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Effect of methods of protein preparation on the separation of urinary proteins from buffaloes at specific stages of estrus cycle by SDS-PAGE

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Abstract
Optimization of the methods for urine preparation and precipitation is an essential step in the identification of protein biomarkers pertaining to the physiological state. One-dimensional sodium dodecyl sulphate-polyacrylamide gel electrophoresis is a proteomic technique which separates proteins based on the molecular weight. Studies have reported that urine precipitation methods can have an influence on the protein recovery. Differences in protein preparation is also found to result in differences in the proteins identified at specific physiological state. In this study, urinary proteins from buffaloes collected at the estrus phase and at diestrus phase were precipitated from smaller volumes using ammonium sulphate, ethanol, acetone and methanol/chloroform followed protein separation by 1D SDS-PAGE. Among the four precipitation methods, ammonium sulphate precipitation is found to be an easy method for the separation of proteins as detected by Coomassie blue staining. With respect to the proteins separated from urine at estrus phase in comparison to the diestrus phase, distinct protein bands were obtained at around 150KDa with ammonium sulphate, ethanol and acetone precipitation indicating the influence of protein preparation and physiological state of sample on the detection of specific proteins separated by SDS-PAGE.

Keywords: Urine, buffalo, estrus, biomarker, protein, precipitation

Introduction
Urine is a non-invasive source of biological fluid and reflects the physiological status of the mammals (Bathla *et al*., 2015) [4]. Though the protein concentration in normal urine is usually very low (Adachi *et al*., 2006) [1], urine is being used for diagnostic applications as it is easy to collect in a non-invasive way when compared to other biological fluids. Moreover, very few studies have been reported on the buffalo urinary proteome as a potential source for biomarker discovery in physiological phases like estrus period and pregnancy (Selvam *et al*., 2017; Archunan, 2020; Nayan *et al*., 2020) [24, 2, 18].
For the separation of complex proteins from biological fluids including urine, techniques like sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) or one-dimensional gel electrophoresis (1-DE) (Patitucci *et al*., 2020; Kshirsagar and Wiggins, 1986) [19, 15], two-dimensional gel electrophoresis (2-DE) (Joo *et al*., 2003) [12] and two-dimensional differential gel electrophoresis (2D-DIGE) (Gozal *et al*., 2009; Lamoureux *et al*., 2013; Rawat *et al*., 2016) [10, 16, 20] are employed. Amongst these, 1-D SDS-PAGE is a simpler and effective technique which can separate specific proteins identified with respect to physiological or pathological state and the proteins identified are found to vary with the methods of protein preparation as reviewed by Kalantari *et al*., 2015 [13]. However, 1-DE also forms a prerequisite for identification and analysis of differentially expressed proteins by western blot, ELISA and microarray techniques (Aslam *et al*., 2017) [3]. Therefore, the present study has been planned with the objective of exploring the influence of different protein precipitation methods on the separation of proteins from buffalo urine at specific stages of estrus cycle i.e. at estrus phase and diestrus phase.

Materials and Methods
Selection of animals
Healthy Murrah buffaloes maintained in the Subramanyam Naidu Dairy Farm, H cross, Vijayapura, Kolar, Bangalore were included in the study. Samples were collected from

animals at the estrus phase and at the diestrus phase by tracking the cycling animals in the herd for vulval edema and reddening, external signs of heat (frequent micturition and bellowing, homosexual mounting behavior). The same animals were examined per rectally for the tonicity of uterus and for the presence of mature dominant follicle or corpus luteum by transrectal ultrasound scanning (TRUS) of the ovaries (Selvam and Archunan, 2017) [23]. Animals in diestrus were examined per rectally for the flaccid uterus and presence of obvious CL and presence of small follicles on the ovaries by TRUS.

Collection of urine samples

Mid-stream urine samples were collected in pre-sterilized polypropylene vials from buffaloes at the estrus phase and at the diestrus phase. The urine samples were immediately strained using a muslin cloth to remove contaminating materials. Any infection/ conditions which could be reflected in the urine was ruled out in the farm itself by using SD fine urine strips. The urine samples examined and free from infection were transported from the farm to the lab in cold chain. The samples were then processed immediately (without adding any preservatives) or stored at -80° C until further analysis by adding phenyl methyl sulfonyl fluoride (PMSF) as a preservative (Zhou *et al*., 2006) [32] at 0.01% (Rawat *et al*., 2016) [20].

Processing of buffalo urine samples, precipitation of total urinary proteins and separation of urinary proteins by SDS-PAGE: A preliminary work

Initially the proteins present in buffalo urine samples (collected at the diestrus phase) were precipitated using both ammonium sulphate (AS) and methanol/chloroform (M/C) precipitation. Total proteins present in 10ml of buffalo urine were precipitated by adding 2.7g of AS (Wai-Hoe *et al*., 2009; Sudha *et al*., 2012; Chacar *et al*., 2017; Wu *et al*., 2018) [28, 26, 6, 30]. The mixture was vortexed on ice for 5min. The solution was then centrifuged at 10,000 g for 20 min at 20 ℃. To the obtained pellet, 80µl of TSE buffer (10mM Tris, 1mM EDTA, 1% w/v SDS, pH 8.8) (Wai-Hoe *et al*., 2009) [28] and 20µl of reducing sample buffer (RSB) (0.5M Tris-Hcl at pH 6.8, 20%v/v glycerol, 10% w/v SDS, 0.5% w/v bromophenol blue and 5% β-mercaptoethanol) were added. The protein pellet was then solubilized and reduced by heating at 100 ℃ for 10 min. Likewise, the urinary protein pellets obtained by the AS precipitation were dissolved in 1x sample loading dye (SLD) which is prepared by adding 5µl of β mercaptoethanol to 95ml of 2x Lamelli buffer, to know the effect of RSB on the separation of urinary proteins by SDS-PAGE.

Buffalo urinary proteins were also precipitated by M/C method (Saito *et al*., 2019) [21] using the method described below. Ten ml of 100% methanol was added to 10ml of urine. To this mixture, 2.52ml of chloroform was added, mixed well and centrifuged at 12,000g for 15 min at 4 ℃. The supernatant was discarded carefully without disturbing the interphase (protein fraction). To this mixture,10ml of 100% methanol was added and mixed gently for 5 min. The mixture was then centrifuged at 12,000g for 15min at 25 ℃. The obtained pellets were dissolved in 100µl of 8M Urea/50mM Tris-Hcl, pH, 8.0. The pellets obtained by M/C precipitation were also dissolved in SLD.

To understand the effect of the above two precipitation methods on the urinary protein extraction and separation on SDS-PAGE, 1ml of neat urine was pipetted into an Eppendorf, 80µl of TSE and 20µl of RSB was added. All the protein lysates were reduced by heating at 100 ℃ for 10 min. A volume of 60µl of the protein lysates were loaded onto 12% gel, separated by SDS-PAGE and visualized by Coomassie brilliant blue (CBB) staining as represented in Figure 1.

Precipitation methods for buffalo urinary protein preparation

Buffalo urine collected from the animals at the estrus phase and diestrus phase of estrus cycle as described in 2.1. were precipitated by four different methods (Saito *et al*., 2019 [21] with slight modifications) from small volumes such that they can be precipitated in Eppendorf tubes as described below.

Ammonium sulphate precipitation

To 1 ml each of urine samples, 0.6g of AS was added and gently vortexed on ice for 5 min. The mixture was then centrifuged at 10,000g for 20 min at 20 ℃. The obtained pellet was dissolved in 180µl of TSE buffer.

Ethanol precipitation

To 250µl each of urine samples, 1250µl of 100% ethanol was added. The solution was mixed well on ice for 5 min, left overnight at -20 ℃ and centrifuged at 12,000g for 15 min. The supernatant was discarded and the pellet was washed with 1ml of 70% ethanol, centrifuged at 12,000 g for 5 min. The obtained pellet was air dried and dissolved in 180µl of TSE buffer.

Acetone precipitation

To 250µl each of urine samples, 1000µl of 100% cold acetone was added. The solution was mixed gently, left at -20 ℃ overnight, centrifuged at 12,000g for 40 min. The supernatant was discarded and the pellet was washed with 1ml of 80% ice cold acetone, centrifuged at 12,000g for 5 min. The supernatant was discarded and the pellet was air dried for dissolving in 180µl of TSE buffer.

Methanol/Chloroform precipitation

To 250µl each of urine samples, 250µl of 100% ethanol and 62.5µl of chloroform was added, mixed well on ice for 5 min and centrifuged at 12,000g for 15 min. The supernatant was removed carefully and 250µl of 100% methanol was added, mixed gently for 5 min and centrifuged at 12,000g for 15min at 25 ℃. The obtained pellet was air dried for dissolving in 180µl of TSE buffer.

Protein profile of the pellets examined by SDS-PAGE

The urinary proteins from buffaloes present at specific physiological state i.e. at estrus phase and diestrus phase of estrus cycle, precipitated by four different methods as described above and dissolved in TSE buffer were reduced with 55µl of RSB and boiled at 95 ℃ for 5 min so as to reduce the Tamm Horsfall protein (THP) which is abundantly present in urine. The samples were then separated by SDS-PAGE (12% gel) along with protein molecular weight marker (as detailed in figures 1 and 2) and the proteins were visualized by CBB staining (Lott *et al*., 1983) [17] to know if there are any qualitative differences in the urinary proteins of buffaloes at different physiological states and if the method of protein precipitation is going to have any influence on the proteins separated on SDS-PAGE.

Results and Discussion

Urinary proteins separated by SDS-PAGE in the preliminary work

From the preliminary work wherein AS and M/C were used for the precipitation of urinary proteins as represented in figure 1 from fresh samples of buffalo, it is evident that both these precipitation methods were effective to extract proteins from buffalo urine. Upon the comparison of the protein bands obtained on SDS-PAGE with CBB staining between the AS and methanol/chloroform precipitation qualitatively, it is evident that the number of proteins detected on SDS-PAGE is maximum with AS precipitation which can be attributed to the either more concentration of particular protein (Gromova and Celis, 2006; Gallagher and Sasse, 1998; Schleicher and Watterson, 1983) [11, 9, 22] or differences in the extraction of wide array of proteins with the precipitation procedure (Beretov *et al*., 2014; Krishnappa *et al*., 2021; Saito *et al*., 2019; Thongboonkerd *et al*., 2002; Sudha *et al*., 2012; Bathla *et al*., 2015; Shourbagy and Ahmed, 1953) [5, 14, 21, 27, 26, 4, 25] .

The use of RSB seems more efficient than SLD in reducing urinary proteins as evident from the separation of protein bands on SDS-PAGE specially with AS precipitation which is in accordance with the findings of Wai-Hoe *et al*., 2009 [28]. The use of RSB for SDS-PAGE analysis of protein extracts dissolved in TSE and stored at -80 ℃ without any protein preservatives was still able to resolve proteins, which the SLD failed to do and this can be attributed to be the strong reducing action of RSB. While the use of RSB for protein analysis from neat urine failed to identify proteins prominently by CBB staining.

The use of TSE buffer and RSB for SDS-PAGE analysis of urinary proteins (Wai-Hoe *et al*., 2009) [28], especially for the THP, which is a 85-90KDa glycoprotein (molecular weight can vary widely based on the glycosylation pattern and the separation of the subunits (Flethcher *et al*., 1970)) [8] seems to be efficient for buffalo species as well and also compatible with M/C precipitation, aged urine sample extracts stored at -80 ℃. However, the proteins predominantly identified on SDS-PAGE by CBB staining were having molecular weights around 75 KDa and between 150 to 100 KDa. Use of silver staining method (Chevallet *et al*., 2006) [7] would have further enhanced the sensitivity of protein detection by SDS-PAGE.

Separation of buffalo urinary proteins by SDS-PAGE varies with the method of protein precipitation and the physiological state

For the separation of urinary proteins from the animals showing visible signs of heat and animals at the diestrus phase on SDS-PAGE as detailed in the figure 2, 60µl of protein samples (extracted by the four different precipitation methods) dissolved in TSE buffer were reduced with 15µl of 6x SLD and a final volume of 60µl each of the samples were loaded into the wells. The proteins detected at estrus phase and diestrus phase by all the precipitation methods included protein bands with the molecular weight ranging between 100 to 75KDa except in estrus sample precipitated by M/C. Interestingly, in the estrus samples precipitated by AS, ethanol and acetone, protein bands were detected around 150 KDa. The results obtained in this study indicated a difference in the proteins identified at specific physiological state in the urine of buffaloes and also the influence of protein preparation methods on the detection of specific proteins which is in accordance with the findings of Beretov *et al*., 2014; Saito *et al*., 2019; Thongboonkerd *et al*., 2002 [5, 21, 27].

To extrapolate the above methods for detection and analysis of immunogenic proteins in urine by techniques like ELISA, the interfering substances in urine like salts needs to be minimized by dissolving the protein pellets with EDTA (Saito *et al*., 2019) [21] such that ions like Ca2+ and Mg2+ present in urine can be chelated and the protein can be quantified by Bradford assay or Lowry assay for loading protein onto the gels and further transfer to the solid support. Desalting can also be done either with commercially available tips (pierce c18 tips from Thermoscientific) or by using modified procedure for protein preparation as described by Yalamati *et al*., 2015 [31] and further quantification of protein by Bicinchoninic acid assay (BCA). Desalting and protein concentration can also be done by modified method described by Wang *et al* 2007 [29].

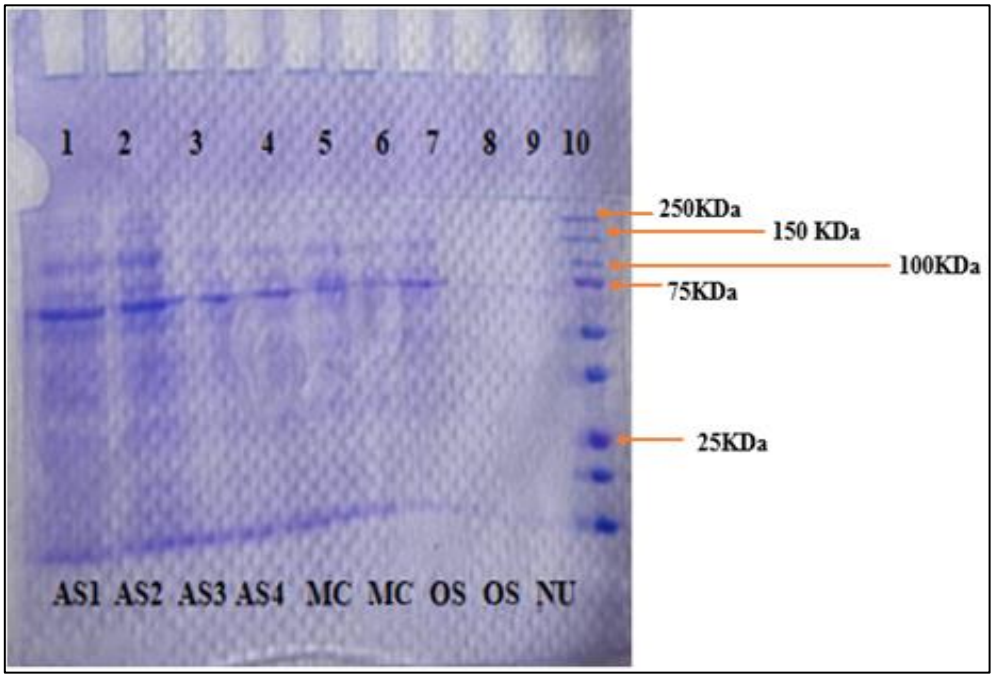

Fig 1: Separation of buffalo urinary proteins by SDS-PAGE

Lanes 1 and 2: Proteins present in pellet and supernatant respectively precipitated by AS, solubilized in TSE buffer and reduced in RSB; Lanes 3 and 4: Proteins present in pellet and supernatant precipitated by AS, solubilized in SLD respectively; Lanes 5 and 6: Total proteins present in the mixture precipitated by M/C, solubilized in TSE buffer and reduced in RSB and SLD respectively; Lanes 7 and 8: Total proteins resolved on SDS-PAGE from the urine samples (precipitated and dissolved in TSE buffer) stored at -80 ℃ without any preservative and reduced by RSB and SLD respectively; Lane 9: Total proteins resolved from neat urine after reducing the sample with RSB; Lane 10: Protein molecular weight marker.

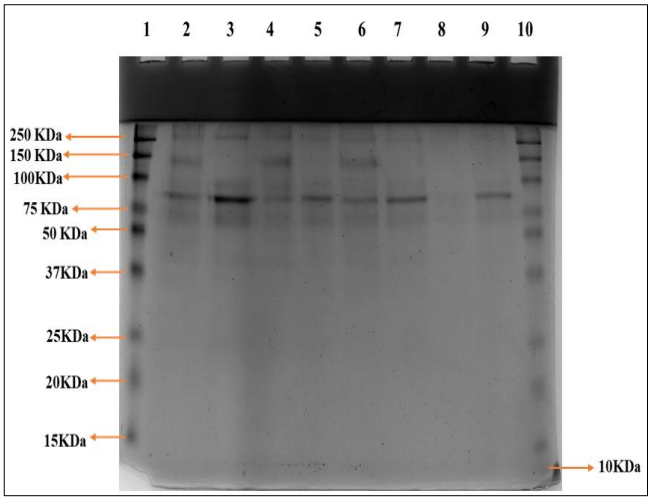

Fig 2: Separation of buffalo urinary proteins precipitated by different methods by SDS-PAGE

Lanes 1 and 10: Protein molecular weight marker; Lanes 2 and 3: Urinary proteins precipitated from samples collected at estrus phase and diestrus phase respectively by using AS; Lanes 4 and 5: Urinary proteins precipitated from estrus and diestrus samples respectively by using ethanol; Lanes 6 and 7: Urinary proteins precipitated from estrus and diestrus samples with acetone; Lanes 8 and 9: Urinary proteins precipitated from estrus and diestrus samples respectively with M/C

Conclusion

From this study, it is evident that the methods of protein preparation and precipitation will have an influence on the proteins separated by SDS-PAGE in a concentration dependent and physiological state dependent manner. And the use of silver staining instead of CBB staining would have detected proteins more sensitively.

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