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Comparative Protein Profiling of Blood and Milk of Early Pregnant Buffaloes Using SDS-PAGE

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ABSTRACT

Conventional methods for detecting pregnancy in buffalo are inefficient before 30 days after artificial insemination. A proteomic approach for high-resolution analysis of blood plasma and milk proteins by SDS polyacrylamide gel electrophoresis (PAGE) is of great importance. Blood and milk samples were collected on days 0 (before insemination) and 20 and 25 after insemination from retrospectively pregnant (n=6) and non-pregnant (n=6) animals. An equal volume of samples was loaded at 12%–4% SDS gel to resolve major plasma and milk proteins. Plasma proteins showed three distinct zones: High: (135-250 kDa), ~208 kDa and ~190 kDa proteins, medium: (60-75 kDa), 5-6 proteins like albumin (~70 kDa), and low molecular weight (MW): (25-35 kDa). In this 75 kDa, and 63 kDa consist of pregnancy-associated glycoproteins, ~73 kDa being dominant. Proteins in milk supernatants showed four distinct zones of proteins: very high; (135-250 kDa), ~208 kDa and ~190 kDa proteins, high: 60-75 kDa, medium: 25-35 kDa, clear bands of milk caseins, and low molecular weight (MW) zones: 11-20 kDa, two thick bands of 18 kDa and 12 kDa. The mean grey values of the 73 kDa protein peak were statistically analysed using GraphPad (version 8.0). Pregnant and non-pregnant statuses defined columns, while time (0-, 20-, and 25-day) defined rows in a repeated measures ANOVA. Results showed higher 73 kDa protein expression (P=0.04) in pregnant buffaloes at 20 and 25 days after insemination in plasma, but no difference in milk. The variation in animals within the groups was significantly higher. The differential expression of ~73 kDa protein in plasma and precise identification of the protein can be a diagnostic marker for early pregnancy detection in buffaloes.

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Introduction

Livestock has a significant impact on total food production and environmental factors. According to the 20th livestock census, the livestock population has shown an increase of 4.8% over the last census. Currently, the country holds 536.76 million populations of the total livestock population. The total buffalo population is 109.85 million as per 2019 statistics, contributing to milk, meat, and other agricultural services (DAHD, 2019). The exceptional adaptability and multifunctionality of domesticated buffaloes, coupled with their significant social and cultural importance, have contributed to their increasing population (Abd El-Salam et al., 2011; Becskei et al., 2020; Noce et al., 2021). These attributes have made them valuable assets in the dairy and agriculture sectors, meeting the rising demand for buffalo products.

The reproductive performance of livestock animals plays a major role in maintaining their production profile. Factors like early detection of pregnancy govern the process of pregnancy; hence, maximizing production by shortening the time interval of the dry period and inter calving by spotting open animals and promptly treating and rebreeding them. Pregnancy diagnosed at an earlier stage can save the farmers from the financial losses caused by the unintended prolongation of the open time of delayed insemination in non-pregnant animals, and the slaughtering of pregnant animals can be prevented by a false pregnancy diagnosis. Therefore, early and faultless pregnancy diagnosis is essential for reducing economic losses (Saadullah et al., 1999; Buragohain et al., 2017).

Widely used methods for pregnancy diagnosis are per-rectal palpation, which accurately detects pregnancy after 32-35 days of artificial insemination (AI); another method is ultrasonography, which detects pregnancy after 25 days of AI (Momont, 1990; Fricke, 2002). Both these methods require a well-equipped veterinarian and also the loss of at least one estrous cycle, as in buffalo, the estrous cycle returns after 21 days and detection at 25 days or later results in the loss of estrous when conception is inevitable; moreover, about 15-73% of buffaloes possess the problem of silent estrous, which is accelerated during the summer season (Zicarelli, 2010; de Carvalho et al., 2016).

There are many other indirect methods of pregnancy diagnosis that have been reported, and each of them has its own merits and demerits (Balhara et al., 2013). There have been studies conducted that reveal proteomic changes in biological fluids during different stages of pregnancy. This study aims towards evaluating the comparative proteomic profile of whole blood plasma and milk serum between pregnant and non-pregnant buffaloes after AI, as during pregnancy several signalling molecules like steroids, progesterone, and other factors associated with pregnancy are secreted (Žvorc et al., 2000).

Materials and Methods

The experiments carried out for the current study were approved by the Institute Animal Ethics Committee (IAEC), Registration No. GADVASU/2021/IAEC/61/05, dated October 13, 2021.

Sample collection

Blood and milk samples were collected from a total (n=12) Murrah buffalo from GADVASU, a dairy farm. Animals were divided into two groups according to their reproductive status: pregnant (n=6) and non-pregnant (n=6). Sampling was done at different time points (0, 20, and 25 days) after timed artificial insemination. Blood samples were collected from the tail vein in a 15-mL falcon tube containing EDTA and immediately transported to the laboratory. Blood was centrifuged at 400 x g for 30 minutes, and plasma was collected and preserved at -20°C until use. Milk samples were collected, defatted by centrifuging at 3000 x g for 15 min to attain milk serum, and stored at -20°C until use.

1D-SDS-PAGE protein profile

Stored blood plasma and milk samples were thawed at room temperature and spun before sample preparation. Each 5 µL of 1:50 diluted blood plasma sample with 1X PBS was combined with a 20 µL 1× loading buffer (Himedia Laboratories, India). In the case of milk, 5 µL of neat milk serum was diluted with 20 µL 1× loading buffer (Himedia Laboratories, India). Both blood plasma and milk serum were placed in a dry bath at 100°C for 5 min to denature the proteins. First, we standardize the protocol of SDS-PAGE regarding, amount of protein samples to be loaded, voltage, run time and temperature. We also empirically tested sample preparation steps, staining of electrophoresed gel, gel % (tested 12, 15 and 18%) and finally reproducibility of the results. Finally, on 12% gel, the denatured proteins were separated using the SDS-PAGE Mini-PROTEAN system (Bio-Rad), 30V for 15 min followed by 100V for 2 h with 4-12% hand-cast gels. Once the run was over, the gel was stained with Commasie Brilliant Blue R250 (Himedia Laboratories, India) for 4 h and destained with destaining solution overnight.

Image analysis

Opened the image (version 1.53t), and after converting to 8-bit grey scale, a background subtraction process is done by using the "Subtract Background" command from the Process menu. Set the rolling ball radius to 50 with the white background checked in. The uncalibrated standard was used to compare bands of gel within a single image. The rectangular selection tool was used to mark the lanes of the band, and plot lanes were generated. Peaks of ~73 kDa protein were selected for the measurement of the mean grey value.

Statistical Analysis

The data of mean grey values were analysed in a two-way ANOVA using GraphPad (version 8.0). Repeated measures of ANOVA were selected where pregnancy status (pregnant and non-pregnant) defined the column factor and time (0-, 20- and 25-day) defined the row factor. The mean of each column was compared with the mean of each row in multiple mean comparisons with Bonferroni corrections. Differences in expression within time points between pregnant and non-pregnant animals were tested using the t-test.

Results

Standardizing SDS-PAGE was crucial to ensure the accuracy and reliability of protein separation and molecular weight determination. Results showed reproducible bands of protein distinctly separated bands that could be analysed (Fig. 1).

The protein patterns of plasma from pregnant and non-pregnant buffaloes were separated by 4%-12% SDS-PAGE gel electrophoresis, as shown in Fig. 2, at three different time points (0, 20, and 25 D) after timed artificial insemination. Plasma proteins were separated into different bands ranging from molecular weights ~245 kDa to ~25k Da (Fig. 2A and 2B) and supernatants of milk proteins (Fig. 2C and 2D). All bands are distributed in three distinct zones based on their molecular weight (MW): high, medium, and low. The high MW zone ranged from 135 to 250 kDa, and within this range, two prominent proteins were visibly present at approximately 208 kDa and 190 kDa. Moving on to the moderate MW zone, which spanned from 60 to 75 kDa, lies albumin (~70 kDa) which is the most abundant protein and creates hindrance in the separation of other minor proteins (Sweety et al., 2020). In the low molecular weight zone (25-35 kDa), a clear band of 27 kDa was visible.

In milk serum SDS-PAGE of pregnant and non-pregnant buffaloes, nine major protein bands between ~245 kDa to ~11 kDa were observed (Fig. 2C and 2D). All protein bands were observed to be distributed across four distinct zones of proteins, namely the very high, high, medium, and low molecular weight (MW) zones. Within the very high MW zone (135-250 kDa), prominent protein bands were detected at approximately 208 kDa and 190 kDa. In the high MW zone (60-75 kDa), several proteins were identified, including lactoferrin (~78.2 kDa), a 73 kDa protein, bovine serum albumin (~66 kDa), and a heavy chain of immunoglobulin (IgG) (~54 kDa). Additionally, ovalbumin was detected at 45 kDa. Moving on to the medium MW zone (25-35 kDa), clear bands of milk caseins were

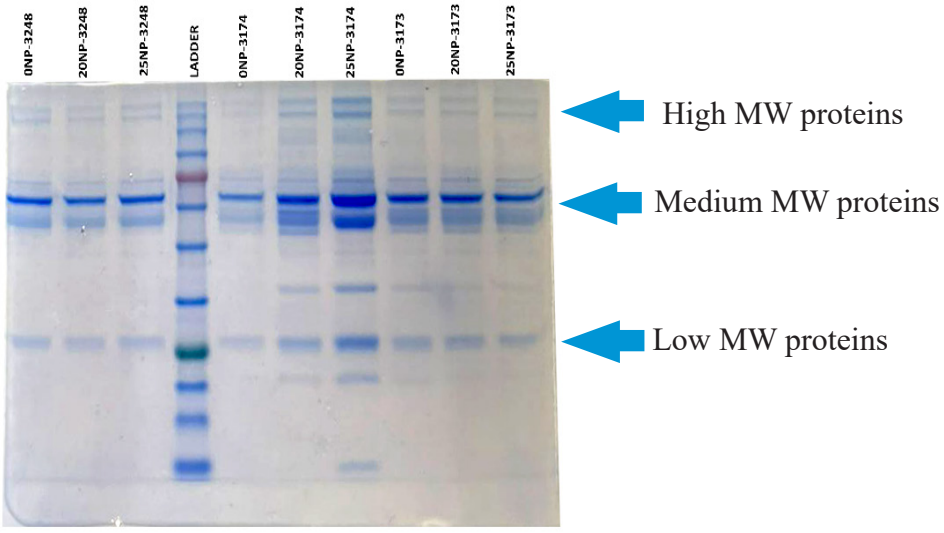

Fig. 1: A representative image of standardized SDS PAGE gel image after staining showing distinct bands of various buffalo proteins in blood plasma. Proteins of high, medium, and low molecular weight of blood plasma are shown.

observed, particularly thick alpha S2 casein at 29 kDa, alongside other caseins. In the low MW zone (11-20 kDa), two prominent bands were visible at 18 kDa and 12 kDa (Kausar et al., 2016; Zhang et al., 2022).

Plasma proteins of pregnant and non-pregnant buffaloes showed distinct bands of protein in SDS-PAGE (Fig. 3A and 3B). In pregnant animals, the band ~73 kDa showed a linear differential expression pattern with increasing band intensity as analysed using ImageJ (version 1.53K) between days 0 and 25 (Fig. 3C) The expression of a ~73 kDa protein in the plasma showed a trend towards significance (P=0.07) between pregnant vs. non-pregnant buffaloes

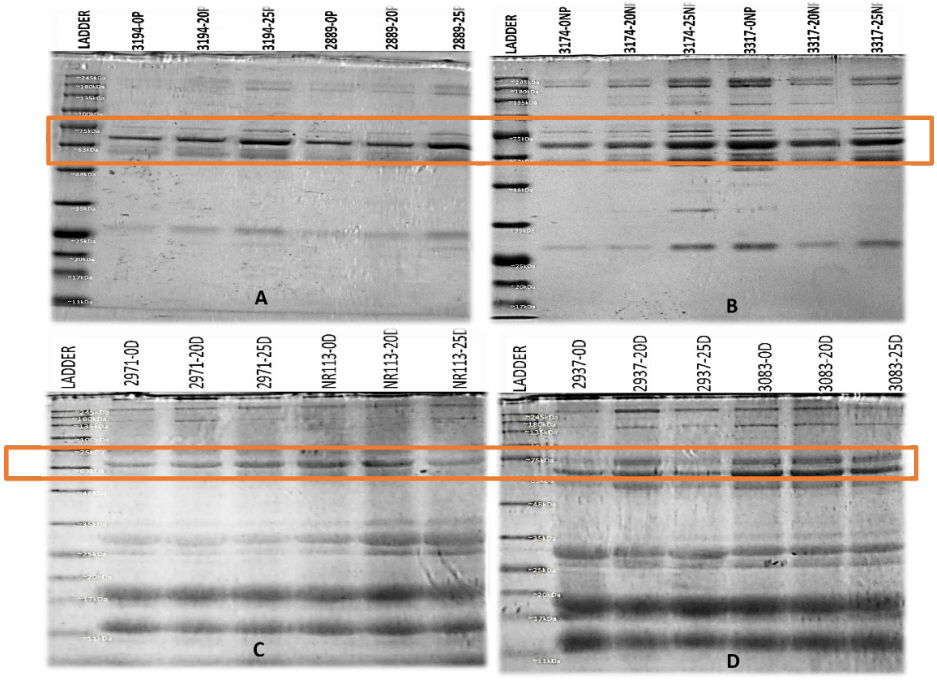

Fig. 2: SDS-electrophoretic analysis of blood plasma of pregnant (A), and non-pregnant (B) buffaloes. Milk after centrifugation, the supernatants of pregnant (C) and non-pregnant (D) buffaloes were subjected to SDS-PAGE. Protein of ~73 kDa band (outlined by rectangular area) is shown on plasma (A-B) and milk supernatant (C-D).

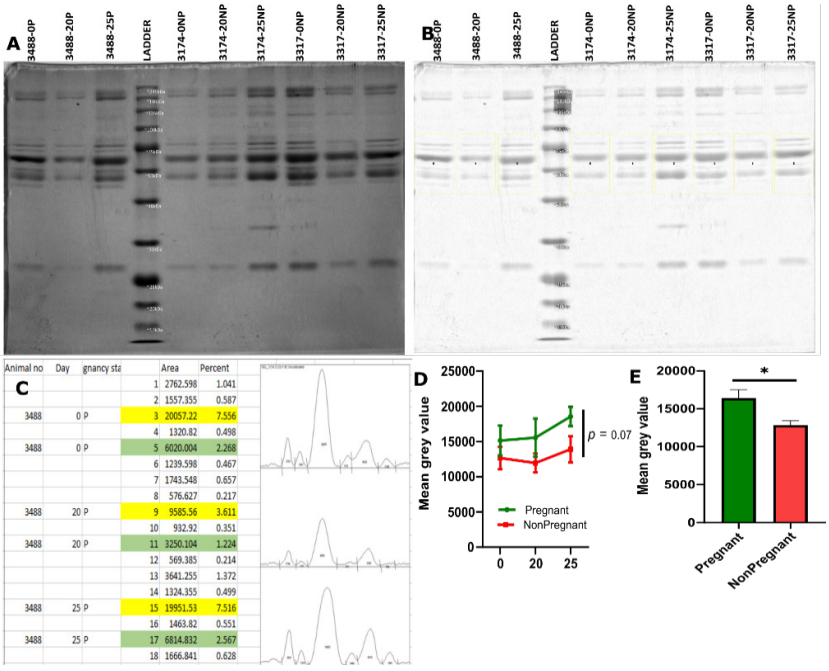

Fig. 3: Blood Plasma SDS-PAGE Gel analysis (A), Raw gel image (B), Gel image with subtracted background (C), Lane profile plots (D), Two-way ANOVA analysis of pregnant and non-pregnant across time points 0th, 20th, and 25th days (E). Unpaired T-test analysis of 20th and 25th days combined between pregnant and non-pregnant animals.

(Fig. 3D). This suggests that there may be a potential association between the presence of this specific protein and pregnancy status in buffaloes, although further investigation is needed to establish a more conclusive relationship. Interestingly, the effects of time were significant (P=0.04) when the expression level of the protein at 20- and 25-day combined and compared between pregnant and non-pregnant animals, keeping the 0-day as a control (Fig. 3E). This indicates significant differences in the expression of the ~73 kDa protein at these defined time points during pregnancy. In non-pregnant animals, no significant difference in protein pattern or band density of blood plasma was observed at 73 kDa between the 0th, 20th, and 25th days.

However, on analysing the expression pattern of 73k Da bands of milk serum SDS-PAGE gel via ImageJ, we did not observe any significant difference in the expression of this specific protein band between the two groups of animals across the various time points studied.

Discussion

Pregnancy alters the expression of proteins in biological fluids like maternal serum. Additionally, the differential variation in protein expression throughout pregnancy is helpful for identifying pregnancy-related biomarkers. Many scientists have reported the elevation of hormones, placenta secreted products and progesterone in blood serum during pregnancy. It has been reported by (Žvorc et al., 2000), around the seventh and eighth month of pregnancy the significant rise in, α-globulin, β-globulin and γ-globulin occurs, which become equal to the level of non-pregnant animal in the ninth month. According to research done by (Mir et al. 2008), mid- and late-gestation cross-bred cows had greater levels of total plasma protein.

Our results are in accordance with Balamurugam et al, 2020, conducted blood serum protein comparison of Murrah buffalo at different reproductive stages which revealed 70kda and 30 Kda bands expressed solely in pregnant animals. Another study conducted by Kalleshwarappa et al., 2009 found secretory protein in the size range >97, 97, 75, 66, 43, 30, 29, 27 and 20 kDa in both bovine oocytes and embryos respectively. Among which 75 kDa and 29kDa was considered to be pregnancy specific. Mid-luteal, estrus, and early pregnant goat serum were shown to include the protein bands of 66, 55, and 45 kDa (embryo), as well as 95 kDa (Malakar and Majumdar, 2005). Similar report by, Singh et al., 2005 that proclaimed six different polypeptide bands from buffalo placental extracts, measuring 78, 67, 53, 42, 33, and 26 kDa. Four glycoproteins (86, 67, 56, and 51 kDa) were found in buffaloes during early pregnancy, while five glycoproteins (86, 75, 67, 56, and 38 kDa) were found in the mid- and late-pregnancy periods. This could be as a result of the fact that more proteins are produced during pregnancy and released into the peripheral circulation. Kumar et al, 2014 investigated the pregnancy associated markers from fetal cotyledon tissues in which 75 kDa band was found to be pregnancy specific, contains two glycoproteins PAG7 and PAG11.

According to a study, placental follicles from the six species (cattle, elk, bison, buffalo, sheep, and goat) on SDS PAGE revealed strongly stained proteins with molecular weights of 45 and 66 kDa (Bella et al. 2007). In comparison to non-pregnant buffalo, (Salam, 2016) found that pregnant buffalo had higher levels of the 66 kDa protein band. Another study discovered a protein band in goat blood with a molecular weight of 55 kDa that was later identified as pregnancy-related glycoproteins (Ningtyas et al., 2019). On days 21 and 28 after the AI, pregnant goats had overexpressed levels of the proteins 42 kDa and 52 kDa (Inyawilert et al., 2019). Also, blood serum protein analysis of cow revealed specific protein band of between 33 and 43 kDa and increasing band density of 66.0 kDa from 16 to 22 day, further nano-LC–MS/MS of 19th day sample suggested lactotransferrin (78kDa), Golgin A4 (259 kDa), MYRIP (90.5 kDa), PKD1 (459 kDa) and PWWP domain containing protein MUM1 (71kda) have potential to be utilised as early pregnancy biomarker in cows (Bahuguna and Sharma, 2022).

While in case of milk, more sensitive and specific technique is needed in order to identify the pregnancy specific protein. Several proteins like lactoferrin, albumin, immunoglobulins and complement protein are present in low amount in milk (Lacy-Hulbert et al., 1999; Korhonen,et al., 2000; ward et al., 2002). The study conducted by Han et al., 2012 revealed that During early pregnancy, the expressions of lactoferrin, lactotransferrin, and alpha-1 G in milk showed an increase, suggesting the expression to be pregnancy associated. Another study has identified potential protein candidate biomarkers of bovine pregnancy present in milk whey samples, including apolipoprotein B, perilipin, spermadhesin-1, the polymeric immunoglobulin receptor, sulfhydryl oxidase, mucin-1, and lymphocyte antigen 96. The research provides novel insights into changes in protein abundances in milk samples associated with early pregnancy. However, it is important to note that the fold change differences between the proteins in the milk whey, between the two physiological states (pregnant and cycling), were not very substantial. Therefore, creating a pregnancy diagnostic test using these proteins that is valid at day 21 post AI may pose challenges due to the relatively small differences observed (Johnston et al., 2018).

Therefore, in this study we found that the protein band expression pattern can be used as a diagnostic tool for identifying the pregnant animals. In blood expression pattern of the 73 kDa band, found to be pregnancy specific while in case of milk no difference can be observed. Hence, suggesting that the more sensitivity technique is required to explore milk for pregnancy biomarker.

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