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Detection of pregnancy associated protein(s) in the sera of pregnant buffaloes

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ABSTRACT

In the present study, attempt has been made to use enzyme linked immuno sorbent assay (ELISA) for the detection of pregnancy-associated proteins in the sera of pregnant buffaloes. The blood samples from pregnant (n=25, 30 days post insemination till term) and non-pregnant buffaloes (n=10) were collected by jugular vein puncture and indirect plate test and dot ELISA was performed with standard protocol using hyper immune sera raised against purified fractions of reproductive fluid from pregnant genital tracts and placental extracts as a primary antibody solution. The optical density in indirect plate ELISA ranged from 0.515 to 0.878 (Mean 0.717), 0.327 to 0.710 (Mean 0.562) for pregnant samples and from 0.251 to 0.469 (Mean 0.338), 0.209 to 0.350 (Mean 0.267) for non-pregnant sample using the anti- purified fraction of reproductive fluid and placental extract sera separately. Indirect plate ELISA revealed higher OD values for pregnant sera samples indicating presence of some proteins associated with pregnancy in the sera samples. In dot ELISA, out of 25 samples from pregnant buffaloes, positive color reaction was observed in 20 (80%) and 17(68%) sera samples using anti-purified fraction of reproductive fluid and placental extract sera separately, however, no color development was observed in 9(n=10, 90%) non pregnant samples. It appears that pregnancy associated proteins can be detected in the sera of pregnant buffaloes further purification of these proteins may enhance the accuracy.

Key words: Pregnancy associated proteins, serum, buffalo, ELISA

An early and accurate diagnosis of pregnancy in farm animals is of considerable economic value. The per-rectal examination of female genital tract, hormonal assays and ultrasonography are the available methods for pregnancy diagnosis in cattle and buffaloes (Noakes, 1985; Prakash and Madan, 1993; Pawshe et al., 1994), however, every method has its own limitations. The diagnosis of pregnancy through conceptus-based substances may be considered as precise and reliable marker of pregnancy than that of maternal origin (Butler *et al.*, 1982; Zoli *et al.*, 1991; Iniguez *et al.*, 1995). The detection of proteins of feto-placental origin in the maternal sera is being used for pregnancy diagnosis in cattle and for the development of specific radio immuno assay (RIA) or enzyme immuno assay (EIA) (Sasser et al., 1986; Threlfall, 1992; Iniguez et al., 1995; Szenci et al., 1998). The accuracy of pregnancy diagnosis by determination of pregnancy specific protein (PSP-B) using RIA in cattle has been reported to be more than 90% at 30 days of gestation (Humblot et al., 1989). Pregnancy associated protein(s) has not been characterized in buffaloes and as such no information is available on the detection of pregnancy using conceptus based substances. In the present study, attempt has been made to use ELISA for the detection of pregnancy-associated proteins in the sera of pregnant buffaloes.

MATERIALS AND METHODS

Collection of sample : The blood samples from pregnant (n=25,30 days post insemination till term) and non-pregnant buffaloes(n=10) were collected by jugular vein puncture. The sera were separated by centrifugation (1500 x g, 4°C, 30 min), aliquoted and stored at -20°C until assayed. The pregnancy in buffaloes studied was confirmed by rectal palpation 60 days post- insemination.

Enzyme Linked immuno sorbent assay (ELISA) : The indirect plate test and dot ELISA was used for the detection of pregnancy associated proteins in test sera as per the protocols of Hudson and Hay (1989) and Tripathi et al. (2001) with some modification. The pregnancy associated proteins in buffaloes were identified and purified from reproductive fluid of pregnant genital tracts and placental extract separately and hyper immune sera raised against purified fractions was used as primary antibody in the present study (Singh et al., 2000, 2001).

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Indirect plate ELISA : The antigen (pregnant and non pregnant sera) at a suitable dilution in coating buffer (pH 9.4, 4mg/well) was coated on to 96 well microtitre plate (Nunc) and incubated in a humid chamber overnight at 4°C. The wells were filled with skimmed milk (5%) to block any remaining protein binding sites at 37°C for 2h. The plates were washed thrice with phosphate buffer saline (pH-7.2) containing 0.05% Tween-20 (PBS-T) after each step. Serial 2-fold dilutions of both primary antibody solution was added separately and allowed to react at 37°C for 2h. Goat anti-rabbit IgG-HRPO conjugate (NII, India) was used at a dilution rate of 1:10,000 and plates were again incubated at 37°C for 1h. The OPD (0.5mg/ml of citrate buffer, pH-5.4) was used as substrate and the enzymatic reaction was stopped by adding 5N H2 SO4 and OD was measured at 492 nm in an ELISA reader.

Dot-ELISA: The dipsticks covered with nitro-cellulose pieces (0.5x 0.5cm) of 45nm pore size bound to one end of the plastic strips (7x0.5cm) were prepared. Approximately 1ml antigen (2mg protein of pregnant as well as non-pregnant sera) was coated in the center of nitro-cellulose pieces and kept at room temperature till dried. The unsaturated sites were blocked by skimmed milk (4%) at 37°C for 2h, washed thrice with PBS-T. The strips were allowed to react separately with diluted primary antibody solution (1:200) and incubated further with goat anti rabbit IgG HRPO conjugate (1:500 in PBS) and rewashed with PBS-T. Finally, the strips were immersed in freshly prepared substrate solution (diaminobenzidine- 0.5gm, PBS- 10 ml, 30% H_2O_2-10ml). The enzymatic reaction was stopped by dipping strips in distilled water. The appearance of brown dot against a white background was indicative of positive reaction.

RESULTS AND DISCUSSION

The indirect plate test and dot ELISA were used for the detection of pregnancy-associated protein(s)in the sera of pregnant buffaloes. Chequer board analysis revealed 40 mg/ml proteins in test sera as an antigen for indirect plate ELISA. End point titre of positive sera was determined by the 2- fold dilution method. A titre of 1:3200 was considered positive. The optical density in plate ELISA ranged from 0.515 to 0.878 (Mean-0.717) and from 0.251 to 0.469 (Mean-0.338) for pregnant and non-pregnant buffalo sera samples respectively, using hyper immune sera raised against purified fractions of reproductive fluids from pregnant genital tracts. However, OD ranged from 0.327 to 0.710 (Mean 0.562) for pregnant and 0.209 to 0.350 (Mean 0.267) for non-pregnant buffaloes using hyper immune sera raised against purified fraction of placental extract (Table 1 and 2). Results of indirect plate ELISA revealed higher OD values for pregnant samples than the non-pregnant one, using both primary antibody solutions, suggestive of presence of some proteins associated with pregnancy in the samples. Dot ELISA results yielded positive color reaction in 20 (80%) and 17 (68%) pregnant sera samples using hyper immune sera raised against purified fractions of genital tracts and placental extracts, respectively. No color development was observed in 9 (90%) non-pregnant samples using both antibody solution (Table3, Fig 1 & 2). The earliest detection of pregnancy-associated proteins was possible at 30-40 days of gestation, which is still comparatively earlier than the conventional methods of pregnancy diagnosis i.e. per-rectal examination of genital tract. The results of the present study are supported by the findings of Sasser et al (1986), Zoli et al. (1991), Klima et al. (1992), Threlfall (1992) and Iniguez et al. (1995), who could also detect pregnancy associated proteins isolated either from placental tissue, pregnant sera or uterine/oviduct flushing in the maternal circulation of cattle earlier than the conventional method by RIA or EIA. In the present study, the pregnancy-associated proteins were detected in good number of buffalo sera samples. The accuracy may be increased by further purification of these pregnancy associated proteins and raising their specific hyper

Table 1. Mean O.D. value at 492 nm of pregnant and non-pregnant test samples using rabbit anti purified fractions from pregnant reproductive fluid sera

Sl. No.	Pregnant	Non-pregnant
1.	0.793	0.302
2.	0.721	0.469
3.	0.725	0.345
4.	0.603	0.370
5.	0.870	0.367
6.	0.878	0.360
7.	0.850	0.287
8.	0.660	0.251
9.	0.680	0.352
10.	0.750	0.285
11.	0.732	-
12.	0.715	-
13.	0.625	-
14.	0.515	-
15.	0.793	-
16.	0.716	-
17.	0.770	-
18.	0.620	-
19.	0.603	-
20.	0.553	-
21.	0.706	-
22.	0.800	-
23.	0.806	-
24.	0.700	-
25.	0.753	-
Mean±SE	0.717±0.094	0.338±0.061

Table2. Mean O.D. value at 492 nm of pregnant and non-pregnant test samples using rabbit anti purified fractions from placental tract sera.

Sl. No.	Pregnant	Non-pregnant
1.	0.628	0.320
2.	0.650	0.317
3.	0.575	0.216
4.	0.525	0.350
5.	0.693	0.260
6.	0.643	0.275
7.	0.710	0.258
8.	0.565	0.226
9.	0.587	0.240
10.	0.519	0.209
11.	0.445	-
12.	0.600	-
13.	0.327	-
14.	0.475	-
15.	0.570	-
16.	0.630	-
17.	0.535	-
18.	0.457	-
19.	0.444	-
20.	0.439	-
21.	0.509	-
22.	0.709	-
23.	0.609	-
24.	0.609	-
25.	0.600	-
Mean± SE	0.562±0.095	0.267±0.048

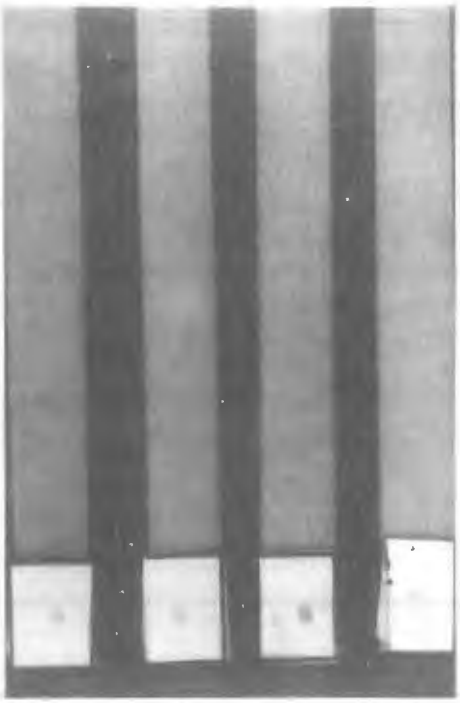

Fig.1 Photograph showing positive color reaction in dot-ELISA

Table 3. Dot ELISA results

	Pregnant buffaloes		Non-pregnant buffaloes (n=10)
	I (First 3 months)	II (4 months till term)	III
Anti-purified from reproductive fluids sera	8	12	9
Anti-purified fractions from placental extract sera	5	12	9

Fig.2 Photograph showing negative color reaction in dot-ELISA

immune serum. It is concluded that in buffaloes there are certain proteins, which are associated with pregnancy and can be detected in peripheral circulation. In future, pregnancy associated proteins may prove as a potential tool for the reduction of calving interval by providing a rapid method of pregnancy diagnosis at an early stages in buffaloes.

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