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Development of Dot-ELISA Technique for Estimation of Milk Progesterone and Pregnancy Diagnosis using PVDF Membrane

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ABSTRACT

An analytical hormone separation method and dot enzyme linked immunosorbant assay (dot-ELISA) for quantitation of hormone are described. For extraction of progesterone from milk chloroform methanol mixture was used. For Dot ELISA, conjugate was prepared by immunoglobulin isolated from whole serum by ammonium sulphate precipitation method and conjugated with Horse radish Peroxidase by modified gluteraldehyde procedure. For immobilization of progesterone to PVDF membrane a number of solvents were tried *viz.,* Methanol, Methanol + Carbonate-bicarbonate buffer, Methanol + PBS, Ethanol and 70 per cent isopropanol. On observation following Dot ELISA procedure, 70 per cent isopropanol was selected as best. Dot ELISA of serially diluted progesterone was performed to get the best titre of conjugate selected for further studies. For standard colour of dot with different progesterone concentration, Dot ELISA with varying concentration of progesterone was performed. The new technique was thus developed for estimation of progesterone using PVDF membrane and 70 per cent isopropanol as solvent. The developed Dot ELISA was also used for estimation of progesterone and pregnancy diagnosis from cattle milk sample and technique was validated. This techniques may be useful for estimation of other steroid hormones.

Key Words:- Anti progesterone antibody, Progesterone, ELISA, Isopropanol, PVDF, PBS

INTRODUCTION

Sterols are hydrophobic compounds which are practically insoluble in aqueous media and poorly adsorb to polystyrene ELISA plates. Determination of the existence, specificity, and quantity of Ab to sterols, such as progesterone, requires a sensitive and reproducible technique for measuring anti-sterol Ab that accommodates the specific hydrophobic properties of sterols. In this paper we describe a dot ELISA technique for both qualitative and quantitative assessment of progesterone using anti progesterone-Ab, its quantitation and utilization for pregnancy determination.

Dot ELISA performed on sheets of nitrocellulose or polyvinylidene are comparatively

simpler to perform, gave results quickly, could be assessed qualitatively and requires less knowledge of technical skills as compared to above all techniques.

In the current scenario for higher milk production in the world the accurate and earliest pregnancy diagnosis plays a vital role for an efficient cattle breeding programme. Increased calving interval leads to serious financial loss in form of low milk yield over whole life of cattle and less no of calves produced, hence, it became very essential to diagnose non pregnant animals as early as possible so that profitable decision could be made about rebreeding / treatment or culling animal. Since the knowledge of the fact that progesterone level in blood or milk reflects the ovarian activity during oestrous cycle or pregnancy (Donaldson *et al, 1970*) its detection around 23-25d after insemination has been extensively used for pregnancy/non pregnancy detection (Heap *et al,1976*). The present study was therefore, conducted to monitor the progesterone level in serum and milk by EIA (HRP enzyme) method at various stages of oestrous cycle (0, 12 and 21 day post A.I. / service). Besides these the

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earliest pregnancy diagnosis at farmer's level/field condition is important in economic development of farmers and as such the work on development of Dot ELISA for early pregnancy diagnosis had been taken that are cheaper (Svobodova *et al,* 2013) requires less technical knowledge and that could be used for development of various kits for on-the-spot estimation of results that appears in form of coloured dot, thus visual estimation of the result could be done under field condition.

MATERIALS AND METHODS

Milk sample

Milk and blood samples were collected from 8 normal cycling healthy cattle during four stages of reproduction *viz.,* 0 day (day of oestrus), 12th day (diestrus) and 21st day after A.I. / natural service (Table 1). All the animals were checked for different stages of oestrous cycle and confirmation of pregnancy was done by per rectal examination (45-60 days after A.I.).

Blood sample

Blood was collected at 8.00 a.m.in sterilized test tube by jugular vein puncture with the help of sterilized needle and it was kept at room temperature in slanting position till serum oozes out. It was then centrifuged at 3000 rpm for 10 minutes to separate the serum. The sera samples were stored at – 20^0 C in sterilized glass stoppered vials. All the animals were watched for any irregularity, repeat breeding or abortion.

Isolation of Immunoglobulin from serum

Immunoglobulin was precipitated from lyophilized whole serum ((Anti progesterone whole serum, sigma, USA) by reconstituting in 1ml distilled water. This was then precipitated with equal volume of saturated ammonium sulphate solution by adding drop by drop with gentle stirring. The precipitate was kept overnight at 4^0C and was centrifuged at 3000 rpm for 30 min at 4^0C. The precipitate containing globulin was resuspended in distilled water to restore 1/3rd of the original serum volume and was reprecipitated as above. The globulin containing precipitate was dialyzed at 4^0C in carbonate bicarbonate buffer pH 6.8 till free from ammonium sulphate as tested by Nessler's reagent (Glaxo, India). Protein estimation of the dialysate was done by the technique of Lowery *et al* (1951). The dialysate was then stored at -20^0C till further analysis.

Conjugation of immunoglobulin with enzyme Horse-radish-peroxidase

Conjugation of immunoglobulin with Horse radish peroxidase (Sigma, U.S.A.) was done by modified gluteraldehyde procedure (Avrameas and Ternynck, 1971 and Prasad, 1983). Horse radish peroxidase (2 mg in 0.095 ml of 0.1 M phosphate buffer pH 6.8) was mixed with 0.005 ml of 25 percent gluteraldehyde and incubated at room temperature for 18 hours. The mixture was dialyzed against one liter of 0.05 M carbonate bicarbonate buffer pH - 9.5 at 4^0C containing 0.1 M NaCl. The dialyzed peroxidase was mixed with 4 mg of anti IgG. It was kept at 4^0C for 24 hours and mixed frequently. The L-lysine was added at the rate of 5mg/ml of HRP immunoglobulin conjugate solution and left for 2 hours at 4^0C. It was dialyzed against 0.01 M

Table 1. Sample collection schedule from cattle during different stages of oestrus cycle.

Sr. No.	Sample	Species	Total Number of Animals	Stages of Oestrus Cycle			
				On the day of Oestrus (0 day)	During luteal phase (day 12)	On day 21 after A.I./service	Pregnancy diagnosis (Day 45)
1.	Milk	Cattle (CB)	8	8	8	5	5
2.	Serum	Cattle (CB)	8	8	8	5	5

phosphate buffer saline (PBS); pH 7.2 with several changes. The volume of dialysate was measured and conjugate was precipitated with equal volume of saturated chilled ammonium sulphate solution at 4^0C. The precipitate was dialyzed against PBS pH 7.2 until the solution was free from ammonium sulphate. The conjugate was stored at 4^0C in amber coloured bottle after addition of bovine serum albumin (BSA) at the rate of 5 %.

Preparation of standard progesterone for plate ELISA

Five milligram of progesterone (Sigma U.S.A.) was dissolved in 50 ml methanol (Glaxo India). It was allowed to equilibrate overnight (stock standard progesterone). Final working standard progesterone solution was prepared containing 1, 5, 10, 20, 30 and 40 ng/ml in methanol.

Assay procedure

Estimation of progesterone from serum and milk was done by plate ELISA as per the method described by Akhtar (1988).

Charging of microtitre plate

Sample comprising 100 - 500 µl of serum or 25-100 µl of milk was extracted using 2 – 10 ml and 0.5 -2 ml of petroleum ether (Glaxo, India), respectively and 0.25 ml of distilled water was added in each test tube. It was mixed thoroughly for 30 seconds. The tube was frozen and ether layer was transferred to another tube and was dried at 45^0C and 0.1 ml of methanol was added in each tube and mixed well. It was transferred to microtitre plate and was air dried. Following this, 100 µl of 0.1 M carbonate-bicarbonate buffer pH 9.6 (coating buffer) was pipetted into each well. The micro titre plate was sealed / covered and kept for 3 hours / overnight at 37^0C in humid chamber.

Immunoassay of progesterone using HRP enzyme conjugate

Previously charged microplate was washed 3 times with 0.1 M phosphate buffer, pH 7.4 containing 0.05% tween 20. The plate was shaken, dried and 100µl anti progesterone conjugate (1:50 dilution with 0.1 M phosphate buffer pH 7.2 with BSA 1%) was added into each well and kept for 6 hours at room temperature (37^0C) in humid chamber. The washing was repeated thrice and subsequently 100µl of freshly prepared substrate (o-phenylenediamine, Sigma U.S.A.) 30 mg and hydrogen peroxide 20 µl, 30% W/V were dissolved in 100 ml 0.2M phosphate buffer pH 6.0) was added and the plate was incubated for one hour at 37^0C. The reaction was stopped by adding 2N H_2SO_4 and reading was recorded colorimetrically at 490 nm (680 micro plate reader, Bio-Rad laboratories).

Preparation of standard curve

Standard curve for each blood and serum immunoassay was prepared. For this 100 µl of standard progesterone of different concentration (5, 10, 20, 40 and 50ng) was poured in microtitre well in duplicate. The well was air dried and 100µl of methanol was added in each well and again air dried. Following this 100 µl of 0.1 M carbonate-bicarbonate buffer pH 9.6 was added to each well. The microplate was sealed and incubated at 37^0C in humid chamber and further the procedure was followed as described earlier. Standard curve was prepared using the optical density at different progesterone concentration.

Development of Dot ELISA technique for progesterone estimation and pregnancy diagnosis

Selection of proper solvent for binding of progesterone to PVDF (Poly vinylidene difluoride) membrane

Experiment was carried out (Table - 3) in different solvent system with respect to its spread on PVDF (pore size 0.2µm, Whatman International U.K.) membrane and development of colour following the dot-ELISA. The different solvents tried were 1 (Methanol), 2 (Methanol + 0.1 M Carbonate-bicarbonate buffer, pH 9.6), 3 (Methanol + 0.1 M PBS, pH 9.6), 4 (Ethanol) and 5 (70 % isopropanol). Based on the trial, 70 % isopropanol

was selected as best as compared to other solvents for dissolving progesterone and binding.

Selection of extraction method for progesterone from milk

The different methods tried was 1 (Ether extraction, 2 ml ether + 1 ml milk + 0.25 ml DW), 2 (Chloroform only, 2 ml chloroform + 1 ml milk + 0.25 ml DW), 3 (Isopropanol only, 2 ml isopropanol + 1 ml milk + 0.25 ml DW), 4 (Chloroform:methanol, 2 ml 2:1 chloroform methanol + 1 ml milk + 0.25 ml DW) and 5 (Chloroform:methanol, Folch *et al* 1957 method) and result were recorded. As per observation two solvent systems were selected.

Extraction of progesterone from milk by ether for Dot-ELISA (Prasad 1983 and Akhtar 1988)

Two milliliter (2 ml) of ether was mixed with 1 ml milk and 0.25 ml of water. It was mixed vigoursly for 30 seconds and left for freezing under deep freeze for 2-3 hr. There after the top layer was decanted / removed in a collecting tube and was air dried / dried in vacuum. The left over substances was reconstituted in 10-15 µl 70 % isopropanol and transferred on PVDF membrane on encircled wells using micropipette slowly by repeated multiple dotting / charging, so that earlier dotted spots got dried up by evaporation of solvents. The result of transferred extract was studied following Dot ELISA procedure.

Extraction of progesterone for Dot-ELISA from milk by Folch *et al* (1957) method

To 19 ml of 2:1 Chloroform methanol (both Glaxo, India) mixture, 1 ml of milk was mixed in a conical glass stoppered flask, then shaken vigorously to dissolve all the lipid substances and breakdown of proteolipids and filtered through filter paper to remove non lipid impurities and left for few minutes for equilibration, then layered with 4 ml of normal saline (0.9 % NaCl). The mixture was again mixed several times by gentle inversion and transferred into a separating funnel and kept overnight for separation of Chloroform and watery layer. Lower chloroform layer was collected carefully in a conical flask and dried in vacuum at 45^0-50^0 C. The content of the flask was then collected with 1 ml of 70% isopropanol by repeated rinsing in a collecting tube (conical bottom). The isopropanol was again evaporated to dryness in vacuum at 45^0-50^0 C and the content was then transferred to PVDF membrane using 5 – 15 µl 70 % isopropanol by multiple dotting as described earlier.

Preparation of standard progesterone solution for Dot ELISA

Standard progesterone (0.001 gm) was mixed in 10 ml of 70% isopropanol to prepare stock progesterone solution. Twenty micro liter (20 µl) of stock progesterone solution was mixed in 1 ml of 70% isopropanol to prepare the working progesterone solution. Working progesterone solution (10 µl) was then further diluted with 990, 490, 240, 190, 90, 40 & 15 µl of 70% isopropanol to get required concentration of 5, 10, 20, 30, 50, 100 & 200ng progesterone in each 5 µl of final solution respectively. The 5 µl of final dilution as described above was used for transferring the different (5, 10, 20, 30, 50, 100 & 200ng) concentration of standard progesterone on to PVDF membrane. Colour intensity of the respective dots was studied further following Dot ELISA procedure.

Dilution (titre) of HRP antiprogesterone conjugate for Dot ELISA

Conjugate (10 µl) was diluted in 190 µl of 0.1 M PBS pH 7.5 to obtain 1:20 times dilution. Further, 10 µl of 1:20 diluted conjugate was diluted with 15, 40, 90, 240, 390 and 490 µl of 0.1 M PBS pH 7.5 buffer to get 1:50, 1:100, 1:200, 1:500, 1:800 and 1:1000 times dilution respectively. The diluted conjugate was used in Dot ELISA to select the best titer using 20ng standard progesterone solution. As per the intensity of colour, the best titre was selected.

Development of Dot ELISA procedure for progesterone estimation

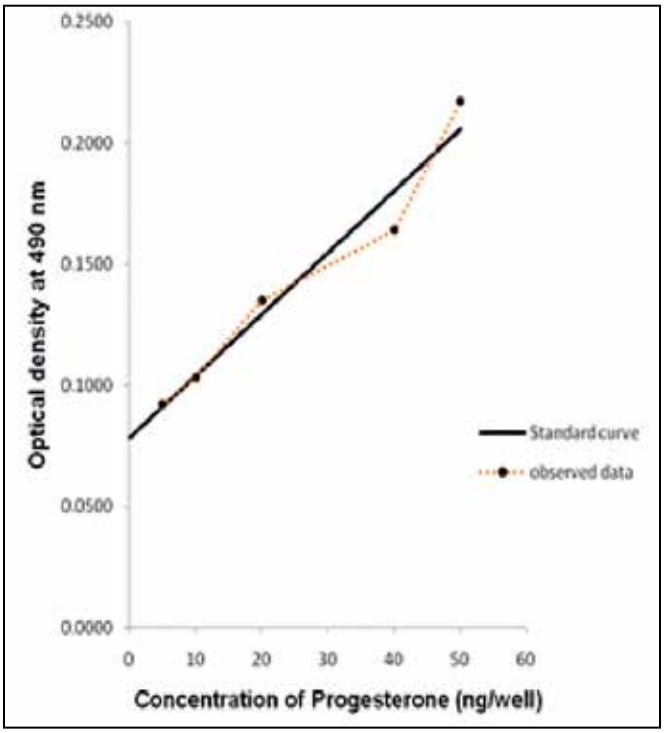
PVDF membrane was cut into ribbon shape and was mounted on a clean grease free glass slide (Fig.

4), to provide it solid support and was fixed on both top and bottom of slide using non reactive rubber based adhesive (Dendrite, Chandra's Chemicals Enterprises (P) Ltd, Kolkata, India) so as to prevent its displacement. Then required number of encircled area (well) was drawn with lead pencil. Each well was then charged with 5 µl each of 70% isopropanol in blank, standard progesterone solution in standard well and extracted progesterone in test wells. The dotting of samples was done slowly by several charging and using small droplets so as to prevent its spread out of well before drying. After complete charging, the strip was left for complete evaporation of isopropanol. Then rest sites around well was blocked with 5 percent BSA (Bovine serum albumin, Sigma, U.S.A.) in 0.1 M PBS pH 7.5 buffer (Intapan *et al 2003)*. The strip was then incubation for 1 hour at room temperature (37^0C) under humid condition. Subsequently 3 washing was given with washing buffer 0.1 M PBS pH 7.5 containing 0.005 percent tween 20 and 1 washing with 0.1 M PBS pH 7.5 buffer. Again, it was left in air till near dryness to remove excess of fluid. Then, 5µl of 1:50 diluted HRP conjugate was added on each well and left for incubation for 2 hr at room temperature, subsequently washing was repeated as above and excess fluid was removed in air till near dryness. Then 5 µl of substrate (0.001 gm diaminobenzedine (Sigma USA) in 10 ml of 0.1 M PBS pH 7.5 and 50 µl of 38 % H_2O_2) was added on each encircled well. This was then left for incubation at room temperature under humid condition till development of colour. Colour starts developing within 2-3 minutes and reached to its maximum intensity up to 15 min, thereafter no much improvement was found. Reaction was stopped after 30 min by washing with distilled water. The results appeared in form of brown dots and intensity of colour was an indicative of concentration of progesterone in the sample, which was compared with dot colour produced by the standard progesterone solution for estimate on the pregnancy diagnosis. Depending upon the intensity of colour of dots + (positive) grades was awarded and result was recorded.

RESULTS AND DISCUSSION

Standard curve for Plate Elisa

The standard curve was drawn with varying concentration of standard progesterone using HRP anti-progesterone conjugate following plate ELISA technique (Akhtar, 1988). The standard curve was drawn using optical density against known concentration of progesterone (Fig.1) The curve appeared almost straight line. While using HRP conjugate, a great precaution was taken as Horse radish peroxidase is a light sensitive enzyme. However, alkaline phosphatase has also been used as label with comparable sensitivity (Stanley *et al,* 1985 and Sauer *et al,* 1986). The quantitation of progesterone upto the level of 4-8 pg/tube obtained by various workers (Arnstadt *et al* 1981, Van-De-Wiel and Koops 1982, Munro and Stabenfeldt, 1984). They reported the use of HRP like our present findings which was found sensitivity of technique comparable to that of RIA.



Progesterone concentration in milk of pregnant cattle

Laing and Heap (1971) were the first to report that progesterone levels in milk of cow reflected the stages of oestrous cycle. On comparison with the standard curve the concentration of milk progesterone was found to be 19.99 ± 0.36 ng/ml as an indicative of pregnancy. While on 0 day and on 12th day it was found to be 2.045 ± 0.11 and 15.02 ± 0.10 ng/ml, respectively. While in serum it was found to be 0.37 ± 0.02, 4.01 ± 0.91 and 6.24 ± 0.12 ng/ml on 0 day, 12th day and on 21st day, respectively (Table 2). As per the present observation 19.99 ± 0.36 ng/ml milk progesterone concentration was taken as standard hormone level for pregnancy diagnosis. Using the above concentration of standard progesterone, the colour intensity of the dot/spot was developed in Dot ELISA for comparison on qualitative basis with respect to the progesterone present in the milk samples.

Fig. 1. Standard curve for enzyme immune assay of progesterone using HRP conjugate

Table 2. Progesterone concentration (ng/ml) in milk and serum at various stages of oestrus cycle.

Progesterone Concentration	Days of oestrus cycle		
	0 day	12th day	21st day
Milk	2.045 ± 0.11	15.02 ± 0.10	19.99 ± 0.36
Serum	0.37 ± 0.02	4.01 ± 0.91	6.23 ± 0.11

The higher milk progesterone level was recorded during luteal phase (Simersky, 2007). The level of progesterone keeps on rising if pregnancy persists. The overall mean serum progesterone concentration was 0.37 ± 0.02 ng/ml on day of oestrous and A.I. The progesterone concentration had been reported to increase from the day after insemination. Unlikely the non pregnant cattle it does not decrease in around 18-20 days in pregnant cows (Akhtar,1988). However, variations in concentration during pregnancy have been reported by Esfandabadi *et al* (2007) who recorded higher serum progesterone concentration in comparison to the present findings. However Choi *et al* (1976) considered the cows to be pregnant when the values exceeded 2.5 and 3.0 ng/ml respectively between 19 – 21d. Sato *et al* (1985) measured serum progesterone concentration by EIA from day of insemination to 22nd day post AI and found that concentration showed an increase from day 2nd post A.I. and remained high up to day 22 in pregnant cows (1.2 to 8.2 ng/ml) which agrees with the present findings. There appears a general agreement among the workers that serum progesterone concentration does not decline to the basal level between day 18 - 22 in pregnant cows as it occurs in non-pregnant subjects.

Keeping all the above points in view during the present investigation 19.99 ± 0.36 ng/ml of milk progesterone was taken as standard hormone level for pregnancy diagnosis.

Selection of solvent for progesterone to PVDF

All the earlier experiments for estimation of progesterone were on ELISA plate or beads (Smersky *et al,* 2007 and Kaygusuzoglu *et al, 2010*). Since no literature was available regarding solvents for progesterone extraction from milk and its binding to PVDF membrane, hence different solvents (1 Methanol), 2 (Methanol + Carbonate-bicarbonate buffer 0.1 M pH 9.6, 3 Methanol + PBS 0.1 M, pH 9.6, 4 Ethanol and 5 70 % isopropanol) were tried (Table 3). And Dot ELISA procedure was followed using 1:10 times diluted conjugate to verify the binding of progesterone on PVDF.

Iit was observed that, when progesterone (standard) dissolved in methanol was transferred on to PVDF membrane, it spreads out to a larger area beyond the well crossing even to other well area and dries up quickly. Following Dot-ELISA no result was obtained indicating there was no progesterone present on the surface. To overcome the binding problem, the carbonate-bicarbonate buffer 0.1M pH 9.6 was applied (Akhtar, 1988). The result was again negative (Table 3). In the next trial the binding of progesterone to PVDF was tried

Table 3. Selection of solvent for progesterone.

Solvent's used	Characteristics studied		
	Spread on PVDF	Development of colour on addition of conjugate and substrate	Results about binding of progester-one to PVDF
Methanol	Spread more and faster	No	-ve
Methanol + 0.1 M Carbonate – bicarbonate buffer pH 9.6	Spread more	No	-ve
Methanol + PBS (0.1 M pH 9.6)	Spread more	No	-ve
Ethanol	Spread more	No	-ve
70% isopropanol	Spread less and slowly	Yes	+ve

by application of same concentration of PBS (0.1 M PBS pH 9.6) after transfer of progesterone to PVDF in methanol. The result was again negative.

Further binding of progesterone was studied by dissolving it in ethanol and transferring on to PVDF, this also results in larger spread area and fast evaporation.

The final trial was conducted using 70% isopropanol as solvent. It spread slowly on to the membrane, which helped in delimiting it to well area only. Its slow evaporation (drying) allowed binding of progesterone to PVDF membrane. Following this, dot-ELISA procedure was followed, which gave result by formation of brown dots, indicating progesterone binding to PVDF membrane (Table 3, Fig. 2 & 3). The present finding was similar to observation of Aniagolu, *et al 1995,* who used in case of cholesterol binding to PVDF membrane. Hence, 70 % isopropanol was selected for further study.

Method for progesterone isolation from milk

Progesterone being fat soluble has to be extracted from milk in order to know the concentration. For this a number of extraction methods were tried (Table 4). In the first trial, Table 5, Fig. 2. Dot Elisa of progesterone extracted by ether extraction method compared to standard progesterone and award of + grades.

	Test result of PVDF Dot Elisa	No of + awarded to each dot based on intensity of chromogen
Blank		-
Standards (20 ng)		+ + + +
Progesterone extracted by chloroform (Folch *et al.* 1957) extraction method		+ + + +

the ether (Table 5, Fig. 2) was used for extraction (Akhtar, 1988). Progesterone was extracted with the formation of two separate layers of liquids. But on evaporation of ether large quantities (10-20µl) of lipidous material were left having oily appearance when transferred to PVDF membrane. It spreads to a larger area and evaporates slowly. On drying and following normal

Dot-ELISA procedure, the result was unsatisfactory. As chloroform was another organic solvent resulted in formation of lower chloroform and top milky

Table 4. Various methods used for extraction of progesterone from milk and its transfer.

	Immediate observation	Final observation after 2-3 hr kept under deep freezing	Finally
Ether extraction (2 ml ether + 1 ml milk + 0.25 ml DW)	Milky layer forms the lower layer	Ether layer separates	Progesterone can be transferred on PVDF
Chloroform only (2 ml chloroform + 1 ml milk + 0.25 ml DW)	Milky layer forms the top layer	Chloroform layer get's entrapped below freezed milky layer, hence separation becomes difficult	-
Isopropanol only (2 ml Isopropanol + 1 mil milk + 0.25 ml DW)	Forms miscible liquid, two liquid do not forms separate layer	Liquid do not freezes	-
Chloroform: methanol (2 ml 2:1 Chloroform: methanol + 1 ml milk + 0.25 ml DW)	Forms separate layer but milky layer thickens due to methanol	Chloroform layer get entrapped below freezed milky layer hence separation becomes difficult	-
Chloroform: methanol (19 ml 2:1 Chloroform: methanol + 1 ml milk)	Extracted as per Folch et al. (1957) where no freezing is required		Progesterone can be transferred on PVDF

layer. On freezing (2-3hr), the chloroform layer got entrapped below frozen milky layer. Hence, separation was difficult. The chloroform was found to be having higher specific gravity, which may be one reason, hence it formed lower layer.

In the third trial, isopropanol was used instead of ether that resulted in the formation of a miscible milky liquid that did not form distinct separate (milky and isopropanol) layer not froze even keeping for longer duration under deep freezing. Hence progesterone was unable to be extracted.

The fourth trial was done with Chloroform:methanol mixture in 2:1 ratio in place of ether. But the result was similar to that obtained with chloroform method. There was formation of an additional middle layer of miscible zone which do not freezes under deep freezing, and hence extraction was not possible.

Finally, trial for extraction was done with Folch *et al* (1957) method. The result appeared in form of very scanty or no lipid substance left in extracted mass. The extracted lipids containing progesterone was able to be transferred to PVDF membrane. Subsequently Dot-ELISA was performed that resulted in formation of brown dots (Table 6 Fig. 3). Since, the chloroform:methanol mixture (2:1) mixed with milk in 19:1 ratio was able to break almost all fat globules, proteolipids, phospholipids and other higher long chain fatty acids. Hence progesterone might appeared in form available for estimation. The result (Table 6, Fig. 3) in the form of figure

Table 6. Fig. 3. Dot-ELISA of chloroform clearly indicated transfer of progesterone to

(Folch et al. 1957) extracted milk progest- PVDF membrane as shown by intensity colour e**rone and award of + grades.** of coloured profile.

	Test result of PVDF Dot Elisa	No of + awarded to each dot based on intensity of chro-mogen
Blank		-
Standard (20 ng)		++++
Progesterone extracted by ether extraction method		+

Development of Dot-ELISA

The Dot ELISA technique developed utilized 100 times less reagents and gave results instantly. The result could be verified visually, poses less/ negligible threat to environment due to waste disposal and could be performed by even layman farmers. And because of all these advantages, it becomes cheaper as compared to other methods. Keeping in view presently the Dot ELISA technique was standardized using 70% isopropanol as solvent, Folch *et al* (1957) method for extraction and HRP-antiprogesterone conjugate using following procedure.

Selection of titer of conjugate

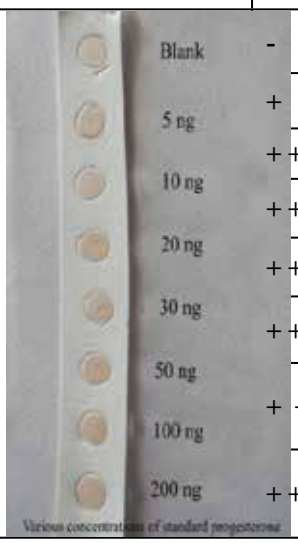
The HRP antiprogesterone (antibody developed in rabbit) conjugate diluted (1:20, 1:50, 1:100, 1:200, 1:500, 1:800 and 1:1000) to obtain best dilution/titer for Dot- ELISA colour (figure 4). On observation1:50 times dilution was the highest dilution giving best result with 20ng standard progesterone following Dot ELISA.

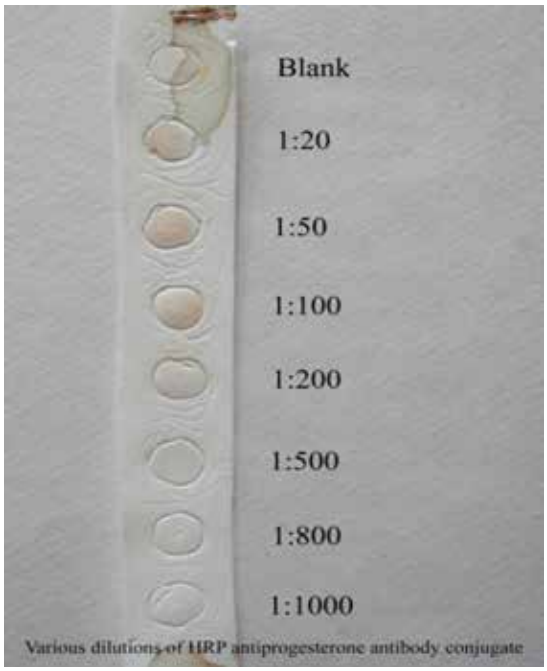
Dot-ELISA for different concentration of standard progesterone and award of + (Positive) grades.

The standard colour for variable concentration was developed using 5ng, 10ng, 20ng, 30ng, 50ng, 100ng and 200ng standard progesterone and + (positive) grades was awarded to each dots depending upon intensity of colour developed (Table 7. Fig. 5). On observation of the results it was found that the lower concentration of progesterone 5 and 10 ng produced fainter colour and got only + (1 positive) and ++ (2 positive grades) respectively. While 20, 30 and 50ng of standard progesterone were found to score ++++ (4 positive grades), ++++++ (6 positive grades), +++++++ (7 positive grades). Subsequently, higher concentration scored only +++++++ (7 positive) grades. For further higher concentrations of progesterone (>50ng) the colour intensity of dots was almost static visually, hence scored same number of positive grades.

Fig. 4. Dot ELISA with serially diluted Table 7, Fig. 5. Dot-ELISA of varying conce-

HRP of standard progesterone conjugate ntration of standard progesterone and award of + grades.

Concen-tr-ation of standard progesterone	Intensity of colour of dot developed	No of + award-ed to dot of each concen-tration based on intensity of chromogen
Blank		-
5 ng		+
10 ng		++
20 ng		++++
30 ng		++++++
50 ng		+++++++
100 ng		+++++++
200 ng		+++++++



Earlier in the experiment, it was recorded that 20ng appox. (19.99 ± 0.36ng/ml) of milk progesterone concentration was considered as pregnant cows milk (Table 2), Earlier workers (Simersky *et al, 2007*) also used 20ng progesterone / ml of milk for pregnant animal which is similar to our present finding with developed Dot ELISA technique. Hence those milk sample getting equal to or more than ++++ (4 positive) grades was considered as pregnant. Those getting +++ (3 positive) grades was considered as suspected/ doubtful and those getting less than +++ (3 positive) grades was considered as non pregnant animal. And ++++ (4 positive) grades was taken as demarcation for pregnant and non pregnant cattle. The pregnancy diagnosis was done accordingly using developed Dot ELISA.

Dot ELISA of confirmed pregnant milk sample

As the 20 ng of standard progesterone was given ++++ (4 positive) grades, it had been further verified with the progesterone isolated from pregnant milk sample. Hence next trial was conducted with early pregnant cattle milk sample (Table 8, Fig. 6) and on perusal of result it was observed that both the pregnant milk sample was giving near intense colour of dot to that produced by 20 ng standard progesterone (Fig. 6). Hence it scored ++++ (4 positive) grades, while one of the negative milk sample one + (1 positive) grades indicating presence of very low progesterone. One negative milk sample was having even lower milk progesterone concentration which had no + grades. Though both the pregnant milk sample and 20 ng standard progesterone scored similar + grades, the intensity of colour of test milk sample appeared slightly lower This might be because of interference due to binding of non progesterone substances on the PVDF membrane. Confirm positive pregnant cattles were verified for pregnancy by rectal palpation which gave the authenticity of the present developed Dot ELISA technique.

Hence it was concluded that Dot ELISA was giving good result with test milk samples also.

Dot-ELISA of test samples and validation

	Test result of PVDF Dot Elisa	No of + awarded to each dot based on intensity of chromogen
Blank		-
Standards (20 ng)		+ + + +
Confirm positive milk sample 1		+ + + +
Confirm positive milk sample 2		+ + + +
Confirm negative milk sample 1		+
Confirm negative milk sample 2		-

As per the technique developed progesterone from the entire test samples were extracted, subjected to Dot-ELISA, awarded respective +

(positive) grades and classified as pregnant, non pregnant and doubtful. Total 65 milk samples from cattle of unknown pregnancy were subjected to pregnancy diagnosis by Dot ELISA procedure. It was found that out of 65 cattle 44,15 and 6 cattle was declared by as pregnant , non pregnant and doubtful respectively. While on per rectal examination only 34 out of 44 (77.27%) was found to be pregnant. All the 15 cattle (100%) declared as non pregnant by dot ELISA was found to be non pregnant. Out of 6 doubtful cases 5 (83.33%) was found to be pregnant. All the pregnant declared cases were also examined per rectally after 45-60 days of pregnancy and birth of fetus. Almost **Table 8, Fig. 6. Showing Dot ELISA of standard progesterone, milk sample of confirm positive and negative pregnant cattle and award of + grades** all pregnant animals gave birth to fetus except few cases of abortions. Work by Heap *et al* (1976) by RIA and Chang and Estergreen (1983) by Paper fibers had reported similar findings with 77.5 and 77.3 per cent accuracy respectively in estimating pregnancy, while for non pregnant animals the accuracy were 85.7-100 and 95-98.3 % respectively. Our present findings was 77.27 per cent.

CONCLUSION

Since, all the pregnant declared animals by per rectal examination were also declared as pregnant by Dot ELISA technique and no pregnant animal was declared as non pregnant, this showed that, the developed technique was a valid technique in estimating pregnancy.

However, it had also showed some false positive results that reduced the accuracy of the method. The false positive results found were might be due to persistent corpus luteum, early abortion or due to pseudo- pregnancy. On the other side, it was 100 % valid for estimating the non pregnant cattle. Hence pregnancy could be diagnosed using developed Dot-ELISA technique with 77.27 % accuracy, while it could be used for diagnosing non pregnant animals with 100 % accuracy.

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