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Biochemical characterization of Urine of Hariana breed of Cattle with special emphasis on presence of various proteins/peptides

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Abstract
Urine is a non-invasive source of biological fluid, which reflects the physiological status of the animals. We have profiled the cow urinary proteome and analyzed its functional significance. The urine collected from two healthy Hariana cows was concentrated by diafiltration (DF) followed by protein extraction using TCA acetone precipitation. The quality of the protein was assessed by two-dimensional gel electrophoresis (2DE) followed by In-gel digestion method. Functional significance of few selected proteins seems to play important role in normal body functions. This study reports the presence of proteins in urine of healthy Hariana cattle for the first time.

Keywords: urine, peptide, cow, two-dimensional gel electrophoresis

Introduction
Urine contains abundant biomolecules including proteins, peptides and metabolites which are the byproducts of the physiological events occuring in the organism. Urine is formed via glomerular filtration of plasma in the kidneys, which act as a filter to retain most of the plasma proteins (Thongboonkerd *et al*, 2002) [21]. Though, many low molecular weight proteins and peptides pass through the glomerular membrane, are catabolized in the proximal tubule and then secreted in the urine. In addition to this, abundant serum proteins like albumin, immunoglobulin light chain, transferrin, myoglobin, and receptor-associated protein, after passing through the glomeruli, are reabsorbed by endocytic receptors in the proximal renal tubules (Kerr *et al*, 1998, Clark *et al*, 1998) [11, 4]. Overall, the protein concentration in normal urine is usually very low (Adachi *et al*, 2006) [1]. Moreover, there is interest in using urine for diagnostic applications due to its simple and non-invasive collection compared to other biological fluids. In particular, many systemic diseases may cause a change in the composition of the urinary constituents, which might be useful for diagnosis and disease monitoring (Adachi *et al*, 2006) [1]. In humans, biomarkers in urine have been used for the diagnosis of kidney and urinary tract diseases (Noiri *et al*, 2009) [14]. However, very few studies have been reported on the bovine urinary proteome as a potential source for biomarker discovery in disease and pregnancy (Shaked *et al*, 2001, Simon *et al*, 2008) [17].
The human urinary proteome has been extensively studied by a variety of methods including two-dimensional gel electrophoresis (2DE) followed by its characterization using matrix assisted laser desorption ionization (MALDI) and liquid chromatography coupled tandem mass spectrometry (LC-MS/MS) (Pieper *et al*, 2004) [15]. In one study, (Sun *et al*, 2005) [20] identified 226 unique human proteins in urine using one-dimensional gel electrophoresis (1D-GE) and multidimensional LC-MS/MS. However, to date, no information is available on the bovine urinary proteome. The aim of the present study was to profile the urinary proteome of Hariana cows. The Hariana breed has adapted well to tropical climate and plays important role in milk production in India (Gurnani *et al*, 1986) [7]. In this proteomic study, a combination of extraction procedures was used to isolate urinary proteins from *Hariana* cows which were then characterized by MS leading to the identification of a many useful proteins. This is the first report of urinary proteome of Hariana cow and will provide a reference database for future studies in biomarker discovery linked to diseases and associated physiological events.

Materials and Methods

Animal selection and sampling

Hariana cows from the cattle farm at Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar, India were maintained under expert veterinary supervision. For this study, urine was collected from two healthy Hariana cattle after screening for the absence of pus cells under microscope to rule out any infection. One liter each of urine sample was then collected aseptically in urine bags from two healthy cows. Just after collection of urine, phenylmethylsulfonyl fluoride (PMSF, 0.01%) was added to prevent proteolytic degradation.

Sample preparation

The freshly collected pooled urine was then centrifuged at 6500 rcf for 30 min to remove insoluble materials. Subsequently, the urine was concentrated by centrifugation using 3 kDa hollow fiber membrane cartridge in Marlow Benchtop System (GE Healthcare, USA). Next, the urine was diafiltered in PBS, pH 7.4 (133 mM NaCl, 2.7 mM NaCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4), (Marimuthu *et al*, 2011) [13]. By this approach, one liter of urine was concentrated to 100 ml to which additional protease inhibitor cocktail (Sigma, USA) was added to prevent proteolysis. Subsequently, samples were stored at − 80 °C until further use.

Precipitation of primary proteins by acetone precipitation

Ten milliliters of diafiltered urine and chilled acetone (− 20 °C) was mixed in a ratio of (1:1 v/v) and centrifuged at 12,000 ×*g* for 30 min. Supernatant was discarded and the pellet was dried and dissolved in solubilizing buffer (7 M Urea, 2 M Thiourea, 4% CHAPS, 30 mM Tris HCL, pH 8.8) and stored at − 80°C until further analysis.

Clean up and protein estimation

Interfering substances such as detergents, salts, lipids and nucleic acids were removed from the precipitated urinary protein preparations using 2D-Clean Up kit (GE Healthcare, USA). The pellet was rehydrated in the same 2D-DIGE lysis buffer and total protein concentration was estimated using 2D-quant kit (GE Healthcare, USA) as per the manufacturer's instruction.

Two dimensional gel electrophoresis (2DE)

2DE was carried out as per the published protocols (Pieper *et al*, 2004) [15], (Janjanam *et al*, 2013). Briefly, 120 µg protein sample was dissolved in 125 µl of rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 30 mM Tris) and rehydrated overnight on IPG strips (7 cm; pH range: 4–7) along with ampholytes. Isoelectric focusing (IEF) was performed in Ettan IPGphor III (GE Health, USA) at 150 V for 70 min, 1000 V for 27 min, 5000 V for 90 min, and 5000 V for 24 min. The IPG strips were equilibrated with 1% w/v DTT in 2.5 ml of equilibration buffer (6 M urea, 50 mm Tris–HCl, pH 8.8, 30% w/v glycerol and 2% w/v SDS) to reduce disulfide bonds followed by 2.5% w/v iodoacetamide in the same buffer to alkylate cysteine residues. The strips were then loaded on top of 12% separating gel and electrophoresis was carried out in standardized conditions of temperature and voltage. For visualization, gels were stained with 2DE compatible silver stain kit followed by destaining. Subsequently, each lane of the gel was cut into 6 equal pieces, further destained using 40% ACN and 40 mM NH_4HCO_3 at a ratio of 1:1 (v/v) and in-gel digestion of protein bands was performed as reported previously (Shevchenko *et al*, 2006) [18], (Fujimoto *et al*, 2006) [6]. In brief, destained bands were reduced with 5 mM dithiothreitol (DTT) in 40 mM NH_4HCO_3 followed by alkylation with 20 mM iodoacetamide in 40 mM NH_4HCO_3. Digestion was carried out overnight using 12.5 ng/µl trypsin (modified sequencing grade; Promega, USA) at 37 °C. Subsequently, peptides were extracted from the gel, lyophilized and desalted using zip-tip (Millipore, Germany) following manufacturer's instruction and stored at − 80 °C until MS analysis.

Image analysis and Spot picking

The spots on gels of different groups were analysed by commercially available densitometric software (PD Quest 8.0.1.Biorad, USA) to identify the differentially expressed proteins and spots were picked manually with the help of a surgical blade under laminar flow. The picked spots were immediately put in sterile 1.5ml microcentrifuge tubes containing 50µl deionized water and stored at -20^{0}C till further analysis.

Mass spectrometry and proteins identification

Spectra for all the spots were generated after trypsin digestion using MALDI-TOF-TOF Ultraflex III Mass Spectrometer (Bruker Daltonics, Germany) at laser wavelength of 337 nm and frequency of 67-100 Hz. The spectra so obtained were searched on the Mascot search engine to identify the probable proteins.

Result and Discussion

A total of 10 spots were selected for the pick list which showed more than 4 times change in percent volume. Spectra for all the spots were generated after trypsin digestion using MALDI-TOF. The spectra so obtained were searched on the Mascot search engine to identify the probable proteins. Results from MS and data search for protein identification are enumerated in table 1. Of the 10 spots for which MS was done, 2 spots (i.e. 1 and 2 were identified with a high confidence level – having 2 or more peptide sets beyond threshold in the Mascot score histograms and the results could be repeated more than once. Three (no. 3, 4 and 5) were identified with single peptide set beyond the threshold in Mascot Score histogram. Five spots with numbers 6, 7, 8, 9 and 10 did not score beyond the threshold and therefore no database match for these spots was reported. This way total 10 spots were identified (Table 1).

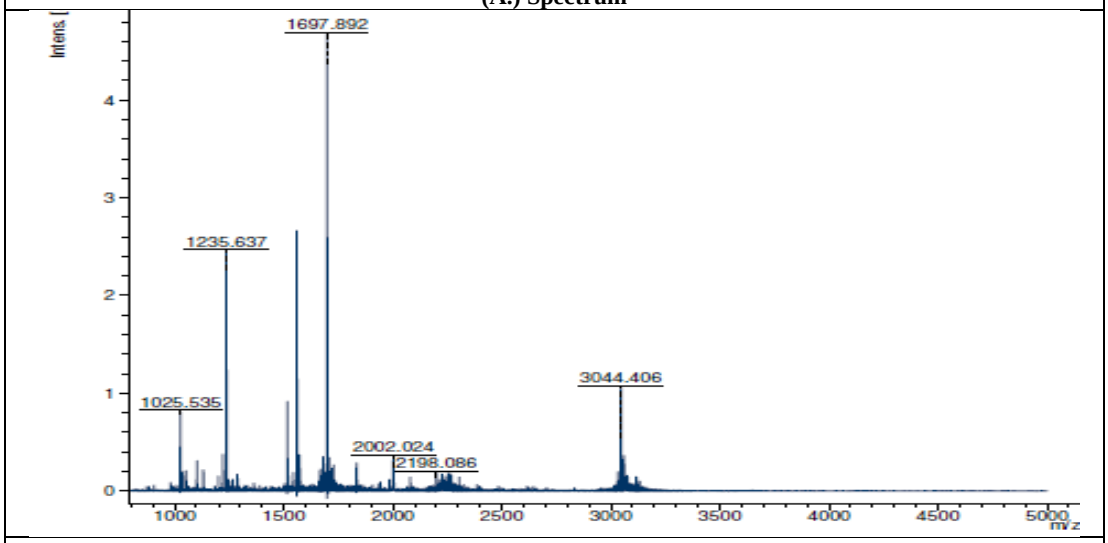
Table 1: MALDI-TOF results after analysis in Mascot search engine

Spot No.	Genomic identifier (gi) No.	Mascot Score	Most related protein
1.	gi 982935812	82	adenomatous polyposis coli protein isoform X4
2.	gi 836710759	101	vinculin
3.	gi 1002832288	79	immunoglobulin heavy chain variable region
4.	gi 61097917	82	cystatin-M precursor
5.	gi 970704850	82	melanoma inhibitory activity protein 3 isoform X2
6.	gi 1032841203	62	ubiquitin carboxyl-terminal hydrolase 32 isoform X1
7.	gi 672019173	63	cyclic nucleotide-binding domain-containing protein 1-like

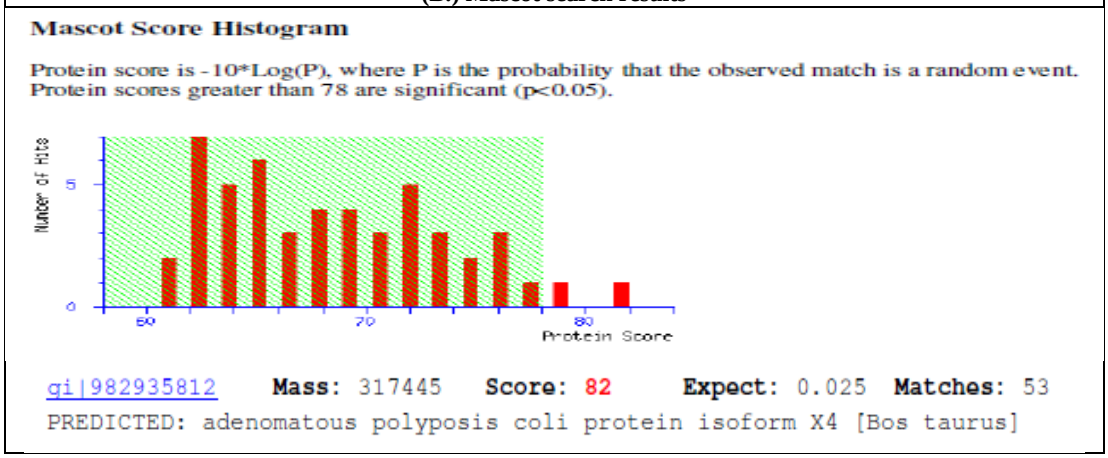
8.	gi 821461580	75	bcl-2-like protein 11 isoform X4
9.	gi 568942208	62	zinc finger protein 786 isoform X1
10.	gi 471368779	73	nebulin-related-anchoring protein

Table 2: Spectrum, Mascot Score histogram results of spot no. 1:

(A.) Spectrum



(B.) Mascot search results



The protein found in spot no. 1 (cattle, Mascot score-82), the adenomatous polyposis coli (APC) is multifunctional, tumour suppressor protein, participating in the canonical Wnt/β-catenin signal transduction pathway, as well as in modulating cytoskeleton function. It was found that APC loss delays radial axonal sorting and PNS myelination. Furthermore, APC loss delays Schwann cell differentiation *in vivo*, which correlates with persistent activation of the Wnt signaling pathway, and results in perturbed Schwann cell processes extension and lamellipodia formation (Elbaz *et al*, 2016) [5]. There has not been any report of presence of this protein in urine of domestic animals. The finding of this protein in urine may be an indicator for development of cytoskeleton function.

Spot number 2 (with Mascot score 101 and 89 respectively) was identified as vinculin proteins. Vinculin is a 116-kDa cytoskeletal protein associated with focal adhesion and adherenes junctions (Rüdiger *et al*, 1998) [16]. It acts as a regulatory bridge between the extracellular matrix (ECM) and the actin cytoskeleton. Vinculin perfoms tissue-specific function of in cartilage, by which it controls chondrocytic differentiation (Koshimizu *et al*, 2012) [12]. Deficiency of this protein leads to the loss of muscular activity and lethality at an early larval stage (Barstead and Waterston, 1989) [2]. There has not been any report of presence of this protein in urine of domestic animals. The finding of this protein in urine may be useful in maintenance of normal muscular activity.

Spot number 4 (Mascot score-98) was identified as cystatin-M precursor. Two notable studies, (Zhang *et al*, 2004) [22] and (Hsu *et al*, 2004) [8] have demonstrated that cystatin M act as tumour suppressor proteins in lung tumors. There has not been any report of presence of this protein in urine of domestic animals. So, this protein may have application in the suppression of the tumour.

Spot number 5 (Mascot score-87) was identified as melanoma inhibitory activity protein 3 isoform X2. Melanoma inhibitory activity (MIA) is a small secreted protein that interacts with extracellular matrix proteins. Its over-expression promotes the metastatic behavior of malignant melanoma, thus making it a potential prognostic marker in this disease (Jamael *et al*, 2005) [9]. There has not been any report of presence of this protein in urine of domestic animals. This can be used as a biomarker in the disease.

Spot number 6 (Mascot score-62) was picked and it was identified as ubiquitin carboxyl-terminal hydrolase 32 isoform X1. This protein has function of catalytic activities like thiol-dependent hydrolysis of ester, thioester, amide, peptide and isopeptide bonds formed by the C-terminal Gly of ubiquitin (a 76-residue protein attached to proteins as an intracellular

targeting signal). There has not been any report of presence of this protein in urine of domestic animals.

Spot number 8 (Mascot score-75) was identified as bcl-2-like protein 11 isoform X4. Bcl-2 family proteins, which include pro- and antiapoptotic members, positively or negatively regulate mitochondrial outer membrane permeabilization, i.e. a barrier to apoptosis induction. There has not been any report of presence of this protein in urine of domestic animals. So, the Bcl-2 family of apoptotic regulators through peptide-based approaches and selective delivery of functional nucleic acids helps in apoptosis regulation.

Spot number 10 (Mascot score-73) was identified as nebulin-related-anchoring protein. Nebulin, a giant, actin-binding protein, is the largest member of a family of proteins (including N-RAP, nebulette, lasp-1 and lasp-2) that are assembled in a variety of cytoskeletal structures, and expressed in different tissues. For decades, nebulin has been thought to act as a molecular ruler, specifying the precise length of actin filaments in skeletal muscle. Nebulin has also been implicated recently in an array of regulatory functions independent of its role in actin filament length regulation (Christopher *et al*, 2011) [3]. There has not been any report of presence of this protein in urine of domestic animals. Proteomics offers great opportunity in investigating the changes in proteome profile associated with urine, as can be seen from the presence of at least 10 spots showing specific appearance in cattle. Some of these identified spots seem to be promising cancer curing, especially melanoma inhibitory activity protein 3(was found in cow urine), which was documented protein having role as anticancerous activity.

Conclusion

In summary, this is the first high-throughput proteomics analysis of bovine urine expressed in healthy cow. Although hampered by the lack of published proteomics information, identification and characterization were possible by cross-species matching and manual validation. Thus, results of this significantly expand our knowledge and emphasize the promising potential of bovine urine as a non-invasive source for discovery of biomarkers against diseases, treatment of many diseases, like hypertension, cancer and maintenance of different physiological disorders. Most of the proteins identified were associated with therapeutic potential or with cytoskeletal function. This study represents the first step in identification of comprehensive proteome map of bovine urine, which will serve as a database for the research community and clinicians.

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