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COMPARISON OF AN IN- HOUSE ELISA KIT WITH A COMMERCIALLY AVAILABLE KIT FOR THE MEASUREMENT OF PREGNANT MARE SERUM GONADOTROPIN (PMSG)

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Abstract: Antiserum to equine Luteinizing Hormone (eLH) raised in a rabbit was used to standardize a competitive ELISA to measure PMSG in native state. The characteristics of this immunoassay developed in-house were compared with those of a commercially available kit. The sensitivity, intra- assay and inter-assay coefficient of variation of both the assays were comparable. Using the in-house assay, immobilized Cibacron Blue, a textile dye, was shown to bind PMSG selectively PMSG. This enabled isolation of PMSG in native condition from pregnant mare serum as well as from enriched serum fractions. It was also observed that a commercial preparation of PMSG gives underestimates when assayed in the commercial kit. Hence there is no correlation between bioassay Units and Immunoassay units when estimated using different antibodies strongly indicating existence of different conformers/isoforms of PMSG.

Key words: Pregnant mare serum gonadotropin; ELISA; eLH antiserum; monoclonal antibody

INTRODUCTION

The presence of a hormone in the blood of pregnant mares was discovered in 1930 by assaying its ability to stimulate ovarian follicle growth in different animal species (1). The hormone was found to be present in the blood beginning at 37days after the fertilizing coitus until approximately 175th day. High amounts were found however between 45 and 80 days of gestation. The native unaltered serum drawn from the mare's jugular vein was capable of exerting clear gonadotropic effects in immature (21-26 days old) mice within a hundred hours following six doses of 0.005 cc. They showed that during the period of high concentration of gonadotropic hormone in the mare's blood, its ovaries exhibited the formation de novo of many corpora lutea, a unique phenomenon and that in the period immediately following this, the fetal gonads exhibited an astonishing hypertrophy caused by the massive appearance of interstitial cells. The measurement of pregnant mare serum gonadotropin (PMSG) in serum by bioassay is useful for detecting pregnancy (2). In 1938, an international standard for PMSG was established. One international unit was defined as activity in 0.25mg of the standard preparation (3). Studies in the early 1970's confirmed that the source of the hormone was fetal chorionic cells that invaded the uterine epithelium to form the endometrial cups (4). This discovery led to renaming the hormone with its current nomenclature, equine chorionic gonadotropin (eCG). Protein analysis demonstrated that eCG is synthesized and secreted as the classic α and β chain heterodimer, as in the case of pituitary gonadotropins. It was also heavily glycosylated (5).

Equine chorionic gonadotropin (eCG) has been characterized as a glycoprotein hormone biochemically with the total molecular weight of 53000 Daltons (6). The unusual high carbohydrate content of eCG which is around 45% in comparison with the 25% carbohydrate content of eFSH and eLH distinguishes the placental eCG. It also possesses 13.5% sialic acid in addition to neutral sugars and hexosamines (5). It is reported that the plasma half-life of eCG was 6 days in geldings and hysterectomized mares, and 24 to 26 days in rabbits (7, 8). Having the same half-life in both geldings and hysterectomized mares suggest that in eCG metabolism, ovaries do not play a major role. Early studies suggested that eCG is not excreted in urine and fully metabolized in the horse (7) but later it was estimated that one seventh of the total circulating eCG is indeed excreted in the urine (9). More detailed studies on plasma half-life or circulating levels required an immunoassay in the place of bioassay as the latter is cumbersome and a large number of samples cannot be handled in one assay.

The most widespread use of eCG, is the exploitation of its FSH activity to induce estrus in immature animals. As the pigs attain puberty at variable time so it has become so it is now industry practice to advance puberty in pigs with a combination of relatively low doses of eCG (400-500 IU) to induce follicle development and 100-200 IU of the hCG to induce ovulation. In the early days of embryo transfer, eCG was employed to induce superovulation in donor animals and its long-life confers the advantage of superovulation by means of a single injection but because it has the tendency to over stimulate the ovary, it results in multiple, unovulated follicle and a yield of viable embryos that is highly variable so the same trait also limits its usefulness (10). The dose response studies showed that the higher doses (3600 IU/ cow) although results in a higher number of ovulations, but produced fewer transferable embryos (12).

Further the related use of eCG has been in synchronization of protocols that have allowed fixed time insemination in cattle (11) and sheep and in synchronization of recipient cows for fixed time embryo transfer (11). Use of eCG proved advantageous in increasing the pregnancy rate for fixed time embryo transfer, independent of the protocol employed for synchronization (12). The problem in all these is to reduce the LH like activity in PMSG. Whether there are naturally occurring isoforms of PMSG with FSH activity but with little LH activity is not clear from existing literature.

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Use of antibodies to probe the surface of proteins to detect different epitopes or isoforms is a good approach to investigate the problem. Hence a preliminary study was undertaken to compare polyclonal antibodies to equine LH generated in our laboratory with a monoclonal antibody available commercially in the form a kit to measure PMSG and to investigate whether there is a differential detection of possible isoforms.

MATERIALS AND METHODS

ELISA Plates were purchased from Symbio Scientific, New Delhi. PMSG was purchased from Sigma Aldrich Chemicals, Bengaluru, India. Primary antibodies like rabbit anti eLH serum, rat anti PMSG serum, rabbit anti oFSH serum and rabbit anti oPRL serum were generated and characterized in our laboratory. Pregnant mare serum, obtained from a commercial source, was subjected to ammonium sulphate fractionation. The 30% saturation pellet and the 30-60% saturation pellet were from our laboratory. Secondary antibodies (Goat anti rabbit-HRP conjugate and rabbit anti mouse-HRP conjugate) were purchased from Brookwood Biomedical, Birmingham, Alabama. Orthophenylene diamine (OPD), Citric acid, trisodium citrate dihydrate, oxalic acid, Polysorbate tween-20, sodium carbonate, sodium bicarbonate and Casein were purchased from Sisco Research Laboratories (SRL), Mumbai, India.. Sandwich ELISA kit for PMSG was purchased from DEMEDITEC Diagnostics GmbH, Kiel, Germany. Blue Sepharose beads were purchased from Sigma Chemicals, USA. All other chemicals used were of AnalaR grade.

Direct-Binding ELISA

This was done as per standard procedures. Briefly, the antigen was coated onto the ELISA plate wells using coating buffer and the plates incubated at 37ºC for 1 hour and then incubated at 4ºC overnight. After washing the plate 3 times using 0.1M phosphate buffer pH-7 the wells were blocked using 1% casein. The primary antibody was used at 1:5000 dilutions. The secondary antibody-enzyme conjugate was used at 1:2000 dilutions. 1M oxalic acid was used to stop the reaction. Absorbance was measured at 490nm in micro plate reader.

Competitive ELISA

Antigen coating and blocking was as earlier described. The primary antibody at 1: 2500 dilutions was pre incubated with standard or unknown samples for an hour and then added into the wells. After incubation for 3 hours at 37ºC, the plate was washed 5 times with 0.1M phosphate buffer. Then the secondary antibody-enzyme conjugate was added into the wells, and then incubated for an hour at 37ºC. The rest of the procedure was as described earlier above.

Pseudo affinity Chromatography on Cibacron-Blue F3G-A Sepharose

A column was filled with Cibacron Blue F3G-A Sepharose beads (Beads+Buffer) so as to obtain a settled volume of 4mL of gel material. The gel was washed and equilibrated with wash buffer (0.1M phosphate buffer, pH-7). The sample (source of PMSG) was loaded onto the gel and left for 15-20 minutes for interaction of the protein with the beads. The gel was then washed with the same buffer till no more protein was coming out of the gel column (A_{280} of the fractions was monitored). Fractions of 1.5mL were collected. The bound material, if any, was eluted with the same buffer but containing 1M NaCl and collected in fractions of 1.5mL each.

RESULTS AND DISCUSSION

When 100mIU of PMSG (Sigma product) was coated in different wells and tested with a variety of antibodies present in our laboratory, rabbit antiserum to equine Luteinizing Hormone (eLH) was found to react and gave an absorbance of 1 at a dilution of 1:5000 in a checker board assay (Fig.1). Hence a direct binding assay for PMSG was standardized. A 1:5000 diluted rabbit antiserum (a/s) to eLH was used to develop coated PMSG coated at different concentrations/amounts. Results (Fig.2) indicate that the antiserum was good enough to detect and measure a range of PMSG concentrations from 10mIU/well to 1000mIU/well of standard PMSG. A competitive ELISA was then standardized to enable measurement of PMSG in natural sources and present in different stages of purity. The calibration curve is shown in Figure 3. The coated antigen (PMSG) in the case of competitive ELISA was used at 200mIU/well constant. A commercial Sandwich ELISA kit was also used (Fig.4). Characteristics of both were compared and the results are indicated under Table 1.

Table 1: Comparison of Characteristics of the two ELISA kits

Competitive ELISA	Sandwitch ELISA
Competitive ELISA	Direct – Binding ELISA
Polyclonal antibody (Rabbit eLH antiserum)	Monoclonal antibody (anti- PMSG)
Ortho-phenylene diamine (OPD) substrate	3,3',5,5' Tetramethylbenzidine (TMB) substrate
Intra-assay CV – 5.93%	Intra-assay CV – 6.69 %
Inter-assay CV – 13.67%	Inter-assay CV – 11.02%
Sensitivity- 8-10mIU/well	25mIU/mL or 5mIU/well

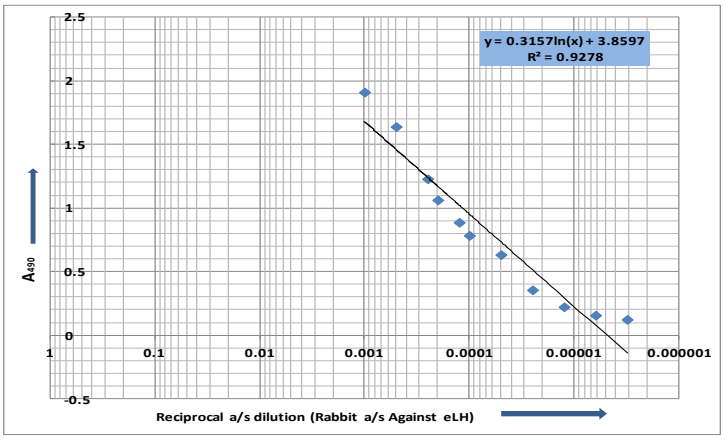

Fig.1: Results of interaction of eLH antiserum with coated PMSG. ELISA plate wells were coated with 100mIU of PMSG and developed with different dilutions of rabbit antiserum to eLH.

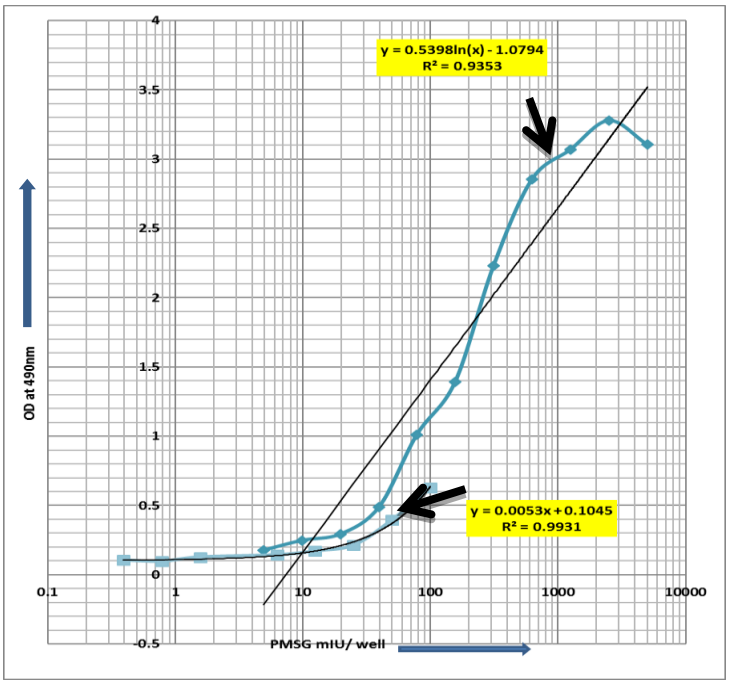

Fig. 2: Results of direct binding assay of PMSG using eLH antiserum. Different amounts of SIGMA make PMSG were coated onto ELISA plate wells and developed with eLH a/s at 1:5000 dilutions.

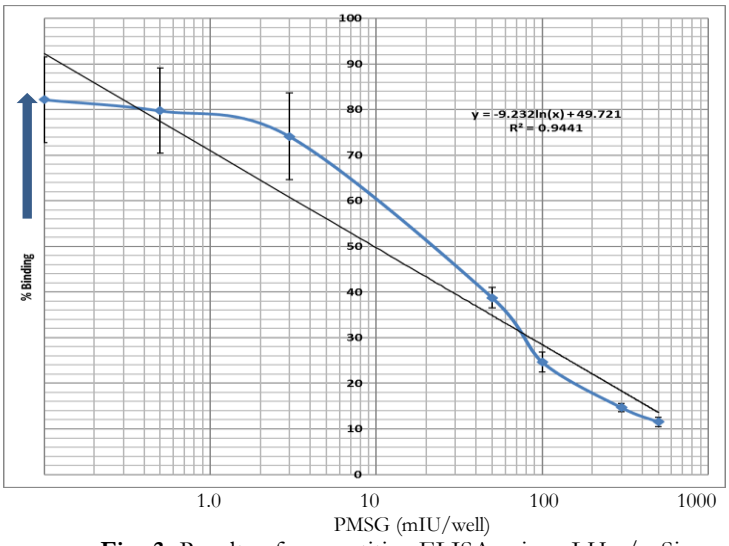

Fig. 3: Results of competitive ELISA using eLH a/s. Sigma PMSG was used as competitor. Details are given under methods section.

The commercial kit was used to estimate PMSG present in different samples (Table 2). The values are in terms of the standard used in that commercial kit. It can be noticed that while the kit was capable of measuring PMSG, a variety of samples gave values which can be interpreted as presence of immuno cross reacting material in those samples. The nature of such material is not known at present. One can guess that probably eLH in normal horse serum, buffalo LH in buffalo serum, PMSG in PMS and its salt fractions (fractionated with ammonium sulphate) do cross react with the monoclonal antibody to PMSG coated on the wells in the commercial kit. But what was surprising was that PMSG obtained from SIGMA Chemical Company used as competitor gave values in terms of the commercial kit standard as units/well but the values do not match with SIGMA units. For example 100mIU and 1000mIU of SIGMA PMSG gave values of 20 and 480mIU respectively. As the kit used a monoclonal antibody which would react with a unique epitope present in their immunogen, SIGMA PMSG giving values less than 100% would only mean that the monoclonal antibody was detecting a sub population of SIGMA PMSG. That would be possible if SIGMA PMSG comprised different populations of PMSG differing in conformation or size or carbohydrate structure. Such a micro heterogeneity in other glycoprotein hormones is known (13).

Table 2: Assay of concentration of PMSG in the different samples using the Sandwich ELISA commercial kit for PMSG.

Samples	OD at 450nm	Concentration of immuno reactive material (mIU)
Pregnant Mare Serum (PMS)	0.082	15mIU/mL
Normal Horse Serum (NHS)	0.216	30mIU/mL
60% ammonium sulphate fraction of PMS	0.054	10mIU/mL
30% ammonium sulphate fraction of PMS	0.057	12mIU/mL
Buffalo serum	0.053	9mIU/mL
100mIU Sigma PMSG	0.121	20mIU
1 IU Sigma PMSG	1.321	480mIU

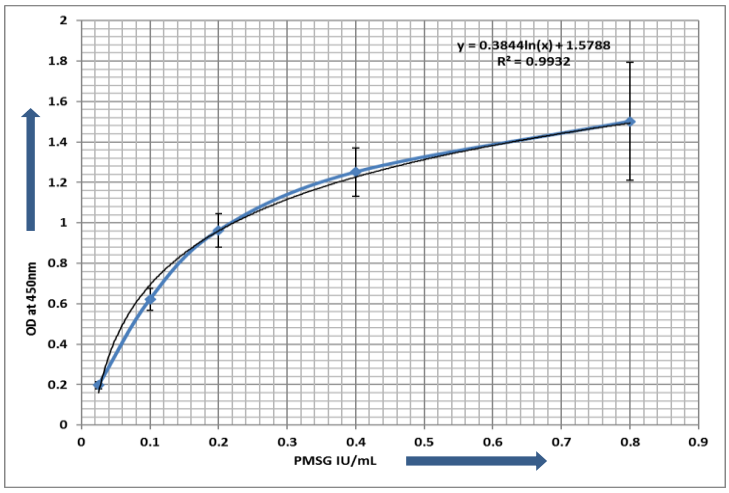

Fig. 4: Calibration curve obtained with the commercially available PMSG kit. The standard used is their own and is part of the kit.

It was then decided to know whether the Laboratory kit can be used to monitor purification of PMSG from its natural source i.e. pregnant mare serum (PMS). PMSG has been reported to have been purified by many groups (14, 15, and 16). All these procedures involve multiple steps and use of more than one chromatographic technique. Pseudo affinity chromatography is a technique useful in reducing the number of operations of purification protocol. If lucky both the yield and purity could be enhanced. Hence immobilized Cibacron Blue, a textile dye was tested for its ability to selectively bind and pick up PMSG. Earlier we have reported that it was very useful in purifying Luteinizing hormone (LH) from buffalo pituitary

crude extracts (17). Original PMS (results not shown) or an ammonium sulphate fraction (30% pellet) was loaded onto a column of Cibacron Blue-Sepharose (Fig. 5).

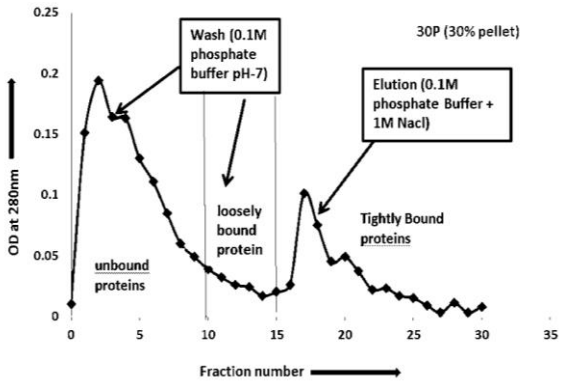

Fig. 5: Chromatography of protein in fractionated pregnant mare serum (i.e. 30% ammonium sulphate precipitate) on Cibacron Blue-Sepharose. Details are given under Methods section.

There was more than 89% recovery of loaded protein from the column. The input O.D. units were 2.883 and 2.579 O.D units were recovered. Part of it was in the eluted fraction thus indicating that PMSG selectively binds to Cibacron Blue. The pooled fractions as indicated in Table 3 were measured for absorbance at 280nm and PMSG content as given in ELISA readings at 490nm. The specific activity was calculated as A490/A280 i.e. PMSG content per O.D. unit of protein. As indicated in Table 3 there was a significant increase in specific activity of the eluted fractions when compared to that of wash fractions (i.e. Column unbound fraction). The fact that PMSG is present in the unbound fraction would only indicate that the loaded sample had more PMSG than the binding capacity of the column used for this experiment. When O.D. data was translated into Units of PMSG from the calibration curve, a clear indication of purification was noticed (Table 4).

Table 3: Increase in specific activity of eluted fractions from Fig. 5

Buffer	Pooled Fractions	A280nm	A490nm	Specific activity A490/A280nm
Wash buffer	2-4	0.165	0.051	0.68
	5-10	0.075		
	11-15	0.031		
Elution buffer	16-19	0.061	0.055	0.9
	20-22	0.025		
	23-27	0.014		
	28-30	0.002		

Table 4: Units of PMSG per O.D.unit of protein in wash and eluted fractions.

Specific Activity (490nm/280nm)	Concentration of PMSG (mIU/ OD unit at 280nm)
0.68	12mIU
0.9	18mIU

CONCLUSION

A competitive ELISA for measurement of PMSG has been standardized using equine LH antiserum raised in rabbits. The assay characteristics compare favorably with those of a commercially available ELISA kit for PMSG. Using the ELISA it was possible to demonstrate the efficacy of immobilized Cibacron Blue to selectively bind PMSG from crude samples of PMSG.

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