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Strategies in sample preparation for proteome analysis of biological fluids from water buffaloes

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ABSTRACT

Livestock species such as water buffalo have some unique characteristics like high fat percentage in milk and plasma, poor sample generation, peculiar behaviour, etc. making it difficult for proteomic analysis of such samples. Additionally, there is a limited database in terms of identified proteins, characterised antibodies and functional attributes of known molecules specific to buffalo. The body fluids like blood, milk, urine and foetal fluids offer a great opportunity in understanding physio-pathological processes and thus aid in developing reliable diagnostics. Standardised sample preparation protocols are key to advancement of proteomics science. Optimising sample preparation for 2-DE proteomics in buffalo serum, milk, and foetal fluids is crucial. Commercially available kits or laboratory methods for clean-up, enrichment, and condition optimising can be applied for getting optimum results. The 2-D clean up strategy is an important step in sample preparation procedures to get rid of inferring substances which hamper IEF. The sample preparation of the buffalo biological fluids (serum, foetal fluids, maternal uterine flushing and milk samples) reported in the present study shall provide valuable information for further research.

Keywords: Buffalo, Proteomics, Sample preparation, 2-DE

The qualitative and quantitative proteomic analysis of a biological sample provide sample information regarding the physiological and pathological status of an animal on the basis of identification of certain diagnostic and prognostic markers in different body fluids regarding any particular condition. The plethora of information generated by various proteomic tools has enlightened several avenues in biological research to make it an indispensable and lucrative machinery of omics era. At the same time, proteomic research demands extreme purity of the samples to be compatible with high end proteomics instrumentation for successful, reproducible and biologically meaningful results from such studies. Proteomic analysis of biological fluids often encounters problems due to high or low abundance proteins in those samples. Serum or plasma, probably the most commonly analysed biological sample, is too difficult to analyse as it has high abundance of albumin and immunoglobulins. Urinary proteomic analysis, on the other hand, face difficulty of low abundance proteins. Although high fat content is the major problem of milk protein analysis but depletion of abundant casein is necessary to obtain quality proteomic result from milk samples.

Two dimensional separations of proteins (based on *p*I and MW) is still one of the most important approaches for separation and visualisation of proteins in biological fluids. Most biological samples from animals are either abundant in few proteins or have some non-protein substances which makes it very difficult to directly analyse these samples. Furthermore, newer technologies in proteome analysis require protein samples to be pure to the highest level. This makes the sample preparation step very crucial to proteomics research. This study describes methods developed for buffalo sample (serum, milk and foetal fluids) preparations using simple tools and common chemicals. Samples from animals especially the large ruminants like buffaloes require additional sample preparation steps, probably due to an altogether different body system. This necessitates trying and developing new strategies for sample preparation for proteomic studies. The objective of these studies was to develop methods for separation of proteins especially which indicate specific conditions and are usually present in low abundance in the sample facilitating their identification by suitable mass spectrometric (MS) technique. Present study is about the methods that has been tried to address sample preparation of buffalo origin and which can be useful for animal researchers involved in proteomic experiments.

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Proteomic analysis of buffalo body fluids has more challenges - high fat percentage in milk and plasma, non-availability of specific antibodies, poor sample generation, etc. The current study was planned to address buffalo body fluids (serum, milk, foetal fluids and urine) specific analytical difficulties and their possible solutions. The methodology will help in developing suitable low-cost methods for high end 'omics' sample preparations of indigenous animals.

MATERIALS AND METHODS

Preparation of body fluid samples with high protein concentration: Body fluids like blood and milk, are characterised by high protein content - few proteins making up vast majority and very low concentration of others. Blood, the transport medium for most bio-molecules (nutrients, hormones, signalling, etc.) is most common body fluid in animals used for diagnosis of pathological conditions. Blood is often called an 'ocean' of proteins where problem of plenty make things very complex for analysis. An adult buffalo on an average contains about 7.1g/dl of serum proteins (Balhara *et al.* 2014), of which the major portion is albumin and immunoglobulins. The low abundant proteins, which are potential biomarkers, are masked by few highly abundant proteins. Similarly, milk is another body fluid with high diagnostic value in dairy animals. Casein, the milk protein makes majority of the protein content of milk. Milk also has high fat content making it necessary to process adopt different procedure for the accomplishing proteomic analysis.

Plasma/Serum sample

Collection of blood and preparation of serum: Approximately 6 ml of venous blood sample in Vacutainer® serum-separator tube (BD Biosciences, USA) was allowed to stand at room temperature for 1 h. Serum was separated by centrifugation at 1500 rpm at 4°C for 20 min and transferred in clean pre-sterilized cryo-vials together with 20-50 µl of working Protease Inhibitor Cocktail (Sigma Life-sciences) and stored at –80°C till further analysis.

Depletion of highly abundant proteins: Three different depletion methods were used for removal of high abundant proteins from the serum samples following manufactures recommendations: (1) ProteoPrep 20 (Sigma, USA); (2) Albumin and IgG removal kit (Quigen, USA); and (3) the ProteoMiner™ large capacity protein enrichment kit (Bio-Rad USA, Catalog #163-3007). Comparative protein depletion by the three methods were analysed in 12% SDS-PAGE.

Two-dimensional gel electrophoresis: Protein concentrations in the depleted serum samples were estimated using 2D Quant kit (Bio-Rad, USA) following manufacturer's protocol. Further removal of nucleic acid, salts and other interfering substances were performed by using 2-D Clean-Up Kit™ (GE Healthcare, USA) according to manufacturer's recommendations. After removal of contaminating substances, the precipitated proteins were resuspended in commercially available rehydration buffer (GE Life science, USA). Approximately 100 µg proteins from each sample was incubated overnight with 7 cm, *p*H 3-10 NL, IPG strips (Immobiline DryStrip gels, Bio-Rad, USA) and loaded into PROTEAN® i12™ IEF System (Bio-Rad, USA) for isoelectric focusing. First dimensional separation of proteins was achieved by using a stepwise gradient with increasing voltage for a total period of 60,000 V-h, following manufacturer's recommendations. After reduction and alkylation, the second-dimension separation was performed on 12% Tris/glycine SDS-PAGE using the Mini-PROTEAN Tetra cell (Bio-Rad, USA) vertical electrophoresis system. The gels were stained with Coomassie R-250 (Bio-Rad, USA) followed by overnight destaining for visualisation of protein spots. Un-depleted serum sample was also run simultaneously in 2-DE to compare the results.

Milk sample: The thick fat layer from milk was removed by centrifugation (12000×g, 4°C for 30 min) in 50 ml conical centrifuge tube. The fat free liquid was then transferred to 15 ml conical centrifuge tubes and *p*H was reduced from the normal *p*H of approximately 6.7 to 4.0, using N/10 HCl solution and 1N NaOH solution for *p*H adjustments. The resulting precipitated material was removed by centrifugation (15000×g, 4°C for 20 min). When the *p*H was again normalised to 6.7, some more precipitates appeared, which were removed by subsequent centrifugation (15000×g, 4°C for 20 min), yielding a clear transparent liquid containing milk proteins. 2-D Clean-Up Kit™ (GE Healthcare, USA) was used to further remove the contaminants from the samples following the manufacturer's instructions.

Preparation of body fluid samples with low protein concentration: Some body fluids act as medium for excretion in the animal body, e.g. urine and foetal fluids. Such fluids are generally rich in metabolites and smaller proteins with high diagnostic value. Since these fluids are excretion mediums, therefore they are rich in molecules which have been broken down in the metabolic pathways and are no longer needed by the body.

Urine samples: Urine samples were collected in sterile containers in presence of Protease Inhibitor Cocktail (Sigma Life-sciences). The samples were filtered twice with Whatman® qualitative filter paper, Grade 1 (Sigma, USA) and subsequently through 0.45 µm Acrodisc® Syringe Filters (Pall Life Sciences, USA) followed by storage at –80°C till further analysis.

Protein precipitation: Protein in the filtered urine samples was precipitated using different precipitation methods to enrich low abundance urinary proteins.

Methanol precipitation: Filtered urine was mixed with ice cold methanol at 1:9 ratio (v/v), vortexed and kept on ice for 10 min. The mixture was centrifuged at 10000×g for 10 min at 4°C and the supernatant was discarded. The pellet was washed with 90% methanol and the supernatant was removed without disturbing the pellet. The pellet was air dried for 10 min.

TCA/acetone precipitation: Equal volume of urine and 20% TCA was mixed, vortexed and kept standing at 4°C for 15 min. The mixture was centrifuged at 10000×g for 10 min at 4°C and the supernatant was discarded without disturbing the pellet. Washing of the pellet was performed by using 0.1 volume of ice-cold acetone twice. The pellet was air dried for 10 min.

Phenol precipitation: Urine sample was mixed at 1:9 ratio (v/v) with extraction buffer containing 500 mM Tris-HCl, 50 mM EDTA,700 mM sucrose, 100 mM KCl, 2% β-mercaptoethanol (β-ME), 1 mM phenylmethylsulfonylfluoride (PMSF)with the *p*H adjusted to 8.0 with HCl. The extraction buffer can be stored for 1 week at 4°C and β-ME and PMSF was added just before use. The mixture was vortexed, and incubated by shaking for 10 min on ice. Equal volume of Tris-buffered phenol (*p*H 8.0) was added to the mixture and was incubated on a shaker for 10 min at room temperature. The mixture was centrifuged at 5500×g for 10 min at 4°C for separation of aqueous and organic phases. The phenolic phase on the top was recovered carefully without disturbing the interphase. This phenolic phase was back-extracted with extraction buffer at 1:9 (v/v) ratio. The phenolic phase still on the top was carefully removed into a fresh tube and mixed by inversion with 4 volume of precipitation solution containing 0.1 M ammonium acetate in cold methanol. The mixture was incubated for at least 4 h or overnight at –20°C. Finally, the protein was pelleted down by centrifugation at 5500×g for 10 min at 4°C. The pellet was washed three times with cooled precipitation solution and final washing was done with ice cold acetone. The pellet was air dried for 10 min.

TCA/ethanol precipitation: Collected urine samples were concentrated by lypholizer (CHRIST Alpha 1-2 LD plus, part no. 101521). Equal volume of concentrated urine, 10% TCA and 1 ml 70% ethanol was mixed, vortexed, kept standing at 4°C for 10 min. The mixture was centrifuged at 10000×g for 10 min at 4°C and the supernatant was discarded without disturbing the pellet. Washing of the pellet was performed by using 0.1 volume of ice-cold acetone twice. The pellet was air dried for 10 min. The interfering substances such as salts, detergents and nucleic acids were removed from the precipitated urinary proteins using 2D-clean up kit and the resulting pellets were rehydrated in lysis buffer. The cleaned-up proteins were analysed by SDS-PAGE with 4% stacking and 12.5% resolving gel. The gel was stained with Coomassie Brilliant Blue G 250 for 1 h and destained. Fig. 2 and Fig. 3 describe the sequence of events involved in TCA/ethanol precipitation.

Solubilization and Quantification of protein: The dried protein pellets were solubilized in rehydration solution containing 7 M urea, 2 M thiourea, 4% CHAPS and 30 mM Tris. The rehydration solution can be stored in aliquots at –20°C for one month. Protein concentrations were measured by Quick Start™ Bradford Protein Assay kit (Bio-Rad, USA). Approximately 1.5 µg of protein was loaded in each well and resolved in 15% Tris/glycine SDS-PAGE. Visualization of protein bands was performed by silver staining using Pierce™ Silver Stain Kit (Thermo Scientific, USA).

Two-Dimensional gel electrophoresis: Three type of columns were used for low abundant protein removal: (1) ProteoPrep 20 (Sigma, USA); (2) Albumin and IgG removal kit (Quigen, USA); and (3) the ProteoMiner™ large capacity protein enrichment kit (BioRad USA, Catalog # 163-3007) (www.bio-rad.com) were tried and results were compared. 12% SDS-PAGE was run to confirm the depletion of abundant proteins. Depleted samples thus prepared were further prepared for 2DE by 2-D Clean-Up Kit™ (GE Healthcare, USA) was used to remove contaminating substances and improve the 2-D electrophoresis pattern as per the manufacturer's protocol (for larger samples with >100 µg proteins).

Foetal fluids: Low protein containing foetal fluids were concentrated using a laboratory developed method in which combination of commercially available products, described in the protocol given in Table 1 was used. Contamination from the samples thus prepared was removed using 2-D Clean-Up Kit™ (GE Healthcare, USA) described above.

RESULTS AND DISCUSSION

Sample preparation which includes sample fractionation, clean-up, enrichment, and sample condition optimization is very important for obtaining good 2-DE based proteomics results. Most of the biological fluids contain few high

Table 1. Protocol for concentrating proteins and removing contaminants in foetal fluids

Step	Remarks	Approximate volume after completion of step
1	Centrifuged (5000×g, 30 min) 30 ml foetal fluid in centricon cut-off filters (10kDa, 50 ml capacity, Novagen USA)	6 ml
2	Collected in 15 ml conical centrifuge tube	6 ml
3	Reduced volume to 1/3rd by centrifugation in SpeedVac	2 ml
4	Added 4 volumes 100% Acetone (chilled, –20°C)	10 ml
5	Shaken slightly	10 ml
6	Kept overnight at –20°C	10 ml
7	Centrifuged (5000×g, 30 min)	10 ml
8	Removed supernatant and dissolved precipites in 50 µl rehydration buffer (DeStreak rehydration solution, GE Healthcare, USA)	60 µl

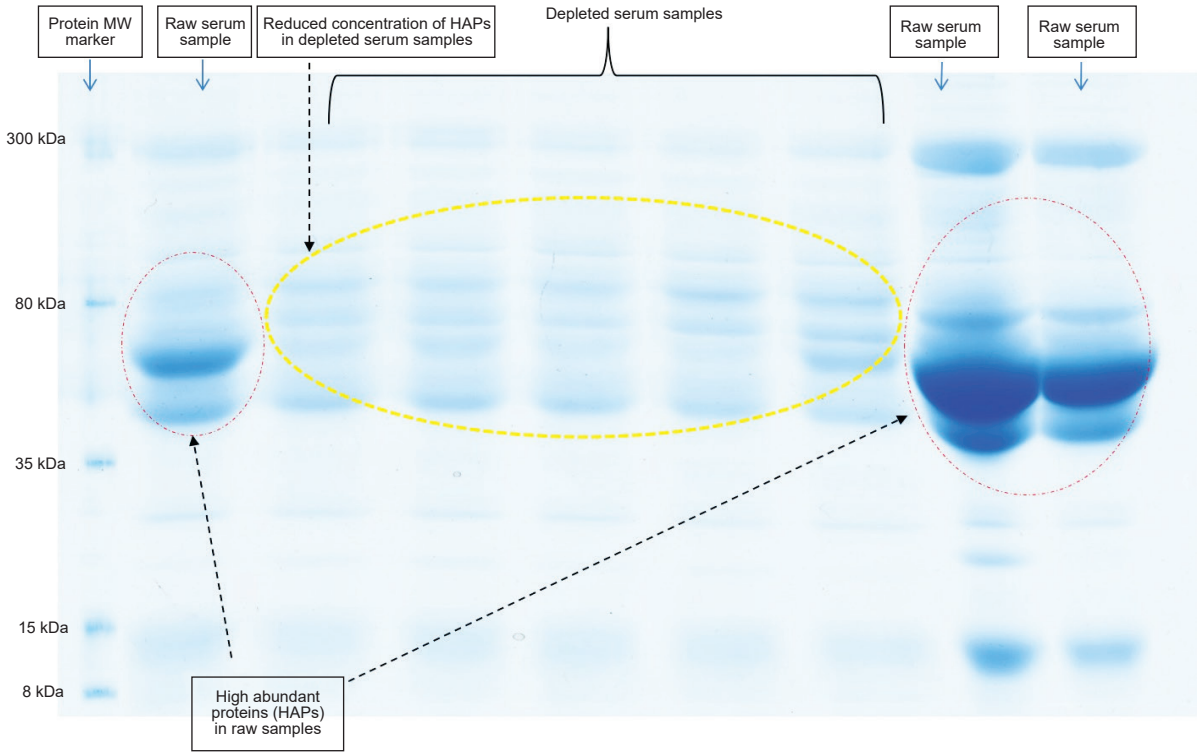

Fig. 1. Depletion of abundant proteins using a library of combinational hexa-peptides columns (each well loaded with 16 µg protein).

abundant proteins (HAPs) or some non-protein inferring substances which suppress the signals from low abundant proteins (LAPs) or prevent good IEF thereby necessitating a prefractionation strategy and/or appropriate sample preparation (Brzeski *et al.* 2003, Steel *et al.* 2003) for proper focussing and detection of proteins from biological fluids (plasma/serum, amniotic fluid, milk).

The commercially available abundant protein removal kits like ProteoPrep 20 (Sigma, USA), and Albumin and IgG removal kit (Quigen, USA) are based on immune-affinity of specific antibodies for removal of abundant proteins. In these kits, antibodies against human antigens are immobilized to the columns. No visible reduction in the HAPs in buffalo serum was observed using these commercially available immune-affinity columns (Supplementary Fig. 1).

Non-depletion of High Abundant Proteins (HAPs) from buffalo serum may be due to non-specificity of human antibodies against buffalo blood proteins. In contrast, the buffalo serum proteins were depleted quite well with a major fraction of serum albumin being depleted by use of the ProteoMiner™ large capacity protein enrichment kit (BioRad USA). Many low abundant proteins got enriched at the same time (Fig. 1). As the area and *p*H range of the gel increases, proteins got greater area for movement and therefore there is better resolution of spots.

The ProteoMiner protein enrichment kit (BioRad USA) uses a library of hexapeptides bound to a chromatographic support to bind all proteins in a protein mixture (Hartwig *et al.* 2009). Because this system is not immunoaffinity based, it has no species issues and works well for the buffalo

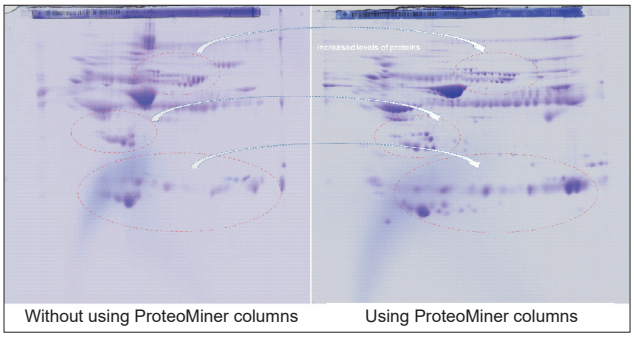

Fig. 2. Increase levels of proteins in 2D gels of serum samples using ProteoMiner depletion columns (7 cm IPG strip at *p*H 4-7).

samples. The ProteoMiner processed serum samples lead to enrichment of low abundant proteins thereby improving the representation of proteins spots on 2-D gels (Fig. 2). The difference led to better visualization of low abundant protein spots in 2-D gel images of ProteoMiner processed and 2-D cleaned up serum samples as compared to raw serum samples. The efficacy of ProteoMiner technology in processing of bovine and porcine samples is documented in literature (Marco-Ramell and Bassols 2010). Present study support the compatibility of ProteoMiner technology with buffalo samples.

The foetal fluid (allantoic and amniotic) recovered from gravid uterus and maternal uterine flushing samples have a lot of interfering substances and relatively low concentration of proteins. Thus, protein precipitation by ice cold acetone improves the visibility of protein bands on SDS–PAGE (Fig. 3). Removal of interfering substances from foetal fluids is a major issue in sample preparation for

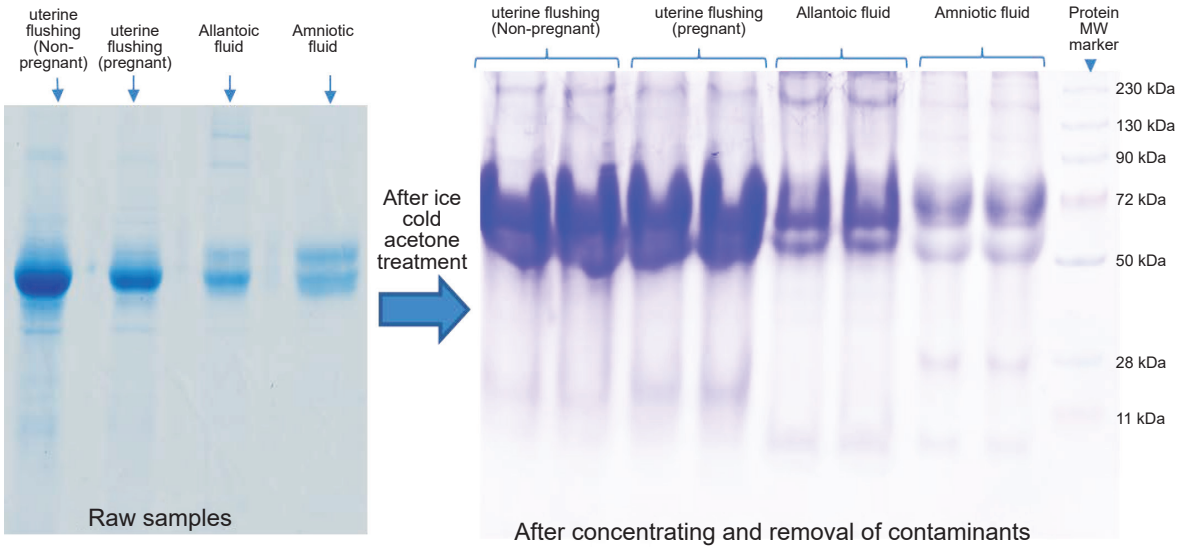

Fig. 3. Comparative SDS-Page gel images of uterine flushings, allantoic and amniotic fluids

proteomic studies (Teng *et al.* 2010).

Urine is one of most useful biofluid for proteomics study and has great diagnostic importance. However, recent urinary proteomics studies have not reached the ideal goal because of difficulty to examine the entire urine proteome (Swensen *et al*. 2021). Lyphilisation is an excellent method for preserving a wide variety of heat-sensitive materials such as proteins, microbes, tissues and plasma. Lypholized urine together with ethanol (70%) and TCA (10%), offer an easy precipitation of proteins due to removal of aqueous binding particles (Afkarian *et al*. 2010). Ethanol is generally good protein precipitant because its lower dielectric constants lowers the solvating power of their aqueous solutions for dissolved proteins, thus helpful to increase protein-protein interaction and decreases protein water interaction and the protein is precipitate out easily (Zhou and Pang 2018). In the present study urinary proteins from four buffaloes were isolated using TCA (10%) and ethanol (70%) treatment in 1:5 dilution (Supplementary Fig. 2).

Buffalo milk has relatively high fat percentage in comparison to cows (normally 7- 8% vs. 3 - 4%, respectively). In the present study, milk samples after removal of fat by centrifugation, were depleted off high abundant protein, casein by adopting a pre-fractionation strategy. *p*H adjustment methodology for casein depletion works well with the buffalo milk samples. Casein depletion allows detection of more number of spots on the 2-DE gel. There are many other interfering substances which has to be removed before running samples for IEF. Uncleaned samples do not complete the IEF run, where as when the samples were 2-D cleaned up, the run was complete and almost ideal (Supplemetary Fig. 3. A, B and C). The 2-D clean-up procedure uses a combination of a unique precipitant and co-precipitant to quantitatively precipitate the sample proteins while leaving interfering substances behind in the solution. 2-D clean up kit has also been been successfully used for sample preparation procedures for 2-DE of *Aspergillus fumigatus* (Kniemeyer *et al.* 2006).

The goal of present study was to find the best approach for getting maximum protein yields from animal fluid samples. Precipitation is the most used method to concentrate and fractionate specific protein in biological fluids of an individual (Zhang *et al.* 2019, Saito *et al.* 2019, Karthik *et al.* 2020, Tkacikova *et al.* 2020, Krishnappa *et al.* 2021). This research provides a standard approach for extracting proteins from biological fluids so that qualitative and quantitative analysis can be accomplished with limited resources. It has a broad range of applications active in proteomics, not only for diagnosis, but also for basic biological research in fields such as physiology, using biomarkers and proteome database.

The sample preparation techniques for proteomic studies are basically aimed at complete solubilization, disaggregation, denaturation, and reduction of the proteins in the sample. Main reasons for poor two-dimensional electrophoresis could be high electric conductivity in samples due to high levels of interfering substances. The pre-fractionation strategy of complex samples could ameliorate the problem of the masking effect of high abundant proteins surpressing the signals from medium to low abundant proteins to an satisfactory level. Cleaning up precipitated proteins helps in removal of inferring substances which hamper iso-electric focussing and thus an important step in sample preperation. Though a preliminary study, sample preparation of the buffalo biological fluids (serum, foetal fluids, maternal uterine flushing and milk samples) reported in the present study shall provide valuable information for researchers working on biomarker discovery for designing novel diagnostic tools.

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