlation to pregnancy and was considered by the authors as a useful diagnostic biomarker of pregnancy in cows.

In 2015, the urine from Karan Fries cows was analyzed by 2DE leading to the identification of more than 1500 proteins (Bathla et al. 2015). These proteins were categorized by gene ontology (GO) classification. On the basis of cellular localization, identified proteins were mainly cytoplasmic (29%), extracellular (20%), or belonged to an organelle fraction (20%). Classification by molecular function indicated that a large majority of these proteins were involved in catalytic activity (32%) and binding (30%); classification by biological process categorization indicated that they were mainly involved in metabolism (25%) and cellular processes (20%). The study of Bathla et al. (2015) was the first high-throughput approach to characterize the bovine urinary proteome which provided a database and considered to be the starting point for future studies focused on biomarker discovery in cows.

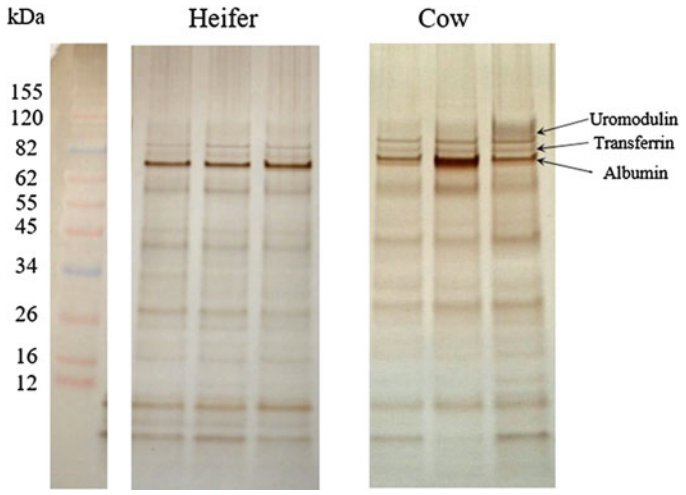

Fig. 4 Examples of urine samples collected from healthy Friesian heifers and cows analyzed by 1D SDS-PAGE. Two μg of proteins were loaded, separated on 4–12% gel in MES buffer, and stained with silver staining. Putative uromodulin, transferrin, and albumin bands, identified on the basis of apparent molecular mass, are indicated

Other studies have exploited and applied the potential of proteomics on urine samples aimed at discovering alternative biomarkers of BSE applicable to the development of diagnostic tests. In 2008, 2D-DIGE lead to the identification of a cluster of 16 spots containing 5 proteins, namely, clusterin, Ig gamma-2 chain C region, similar to uroguanylin, cystatin E/M, and cathelicidin 1, with differential abundance in urine of control versus BSE-infected cows (Simon et al. 2008). These proteins were considered specific for monitoring disease progression, and the authors suggested that they may also provide new information on the biochemical basis of BSE infection. Subsequently, the influence of sex, breed, and age was investigated. Results demonstrated that, at the clinical stage of the disease, these factors significantly affected the urinary proteome, through a specific panel of proteins which showed high accuracy to discriminate between control and infected cattle (Plews et al. 2011). In addition to BSE, ketosis, an important metabolic disorder affecting dairy cows during the parturition/first days of lactation periods, is a big issue in farm animal medicine that still needs early predictors. The study of Xu et al. (2015) was the first to report SELDI-TOF-MS application to the proteome of urine in large animals, leading to the identification of 11 proteins underrepresented in urine of cows affected by ketosis. Fibrinogen, C1 inhibitor, and osteopontin were associated with the inflammatory response, VGF nerve growth factor inducible and amyloid β were associated with the neuronal function, while serum amyloid A and apolipoprotein CIII were associated with lipid metabolism; the remaining four were cystatin C, transthyretin, hepcidin, and human neutrophil peptides.

Few studies have applied proteomics on urine samples from goats (*Capra hircus*) and sheep (*Ovis aries*). Ozgo et al. (2009) studied the urine proteins in young goats by SDS-PAGE to evaluate the maturation of the glomerular filtration barrier. Neonatal proteinuria and its changes during the first month were related to the presence of low molecular mass proteins (<67 kDa). The authors did not identify these proteins, but hypothesized that proteinuria was due to an adaptive mechanism for the removal of excess protein taken up with colostrum as also reported in foals (Jeffcott and Jeffcott 1974).

In 2012, Palviainen et al. (2012a) applied 2DE to urine from 12 sheep to search for novel biomarkers of acute kidney injury (AKI). AKI was induced in sheep by the administration of ketoprofen via catheterization, and urine samples were collected and analyzed by 2DE. Calbindin-D28k, retinol-binding protein, and antigen-presenting glycoprotein CD1d were detected only in the urine of the AKI group. The presence of these proteins in urine confirmed the tubular involvement during AKI in sheep, suggesting that calbindin-D28k and antigen-presenting glycoprotein CD1d proteins may represent sensitive and specific biomarkers of kidney injury. Sheep, similar to cows and humans, is a target of transmissible spongiform encephalopathies, which in this species is known as scrapie. Andrievskaia et al. (2008) applied SDS-PAGE and Western blotting on urine samples from healthy and naturally scrapie-infected sheep to detect and quantify the physiological prion protein (PrP^C). PrP^C was identified in very low levels in urine of healthy control animals (0.04 ng/mL), while 21% of scrapie-infected sheep presented elevated concentrations (0.6–4.7 ng/mL).

1.5.2 The Urinary Proteome of Other Farm Animals

Though the pig (*Sus scrofa*) is an economically important species and a consolidated model for translational medicine, data on the characterization of its urinary proteome is lacking. Our preliminary data on pig urine proteome is reported in Fig. 5 (Forni and Isani, unpublished results). Noteworthy is the low abundance of high molecular mass proteins and the presence of putative albumin at 67 kDa. The putative uromodulin at 90–100 kDa is also present in urine from healthy specimens along with many different low molecular mass proteins.

Studies on the characterization of the urinary proteome from horses (*Equus caballus*) are scarce. SDS-PAGE was applied for qualitative and quantitative evaluation of urine proteins in healthy and diseased horses (Halbmayr and Schusser 2002) and before and after exercise (Scarpa et al. 2007). Data were quite similar to those obtained by the authors, who analyzed urine from healthy and diseased horses (Isani et al., unpublished results) (Fig. 6). Noticeable is the presence of an abundant band at 90–100 kDa attributable to uromodulin on the basis of molecular mass, followed by putative transferrin and albumin. The presence of different thin bands between 73 and 61 kDa is intriguing; they might be albumin isoforms due to posttranslational modifications as reported in human urine (Barratt and Topham 2007). The bands at 58 and 27 kDa might be assigned to the heavy and light chains

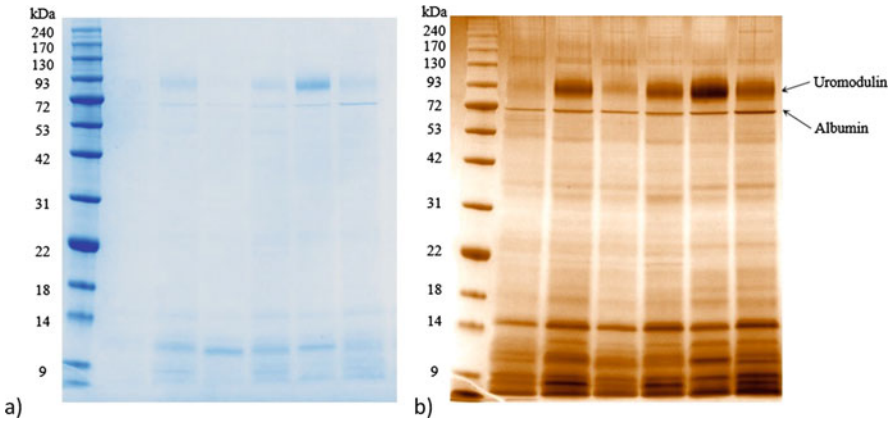

Fig. 5 SDS-PAGE of urine samples collected from piglets (3 months old). Three μg of proteins were loaded on a 12% gel in MOPS buffer and stained with (a) Coomassie brilliant blue staining or (b) silver staining. Putative uromodulin and albumin bands, identified on the basis of apparent molecular mass, are indicated

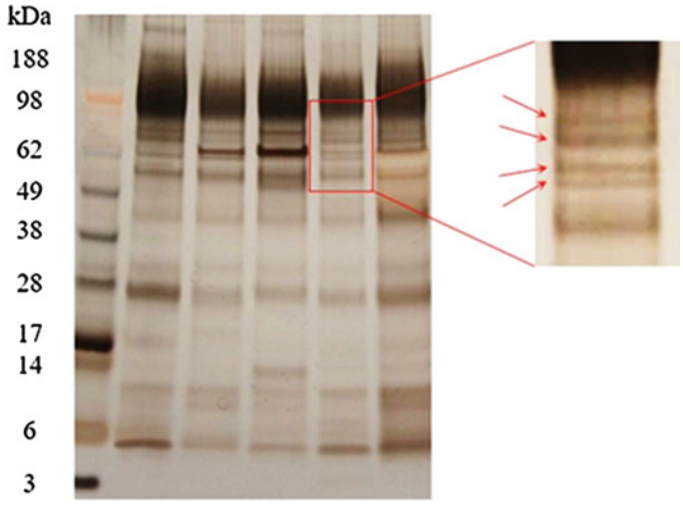

Fig. 6 SDS-PAGE of urine samples collected from healthy horses. Two μg of proteins were loaded on 4–12% gel in MES buffer and stained with silver staining. The magnification highlights the thin bands between 73 and 61 kDa

of IgG, respectively. In specimens affected by renal, gastrointestinal, and orthopedic diseases, the concentration of putative uromodulin declines, similar to observations reported also by Halbmayr and Schusser (2002). Also in horses, the decrease of uromodulin in urine seems to be a common sign of renal impairment, as reported in humans and dogs (Lhotta 2010; Raila et al. 2014). Other studies applied acetate and agarose gel electrophoresis to equine urine as a diagnostic tool

for the evaluation of peripartum stress and multiple myeloma. Jeffcott and Jeffcott (1974) analyzed proteinuria in foals during the first 24 h of life reporting the presence of low molecular mass proteins and the absence of gamma globulins. Geelen et al. (1997) evidenced an increase of IgG, whereas Pusterla et al. (2004) reported that the electrophoretic pattern presented peaks in the albumin and gamma globulin zones and that the gamma globulin fraction consisted mainly of IgA.

1.5.3 The Urinary Proteome of Companion Animals

Companion animals, though less investigated than humans, can be considered good models for future application of proteomic techniques to urine of farm animals. In recent years, research has shed light on the urinary proteome of a man's best friend. Urine of healthy dogs (*Canis familiaris*) is characterized by the presence of a few abundant proteins, namely, albumin, uromodulin, and immunoglobulin light chains, and in entire male adult dogs, of the prostate-specific proteins (Miller et al. 2014). Differently from other animal species, sex-specific proteins, e.g., arginine esterase similar to human prostate-specific antigen, represent most of total urine proteins in adult entire males, in contrast to females and castrated males. Similarly, in human urine, a total of 20 protein spots with more than a twofold change were identified and related to the prostate origin in males (Guo et al. 2015). The most complete characterization of the normal urinary proteome in dogs was recently performed by Brandt et al. (2014), who investigated exosomal and soluble proteins from dog urine by 1D SDS-PAGE combined with LC-MS/MS. Authors identified 391 and 214 proteins in exosomal pellets and urine supernatants, respectively. Interestingly, 52% of the exosomal fraction and 62% of soluble proteins identified in this study have been already identified in the human urinary proteome, and some of these proteins, e.g., uromodulin and retinol-binding protein, are already being used as urinary biomarkers for renal and extrarenal diseases in humans, pointing out the importance of the dog as an animal model for translational medicine.

Different studies have also applied proteomic techniques to dog urine to identify novel biomarkers of disease. In particular, the urinary proteome was investigated in dogs affected by nephropathy (Miller et al. 2004, 2014; Forterre et al. 2004; Nabity et al. 2011), different types of urinary stones (Forterre et al. 2006), transitional cell carcinoma (Bracha et al. 2014), envenomation by the bite of *Vipera berus berus* (Palviainen et al. 2012b), or infected by *Dirofilaria immitis* (Hormaeche et al. 2014) and by *Leishmania infantum* (Zaragoza et al. 2003; Ferlizza et al. 2013).

On the other hand, urine proteome in the domestic cat is still to be completely characterized. Only in recent years, two studies have analyzed the urinary proteome of healthy cats by 1D SDS-PAGE and 2DE (McLean et al. 2007; Ferlizza et al. 2015), yielding a preliminary proteomic map. The most abundant proteins are albumin, transferrin, uromodulin, and, in entire males, cauxin. Moreover, other low molecular mass proteins (<70 kDa) were identified as haptoglobin and Ig light chains and high molecular mass proteins (>70 kDa) as alpha-2-macroglobulin. Finally, few studies applied proteomic techniques to search for novel biomarkers in

urine of cats affected by chronic kidney disease (Ferlizza et al. 2015), tubular nephropathy (Miyazaki et al. 2007), urinary tract infection, idiopathic cystitis and urolithiasis (Lemberger et al. 2011), and azotemia (Jepson et al. 2013).

2 Proteomics of Saliva and Other Bodily Fluids in Farm Animals

As with urine, a range of bodily fluids are amenable to proteomics tools. The identification and characterization of the protein content of such fluids are being used to provide novel insights into the evolutionary adaptations of farm and domestic animal species and to characterize their normal physiological state. Results can be used to understand animal physiology, improve livestock productivity and welfare, and provide biomarkers of stress and animal disease.

2.1 Proteomics of Saliva

The identification and characterization of the proteome of saliva has been pioneered in human samples, to define pathophysiological-related changes relevant to disease pathogenesis and diagnostics. Such studies provide insights into the proteome and diagnostic potential of saliva and identify it as an amenable sample readily available from farm and domestic animals for detailed proteomic analysis (Mavromati et al. 2014). However, it is clear that there is significant variation in the protein content of saliva from different mammalian species, which likely reflects distinct evolutionary and environmental lineages (de Sousa-Pereira et al. 2013).

Gland-specific saliva can be collected directly from individual salivary glands which include the parotid, submandibular, sublingual, and minor salivary glands. Their respective characterizations allow for insights into gland-specific pathology. Whole (mixed) saliva comprises secretions from each of these salivary glands in addition to constituents of non-salivary origin readily detected in the oral cavity; these include gingival crevicular fluid, expectorated bronchial and nasal secretions, serum and blood derivatives from oral wounds, bacteria and their respective products, viruses, fungi, cellular components, and food debris. Collectively, the components of whole saliva comprise a bodily fluid essential for the health of the oral cavity and which functions to facilitate maintenance of homeostasis, food digestion, and protection from pathogens. Saliva comprises a complex hypotonic aqueous solution composed of more than 99% water that contains a range of proteins, peptides, enzymes, hormones, sugars, lipids, electrolytes, and a plethora of other compounds. Saliva is a relatively easy sample to collect; collection is noninvasive and stress-free. It can be performed multiple times on the same animal and in a relatively short time. Collectively, these factors make it an ideal candidate to screen for the identification of protein/peptide biomarkers of health status.

A cross-species comparison of the proteome of saliva was performed on seven mammalian species classified into four major evolutionary groups: ungulates (which include the order Artiodactyla, sheep and cattle, and the order Perissodactyla, horse), glires (which include rodents and lagomorphs: rat and rabbit), carnivores (dog), and primates (human) (de Sousa-Pereira et al. 2015). Distinct protein profiles were observed for each group, and only seven protein families were common to all groups; common protein families included carbonic anhydrase, albumin, polymeric immunoglobulin receptor, prolactin-inducible protein, lactoperoxidase, glutathione-S-transferase P, and keratin. Collectively, these protein families have antibacterial activity, buffer capacity, and lubrication activity. This comparative approach is defining a proteomic signature characteristic of each species and their respective environmental adaptation. For example, amylase, which plays a key role during digestion since it catalyzes the hydrolysis of glycosidic bonds from diet polysaccharides such as starch, was identified in the saliva of rodents, lagomorphs, carnivores, and ungulates. In contrast, latherin was identified only in horse saliva; this is a protein family that helps mastication of large quantities of dry food and reflects an adaptive mechanism to environmental constraints for horses. Goats have a higher tolerance than sheep to plant allelochemicals in their diet which has been hypothesized to correlate with the existence of tannin-binding proteins identified in goat, but not sheep, saliva (Austin et al. 1989; Narjisse et al. 1995).

Although saliva is amenable to the tools of proteomics, several studies have highlighted the importance of standardizing the collection and handling of saliva prior to analysis. Inherent variability and experimental artifacts during sample preparation and analysis have led to the proposal and development of standardized methodologies to collect, process, and extract samples, e.g., centrifugation for removal of food debris (Vitorino et al. 2012a, b; de Sousa-Pereira et al. 2015). Consideration must also be given to the fact that the secretion of saliva into the oral cavity results in the exposure of saliva to a large number of proteolytic activities that can be of endogenous and exogenous origin and that can rapidly process and modify salivary proteins. Protease inhibitors can be advantageous, but their use can also increase sample complexity and interfere with mass spectrometry. These variables are further compounded in animal studies compared to human studies when one must also consider the variation of animal diets, the mode of digestion, and the logistics of sample collection. Such considerations include inter- and intraindividual variability (Jehmlich et al. 2013), response to age and different environmental conditions, variability due to genetic polymorphisms, and the collection of stimulated versus unstimulated samples. Saliva composition is mainly under autonomic control and changes rapidly in response to a wide range of different chemical and mechanical stimuli (D'Amato et al. 1988; Edwards and Titchen 2002, 2003; Scocco et al. 2011). All samples of saliva should be processed immediately upon collection. Analysis of porcine saliva showed that its protein composition changes during storage at −20 °C, so preservation of samples at −80 °C is recommended (Messana et al. 2008; Gutiérrez et al. 2011a).

Porcine saliva has long been used as an analytical sample for specific pathogen disease diagnosis, either by direct detection of pathogen or indirectly via the

detection of specific antibodies (Gutiérrez et al. 2014). Multiple analytes have been detected and measured in porcine saliva and include haptoglobin (Hiss et al. 2003), cortisol (Ruis et al. 1997), estrone sulfate (Ohtaki et al. 1997), progesterone (Moriyoshi et al. 1996), and immunoglobulin (Van der Stede et al. 2001; Devillers et al. 2004). With the advent of increasingly sensitive instrumentation, C-reactive protein (CRP) was quantified in samples from healthy compared to diseased pigs as an indicator of pig health and welfare monitoring. The limit of detection for salivary CRP was 0.47 ng/mL, and salivary concentrations were ~322-fold lower than that of serum. Saliva samples could be stored at 20 °C for several days without compromising test results, and levels of CRP could differentiate healthy pigs from those with a variety of inflammatory conditions (Gutiérrez et al. 2009). Subsequent studies determined that while amounts of CRP were not influenced by circadian pattern, the amounts of other acute phase proteins such as haptoglobin differed according to early morning versus late afternoon sample collection; this highlights another variable (circadian rhythms) to consider for sample analysis as well as the need to report components of saliva as ranges rather than absolute values (Gutiérrez et al. 2013a). Initial studies using one- and two-dimensional electrophoresis and immunoblotting confirmed the complex dynamic nature of the porcine salivary proteome and provided 2D gel proteomic maps of salivary proteins including immunoglobulins (Gutiérrez et al. 2011b). Multiple protein isoforms are attributed to fragmentation, bacterial degradation, endogenous truncation, glycosylation, and phosphorylation. Thirteen proteins were identified by mass spectrometry (Gutiérrez et al. 2011b). Improved databases and comparative analyses increased this to 20 unique proteins and provided normalization strategies to identify a panel of biomarkers that could discriminate clinically infected pigs compared to their healthy counterparts (Gutiérrez et al. 2013b). Porcine saliva and serum share several proteins highlighting the potential for these to act as markers of general health. The most abundant porcine salivary proteins include lipocalins, in contrast to amylase which comprises up to 25% of the human salivary proteome. Porcine saliva also contains proline-rich proteins, but note that their detection on gels is stain dependent (Miller et al. 2006). Enrichment for glycoproteins using boronic acid allowed for the detection of differential expression of carbonic anhydrase VI, α-1-antichymotrypsin, and haptoglobin by 2D gel electrophoresis of saliva derived from pigs suffering rectal prolapse; interestingly, glycopatterns of haptoglobin in saliva differed to that of haptoglobin derived from serum (Gutiérrez et al. 2016).

Cows secrete on the order of 150 liters of saliva per day (Bailey 1961). A comprehensive proteome analysis of bovine saliva identified 402 salivary proteins and 45 N-linked glycoproteins using three different strategies: nontargeted, targeted, and glycocapture (Ang et al. 2011). In the nontargeted approach, four prefractionation methodologies were performed based on different physiochemical properties and included SDS-PAGE (electrophoretic mobility), off-gel fractionation (protein isoelectric point), reverse-phase HPLC (protein hydrophobicity), and strong cation exchange HPLC (peptide charge). This nontargeted approach facilitated the identification of 396 proteins by MS, only 10% (42) of which were identified in all approaches, highlighting the need for multiple complementary strategies for comprehensive

analyses. The targeted approach used publicly available information to create a list of peptides for identification by mass accuracy and resolution on the mass spectrometer; it is limited by the magnitude of selected databases used to generate a user list. The glycocapture approach utilized hydrazide coupling methodology to enrich for glycosylated peptides prior to MS and provides the complete N-glycoprotein profile of bovine saliva. The most abundant proteins in bovine saliva are similar to that of human with the notable absence of amylase and the presence of short palate, lung and nasal epithelium carcinoma-associated protein 2A (BSP30), and odorant-binding protein (Ang et al. 2011). In buffalo, protein content of whole saliva has been monitored through all stages of the estrus cycle (Muthukumar et al. 2014). Collectively, 179 proteins were identified from saliva taken at different stages of the estrus cycle, but 37 of these were expressed exclusively during estrus; the expression of β-enolase and TLR 4 in saliva was validated by immunoblot as an indicator of estrus in buffalo (Muthukumar et al. 2014).

Sheep secrete on the order of 10 liters of saliva per day (Kay 1960). Separation of the ovine salivary proteome by SDS-PAGE followed by MS identified 319 protein families and a predominance of proteins associated with metabolism and inflammation (de Sousa-Pereira et al. 2015). Three hundred and twenty-four unique proteins were identified compared to only 74 unique proteins that were identified in bovine saliva when processed in the same way; for example, CATHL1 and CATHL2, members of the cathelicidin family, were identified in sheep saliva, whereas only CATHL4 was identified in bovine saliva, suggesting differential evolutionary pressures. Analysis of ovine saliva collected directly from the parotid gland comprised 260 protein spots evident by 2D gel electrophoresis, 106 of which were identified using MALDI-TOF MS and 11 by LC-MS/MS totaling 26 different proteins, several of which show evidence of phosphorylation and/or glycosylation (Lamy et al. 2009). Identified proteins function primarily as transporters (i.e., annexin, apolipoprotein, haptoglobin, serum albumin, serotransferrin, transthyretin, vitamin D-binding protein, hemoglobin, lactoferrin, lactoglobulin, casein) followed by immune modulation (e.g., immunoglobulin and antimicrobials). As with all complex protein samples, multiple fractionation and identification strategies are advised for a more comprehensive analysis of protein content. Comparison of sheep parotid gland secretions with that of goats was very similar by 2DE; only three proteins were identified as unique to goats, apolipoprotein A-IV, hemoglobin, and cathelicidin-3 precursor, compared to two proteins which were unique to sheep, clusterin and transthyretin precursor (Lamy et al. 2009). Sheep and goats that were conditioned on Quebracho tannin extract showed increased total protein content and proteomic changes in parotid saliva (Lamy et al. 2011); however, no constitutive changes in tannin-binding salivary proteins were detected.

LC-MS/MS has also been applied to saliva derived from horses (de Sousa-Pereira et al. 2015). Of the 195 unique proteins identified, 57 were found only in saliva from horses with systemic inflammation which included acute phase proteins such as serum amyloid A, fibrinogen, haptoglobin, and alpha1-acid glycoprotein (Jacobsen et al. 2014).

2.2 Proteomics of Other Bodily Fluids

Proteomic analysis has been pioneered on an assortment of alternative bodily fluids in a subset of domestic animal species including cerebrospinal fluid, bronchoalveolar lavage fluid, and amniotic fluids.

Cerebrospinal fluid (CSF) is a potential source of biomarkers of aging and neurodegenerative disorders which can be detected and characterized using the tools of proteomics. Since CSF surrounds the brain and spinal cord and acts as an intermediate between blood and nervous tissue, its proteomic content reflects the metabolic state of normal versus diseased brain tissue. Analysis of bovine CSF from 75 suspect cases of bovine spongiform encephalopathy (BSE) compared with 38 normal cattle by 2D gel electrophoresis showed consistent differences including the presence of apolipoprotein E and two unidentified proteins of 35 and 36 kDa with a pI of 5.5 (Jones et al. 1996) and as similarly observed by Hochstrasser et al. (1997). A comprehensive reference map of bovine CSF proteins has been generated and includes the identification of 66 different proteins, 58 of which had not been previously identified in bovine CSF (Brenn et al. 2009). Charge isoforms were identified for nearly all proteins. The high salt content and low amounts of protein in CSF require optimal sample preparation. The effect of four different sample treatments to ovine CSF was compared; protein precipitation with acetone or using a 2D cleanup kit showed best sample recovery in terms of protein gel spots of 2DE gels compared to the use of two different spin columns. However, the 53 kDa transthyretin tetramer was not identified in samples treated with the 2D cleanup kit but was retained in samples treated with acetone or spin columns (Chen et al. 2006). Ovine CSF has been used to study age-related changes in composition; results indicate that there is an age-related reduction in CSF turnover which has a concentrating effect, and therefore, CFS protein concentrations should be normalized according to their age-specific turnovers before comparison with samples from different age groups (Chen et al. 2010). Among 103 proteins identified in ovine CSF, 41 were differentially regulated according to photoperiod; 18 were more abundant during long days, and 23 were more abundant in short days (Teixeira-Gomes et al. 2015). Equine CSF had been analyzed for the presence of apolipoproteins associated with high-density lipoproteins; as is the case in plasma, apo A-II circulates as a homodimer, whereas there appears to be a higher percentage of acylated apo A-I in CSF compared to plasma (Puppione et al. 2012).

The respiratory health of domestic farm animals can be determined via the proteome content of bronchoalveolar lavage fluid (BALF), which contains secreted peptides and proteins derived from airway mucosa and alveolar surfaces that contribute to host defenses. The antimicrobial peptides, prophenin-2 and PR-39, and the calcium-binding protein calgranulin C were reproducibly upregulated in BALF of pigs chronically infected with *Actinobacillus pleuropneumoniae* (Hennig-Pauka et al. 2006). Concentrations of PR-39 were significantly ($P < 0.05$) increased in BALF (median of 4.8 nM) but not in serum (median of 2.5 nM) on day 21 after infection. With the advent of the *Sus scrofa* genome database, a

comprehensive analysis of porcine BALF and methacholine-induced tracheal secretions identified 3858 porcine-specific proteins that encompass a diverse array of functions that include host defense, molecular transport, cell communication, and cytoskeletal and metabolic functions (Bartlett et al. 2013). Cattle that are subjected to stressors such as transportation, weaning, and comingling demonstrate an increased susceptibility to bacterial pneumonia which in turn is associated with elevated levels of endogenous glucocorticoids. Consistent with the initiation of an acute phase response, dexamethasone-treated cattle have increased levels of alpha-1-acid glycoprotein and alpha-1-antitrypsin in BALF which was detected by 2DE, while levels of alpha-HS-glycoprotein were decreased (Mitchell et al. 2007). Treatment with dexamethasone also induced adipocyte fatty acid-binding protein and odorant-binding protein as well as alpha-enolase, cofilin-1, and immunoglobulin J chain. Similar results were obtained in BALF samples collected from weaned and transported calves (Mitchell et al. 2008). LC-MS/MS of bovine BALF identified 88 unique proteins, of which 20 were only detected in samples collected from steers with experimentally induced clinical pneumonia with *Mannheimia haemolytica* (Boehmer et al. 2011). Differences in protein pattern/concentration were detected between sham- and *M. haemolytica*-infected steers for haptoglobin, as well as the antimicrobial peptides cathelicidin-1 and cathelicidin-4 and inter-α-trypsin inhibitor heavy chain-4. A genetic linkage study linked eight candidate genes with proteins in BALF collected from healthy and recurrent airway obstruction-affected horses (Racine et al. 2011). The equine genome allowed for the identification of 582 proteins in normal cell-free equine BALF confirming genome annotation and providing functional annotation and as well as a framework for continued analysis of the biological significance of BALF protein content (Bright et al. 2011).

Characterization of the equine amniotic fluid proteome is a prerequisite to study changes during disease associated with pregnancy and thus to identify biomarkers of health status or embryonic abnormalities (Isani et al. 2016). Thirty-four proteins were identified of which 12 were associated with the extracellular matrix highlighting their important role during the development of fetal tissues. The three most abundant proteins were albumin, major allergen Eqc1, and fibronectin. No immunoglobulin was detected in equine amniotic fluid. A reference map for the equine amniotic membrane has been established (Galera et al. 2015). From gel-based proteomics, 49 spots were excised and 43 proteins were identified by LC-MS/MS. Shotgun proteomics allowed the identification of 116 proteins; only 10 protein identifications were common to both analyses. A comparison of the proteomic profile of the chorioamnion and corresponding caruncle for buffalo embryos associated with normal or retarded development by 2D-DIGE identified decreased concentration of proteins involved with protein folding (HSP 90-alpha, calreticulin), calcium binding (annexin A1, annexin A2), and coagulation (fibrinogen alpha-chain) ($P < 0.05$), whereas proteins involved in protease inhibition (alpha-1-antiproteinase, serpin H1, serpin A3-8), DNA and RNA binding (heterogeneous nuclear ribonucleoproteins A2/B1 and K), chromosome segregation (serine/threonine-protein phosphatase 2A), cytoskeletal organization (ezrin), cell

redox homeostasis (amine oxidase-A), and hemoglobin binding (haptoglobin) were upregulated ($P < 0.05$) (Balestrieri et al. 2013). The identity of 139 individual protein species was confirmed in bovine amniotic and allantoic fluids collected at day 45 of gestation using two strategies: first, 2DE combined with MALDI-TOF-MS/MS and LC-ESI-MS/MS analysis of individual protein spots and, second, a global protein snapshot of the enriched 5–50 kDa protein fraction by LC-ESI-MS/MS and LC-MALDI-TOF-MS/MS (Riding et al. 2008). Immunoglobulin was identified in bovine allantoic fluid at day 45 of gestation. Amniotic and allantoic fluids collected from pregnant ewes experimentally infected with *Chlamydophila abortus* or *Toxoplasma gondii* also contain immunoglobulin specific for antigens derived from their respective infectious agent (Marques et al. 2011, 2012). In both cases, results suggest both a maternal and fetal source of immunoglobulin.

3 Conclusions

Proteomics provides for the characterization of complex biological samples. Bodily fluids, including urine, saliva, CSF, and BALF, and amniotic fluids are readily amenable to the tools of proteomics. Though farm animals are mammals that are characterized by high homology at the genomic, proteomic, and metabolic level, it is clear that they are readily discriminated by differences in their respective proteomes. This is exemplified by the characterization of urine and saliva which provides unique insight into an animal's normal physiological state. The continued characterization of such fluids can be used to further provide insights into how animals respond to environmental pressures, as well as defining biomarkers of pathophysiological-related changes to health and fitness.

References

Abate O, Vittone V, Zanatta R et al (2005) Valutazione qualitativa della proteinuria mediante SDS-AGE ai fini della localizzazione del danno renale nel cane e nel gatto. Veterinaria 19:9–14

Adachi J, Kumar C, Zhang Y et al (2006) The human urinary proteome contains more than 1500 proteins, including a large proportion of membrane proteins. Genome Biol 7:R80. https://doi.org/10.1186/gb-2006-7-9-r80

Alhaider A, Bayoumy N, Argo E et al (2012) Survey of the camel urinary proteome by shotgun proteomics using a multiple database search strategy. Proteomics 12:3403–3406. https://doi.org/10.1002/pmic.201100631

Almeida AM, Bassols A, Bendixen E et al (2015) Animal board invited review: advances in proteomics for animal and food sciences. Animal 9:1–17. https://doi.org/10.1017/S1751731114002602

Andersen S, Mischak H, Zürbig P, Parving HH, Rossing P (2010) Urinary proteome analysis enables assessment of renoprotective treatment in type 2 diabetic patients with microalbuminuria. BMC Nephrol 11:29. https://doi.org/10.1186/1471-2369-11-29.

Anderson NG, Anderson NL, Tollaksen SL et al (1979) Analytical techniques for cell fractions: XXV. Concentration and two-dimensional electrophoretic analysis of human urinary proteins. Anal Biochem 95:48–61. https://doi.org/10.1016/0003-2697(79)90184-2

Andrievskaia O, Algire J, Balachandran A, Nielsen K (2008) Prion protein in sheep urine. J Vet Diagnostic Investig 20:141–146. https://doi.org/10.1177/104063870802000201

Ang CS, Binos S, Knight MI et al (2011) Global survey of the bovine salivary proteome: integrating multidimensional prefractionation, targeted, and glycocapture strategies. J Proteome Res 10:5059–5069. https://doi.org/10.1021/pr200516d

Austin PJ, Suchar LA, Robbins CT, Hagerman AE (1989) Tannin-binding proteins in saliva of deer and their absence in saliva of sheep and cattle. J Chem Ecol 15:1335–1347. https://doi.org/10.1007/BF01014834

Bailey CB (1961) Saliva secretion and its relation to feeding in cattle. 3. The rate of secretion of mixed saliva in the cow during eating, with an estimate of the magnitude of the total daily secretion of mixed saliva. Br J Nutr 15:443–451. https://doi.org/10.1079/BJN19610062

Balestrieri ML, Gasparrini B, Neglia G et al (2013) Proteomic profiles of the embryonic chorioamnion and uterine caruncles in buffaloes (*Bubalus bubalis*) with normal and retarded embryonic development. Biol Reprod 88:1–14. https://doi.org/10.1095/biolreprod.113.108696

Barratt J, Topham P (2007) Urine proteomics: the present and future of measuring urinary protein components in disease. Can Med Assoc J 177:361–368. https://doi.org/10.1503/cmaj.061590

Bartlett JA, Albertolle ME, Wohlford-Lenane C et al (2013) Protein composition of bronchoalveolar lavage fluid and airway surface liquid from newborn pigs. Am J Physiol Lung Cell Mol Physiol 305:L256–L266. https://doi.org/10.1152/ajplung.00056.2013

Bathla S, Rawat P, Baithalu R et al (2015) Profiling of urinary proteins in Karan Fries cows reveals more than 1550 proteins. J Proteomics 127:193–201. https://doi.org/10.1016/j.jprot.2015.05.026

Boehmer JL, Degrasse JA, Lancaster VA et al (2011) Evaluation of protein expression in bovine bronchoalveolar fluid following challenge with *Mannheimia haemolytica*. Proteomics 11:3685–3697. https://doi.org/10.1002/pmic.201000710

Bonfanti U, Zini E, Minetti E, Zatelli A (2004) Free light-chain proteinuria and normal renal histopathology and function in 11 dogs exposed to *Leishmania infantum*, *Ehrlichia canis*, and *Babesia canis*. J Vet Intern Med 18:618–624. https://doi.org/10.1111/j.1939-1676.2004.tb02596.x

Bracha S, McNamara M, Hilgart I et al (2014) A multiplex biomarker approach for the diagnosis of transitional cell carcinoma from canine urine. Anal Biochem 455:41–47. https://doi.org/10.1016/j.ab.2014.03.017

Brandt LE, Ehrhart EJ, Scherman H et al (2014) Characterization of the canine urinary proteome. Vet Clin Pathol 43:193–205. https://doi.org/10.1111/vcp.12147

Brenn A, Karger A, Skiba M et al (2009) A comprehensive proteome map of bovine cerebrospinal fluid. Proteomics 9:5199–5205. https://doi.org/10.1002/pmic.200800906

Bright LA, Mujahid N, Nanduri B et al (2011) Functional modelling of an equine bronchoalveolar lavage fluid proteome provides experimental confirmation and functional annotation of equine genome sequences. Anim Genet 42:395–405. https://doi.org/10.1111/j.1365-2052.2010.02158.x

Candiano G, Santucci L, Petretto A et al (2010) 2D-electrophoresis and the urine proteome map: where do we stand? J Proteomics 73:829–844. https://doi.org/10.1016/j.jprot.2009.12.003

Chen RL, Sage EA, Dunn MJ et al (2006) Optimising ovine cerebrospinal fluid preparation for two-dimensional gel electrophoresis. Proteomics 6:3170–3175. https://doi.org/10.1002/pmic.200500623

Chen CPC, Chen RL, Preston JE (2010) The influence of cerebrospinal fluid turnover on age-related changes in cerebrospinal fluid protein concentrations. Neurosci Lett 476:138–141. https://doi.org/10.1016/j.neulet.2010.04.015

D'Amato M, De Beurme FA, Lefebvre RA (1988) Comparison of the effect of vasoactive intestinal polypeptide and non-adrenergic non-cholinergic neurone stimulation in the cat gastric fundus. Eur J Pharmacol 152:71–82

de Sousa-Pereira P, Abrantes J, Colaço B et al (2013) Characterization of thymosin β4 in mammals' saliva. Peptides 40:1–7. https://doi.org/10.1016/j.peptides.2012.12.007

de Sousa-Pereira P, Cova M, Abrantes J et al (2015) Cross-species comparison of mammalian saliva using an LC-MALDI based proteomic approach. Proteomics 15:1598–1607. https://doi.org/10.1002/pmic.201400083

Decramer S, Gonzalez de Peredo A, Breuil B et al (2008) Urine in clinical proteomics. Mol Cell Proteomics 7:1850–1862. https://doi.org/10.1074/mcp.R800001-MCP200

Devarajan P (2011) Biomarkers for the early detection of acute kidney injury. Curr Opin Pediatr 23:194–200. https://doi.org/10.1097/MOP.0b013e328343f4dd.

Devillers N, Farmer C, Mounier AM et al (2004) Hormones, IgG and lactose changes around parturition in plasma, and colostrum or saliva of multiparous sows. Reprod Nutr Dev 44:381–396. https://doi.org/10.1051/rnd:2004043

Di Meo A, Batruch I, Yousef AG et al (2017) An integrated proteomic and peptidomic assessment of the normal human urinome. Clin Chem Lab Med 55:237–247. https://doi.org/10.1515/cclm-2016-0390

Eckersall PD (2008) Proteins, proteomics, and the dysproteinemias. In: Kaneko JJ, Harvey JW, Bruss ML (eds) Clinical biochemistry of domestic animals, 6th edn. Elsevier Academic Press, Amsterdam, pp 117–155

Edwards A, Titchen D (2002) The effect of parasympathetic postganglionic denervation on parotid salivary protein secretion in anaesthetized sheep. Auton Neurosci 100:50–57. https://doi.org/10.1016/S1566-0702(02)00148-0

Edwards A, Titchen D (2003) Autonomic control of protein production by the parotid gland of the sheep. Auton Neurosci 103:38–49. https://doi.org/10.1016/S1566-0702(02)00147-9

Ferlizza E, Buono A, Fracassi F et al (2013) Urine proteome in dogs affected by leishmaniasis and Cushing's syndrome. Biochim Clin 37, Special Supplement. S219

Ferlizza E, Campos A, Neagu A et al (2015) The effect of chronic kidney disease on the urine proteome in the domestic cat (*Felis catus*). Vet J 204:73–81. https://doi.org/10.1016/j.tvjl.2015.01.023

Ferlizza E, Dondi F, Andreani G et al (2017) Validation of an electrophoretic method to detect albuminuria in cat. J Feline Med Surg 19:860–868. https://doi.org/10.1177/1098612X16664112

Filip S, Vougas K, Zoidakis J et al (2015) Comparison of depletion strategies for the enrichment of low-abundance proteins in urine. PLoS One 10:e0133773. https://doi.org/10.1371/journal.pone.0133773

Forterre S, Raila J, Schweigert FJ (2004) Protein profiling of urine from dogs with renal disease using ProteinChip analysis. J Vet Diagn Invest 16:271–277

Forterre S, Raila J, Kohn B et al (2006) Protein profiling of organic stone matrix and urine from dogs with urolithiasis. J Anim Physiol Anim Nutr (Berl) 90:192–199

Galera PD, Ribeiro CR, Sapp HL et al (2015) Proteomic analysis of equine amniotic membrane: characterization of proteins. Vet Ophthalmol 18:198–209. https://doi.org/10.1111/vop.12190

Geckle M (2007) Renal albumin handling: a look at the dark side of the filter. Kidney Int 71:479–481. https://doi.org/10.1038/sj.ki.5002123

Geelen SNJ, Bernadina WE, Grinwis GCM, Kalsbeek HC (1997) Monoclonal gammopathy in a Dutch warmblood mare. Vet Q 19:29–32. https://doi.org/10.1080/01652176.1997.9694734

Gerber B, Eichenberger S, Haug K et al (2009) Association of urine protein excretion and infection with *Borrelia burgdorferi* sensu lato in Bernese Mountain dogs. Vet J 182:487–488. https://doi.org/10.1016/j.tvjl.2008.08.022

Giori L, Tricomi FM, Zatelli A et al (2011) High-resolution gel electrophoresis and sodium dodecyl sulphate-agarose gel electrophoresis on urine samples for qualitative analysis of proteinuria in dogs. J Vet Diagn Invest 23:682–690. https://doi.org/10.1177/1040638711407900

Guo Z, Zhang Y, Zou L et al (2015) A proteomic analysis of individual and gender variations in normal human urine and cerebrospinal fluid using iTRAQ quantification. PLoS One 10: e0133270. https://doi.org/10.1371/journal.pone.0133270

Gutiérrez AM, Martínez-Subiela S, Eckersall PD, Cerón JJ (2009) C-reactive protein quantification in porcine saliva: a minimally invasive test for pig health monitoring. Vet J 181:261–265. https://doi.org/10.1016/j.tvjl.2008.03.021

Gutiérrez AM, Martínez-Subiela S, Cerón JJ (2011a) Evaluation of changes in haptoglobin and C-reactive protein concentrations caused by freezing of saliva and meat juice samples collected from healthy and diseased pigs. Am J Vet Res 72:11–17. https://doi.org/10.2460/ajvr.72.1.11

Gutiérrez AM, Miller I, Hummel K et al (2011b) Proteomic analysis of porcine saliva. Vet J 187:356–362. https://doi.org/10.1016/j.tvjl.2009.12.020

Gutiérrez AM, Escribano D, Fuentes M, Cerón JJ (2013a) Circadian pattern of acute phase proteins in the saliva of growing pigs. Vet J 196:167–170. https://doi.org/10.1016/j.tvjl.2012.10.003

Gutiérrez AM, Nöbauer K, Soler L et al (2013b) Detection of potential markers for systemic disease in saliva of pigs by proteomics: a pilot study. Vet Immunol Immunopathol 151:73–82. https://doi.org/10.1016/j.vetimm.2012.10.007

Gutiérrez AM, Cerón JJ, Fuentes-Rubio M et al (2014) A proteomic approach to porcine saliva. Curr Protein Pept Sci 15:56–63. https://doi.org/10.2174/1389203715666140221115704

Gutiérrez AM, Miller I, Kolarich D et al (2016) Detection and first characterization of an uncommon haptoglobin in porcine saliva of pigs with rectal prolapse by using boronic acid sample enrichment. Animal 1860:1–9. https://doi.org/10.1017/S1751731116002159

Halbmayr E, Schusser GF (2002) Quantitative and qualitative urinary protein excretion in healthy and sick horses. Tierarztl Prax Ausgabe G Grosstiere - Nutztiere 30:115–123

He W, Huang C, Luo G et al (2012) A stable panel comprising 18 urinary proteins in the human healthy population. Proteomics 12:1059–1072. https://doi.org/10.1002/pmic.201100400

Hennig-Pauka I, Jacobsen I, Blecha F et al (2006) Differential proteomic analysis reveals increased cathelicidin expression in porcine bronchoalveolar lavage fluid after an *Actinobacillus pleuropneumoniae* infection. Vet Res 37:75–87. https://doi.org/10.1051/vetres:2005043

Hiss S, Knura-Deszczka S, Regula G et al (2003) Development of an enzyme immuno assay for the determination of porcine haptoglobin in various body fluids: testing the significance of meat juice measurements for quality monitoring programs. Vet Immunol Immunopathol 96:73–82. https://doi.org/10.1016/S0165-2427(03)00141-7

Hochstrasser DF, Frutiger S, Wilkins MR et al (1997) Elevation of apolipoprotein E in the CSF of cattle affected by BSE. FEBS Lett 416:161–163. https://doi.org/10.1016/S0014-5793(97)01191-5

Hormaeche M, Carretón E, González-Miguel J et al (2014) Proteomic analysis of the urine of Dirofilaria immitis infected dogs. Vet Parasitol 203:241–246. https://doi.org/10.1016/j.vetpar.2014.01.025

Isani G, Ferlizza E, Cuoghi A et al (2016) Identification of the most abundant proteins in equine amniotic fluid by a proteomic approach. Anim Reprod Sci 174:150–160. https://doi.org/10.1016/j.anireprosci.2016.10.003

Jacobsen S, Top Adler DM, Bundgaard L et al (2014) The use of liquid chromatography tandem mass spectrometry to detect proteins in saliva from horses with and without systemic inflammation. Vet J 202:483–488. https://doi.org/10.1016/j.tvjl.2014.08.032

Jeffcott LB, Jeffcott TJ (1974) Studies on passive immunity in the foal: III. The characterization and significance of neonatal proteinuria. J Comp Pathol 84:455–465

Jehmlich N, Dinh KHD, Gesell-Salazar M et al (2013) Quantitative analysis of the intra- and inter-subject variability of the whole salivary proteome. J Periodontal Res 48:392–403. https://doi.org/10.1111/jre.12025

Jepson R, Coulton G, Cowan M et al (2013) Evaluation of mass spectrometry of urinary proteins and peptides as biomarkers for cats at risk of developing azotemia. Am J Vet Res 74:333–342

Jones V, Martin TC, Keyes P, Dawson M (1996) Protein markers in cerebrospinal fluid from bse-affected cattle. Vet Rec 139:360–363

Kay RNB (1960) The rate of flow and composition of various salivary secretions in sheep and calves. J Physiol 150:515–537. https://doi.org/10.1113/jphysiol.1960.sp006402

Khan A, Packer NH (2006) Simple urinary sample preparation for proteomic analysis. J Proteome Res 5:2824–2838. https://doi.org/10.1021/pr060305y

Lamy E, da Costa G, Santos R et al (2009) Sheep and goat saliva proteome analysis: a useful tool for ingestive behavior research? Physiol Behav 98:393–401. https://doi.org/10.1016/j.physbeh.2009.07.002

Lamy E, Da Costa G, Santos R et al (2011) Effect of condensed tannin ingestion in sheep and goat parotid saliva proteome. J Anim Physiol Anim Nutr 95:304–312. https://doi.org/10.1111/j.1439-0396.2010.01055.x

Lavoué R, Trumel C, Smets PMY et al (2015) Characterization of proteinuria in dogue de bordeaux dogs, a breed predisposed to a familial glomerulonephropathy: a retrospective study. PLoS One 10:e0133311. https://doi.org/10.1371/journal.pone.0133311

Lemberger SIK, Deeg CA, Hauck SM et al (2011) Comparison of urine protein profiles in cats without urinary tract disease and cats with idiopathic cystitis, bacterial urinary tract infection, or urolithiasis. Am J Vet Res 72:1407–1415. https://doi.org/10.2460/ajvr.72.10.1407

Lhotta K (2010) Uromodulin and chronic kidney disease. Kidney Blood Press Res 33:393–398. https://doi.org/10.1159/000320681

Li QR, Fan KX, Li RX et al (2010) A comprehensive and non-prefractionation on the protein level approach for the human urinary proteome: touching phosphorylation in urine. Rapid Commun Mass Spectrom 24:823–832. https://doi.org/10.1002/rcm.4441

Liu X, Shao C, Wei L et al (2012) An individual urinary proteome analysis in normal human beings to define the minimal sample number to represent the normal urinary proteome. Proteome Sci 10:70. https://doi.org/10.1186/1477-5956-10-70

Marimuthu A, O'Meally RN, Chaerkady R et al (2011) A comprehensive map of the human urinary proteome. J Proteome Res 10:2734–2743. https://doi.org/10.1021/pr2003038

Marques PX, O'Donovan J, Souda P et al (2011) Amniotic and allantoic fluids from experimentally infected sheep contain immunoglobulin specific for *Chlamydophila abortus*. Vet Immunol Immunopathol 140:1–9. https://doi.org/10.1016/j.vetimm.2010.11.002

Marques PX, O'Donovan J, Williams EJ et al (2012) Detection of *Toxoplasma gondii* antigens reactive with antibodies from serum, amniotic, and allantoic fluids from experimentally infected pregnant ewes. Vet Parasitol 185:91–100. https://doi.org/10.1016/j.vetpar.2011.10.028

Martin-Lorenzo M, Gonzalez-Calero L, Zubiri I et al (2014) Urine 2DE proteome analysis in healthy condition and kidney disease. Electrophoresis 35:2634–2641. https://doi.org/10.1002/elps.201300601

Mavromati J, Cash P, Restelli L, Soler L (2014) Proteomics and protein analyses of ovine and caprine body fluids: current studies and future promises. Curr Protein Pept Sci 15:45–55. https://doi.org/10.2174/1389203715666140221113544

McLean L, Hurst JL, Gaskell CJ et al (2007) Characterization of cauxin in the urine of domestic and big cats. J Chem Ecol 33:1997–2009. https://doi.org/10.1007/s10886-007-9354-6

Messana I, Inzitari R, Fanali C et al (2008) Facts and artifacts in proteomics of body fluids. What proteomics of saliva is telling us? J Sep Sci 31:1948–1963. https://doi.org/10.1002/jssc.200800100

Miller I, Teinfalt M, Leschnik M et al (2004) Nonreducing two-dimensional gel electrophoresis for the detection of Bence Jones proteins in serum and urine. Proteomics 4:257–260. https://doi.org/10.1002/pmic.200300546

Miller I, Crawford J, Gianazza E (2006) Protein stains for proteomic applications: which, when, why? Proteomics 6:5385–5408

Miller I, Preßlmayer-Hartler A, Wait R et al (2014) In between - Proteomics of dog biological fluids. J Proteomics 106:30–45. https://doi.org/10.1016/j.jprot.2014.04.016

Mitchell GB, Clark ME, Caswell JL (2007) Alterations in the bovine bronchoalveolar lavage proteome induced by dexamethasone. Vet Immunol Immunopathol 118:283–293. https://doi.org/10.1016/j.vetimm.2007.05.017

Mitchell GB, Clark ME, Siwicky M, Caswell JL (2008) Stress alters the cellular and proteomic compartments of bovine bronchoalveolar lavage fluid. Vet Immunol Immunopathol 125:111–125. https://doi.org/10.1016/j.vetimm.2008.05.005

Miyazaki M, Soeta S, Yamagishi N et al (2007) Tubulointerstitial nephritis causes decreased renal expression and urinary excretion of cauxin, a major urinary protein of the domestic cat. Res Vet Sci 82:76–79. https://doi.org/10.1016/j.rvsc.2006.06.009

Molina L, Salvetat N, Ben AR et al (2011) Analysis of the variability of human normal urine by 2D-GE reveals a "public" and a "private" proteome. J Proteomics 75:70–80. https://doi.org/10.1016/j.jprot.2011.06.031

Moriyoshi M, Tamaki M, Nakao T, Kawata K (1996) Early pregnancy diagnosis in the sow by saliva progesterone measurement using a bovine milk progesterone qualitative test EIA kit. J Vet Med Sci 58:737–741

Muthukumar S, Rajkumar R, Rajesh D et al (2014) Exploration of salivary proteins in buffalo: an approach to find marker proteins for estrus. FASEB J 28:4700–4709. https://doi.org/10.1096/fj.14-252288

Nabity MB, Lees GE, Dangott LJ et al (2011) Proteomic analysis of urine from male dogs during early stages of tubulointerstitial injury in a canine model of progressive glomerular disease. Vet Clin Pathol 40:222–236. https://doi.org/10.1111/j.1939-165X.2011.00307.x

Nagaraj N, Mann M (2011) Quantitative analysis of the intra-and inter-individual variability of the normal urinary proteome. J Proteome Res 10:637–645

Narjisse H, Elhonsali MA, Olsen JD (1995) Effects of oak (*Quercus ilex*) tannins on digestion and nitrogen balance in sheep and goats. Small Rumin Res 18:201–206. https://doi.org/10.1016/0921-4488(95)00700-0

Oh J, Pyo JH, Jo EH et al (2004) Establishment of a near-standard two-dimensional human urine proteomic map. Proteomics 4:3485–3497. https://doi.org/10.1002/pmic.200401018

Ohtaki T, Moriyoshi M, Nakada K et al (1997) Radioimmunoassay of saliva estrone sulfate in pregnant sows. J Vet Med Sci 59:759–763. https://doi.org/10.1292/jvms.59.759

Ozgo M, Skrzypczak W, Drzezdzon D et al (2009) Urinary excretion of low molecular weight proteins in goats. J Physiol Pharmacol 60:119–125

Paltrinieri S, Sironi G, Giori L et al (2015) Changes in serum and urine SAA concentrations and qualitative and quantitative proteinuria in abyssinian cats with familial amyloidosis: a five-year longitudinal study (2009-2014). J Vet Intern Med 29:505–512. https://doi.org/10.1111/jvim.12561

Palviainen M, Raekallio M, Rajamäki MM et al (2012a) Kidney-derived proteins in urine as biomarkers of induced acute kidney injury in sheep. Vet J 193:287–289. https://doi.org/10.1016/j.tvjl.2011.10.004

Palviainen M, Raekallio M, Vainionpää M et al (2012b) Proteomic profiling of dog urine after European adder (*Vipera berus berus*) envenomation by two-dimensional difference gel electrophoresis. Toxicon 60:1228–1234. https://doi.org/10.1016/j.toxicon.2012.08.010

Pastushkova LK, Kononikhin AS, Tiys ES et al (2016) Characteristics of age-dependent changes in urine proteome in healthy men. Adv Gerontol 6:123–128. https://doi.org/10.1134/S2079057016020107

Plews M, Lamoureux L, Simon SL et al (2011) Factors affecting the accuracy of urine-based biomarkers of BSE. Proteome Sci 9:6. https://doi.org/10.1186/1477-5956-9-6

Puppione DL, Della Donna L, Bassilian S et al (2012) Mass spectral measurements of the apoHDL in horse (*Equus caballus*) cerebrospinal fluid. Comp Biochem Physiol Part D Genomics Proteomics 7:172–174. https://doi.org/10.1016/j.cbd.2012.02.002

Pusterla N, Stacy BA, Vernau W et al (2004) Immunoglobulin A monoclonal gammopathy in two horses with multiple myeloma. Vet Rec 155:19–23. https://doi.org/10.1136/vr.155.1.19

Pyo J, Hwang SI, Oh J et al (2003) Characterization of a bovine pregnancy-associated protein using two-dimensional gel electrophoresis, N-terminal sequencing and mass spectrometry. Proteomics 3:2420–2427. https://doi.org/10.1002/pmic.200300618

Racine J, Gerber V, Feutz MM et al (2011) Comparison of genomic and proteomic data in recurrent airway obstruction affected horses using ingenuity pathway analysis®. BMC Vet Res 7:48. https://doi.org/10.2746/042516401776767412

Raila J, Aupperle H, Raila G (2007) Renal pathology and urinary protein excretion in a 14-Month-old Bernese mountain dog with chronic renal failure. J Vet Med A 54:131–135

Raila J, Schweigert FJ, Kohn B (2014) Relationship between urinary Tamm-Horsfall protein excretion and renal function in dogs with naturally occurring renal disease. Vet Clin Pathol 43:261–265. https://doi.org/10.1111/vcp.12143

Riding GA, Jones A, Holland MK et al (2008) Proteomic analysis of bovine conceptus fluids during early pregnancy. Proteomics 8:160–177. https://doi.org/10.1002/pmic.200700465

Ruis MAW, Te Brake JHA, Engel B et al (1997) The circadian rhythm of salivary cortisol in growing pigs: effects of age, gender, and stress. Physiol Behav 62:623–630. https://doi.org/10.1016/S0031-9384(97)00177-7

Scarpa P, Di Fabio V, Ramirez C et al (2007) Proteinuria, GGT index and fractional clearance of electrolytes in exercising athletic horses. Vet Res Commun 31:339–342

Schaefer H, Kohn B, Schweigert FJ, Raila J (2011) Quantitative and qualitative urine protein excretion in dogs with severe inflammatory response syndrome. J Vet Intern Med 25:1292–1297. https://doi.org/10.1111/j.1939-1676.2011.00829.x

Schellenberg S, Mettler M, Gentilini F et al (2008) The effects of hydrocortisone on systemic arterial blood pressure and urinary protein excretion in dogs. J Vet Intern Med 22:273–281

Scocco P, Aralla M, Catorci A et al (2011) Immunodetection of aquaporin 5 in sheep salivary glands related to pasture vegetative cycle. Folia Histochem Cytobiol 49:458–464. https://doi.org/10.5603/FHC.2011.0065

Simon SL, Lamoureux L, Plews M et al (2008) The identification of disease-induced biomarkers in the urine of BSE infected cattle. Proteome Sci 6:23. https://doi.org/10.1186/1477-5956-6-23

Teixeira-Gomes AP, Harichaux G, Gennetay D et al (2015) Photoperiod affects the cerebrospinal fluid proteome: a comparison between short day– and long day–treated ewes. Domest Anim Endocrinol 53:1–8. https://doi.org/10.1016/j.domaniend.2015.04.003

Thongboonkerd V, Chutipongtanate S, Kanlaya R (2006) Systematic evaluation of sample preparation methods for gel-based human urinary proteomics: quantity, quality, and variability. J Proteome Res 5:183–191. https://doi.org/10.1021/pr0502525

Van der Stede Y, Cox E, Van den broeck W, Goddeeris BM (2001) Enhanced induction of the IgA response in pigs by calcitriol after intramuscular immunization. Vaccine 19:1870–1878. https://doi.org/10.1016/S0264-410X(00)00440-0

Vitorino R, Barros AS, Caseiro A et al (2012a) Evaluation of different extraction procedures for salivary peptide analysis. Talanta 94:209–215. https://doi.org/10.1016/j.talanta.2012.03.023

Vitorino R, Guedes S, Manadas B et al (2012b) Toward a standardized saliva proteome analysis methodology. J Proteomics 75:5140–5165. https://doi.org/10.1016/j.jprot.2012.05.045

Xu C, Shu S, Xia C et al (2015) Mass spectral analysis of urine proteomic profiles of dairy cows suffering from clinical ketosis. Vet Q 35:133–141. https://doi.org/10.1080/01652176.2015.1055352

Zaragoza C, Barrera R, Centeno F (2003) SDS-PAGE and Western blot of urinary proteins in dogs with leishmaniasis. Vet Res 34:137–151. https://doi.org/10.1051/vetres