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Changes in sera proteome in relation to day of pregnancy in early pregnant buffaloes

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ABSTRACT

The present study was planned for studying the sequential changes in early pregnant buffalo for the development of diagnostics for early pregnancy. In the present study, sera samples obtained at weekly intervals from early pregnant (day 0 to day 42 post-AI) buffaloes, and on days 0, 7 and 14 from non-pregnant cyclic buffaloes, were subjected to depletion of high abundant proteins followed by 2-dimensional gel electrophoresis and densitometric analysis. At least 65 2-D gel spots exhibited up-regulation, down-regulation or specific appearance at a specific stage during early buffalo pregnancy, except for the spots correlating with the high abundant proteins' location. Comparison with ExPASy and NCBI databases matched 48 of these spots with known proteins, but with varying degrees of confidence in terms of Mascot score and the species. Although high abundant proteins were depleted before 2-D electrophoresis, yet in some of the picked spots isoforms of common abundant proteins, viz. serum albumin, igg, serrotransferrin, complement and mhc molecules, were found. synaptojanin-1, apolipoprotein a-1, apolipoprotein b, keratin 10 and von Willebrand factors were some of the proteins identified in these spots, which have a documented role in embryogenesis and early pregnancy.

Key words: Buffaloes, Early pregnancy, Proteomics, Serum

An early and accurate diagnosis of reproductive dysfunctions or aberrations is the key to better reproductive management. Though studies on levels of progesterone, pregnancy associated glycoproteins (PAGs), and early pregnancy factor are some of the clinically practised pregnancy detection methods in bovines, yet none of the bio-molecules identified for pregnancy till date qualifies as an ideal biomarker for early pregnancy. In the absence of any such reliable bench top pregnancy diagnosis method in large dairy animals, the quest for suitable early pregnancy biomarkers continues, while the traditional method of rectal palpation continues to be the undisputed method of choice the world over.

With the advent of post-genomic technologies like proteomics during the last decade and its anticipated applications in varied areas of biological science, it has now become possible to study differential analysis of several protein components (numbering in thousands) in a complex mixture of proteins in body fluids like serum, plasma, milk, urine etc. It is presumed that the monitoring of sequential changes in blood proteome profile from the day of estrus to successful conception and through progression of gestation, can lead to discovery of molecules, which will perhaps be novel and specific to the physiological stage of the animal. The present study was planned for studying the sequential changes in early pregnant buffalo sera proteome vis-à-vis day post insemination and non- pregnancy.

MATERIALS AND METHODS

The experimental Murrah buffaloes (second lactation, approximately similar age and bodyweight) were maintained under uniform management conditions at the Central Institute for Research on Buffaloes, Hisar. The females that were reported in estrus through visual observations aided by a teaser bull, were examined for the presence of cyclic ovarian structures with 'B mode' ultrasound scanner equipped with an intra-operative 7.0 MHz micro-convex multi-frequency transducer. Buffaloes (16) that were confirmed in estrus were selected and inseminated artificially using frozen semen from the same fertile bull. The day of estrus / AI was designated as day 0. After insemination, the females were maintained under observation for returns to estrus and scanned ultrasonographically to determine gravid status. Pregnant females were further scanned for monitoring progress of gestation. Six inseminated females, which continued

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pregnancy across to day 42, were grouped as pregnant females for further analysis of samples, and the rest were excluded from the 'pregnant' experimental group. Six normal cyclic buffaloes, which repeated their cyclic estrous activity after 21 days of last estrus with good behavioural manifestation of estrus, were selected as the 'non-pregnant' group animals. Peripheral venous blood samples were collected both from the pregnant and non-pregnant animals at weekly intervals (day 0, 7, 14, 21, 28, 35 and 42 in pregnant and day 0, 7 and 14 in non-pregnant animals).

Blood collection, preservation and pooling: Venous blood sample (6 ml) was collected in serum-separator tube by jugular venipuncture from experimental animals at weekly intervals. Serum was separated by centrifugation at 1,500 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 and stored at –80°C till further analysis. For proteomic analysis pooling of equal volumes of serum from each animal in a group were taken to make a pooled sample for specific stage of pregnancy or non-pregnant group.

Sample preparation: Optimal sample preparation procedures were adopted for complete solubilisation, disaggregation, denaturation and reduction of proteins in a sample. Low abundant proteins were enriched with simultaneous depletion of the high abundant proteins using the large capacity protein enrichment kit following the manufacturer's instruction. Kit was used to remove contaminating substances and improve the 2-D electrophoresis pattern. The protein concentration in all the prepared samples was estimated using kit following manufacturer's protocol.

2D Electrophoresis: IEF was accomplished with isoelectric focussing system in 13 cm, pH 3–10 NL, IPG strips. A ready to use rehydration solution, as per the manufacturer's guidelines, in appropriate amounts samples were mixed with rehydration buffer, to make up the total volume (250 µl) for loading into the IPG strips. Volume and protein quantity loaded per IPG strip were 250 µl and 1,000 µg, respectively. The protein samples, mixed in appropriate amounts of rehydration buffer were loaded into IPG strips (13 cm, pH 3–10NL) and left undisturbed for 16–20 h to complete the rehydration process, passively. Protein load and rehydration volume per strip was kept at 1,000 µg and 250 µl, respectively. IEF was run at a total of 18000Vhs in 4 steps with 500, 800, 11,300 and 5400 Vh in respective steps.

After completion of IEF, gels were stored in 25 × 200 mm screw cap culture tubes at –80°C till the second dimension SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using vertical electrophoresis system as described by Gorg (2004). Briefly, IEF run IPG strips were equilibrated, first with SDS buffer containing DTT (100 mg per 10 ml) and then with SDS buffer containing iodoacetamide (250 mg / 10 ml). The equilibrated IPG strips, washed in sufficient amounts of running buffer solution, were placed on the top of casted trip gels and gently pushed down to the gel surface. SDS PAGE was run under temperature controlled (25°C) in vertical electrophoresis system. A current of 15mA per gel was given for 15 min (pre-run) and then 60mA till dye reached the lower end of plates. Gels stained in commassie blue (R–350, 0.1% working solution, 2 h) were destained (solution of glacial acetic acid, methanol, and distilled water mixed in 1:3:6 ratio, 2–3 h) and washed in ultrapure water for 10–15 min for image acquisition.

Image acquisition, analysis and spot selection: The destained 2-DE gels were washed in ultrapure water for 15 min and then put in fresh ultrapure water. The gels were placed on clean glass surface to capture images using software for image acquisition at 300 dpi resolution. All gel images of different stages of pregnancy and non-pregnancy were brought into image pool and set at a common saliency and calibration level. This along with excluding spots at boundaries of the gel significantly reduced the number of spots being detected by the software. The scanned image was saved in *.TIFF or *.mel format. Saved images were uploaded into the image master 2D platinum software for analysis. These gel images from the image pool were brought into a single match set and different classes for comparisons were made. Subsequently, reference gel image was generated and different gels were compared with reference and between themselves in 14 classes. For convenience this reference has been named as D0 gel. The spots were detected by selecting appropriate values for saliency, smoothness and minimum area. Ratio of % volume was taken as criterion for determining the parameters of the spots in the gel. Subsequently, a pick-list of the spots was prepared in which spots showing a change of < 5 times and / or spots with no match were selected to be picked-up.

Mass spectrometric analysis and protein identification: Picked-up gel spots (as described in part I) were destained and then 200 mM ammonium bicarbonate was added to cover the gel spot for 20 min and then discarded. The spots were then treated with 10mM DTT and 55mM IAA. Subsequently, each picked-up gel spot was cut into small pieces, washed with water, and dehydrated repeatedly with changes of acetonitrile until the gel pieces turned opaque white. These were then dried in a vacuum centrifuge for 30 min. The digestion was performed with 10 ng/mL of trypsin and 50 mM ammonium bicarbonate and incubated overnight at 37°C. Following enzymatic digestion, the resultant peptides were extracted 3 times with 20µl of 1% trifluoroacetic acid in 50% acetonitrile and concentrated in Speedvac. All MS analyses were performed using a spectrometer operated in the positive ion linear and reflector mode with the accelerating voltage of 25 kV (Ion source 1) and 21.85 kV (Ion source 2), respectively. The processing was done at laser wavelength of 337 nm, frequency of 67–100 Hz and percentage set to 25%. Peptide mixtures were analyzed for PMF and MS-MS using a saturated solution of a-cyano-4-hydroxycinnamic acid in 50% acetonitrile / 0.1% trifluoroacetic acid. Peptides were selected in the mass

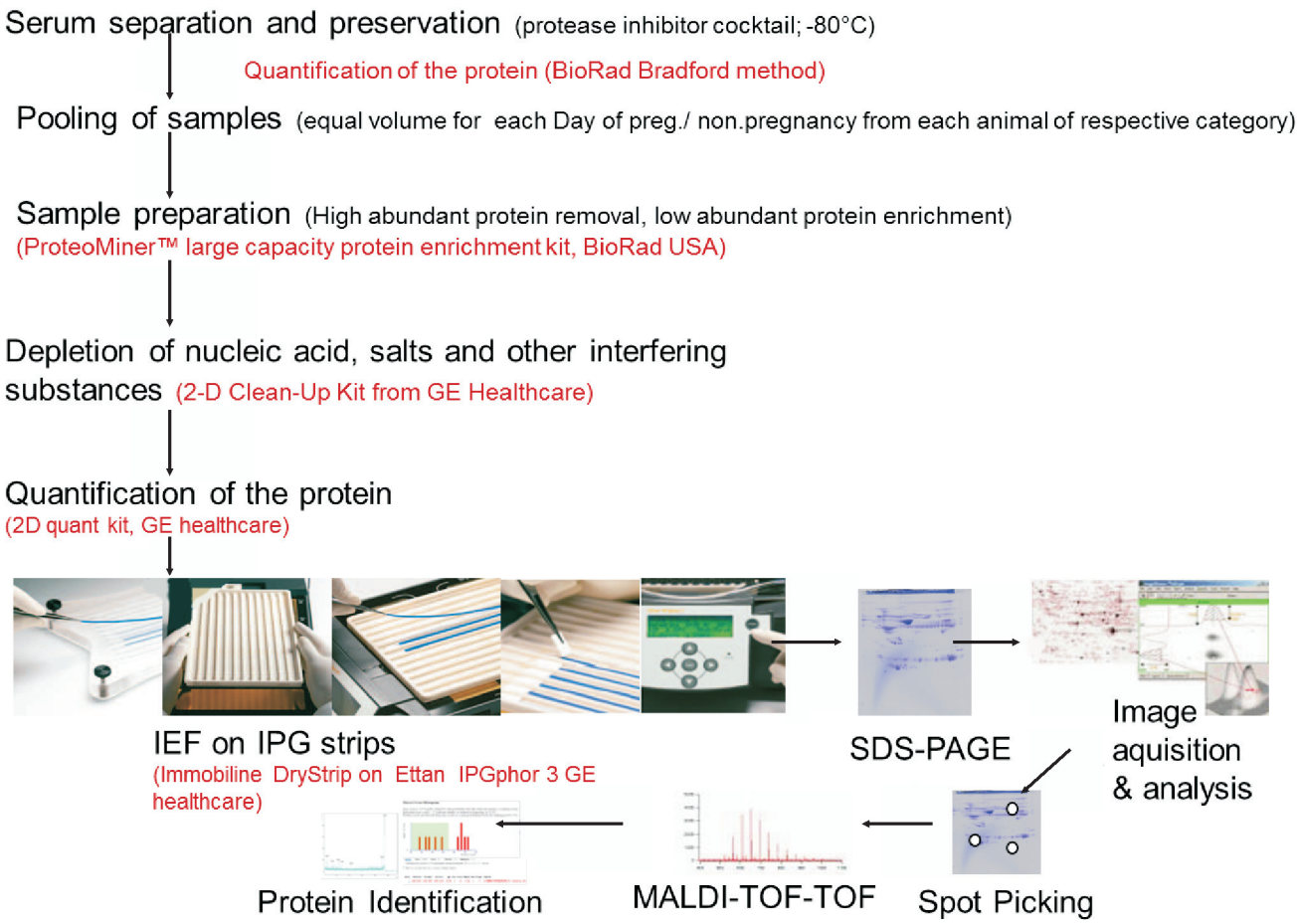

Fig. 1. Work flow: Buffalo sera proteomic analysis.

range of 800±4000Da. Spectra were calibrated using a Pepmix II as external standard.

Peptide masses were searched against SIB based ExPASy and NCBI databases employing Mascot (in-house MASCOT-server) for protein identification. Database searches were performed taking into account carbamidomethyl modification of cysteines and possible oxidation of methionine and allowing one missed cleavage and selecting mammals for species. The mass accuracy required for PMF and MS-MS was basically chosen according to peptide mass-tolerance for peptide mass fingerprint and ion search, respectively, to obtain a significant score ($P<0.05$) of matched peptides to select protein entry. Isoelectric point (PI) and molecular weight (MWt), for whole protein, and not fragment, were calculated on the basis of bioinformatics calculator available at SIB based ExPASy bioinformatics resource portal (http://web.expasy.org/cgi-bin/compute_pi/pi_tool).

RESULTS AND DISCUSSION

Densitometry of the gels and spot picks

All gel images were brought into a single match set and 14 different classes for comparisons between different days of pregnancy and non-pregnancy (Fig 2). Gel image corresponding to day 0 was taken as the reference gel. In 243 match sets 2,924 spots were considered for comparison of the level of protein expression in 9 gels run after critically examining in 3D view using ImageMaster 2D Platinum 7.0 software. A total of 65 spots were selected for the pick list (Table 1), 55 showed more than 5 times change in percent volume with respect to one or more comparing gel of a different day(s) of pregnancy and/or non-pregnancy. Among the spots picked-up on the basis of specific appearance at a particular stage, the maximum 4 spots were unique to day 42 gel, 2 to day 21 and 1 to day 28 gels. Maximum 27 spots were picked from day 0 gels, corresponding to day of estrus / AI and common to both pregnant and non-pregnant buffaloes. This was followed by day 28 of pregnancy with a pick of 15 spots, day 14 and day 42 of pregnancy with 9 spots each, day 21 of pregnancy with 4 spots and day 7 of pregnancy with a single pick. No spot was picked from 2D gels corresponding to day 35 of pregnancy and day 7 and 14 of non-pregnant animals. The picked spots are depicted in Fig. 3 (A-I). Since all the 2D gels were Comassie stained, so it was presumed that each spot contained at least 10 mg protein.

Mass spectrometry and proteins identification

Spectra for all the spots were generated after trypsin digestion using MALDI-TOF-TOF Ultraflex III Mass Spectrometer at laser wavelength of 337 nm and frequency of 67–100 Hz. The spectra so obtained were searched on

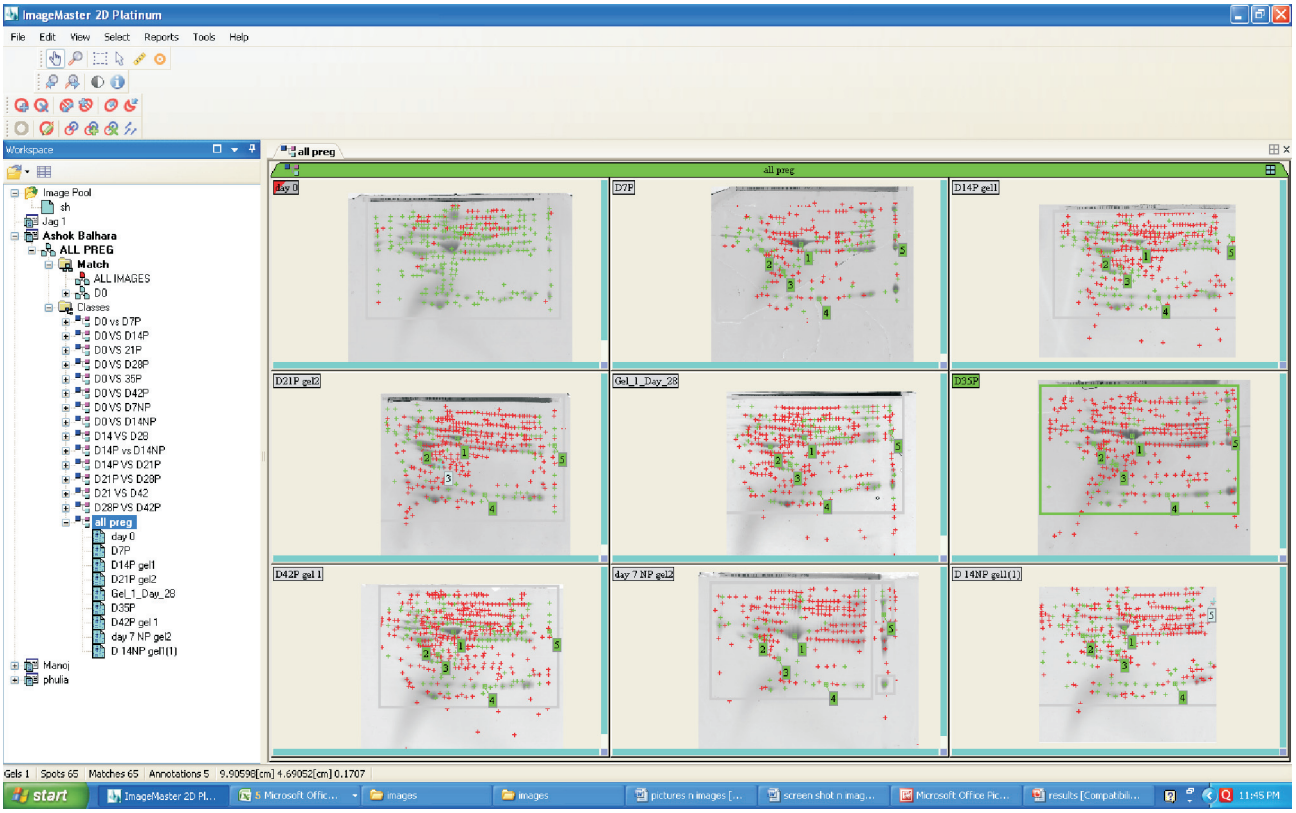

Fig. 2. Screen shot showing image pool, matches, classes and the spots detected after viewing in 3D using ImageMaster 2D Platinum 7.0 software

the Mascot search engine to identify the probable proteins. Of the 65 spots for which MS was done, 26 spots (i.e. 2, 5, 8, 10, 13, 17, 21, 22, 23, 25, 26, 28, 31, 35, 36, 37, 40, 41, 42, 47, 57, 58, 60, 62, 63 and 65) 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. Twenty-two spots (no. 4, 6, 14, 18, 27, 29, 33, 34, 38, 43, 44, 45, 46, 48, 50, 51, 53, 54, 55, 56, 59, 64) were identified with single peptide set beyond the threshold in Muscot score histogram. Four spot with numbers 15, 16, 24 and 52 did not score beyond the threshold and therefore no database match for these spots was reported. Eleven spots (1, 3, 7, 9, 11, 12, 19, 20, 30, 32 and 39) generated spectra which did not match any protein in the available database due to which no score was actually possible. Spot number 49 probably had heavy keratin contamination and therefore could not be processed to any level of MS analysis. This way out of 65 spots only 48 spots could be identified.

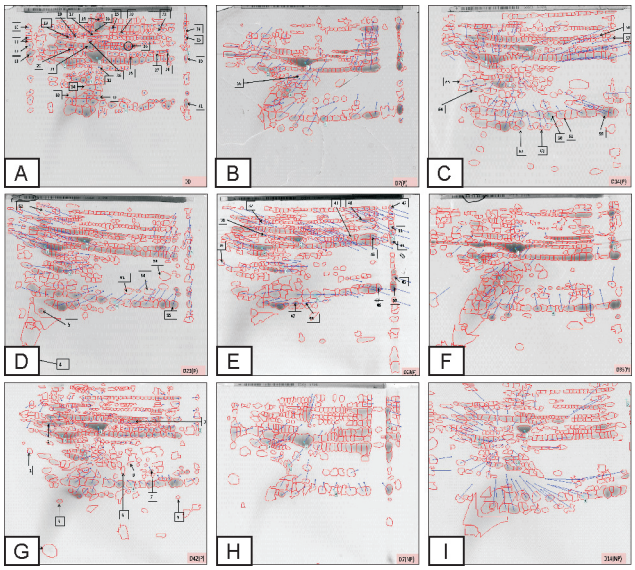

Fig. 3. (A-I) Gel images generated by ImageMaster 2D Platinum 7.0 software showing the assigned spot numbers of spots picked for MS analysis, on different days of pregnancy and non-pregnancy, which showed more than 5 times change in % volume. The numbers 1 to 5 in small boxes are land-marks in all the images. D0, D7P, D14P, D21P, D28P, D35P, D42P, D7 (NP) and D14 (NP) correspond to 2D gel images on day 0 viz. day of estrus / insemination, day 7, 14, 21, 28, 35 and 42 of pregnancy; and day 7 and 14 of non-pregnant cyclic animals, respectively.

Of the 48 (26+22) identified proteins, 38 (approximately 77%) matched *Bos taurus* (bovine) proteins from SIB based ExPASy and NCBI databases (Tables 2 (A) and (B)). Among the remaining 10 proteins identified, protein corresponding to spot number 10 matched with protein of *Monodelphis domestica,* 13 with *Bos indicus,* 23 with *Cervus nippon* (sika deer), 29 with *Bubalus bubalis* (domestic water buffalo), 42 with *Canis famililiaris*, 48 with *Homo sapiens,*56 with *Diceros bicornis (Black rhinoceros)* and 59 with *Crocuta crocuta* (Spotted Hyena). In spots 33 and 43, no database match could be found. The up-/down-regulated proteins were in the pI range of 5.1 to 11.0 and M Wt of 10 to 245

Table 1. List of spots picked for MS analysis

Assigned spot number for MS	Match ID	Spot picked up for MS analysis			Spot to which comparison is made			Change in % volume
		Sample day post AI	Spot ID	% volume	Sample day post AI	Spot ID	% volume	
1	59	D42P	1544	0.615	D0	724	0.098	6.3
2	56	D42P	1542	1.014	D28P	1893	0.090	11.3
3	143	D42P	1537	1.488	D28P	1882	0.285	5.2
4	157	D42P	1722	0.253	D0	675	0.043	5.9
5	Nil	D42P	1762	0.199	-	-	-	-
6	Nil	D42P	1703	0.032	-	-	-	-
7	85	D42P	1702	0.662	D0	2012	0.202	3.3
8	Nil	D42P	1684,	0.075	-	-	-	-
9	Nil	D42P	1751	0.199	-	-	-	-
10	105	D0	580	0.174	D21P	2086	0.022	7.9
11	107	D0	568	0.308	D42P	1430	0.020	15.4
12	99	D0	677	0.484	D21P	2083	0.017	28.5
13	95	D0	723	1.047	D21P, D42P	2080,1532	0.045,0.130	23.3, 8.1
14	175	D0	544	0.107	D42P	1818	0.013	8.2
15	80	D0	595	0.297	D21P	1654	0.056	5.3
16	238	D0	612	0.488	D14P	1466	0.097	5.0
17	162	D0	633	0.691	D14P, D42P	1484, 1458	0.076, 0.084	9.1, 8.2
18	160	D0	645	0.097	D42P	1816	0.007	13.9
19	70	D0	653	0.112	D42P	1460	0.014	8.0
20	61	D0	714	0.371	D42P	1803	0.018	20.6
21	64	D0	700	2.461	D14P, D28P	1551, 1876	0.387, 0.162	6.4, 15.2
22	212	D0	620	0.086	D28P	2870	0.013	6.6
23	79	D0	606	0.089	D42P	1779	0.002	44.5
24	103	D0	601	0.304	D21P	1749	0.055	5.5
25	101	D0	634	0.085	D42P	1473	0.015	5.7
26	144	D0	712	0.451	D28P	1892	0.064	7.0
27	148	D0	722	1.037	D7P	2217	0.578	1.8
28	142	D0	717	0.175	D14P	1837	0.033	5.3
29	135	D0	738	0.105	D42P	1791	0.019	5.5
30	199	D0	746	0.534	D14P	1840	0.076	7.0
31	114	D0	859	1.080	D21P, D42P	2012, 1724	0.214, 0.096	5.0,11.3
32	22	D0	846	0.575	D21P, D42P	1982, 1711	0.083, 0.064	6.9, 9.0
33	178	D0	844	0.574	D14P, D21P	1757, 1971	0.069, 0.074	8.3, 7.8
34	118	D0	826	0.541	D14P, D42P	1729, 1686	0.091, 0.090	5.9, 6.0
35	40	D0	775	0.226	D42P	1617	0.018	12.6
36	159	D0	666	0.126	D42P	1814	0.010	12.6
37	160	D28P	1832	0.096	D42P	1816	0.007	13.7
38	61	D28P	1894	0.288	D42P	1803	0.018	16.0
39	33	D28P	2237	0.105	D42P	1654	0.021	5.0
40	211	D28P	1812	1.122	D0	636	0.216	5.2
41	153	D28P	1845	0.434	D42P, D0	1520, 682	0.060, 0.060	7.2,7.2
42	107	D28P	1750	0.240	D42P	1430	0.020	12.0
43	142	D28P	1895	0.913	D0, D14P	717, 1837	0.175, 0.033	5.2, 27.7
44	199	D28P	1937	0.759	D14P	1840	0.076	10.0
45	196	D28P	2077	0.646	D14P	1845	0.072	9.0
46	115	D28P	2104	0.791	D42P	1708	0.054	14.6
47	195	D28P	2139	0.885	D0	883	0.052	17.0
48	4	D28P	2142	0.114	D0	881	0.022	5.2
49	Nil	D28P	2190	0.037	-	-	-	-
50	114	D28P	2098	2.505	D42P	1724	0.096	26.1
51	158	D28P	1846	0.257	D42P	1506	0.036	7.1
52	171	D21P	1644	0.219	D0	582	0.028	7.8
53	Nil	D21P	2089	0.063	-	-	-	-
54	Nil	D21P	2095	0.076	-	-	-	-
55	115	D21P	2011	1.595	D0	853	0.225	7.1
56	40	D7P	1931	0.270	D7NP	8993	0.038	7.1
57	156	D14P	1538	0.243	D0	676	0.048	5.1
58	157	D14P	1527	0.290	D0	675	0.043	6.7
59	176	D14P	1774	2.271	D0	869	0.234	9.7
60	11	D14P	1784	1.211	D0	858	0.242	5.0
61	87	D14P	1783	0.620	D0	867	0.103	6.0
62	6	D14P	1793	0.722	D0	879	0.078	9.3
63	4	D14P	1806	0.109	D0	881	0.022	5.0
64	27	D14P	1716	0.344	D0	815	0.038	9.1
65	30	D14P	1698	0.477	D28P, D0	2031, 806	0.054, 0.075	8.8, 6.4

Table 2 (A). MALDI-TOF-TOF results with 2 or more peptide match set

Assigned Spot number	m/z ratio	PI,MWt (kDa)	Accession No.	Mascot Score	Most related protein	Related species
2	1480	5.8, 69.3	ABBOS	104	Serum albumin precursor	*Bos taurus*
5	3971	7.4, 147	SYNJI_BOVIN	158	Synaptojanin-1(synaptic insitol-1,4,5-trisphosphate 5-phosphatase)	*Bos taurus*
8	2384	5.9, 139	A 28822	30	1 phosphatidylinisitol- 4,5-biphosphate phosphodiesterase	*Bos taurus*
10	1947	11.0, 245	AAD54482	33	Small nuclear ribonucleoprotein-associated protein B	*Monodelphis domestica*
13	1479	5.8,69	Q3I349_BOSIN	103	Serum Albumin	*Bos indicus*
17	2170	9.1,47	Q32P72_BOVIN	88	Ferrochelatase (Fragment)	*Bos taurus*
21	1633	Not Kown	Q1RMK2_BOVIN	105	Hypothetical protein	*Bos taurus*
22	1685	6.3, 106	AAD 41665	48	Hypothetical protein	*Bos taurus*
23	1313	11.8, 78	Q2TQL0_CERNI	69	MHC Class I antigen (fragment)	*Cervus nippon* (sika deer)
25	1946	6.8, 142	AAA 30436	33	Collagen alpha-1(II) chain	*Bos taurus*
26	2273	6.7, 78	A60166	50	Serotransferrin	*Bos taurus*
28	2166	7.0, 201	AAC 48518	34	Tuberous sclerosis 2 protein (TSC2)	*Bos taurus*
31	1791	9.5, 26	Q2KIV9_BOVIN	39	Complement C1q subcomponent subunit B	*Bos taurus*
35	1567	5.8,69	1::ABBOS	80	Serum albumin precursor or serum albumin (fragment)	*Bos taurus*
36	1221	8.0, 76	Q693V9_BOVIN	59	Complement component C3d (fragment)	*Bos taurus*
37	1865	5.3, 67	2::gi |329663230	42	DNA polymerase alpha catalytic subunit	*Bos taurus*
40	1866	8.0, 76	AAI 12453	114	Complement component C3	*Bos taurus*
41	1855	8.2, 41	1::AAC48761	89	IgG 3 heavy chain constant region (fragment)	*Bos taurus*
42	2318	Not Kown	2::gi |73955386	151	Hypothetical protein similar to Acyl-CoA dehydogenase very long chain specific mitochondrial precursor (VLCAD) isoform	*Canis famililiaris*
47	2877	Not Kown	2::gi |297476818	112	Hypothetical protein similar to lethal giant larvae homolog 1	*Bos taurus*
57	1865	6.4, 187	Q2UVX4	39	Complement C3	*Bos taurus*
58	1865	6.4, 187	Q2UVX4	39	Complement C3	*Bos taurus*
60	1697	6.8, 20	O78058--_BOVIN	27	MHC Class I heavy chain	*Bos taurus*
62	2380	5.7, 30	A56858	149	Apolipoprotein A-I precursor	*Bos taurus*
63	1614	Not Kown	AA112732	46	Hypothetical protein	*Bos taurus*
65	1439	6.0, 60	Q58CV7_BOVIN	24	Hypothetical protein	*Bos taurus*

Table 2 (B). MALDI-TOF-TOF results with a single peptide match set

Assigned Spot number	m/z ratio	PI, MWt (kDa)	Accession No.	Mascot Score	Most related protein	Related species
4	1368	7.8, 11	JC 5734	56	Apolipo-protein A – II	*Bos taurus*
6	1708	5.1, 55	Q6EIZ0	45	Keratin, Type 1 cytoskeletal 10	*Bos taurus*
14	1439	5.8,69	AAI02743	60	Serum albumin precursor	*Bos taurus*
18	1183	7.5, 71	Q2T9P1_BOVIN	45	Protein regulator of cytokinesis 1	*Bos taurus*
27	2570	Not known	AAC 18410	28	Immunoglobulin heavy chain variable region (fragment)	*Bos taurus*
29	1661	8.9, 10	Q599X0_BUBBU	30	Putative glucose transporter isoform 1 (EC 6.3.2.19) (fragment)	*Bubalus bubalis* (Domestic water buffalo)
33	1488	Not known	Not known (gene identifier is 2::gi|126304187)	41	Cannot be retrieved	Not known
34	1479	8.8, 64	Q2TBR1_BOVIN	29	5'-nucleotidase, cytosolic IB	*Bos taurus*
38	1060	5.2, 73	A5D7S4_BOVIN	48	Interleukin – 27 subunit- alpha (IL27RA) protein	*Bos taurus*
43	2166	Not known	Not known (locus I47194)	94	unclassified	Not known
44	2318	6.0, 26	Q1JQ90_BOVIN	86	Glutathione S-transferase	*Bos taurus*
45	2158	4.5, 155	CAA 61769	31	Cyclic nucleotide-gated cation channel beta-1	*Bos taurus*
46	2181	9.0, 44	A40057	81	Transforming growth factor beta-1 precursor (fragment)	*Bos taurus*
48	1898	6.8, 165	Q92626	81	Peroxidasin homolog	*Homo sapiens*
50	2181	5.1, 129	Q9NPG4	125	Protocadherin-12	*Bos taurus*
51	1711	6.42, 27	Q32S21_BOVIN	69	Hydroxysteroid (17-beta) dehydrogenase 8 (FabG-like protein)	*Bos taurus*
53	1615	Not known	Q3MHV6_BOVIN	70	SLAIN motif-containing protein 2	*Bos taurus*
54	1699	9.4, 77	Q2KIA6	68	U4/U6 small nuclear ribonucleoprotein Prp3 protein or PRPF3 protein	*Bos taurus*
55	2279	5.6, 27	Q2TBQ8	110	6-phosphogluconolactonase	*Bos taurus*
56	1327	7.8, 11	Q7YR10_DICBI	40	Apoliloprotein B	*Diceros bicornis* (Black rhinoceros)
59	2454	6.6, 47	Q2MDL7_CROCR	103	Von Willebrand factor (fragment)	*Crocuta crocuta* (Spotted Hyena)
64	1363	8.4, 82	AAI08096	35	Origin recognition complex subunit 3	*Bos taurus*

kDa, covering a wide range of proteins. The Mascot score for the identified proteins varied from a low of 24 for spot number 65 (a hypothetical protein) to a high of 158 for spot number 5, identified as Synaptojanin-1. Table 2 (A) contains MALDI-TOF-TOF results with 2 or more peptide match set. Spot numbers 2, 13 and 35 contain the same protein i.e. serum albumin, either whole or as a fragment or its precursor. Similarly, spot numbers 36, 40, 57 and 58 contained complement component C3 either as whole or a fragment of it. Spots 23 and 60 are reportedly having same polypeptide i.e. MHC Class I with its fragment matching *Cervus nippon* (sika deer) in former and whole molecule of same protein in *Bos taurus* in the later case. The remaining spots did not match with each other and, therefore, were specifically expressed in that particular spot / position only. However, 6 proteins in spots 21, 22, 42, 47, 63 and 65 remained uncharacterized hypothetical proteins.

Table 2 (B) contains MALDI-TOF-TOF results with a single peptide match set. Here the reported results do not match with each other and therefore were specifically expressed in that particular spot only. However, 2 spots, 33 and 43 are completely unclassified and uncharacterized hypothetical proteins with only gene identifier number known for the former and gene locus only for the later.

Proteomics approach for addressing researchable issues in animal production systems is still in its infancy and, therefore, only limited information is available on the bovine proteome in general, and bovine pregnancy, in particular. Serum contains innumerable proteins and the study of serum proteome with differential expression of proteins under various physiological conditions, including pregnancy, is one of the most challenging areas in proteomics. The present study reports proteome analysis of pregnant and non-pregnant buffalo sera for the first time. Due to these limitations and almost nonexistent buffalo protein database, an exact comparison of the results is not possible. Hence, proteins identified in buffalo serum were analysed based on the information available for other mammalian species, especially in relation to pregnancy and early embryo development.

The early embryonic period in cattle has been described to be lasting for approximately 42 days post insemination (Committee on Reproductive Nomenclature, 1972), encompassing a series of events starting with fertilization and culminating in implantation (Table 3). Post-implantation, embryonic losses due to non-infectious causes are rare and the pregnancy becomes more secure (Ayalon 1978, Morris and Diskin 2008).

The events desribed above, if generalized for bovine species including buffaloes, can help in understanding the differential expression of the proteins resulting in varied densitometry for each stage of pregnancy. Development of buffalo embryo during early stages is faster than that of cattle embryo. By day 5.5 to 6, bubaline embryo is an expanded blastocyst that hatches by day 7 post-AI. During development bovine embryo has to undergo transition from total reliance on maternal regulatory machinery to its own genome activation by breaking the developmental block, at around 8 cell stage (Meirelles *et al*. 2004, Telford *et al*. 1990). Though whole mechanism has not yet been fully deciphered, it is assumed that the transition is accompanied by deactivation of maternal mRNA and significant changes in the embryonic protein expression patterns.

Table 3. Embryonic development timeline during early bovine pregnancy

Day of pregnancy	Event
Day 0-1	Fertilization, single-cell embryo (zygote) in oviduct
Day 2	Early cleavages in the oviduct (up to 8 cell stage), activation of embryonic genome
Day 3-4	Embryo enters the uterus
Day 5-6	16-32 cell zona-enclosed embryo progressing into compact morula stage
Day 7-8	Formation of a blastocoele with differentiation of embryonic cells
Day 9-10	Blastocyst expansion and hatching from the zona pellucida
Day 11-15	Blastocyst elongation from tubular to a filamentous structure
Day 14-19	Maternal recognition of pregnancy
Day 19-20	Implantation begins
Day 21	Caruncles–cotyledons appear
Day 22-41	Implantation progresses
Day 42	Implantation completed

Compiled from available information (Morris and Diskin 2008, Hafez 1993).

In the present study, densitometric analysis detected significant differences in the numbers and patterns of spots exhibited in 2D gels on day 0 vis-à-vis subsequent stages of pregnancy, suggesting proteome profile dynamics change with advancing gestation. However, in order to decipher significant differences between different stages of pregnancy / non-pregnancy, the criteria selected was an up- or down-regulation of same protein by at least 5 folds, besides unique protein(s) identified at a specific stage of pregnancy.

From 2D gel corresponding to day 7 of pregnancy, spot number 56 (spot ID 1931) was picked and it was identified as apolipoprotein B (accession number Q7YR10_DICBI, Mascot score 40) from a distant species *Diceros bicornis* (black rhinoceros). No documented evidence for up-regulation of apolipoprotein B in sera of pregnant buffalo is available, though apolipoproteins (apolipoprotein A-1 and E) are present in cattle conceptus fluids (Riding *et al*. 2008) and assigned functions as a transporter of iron and lipids (Roy *et al*. 1994). There are no reports on the presence of these proteins in sera of pregnant cows and their relative expression. The presence of this protein in early buffalo pregnancy indicates increased demand of iron and lipids for the developing embryo in uterine cavity. Presence of another apolipoprotein isomer, apolipoprotein A-II (accession number JC 5734, Mascot score 56), in spot number 4 (ID 1722) of day 42P gel, further supports the

inference that this protein is expressed during early buffalo embryonic development and further increases as pregnancy progresses from day 7 to day 42. Functional analysis of proteins further demonstrates interdependence between the two apolipoprotein isomers. Apolipoprotein B (day 7), which appeared before apolipoprotein A-II (day 42) in the present study, was found to directly regulate expression of later.

By day 14 of pregnancy, hatched embryo is rapidly elongating and signalling for maternal recognition of pregnancy while remaining in intimate communication with the uterine wall. This phase must trigger several metabolic changes in the dam, which was reflected by the present findings of significant changes (<5 times change in% volume) in at least 9 proteins at this stage (numbers 57 to 65). Of these, the identified proteins Complement C3 (spot number 57), Von Willebrand factor (VWF, spot number 59), MHC Class I heavy chain (spot number 60) and apolipoprotein A-I precursor (spot number 62) are documented in other species with some relation suggested in embryo development. Complement molecules, in general, are involved in immune system regulation and animals with low level of complement C3 have less developed immunity (Authie and Pobel 1990). Therefore, complement C3 may facilitate implantation through immunomodulation in the dam. As an established endothelial cell marker, VWF was up-regulated by day 16 of pregnancy in cattle and then down-regulated until day 40, possibly to prepare for maternal recognition of pregnancy (Beindorff *et al*. 2010). A strong functional interaction reportedly exists between complement C3 (C3), Von Willebrand factor (VWF) and apolipoprotein A-I precursor (APOA1). MHC Class I heavy chain molecule is down-regulated in the cattle placenta and up-regulated in binucleate trophoblast cells. The downregulations help in avoiding immunogenic rejection by the mother, while the up-regulation in binucleate cells help in maternal recognition of fetus; exact function, however, is not known (Bainbridge *et al*. 2001). Other spots could not be identified probably due to the absence of suitable database match; however there may be some unique proteins in these spots.

At day 21, the implantation process has already started and metabolic pathways are highly active, leading to abundant changes in the proteome profile. Densitometric analysis supports this assumption, since as many as 28 spots were detected between Day 21 to 42 of pregnancy, which were either unique in a particular stage of pregnancy or showed a significant change in expression level. Spots 53, 54 and 55, identified as SLAIN motif-containing protein 2, U4/U6 small nuclear ribonucleoprotein Prp3 (PRPF3) protein and 6-phosphogluconolactonase, respectively, in gel corresponding to day 21 of pregnancy were all uncharacterized hypothetical proteins with unknown functions. They may, however, be involved in cell differentiation through nucleic acid and protein synthesis pathways.

From gel corresponding to day 28, a total of 15 spots (numbers 37 to 51) were picked for their uniqueness and analysed by MS. Spot number 37, identified as DNA polymerase alpha catalytic subunit, is reportedly required for DNA replication in early mammalian embryogensesis (Uchimura *et al*. 2009), though specific function in early pregnancy and / or embryogensis in bovine has not yet been explored. Spot number 38, identified as interleukin-27 subunit-alpha (IL27RA) protein, has no defined role in bovine (Atsunobu *et al*. 2003). Spot number 40, identified as acyl-CoA synthetase long chain family member 5, is probably involved in increased transportation requirement of the developing embryo due to presence of acyl-CoA-binding protein in cattle placental tissue (Kim *et al*. 2010). It also blocks cell proliferation machinery to function as an up-regulator of genes involved in lactation (Finucane *et al*. 2008). Spot number 41, identified as IgG_3 heavy chain molecules, were present in cattle conceptus fluids where they help in antigen binding for development of immune system (Riding *et al*. 2008). The heightened level of enzyme glutathione S-transferase (GST), spot number 44 (accession number Q1JQ90_BOVIN), is perhaps manifestation of the antioxidant system activation. Glutathione reductase was reported in bovine placenta (Kim *et al*. 2010) where it contributes to oxidation-reduction processes of the antioxidant mechanism. GST supergene family members encode for iso-enzymes responsible for developing resistance against oxidative stress (Hayes and Strange 1995). The role of the enzyme GST is not clearly defined in early pregnancy in bovine species, although it is well documented that oxidative stress affects both implantation and early embryo development in human (Agarwal *et al*. 2006). Cyclic nucleotide-gated cation channel beta-1 (spot number 45), transforming growth factor beta-1 precursor (spot number 46), peroxidasin homolog (spot number 48), protocadherin-12 (spot number 50) and hydroxysteroid (17-beta) dehydrogenase 8 (FabG-like protein, spot number 51) were other proteins identified from day 28 pregnancy serum, but the functions of these are not known. Most of these appear to be growth factors required for embryo development and continuation of pregnancy, though the supporting evidence from literature is not available.

Gel corresponding to day 42 of pregnancy, when implantation should have been completed, offers most interesting profile of the proteins. Four proteins, apolipoprotein A-II, synaptojanin-1 (synaptic insitol-1, 4, 5–trisphosphate 5 phosphatase 1 p150), keratin type 1 cytoskeletal 10 and 1 phosphatidylinisitol-4, 5 biphosphate phosphodiesterase were identified in spot numbers 4, 5, 6 and 8, respectively. These spots became more visible after day 28 of buffalo pregnancy, as evident from densitometric analysis carried out in the present study. As reported earlier, apolipoprotein isoforms are reportedly present in the bovine conceptus fluids with binding and transporter functions assigned to them. Synaptojanin-1 is an uncharacterized molecule in bovine species but proteomic studies in human cases have proved that this protein has a definite role in central nervous system development especially

astrogliogenesis during embryonic development and a down-regulation is associated with Down's syndrome (Herrera *et al*. 2009). 1 phosphatidylinisitol-4, 5-biphosphate phosphodiesterase was proposed to have role in regulating life-span and function of mammalian CL (Niswender *et al*. 2000). Protein keratin, type 1 cytoskeletal 10 being a structural constituent of cytoskeleton, is an established indicator of embryonic development and was also reported in the bovine conceptus fluid proteome (Riding *et al*. 2008).

To conclude, proteomics offer great opportunity in investigating the changes in proteome profile associated with early pregnancy, as can be seen from the presence of at least 65 spots showing up-regulation, down-regulation or specific appearance at some specific stage of early buffalo pregnancy. Some of these identified spots seem to be promising pregnancy bio-markers, especially synaptojanin-1, apolipoprotein A-1, apolipoprotein B, keratin 10 and Von Willebrand factors, which are documented proteins having role in embryogenesis and early pregnancy. Limitation commonly encountered in proteomic analysis of complex protein samples like serum, is the abundance of a few proteins (about 20 proteins constitute 99% of the total protein mass in serum), which masks presence of the remaining low abundant proteins, though they could be the most potent candidates for biomarker discovery. When high abundant proteins are depleted in a biological sample, many low abundant proteins are also lost simultaneously. Present instrumentation, especially IEF and MS, require high purity protein samples, for which sample has to pass through multi-step biochemical procedures, increasing the probability that the protein of our interest may be lost midway. Further, another important limitation at this point of time is the non-availability of a large database on the livestock in general, and buffalo in particular. Considering that proteomics in animal research is still in its infancy, it is a long way before practical applications of such experiments could actually be realized.

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