Preliminary Study on Urine Chemistry and Protein Profile in Cows and Heifers

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ABSTRACT

Urinalysis offers important clinical information regarding not only the kidney function but also about the general health status of an organism. The aims of this research were to obtain preliminary data on urine chemistry and electrophoretic protein profile from cows and heifers, to compare electrophoretic profiles of not pregnant with pregnant animals and to evaluate their changes as the pregnancy progresses. Eight heifers and six cows were included in the study and 97 urine samples were collected. Complete urinalysis was performed and urinary proteins were separated by electrophoresis. Considering the pregnancy as a source of variability, significant differences were reported between pregnant and not pregnant heifers for the urine specific gravity (P=0.005), urine total proteins (P=0.009) and urine total proteins to urine creatinine ratio (UPC) (P=0.008). The majority of urine samples analysed in this study showed common protein bands. A mean of protein bands of 17±3 was detected in heifers, while a mean of 13±3 protein bands was recorded in cows. The putative proteins were uromodulin, transferrin, albumin, heavy and light chains of immunoglobulins. The comparison between pregnant and not pregnant animals showed qualitative differences, with the absence of three bands in not pregnant cows including the putative alpha-fetoprotein. In conclusion, urinalysis is an economical and a non-invasive diagnostic protocol, which should be routinely used for the clinical evaluation of large animals. The data reported in the present study could be considered suggestive of healthy animals and they confirmed those previously reported in the literature.

INTRODUCTION

By the evaluation of color, transparency, chemical composition and urinary sediment, urinalysis offers important clinical information regarding not only the kidney function but also the general health status or physiological condition (e.g. pregnancy) of an organism (Rawat et al., 2016; Piech and Wycislo, 2019). Of the myriad of the urinary molecules, proteins are of great interest due to their role in pathophysiological processes. The importance of the study of the urinary proteome was recently highlighted in farm and companion animals (Miller et al., 2014; Ferlizza et al., 2015; Isani et al., 2018).

In particular, urine reference values of cattle, as well as the composition of the bovine urinary proteome have been previously reported (Pyo et al., 2003; Bathla et al., 2015; Mokbul et al., 2016; Rawat et al., 2016; Herman et al., 2019). These studies aimed at the discovery of biomarkers, which could help the farmer in the early pregnancy diagnosis as well as in the detection of the parturition’s onset. An early pregnancy diagnosis is essential for the reduction of the interval between the gestation and the reintroduction of cows in the breeding program (Mokbul et al., 2016). Moreover, the determination of the onset of parturition is important to give proper care of calves, avoiding complications during the parturition (Wawrzykowski et al., 2018). Consequently, many studies have been focused on pregnancy, given its key role in the dairy industry, while few research projects have been performed to identify the physiological urinary proteins of cattle. Indeed, the urinary proteome of farm and companion animals was studied to a lesser extent than the human proteome.
(Adachi et al., 2006; Candiano et al., 2010; Brandt et al., 2014; Almeida et al., 2015; Ferlizza et al., 2015; Rawat et al., 2016; Isani et al., 2018). Different protocols have been proposed for the study of the urinary proteome, including one and two-dimensional gel electrophoresis followed by the identification of the most important proteins by mass spectrometry. Considering the paucity in the literature of studies on cow urine characterization, the aims of this research were to obtain preliminary data on urine chemistry and electrophoretic protein profile from cows and heifers, to compare electrophoretic profiles of not pregnant with pregnant animals and to evaluate their changes as the pregnancy progresses.

**MATERIALS AND METHODS**

**Study subjects and sample collection:** Ninety-seven urine samples were obtained from eight heifers (62 samples) and six cows (35 samples) hosted at the farm of the Department of Veterinary Medical Sciences (University of Bologna) (Table 1). The barn was equipped with automated cooling systems and made up of a resting area with birth-barns with straw, arranged in double row system tail to tail. The floor was made up of striped concrete and was cleaned with automatic scraper systems. The heifers and the cows during the dry period were allocated in a good health status based on periodic routine physical examination and blood analyses and were not affected by diseases during this study.

**Urinalysis, urine chemistry and urine protein to creatinine ratio calculation:** The samples were subjected to routine urinalysis, including urine specific gravity (USG), semi-quantitative dipstick test (Dirui® H10 Urine Analysis Strips; Dirui analyser H-500), centrifugation at 1,500 g for 10 minutes and microscopic sediment analysis (Ferlizza et al., 2015, 2017). The supernatants were stored at -80°C for subsequent analyses. The microscopic sediment analysis was performed within 5 hours from collection. Two drops (30 µL each) of sediment (uncoloured and coloured with fuchsine) were examined under microscopic low power field (100X) and high-power field (400X) searching for the presence of casts, crystals, urinary tract cells, red blood cells and leucocytes.

On urine supernatants, urine total proteins (uTP) and urine creatinine (uCrea) were determined using commercial kits (Urine/CSF Protein, OSR6170, and Creatinine OSR6178, Olympus/Beckman Coulter) on an automated chemistry analyser (AU 400, Olympus/Beckman Coulter). The uTP to uCrea ratio (UPC) was calculated using the following formula: UPC = uTP (mg/dL)/uCrea (mg/dL).

**SDS-PAGE:** Eighty-four samples were subjected to sodium-dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as previously reported (Ferlizza et al., 2015; Isani et al., 2016). Two micrograms of urine proteins were separated on precast gels (10%, 12%, 4-12%; NuPAGE®, Thermo Fisher Scientific, Waltham, Massachusetts, USA) in buffers containing SDS (MES; MOPS; Thermo Fisher Scientific) and reducing conditions. The gels were stained with silver staining (SilverQuest™ Staining Kit Invitrogen), then digitalised and the pherograms were obtained using GelAnalyzer 2010 software.

**Statistical analysis:** Statistical analysis was performed using the software R® (Rx64 3.4.3) and RStudio. The normal distribution of the data was tested by the Shapiro-Wilk normality test. A P>0.05 was considered indicative of a normal distribution. Data are reported as mean±standard deviation or median and range (minimum-maximum value) depending on normal or not normal distribution, respectively. Mean values of repeated measures from the same animal were calculated (Petrie and Watson, 2013) in each group (cows vs heifers) and/or condition (pregnant vs not pregnant). Normally distributed variables were analysed by t-test to determine the differences between heifers and cows and, within each group, the differences between the pregnant and not pregnant animals. The variables without normal distribution were tested by Wilcoxon rank sum test. The significance level was set at P<0.05.

**RESULTS**

**Urinalysis:** *Heifers* - Heifers were 1-2 years old and, out of eight individuals, seven were pregnant. Three specimens (ID: 266, 283 and 290) were followed throughout the pregnancy and one of them (ID: 290) was sampled also at the time of oestrus. The other three heifers (ID: 236, 247 and 250) were followed in the second and third trimester of pregnancy as well as in the post-partum period. One heifer (ID: 266) was followed starting from before the pregnancy up to the 232nd day. One heifer (ID: 296) was never pregnant and another one (ID: 283) had a miscarriage in the sixth month of the first pregnancy and, consequently, the next gestation was monitored. All urine samples were clear and pale yellow colour. Urine pH ranged from 8.5 to 9, while the USG had a mean value of 1.032±0.002. Complete data are reported in Table 2.

The semi-quantitative dipstick test resulted positive for proteins (30-500 mg/dL) in 59 samples, for erythrocytes (50 Ery/µL) in one sample, for ketones (150 mg/dL) in one sample collected 24 days after the parturition, and negative in all samples for leucocytes, glucose and bilirubin. The microscopic evaluation of urine sediments revealed epithelial cells in all the samples analysed, while bacteria and casts (hyaline, granular and fatty) were present in three urine samples. Crystals were found in 15 urine samples, in particular, eight samples showed triple phosphate crystals, six samples calcium phosphate crystals and two urines calcium carbonate crystals (Fig. 1).
Two animals were examined; heifers, significant differences were found for pH examination of urine sediment revealed rare epithelial cells. Two samples presented positivity to ketones (15 mg/dL) in urines collected 13 days and 33 days after the parturition. The microscopic examination of urine sediment revealed rare epithelial cells.

Considering the comparison between cows and heifers, significant differences were found for pH (P=0.003), USG (P=0.0001) (Fig. 2) and proteins mg/dL (P=0.003). USG was significantly different also between pregnant and not pregnant heifers (P=0.005) (Fig. 3).

Cows - Cows were 2-5 years old and, out of the six animals, five were pregnant. Three specimens (ID: 61, 62 and 185) were followed through the pregnancy up to the post-partum period and two cows (ID: 191 and 193) from the second and third month of pregnancy up to the seventh and eighth month, respectively. Two animals were sampled also at oestrus (ID: 61 and 242), one of them (242) was never pregnant.

Cow urines appeared light to medium yellow and clear, with a urine pH ranging from 8.5 to 9. The USG had a mean value of 1.027±0.001. Complete data are reported in Table 2. Dipstick test results were negative for erythrocytes, leukocytes, glucose and bilirubin in all samples, while positivity to proteins was recorded in 30 samples analysed (30-100 mg/dL). Two samples presented positivity to ketones (15 mg/dL) in urines collected 13 days and 33 days after the parturition. The microscopic examination of urine sediment revealed rare epithelial cells.

Considering the comparison between cows and heifers, significant differences were found for pH (P=0.003), USG (P=0.0001) (Fig. 2) and proteins mg/dL (P=0.003). USG was significantly different also between pregnant and not pregnant heifers (P=0.005) (Fig. 3).

**Table 1: Animals’ breed, group, age and lactation number**

| ID   | Bovine breed | Group | Age     | Lactation |
|------|--------------|-------|---------|-----------|
| 61   | Holstein-Friesian | Cow   | 5-y and 2-mo | 3*        |
| 62   | Holstein-Friesian | Cow   | 5-y and 2-mo | 3*        |
| 185  | Holstein-Friesian | Cow   | 3-y and 1-mo | 1*        |
| 191  | Holstein-Friesian | Cow   | 3-y and 7-mo | 2nd       |
| 193  | Holstein-Friesian | Cow   | 3-y and 5-mo | 2nd       |
| 242  | Holstein-Friesian | Cow   | 2-y and 3-mo | 1*        |
| 236  | Holstein-Friesian | Heifer| 2-y and 4-mo | 0         |
| 247  | Holstein-Friesian | Heifer| 2-y     | 0         |
| 250  | Holstein-Friesian | Heifer| 1-y and 10-mo | 0         |
| 251  | Holstein-Friesian | Heifer| 1-y and 10-mo | 0         |
| 266  | Holstein-Friesian | Heifer| 1-y and 7-mo | 0         |
| 283  | Holstein-Friesian | Heifer| 1-y and 5-mo | 0         |
| 290  | Holstein-Friesian | Heifer| 1-y and 2-mo | 0         |
| 296  | Holstein-Friesian | Heifer| 1-y and 2-mo | 0         |

*As at the time of the start of the study.

**Table 2: Urinalysis (USG, pH and dipstick), urine total proteins (uTP), urine creatinine (uCrea) and UPC of cows and heifers.** Data are reported as mean±SD or median and range (min - maximum values) depending on normal or not normal distribution, respectively

| Variable          | Cows (N=6; n=35) | Heifers (N=8; n=62) | p*  |
|-------------------|------------------|---------------------|-----|
| USG               | 1.027±0.001      | 1.032±0.002         | 0.0001 |
| pH                | 8.56±0.08        | 8.75±0.09           | 0.003 |
| Leu (cell/µL)     | 0                | 0                   | NA   |
| Pro (mg/dL)       | 62.1±20.5        | 120.6±37.0          | 0.003 |
| Glu (mg/dL)       | 0                | 0                   | NA   |
| Ket (mg/dL)       | 0 (0-5)          | 0 (0-21.4)          | 0.5  |
| Bil (mg/dL)       | 0                | 0                   | NA   |
| Ery (cell/µL)     | 0                | 0.1±0.1             | 0.5  |
| uTP (mg/dL)       | 13.7±2.8         | 20.8±2.6            | 0.0005 |
| uCrea (mg/dL)     | 94.3±28.4        | 177.7±23.4          | 0.0008 |
| UPC               | 0.16±0.04        | 0.12±0.01           | 0.09  |

N = number of specimens; n = number of urine samples; USG = urine specific gravity; Leu= Dipstick Leukocytes; Pro = Dipstick Proteins; Glu = Dipstick Glucose; Ket = Dipstick Ketones; Bil = Dipstick Bilirubin; Ery = Dipstick Erythrocytes; uTP = urine total proteins; uCrea = urine creatinine; UPC = urine protein to urine creatinine ratio; NA = not applicable. *P refers to the comparison between cows and heifers.

**Urine total proteins, urine creatinine and UPC:** Complete data are reported in Table 2. Mean values for uTP, uCrea and UPC in heifer urine were 20.8±2.6 mg/dL, 177.7±23.4 mg/dL and 0.12±0.01, respectively. Mean values for uTP, uCrea and UPC in cow urine were 13.7±1.6 mg/dL, 94.3±28.4 mg/dL and 0.16±0.04, respectively.
The majority of the samples presented the protein bands analysed and a mean of 17 ± 3, 27, 23, 21 kDa and less than 13 kDa. Complete data bands with molecular weight (MW) of 97, 86, 78, 70, 67, 59, 62 and 58 kDa.

Indeed, the band appearing at the last month of the pregnancy presented a number of protein bands higher than that determined at the initial stages.


cows: Twenty-nine urine samples were analysed from five cows and a mean of 13 ± 3 protein bands were recorded. The majority of the samples presented a common profile characterized by protein bands at MW of 97, 86, 78, 70, 67, 59, 62 and 58 kDa.

In the final stages of the pregnancy, some differences were detected: urinary proteins with MW less than 9 kDa were increased in number, while the band with MW of 11 kDa increased in intensity as gestation progresses. Moreover, urinary proteins with MW of 18 and 27 kDa appeared at the last month of the pregnancy. As reported for the heifers, the band with MW of 67 kDa was visible during the entire gestation and in the post-partum period.

Urine samples collected in the oestrus time presented a urinary proteome similar to the pregnant animals. Indeed, the bands with MW of 97, 86, 78, 70, 67, 59, 58, 41, 33 and 18 kDa were present. Moreover, two additional bands with MW of 15 and 16 kDa were detected. Conversely, in these samples, the band with MW of 11 kDa was absent.

In not pregnant animals, the urinary proteome was mainly characterised by the following bands: 86, 78, 70, 59, 38, 27, 22, 11 and 9 kDa. The band at 97 kDa was scarcely evident, while the bands at 67 and 13 kDa were not present.

**DISCUSSION**

Considering the importance of cattle as farm animals, previous studies were focused on the productive aspects and the quality of meat and milk as well as the strategy for their improvement (Toral et al., 2018). Urinalysis was used for the evaluation of urinary nitrogen and the diagnosis of ketosis as well as for the detection of biomarkers of pregnancy and parturition (Nennich et al., 2006; McArt et al., 2012; Rawat et al., 2012; Spek et al., 2012). Despite the non-invasive sampling and the affordable price, urinalysis is still rarely used as a routine...
clinical diagnostic tool in farm animals. For this reason, this study investigated the urine chemistry and the urinary proteome to obtain preliminary data on cows and heifers, to compare the electrophoretic profiles of pregnant and not pregnant animals and to evaluate their changes during the pregnancy.

Urinalysis and UPC: The USG value determined in this study was similar to those reported in the literature for adult cows (Herman et al., 2019). In our study, the USG value was higher in heifers than in cows as well as in pregnant than in not pregnant heifers. These results could be explained considering the possible correlation between the USG and the creatinine urinary concentration, which was also higher in heifers. Indeed, the creatinine concentration is able to affect the USG value, as previously reported (Herman et al., 2019). Hermann et al. (2019) found a strong positive correlation between the USG and the urinary creatinine concentration in beef cows. Accordingly, in our study, heifers showed a urinary creatinine concentration almost double than cattle, confirming the results reported in the literature (Herman et al., 2019). The creatinine excretion through the milk could have also contributed to the differences between cows and heifers. Indeed, the cows were in lactation, while the heifers, being at their first gestation, still had to start producing milk.

Cattle urine samples had an alkaline pH, between 8 and 9, confirming the results previously reported by other authors (Herman et al., 2019; Ihedioha et al., 2019).

The results of the dipstick test were negative for almost all of the variables with few exceptions. In particular, the dipstick positivity to proteins should be considered with attention. The alkaline urinary pH of the cattle could have determined false positive results; therefore, quantitative measurement of proteinuria is recommended (Herman et al., 2019). However, traces of proteins in bovine urine were previously reported in the literature (Mokbul et al., 2016; Herman et al., 2019; Ihedioha et al., 2019).

Special attention should be paid also to the positivity to ketones, which was recorded in three urine samples in the post-partum period. These findings are in accordance with data previously reported in the literature for pregnant cows (Mokbul et al., 2016; Ihedioha et al., 2019). A metabolic switch of glucose metabolism for lactose synthesis and milk production may cause the presence of ketones in the urine (McArt et al., 2012; Ihedioha et al., 2019). However, the positivity determined in our study might also be originated from ruminal ketone bodies as suggested by Herman et al. (2019).

Regarding sediment analysis, crystals in urines, especially calcium phosphate ones, were previously reported in bovine, as well as casts and leucocytes (Mokbul et al., 2016). The presence of crystals should be considered with attention and urine samples monitored to avoid obstructive urolithiasis. Indeed, in ruminants, the formation of uroliths is related to several factors, including high concentrate ration and plant rich in oxalate and phytoestrogen (Thakur et al., 2019). Although crystals seemed to be frequent in urine, obstructive urolithiasis should be prevented by balancing the feeding and encouraging the animals to drink water (Parrah et al., 2010).

In this study, the mean urine total protein concentrations of 13.7±1.6 mg/dL determined in cows and 20.8±2.6 mg/dL in heifers fall within the reference interval reported by Herman et al. (2019) for healthy cows; therefore, the animals included in our study should be considered as non-proteinuric (Herman et al., 2019). Comparing the cows with the heifers, the uTP value was significantly different between the two groups (P=0.0005). According to data reported in the literature, the lactation number influences the UPC values, which was higher in cows during the lactation than in dry animals (Herman et al., 2019). However, in our study, only the uTP value was statistically different between cows and heifers suggesting that further studies are required to investigate the changes in urine total proteins as well as in the UPC value.

SDS-PAGE electrophoresis: In the samples examined, common bands with apparent MW of 97, 86, 78, 70, 59, 38, 27, 23, 21 kDa and between 13 and 9 kDa were separated. The identity of some of the bands can be hypothesised basing on the apparent MW and comparing the data with those previously reported in humans and animals (Pyo et al., 2003; Serafini-Cessi et al., 2003; Ozgo et al., 2009; Candiano et al., 2010; Alhaider et al., 2012; Balhara et al., 2013; Brandt et al., 2014; Miller et al., 2014; Bathla et al., 2015; Ferlizza et al., 2015; Rawat et al., 2016). The proteins could be identified as uromodulin (band with MW of 97 kDa), transferrin (band with MW of 78 kDa), albumin (band with MW of 70 kDa), heavy and light chains of immunoglobulins (band with MW of 59 and 27 kDa, respectively). Additionally, other proteins can be hypothesised as haptoglobin (band with MW about 38 kDa), retinol binding protein (RBP) and/or bPAP (bovine pregnancy-associated protein) (band with MW of 21-22 kDa), lysozyme and β2-microglobuline (band with MW of 13 and 9 kDa, respectively).

Uromodulin (Tamm-Horsfall protein), one of the most abundant urinary proteins in healthy mammals, was described in the urine of camel, dog, cat and humans (Serafini-Cessi et al., 2003; Brandt et al., 2014; Miller et al., 2014; Ferlizza et al., 2015). It is the main tubular protein of canine urine (Miller et al., 2014) and it has also been reported in cows, where elevated concentration of uromodulin has been suggested as a pregnancy biomarker (Bathla et al., 2015).

In our study, the band with MW of 78 kDa could be hypothetically identified as transferrin, which appeared as a very thin band without variation between cows and heifers and pregnant and not pregnant animals. This protein was reported also in dog urine (Brandt et al., 2014). Albumin was described in many species, including humans, dog, cattle, cat, goats and camels (Pyo et al., 2003; Ozgo et al., 2009; Candiano et al., 2010; Alhaider et al., 2012; Brandt et al., 2014; Miller et al., 2014). In our study, the band with MW of 70 kDa might be identified as albumin, which was present as an evident band in all the urine samples analysed.

Haptoglobin and RBP were described in feline urine and in urine of pregnant cows (Ferlizza et al., 2015; Rawat et al., 2016). Haptoglobin originates from plasma and it is considered a common component of healthy humans’ urine, but it was also detected in urine of pregnant cows and it is a glycoprotein expressed in uterine epithelium during
implantation period and increases significantly in the early days of gestation (Candiano et al., 2010; Rawat et al., 2016). The bovine pregnancy-associated protein (bPAP), with MW of 21 kDa, is a protein detected in the urines of pregnant cows (Pyo et al., 2003).

The comparison between pregnant and not pregnant specimens showed qualitative differences, with the appearance of three bands with MW of 67, 41 and 33 kDa, which were absent in not pregnant animals. In particular, the band with MW of 67 kDa was present in all the urine samples of pregnant cows and heifers. This band might correspond to the alpha-fetoprotein (AFP), a protein produced by foetal tissues by a family of genes phylogenetically related to serum albumin (Mizejewski, 2004). The bands at 67 and 41 kDa might also be the different isoforms of Pregnancy Associated Glycoproteins (PAGs) reported in milk and serum of pregnant cows by Balhara et al. (2013) and suggested as useful biomarkers for early pregnancy diagnosis in bovine. However, more studies are required not only to confirm the presence of PAGs in bovine urines, but also to increase the knowledge about bovine pregnant proteome.

Conclusions: Urinalysis is a simple and non-invasive diagnostic protocol, which should be routinely used for the clinical evaluation of large animals. The data reported in the present study could be considered suggestive of healthy animals and they confirmed those previously reported in the literature. Proteinuria should be also investigated by quantitative methods, to exclude dipstick false positive. SDS-PAGE can be considered a useful tool to study the renal function and it might be proposed for the identification of pregnancy biomarkers. However, further studies are required to improve knowledge about the bovine urine proteome.

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Authors contribution: GI and GA conceived and designed the study. EF and SF executed the experiments and analysed the samples. GI, EF, SF, DC, MB interpreted the data. SF and EF drafted the manuscript. All authors critically revised the manuscript for important intellectual contents and approved the final version.

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