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Development of equine chorionic gonadotrophin (eCG) based sandwich ELISA for pregnancy diagnosis and fetus viability in mares

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ABSTRACT

An equine chorionic gonadotrophin (eCG) based sandwich ELISA (sELISA) was developed for confirmation of early conception in pregnant mares around 30 days of gestation and fetus viability thereafter. This serum based quantitative assay is quite sensitive, specific, precise and efficient. Serum eCG content higher than 10 IU/ml during early gestation was used for confirmation of conception while eCG content more than 40 IU/ml was taken as cut off for fetus viability between 40 – 60 days of gestation. Assay was performed with more than 4,000 field serum samples and compared with equivalent commercial pregnancy diagnostic kit with its better sensitivity. Assay showed little cross reactivity with serum of non pregnant mares, jennies, and other animal species. Confirmation of fetus viability sELISA helped in re-servicing the mares which are observed to empty after conception failure or fetal loss.

Key words: eCG quantification, Fetus viability, Pregnancy diagnosis, Sandwich ELISA, Sensitivity, Specificity

In equines, pregnancy diagnosis is a complex issue as a pregnant mare may show false heat symptom. Normally the cessation of estrus is considered as the criteria for pregnancy in animals but in mares, neither the cessation nor the occurrence of estrus confirms the pregnancy (Lewis 1995). Early pregnancy diagnosis is of considerable economic importance, as it helps in reducing the inter-foaling interval to get maximum number of foals from a healthy broodmare in its life-time. A simple and animal friendly method of pregnancy diagnosis can improve the reproductive efficiency of mares, by helping in the early identification of non-pregnant mares, and re-inseminate them as early as possible. Beside pregnancy status, confirmation of fetus viability or early embryonic loss in mares is also a quite important factor in getting optimum foal production, as the later accounts for 5–24% loss in mares due to intrinsic, extrinsic and embryonic factors (Ginther *et al.* 1985, Papa *et al.* 1998).

Serum based ELISA is an important tool for equine owners who do not have other diagnostic tools like ultra sound scanning or rectal examination facilities within their reach. It is considered as an animal friendly and safe method as it does not involve fetus loss due to rectal examination (Alexander *et al*. 1995, Van Niekerk 1965). Equine chorionic gonadotrophin (eCG), also earlier known as pregnant mare serum gonadotrophin (PMGS), is used as an indicator for developing an eCG based ELISA for pregnancy diagnosis in mares. This hormone is secreted by the endometrial cups of the pregnant mares after 30 - 45 days of gestation and presence of this hormone in serum was used for confirming conception in mares (Clegg *et al*. 1954, Allen 1969, Allen *et al*. 1973). A number of commercial kits are available in market for pregnancy diagnosis in mares but all are silent about early fetus loss in pregnant mares. A quantitative sandwich ELISA which has an edge over commercial kits in terms of its sensitivity and specificity, was planned to confirm pregnancy in mares along with fetus viability for the benefit of equine owners.

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MATERIALS AND METHODS

Raising of hyper immune serum against eCG: Hyper immune sera were raised against eCG, both in healthy poultry bird and rabbit using the immunization schedule as adopted by Relan (2001). Antibodies (IgG) present in each serum were partially purified using affinity column protein A-CL agarose as per standard protocol (Harlow and Lane 1988). Purified antibodies labeled as Ab-P (raised in poultry bird) and Ab-R (in rabbit), were stored at –20°C in small aliquots.

Serum samples collected and used: Blood samples (5–10 ml) were collected in purified in 15 ml fresh centrifuge tubes from four non-pregnant mares and eight pregnant mares (between 75 to 85 days of gestation) and their serum samples were used as negative control serum (NCS) and positive control serum (PCS), respectively. Besides this**,** blood samples (8 ml) were also collected from 25 pregnant

mares from organized equine farm on alternate days between 15 to 35 days post-service. Thereafter, samples were collected twice a week till 150 days of gestation. Blood serum samples (250) were also collected from 25 non-pregnant mares (NPM) during two consecutive estrous cycles each, on alternate days between 8.00 to 9.00 AM for this study. Further serum samples of horse mares bred for mule production, non pregnant and pregnant donkey mares (between 60 to 90 days of gestation), pregnant sheep, buffaloes and cattle etc. were also collected for standardization of this assay. Serum was separated from each blood sample and stored at –40°C till further use.

About 4,000 serum samples (between 20 to 180 days of gestation) collected or received from field cases were used for assessing the pregnancy status in them. Blood samples from 110 problematic mares which showed heat symptoms after 40–50 days of gestation, were also collected at regular weekly intervals till 90 days of gestation.

Checker board ELISA protocol: Different simple indirect antigen capture checker board ELISAs were carried out using different dilutions of serum samples, antibodies (Ab-P and Ab-R), conjugates to assess the appropriate dilution of antibodies (Ab-R and Ab-P), conjugate, serum samples etc for their further use in sandwich ELISA (Relan 2001). Serum of non pregnant mare was used as blank for deciding the cut off value in assay.

Standardization of Sandwich ELISA (*sELISA*)*:* ELISA plate or modules were coated with 200 µl of Ab-P antibodies (1: 5000 diluted in coating buffer). Plate was incubated overnight at 4°C followed by four washings with 300 µl of freshly prepared wash buffer. Thereafter, blocking of free spaces was done using BSA solution as mentioned above. After discarding excess of blocking solution and washing thrice with 300 µl washing buffer, wells in duplicate were marked for 5 eCG standards (10, 20, 60, 100 and 140 mIU/ 200 µl / well), negative (NCS) and positive (PCS) control serum samples. 200µl volume of each standard eCG solutions and control sera (1: 250 dilution of PCS and NCS) were added in duplicate. In rest of the wells, 200µl volume of diluted field / known serum sample was added in duplicate for their testing. Plate was incubated at 37°C for 1 h followed by washing thrice with 300µl of wash buffer. Second antibody (Ab-R) at appropriate dilution was added @ 200µl / well to sandwich the eCG antigen present in control/ sample, followed by incubation at room temperature for 1 h. After 3 washings, 200µl of diluted goat anti- rabbit HRPO conjugate was added to each well and plate was again incubated for 1 h at room temperature. After decanting unused conjugate and washing plate thrice, 200µl volume of diluted TMB substrate was added per well followed by incubation for 15–30 min in dark. Reaction was stopped by adding STOP solution (100 µl of 1N H_2SO_4/ well). The plate was read at 450 nm in an ELISA reader for optical density of colour developed in different wells. A standard curve using eCG content vs optical density was plotted for pregnancy diagnosis in terms of eCG contents at a given gestation interval between 35 to 150 days of gestation. Regression equation was also derived from this data for calculating eCG contents in serum samples.

Precision of sELISA: Both inter and intra precision assay of sELISAs were carried out using 5 and 7 serum samples of pregnant mares, respectively, in 6 replications each in 3 plates. Each serum sample was diluted (1:250) in wash buffer before use and eCG contents were evaluated as per sELISA protocol. Coefficients of variance were calculated as per the following standard statistical tool.

i). Inter assay precision

$$CV = \frac{\text{Standard deviation of the means of the replicates}}{\text{Grand mean of the replicates}} \times 100$$

ii). Intra assay precision

$$CV = \frac{\text{Mean of the standard deviations of the replicates}}{\text{Grand mean of the replicates}} \times 100$$

Sensitivity and specificity of sELISA: To evaluate sensitivity of this assay in terms of minimum detectable quantity of eCG, eight different concentrations (2, 5, 10, 20, 40, 60, 100 and 140 mIU / 200 µl in diluted NPMS / well) of standard eCG were used for coating the plates in triplicate in two plates**.** NPMS diluted (1:250) in coating buffer was used as blank or control. Rest of the protocol was the same as for standard sELISA.

For specificity and sensitivity of ELISA, 175 serum samples of confirmed pregnant and non-pregnant mares from organized farms, were also tested for their eCG contents in three different plates using 1: 250 dilution of each sample. Sensitivity and specificity were deduced by the method of Tyler and Cullor (1989).

Comparison of sandwich ELISA test with commercial kit: A comparative study was carried out by testing 66 sera samples from confirmed non pregnant (30) mares and pregnant between 30 to 125 days of gestation (36) by eCG based kit and sELISA. As per kit, 50 IU/ml or more eCG content in serum was taken as positive for pregnancy status while eCG content more than 10 IU/ml by sandwich ELISA, was taken as cut off value for declaring pregnant status.

eCG quantification for pregnancy confirmation and fetus viability: Serum samples collected from 25 confirmed pregnant mares at different gestation intervals between 20 to 180 days of gestation were diluted (1:250) before evaluating their eCG contents to find out a early cut-off date for pregnancy confirmation. Further, serum samples (200) collected at different intervals during estrous cycle from 25 non-pregnant mares were also assessed for eCG in them. Beside this, serum samples collected from fillies, pregnant and non-pregnant jennies, mules and other animal species were also evaluated for eCG content or similar activity (FSH or LH) in them. All the 4,000 field samples were also tested for pregnancy diagnosis and fetus viability in terms of eCG contents. Serum samples of about 110 problematic mares were assessed by sELISA for eCG contents in them.

RESULTS AND DISCUSSION

Checker board ELISA's: Indirect antigen capture checker board ELISAs revealed optimum dilutions of (Ab-P) and (Ab-R) antibodies as 1:8000 and 1:4000, respectively. Goat anti-rabbit and anti-poutry IgG –HRPO conjugates were observed to give optimum colour at 1:10,000 dilution. Serum diluted at 1:250 dilution was observed to give optimum eCG concentration irrespective of gestation interval between 30 to 150 days (data can be provided if required). The eCG content in serum was derived both by standard curve (eCG content vs optical density) and regression equation method without any significant difference in its values.

Sandwich ELISA: In sandwich ELISA (sELISA), instead of wash buffer as diluent, 1:250 dilution of NPMS was used for preparing different eCG standards for standard curve. This avoided the problem of subtracting the optical density (OD) values of NPMS from test sample and fixing cut off value for deciding pregnancy status and fetus viability.

a) *Inter and intra assay precision of sELISA: Both inter and intra-assay precision were* evaluated in terms of coefficient of variance, which ranged from 8.18 to 13.70% in inter assay precision while in intra assay precision, it ranged from 5.61 to 8.38%. Both the ranges were well within the prescribed limit of <15% and 10% acceptability, respectively (supplementary data).

b) *Sensitivity and specificity of sELISA: The eCG content in pregnant mares va*ried from 40 to 150 IU/ml while in non pregnant mares, it was nil. Out of 175 mares, 75 mares were found pregnant on the basis of their eCG content while rest were non pregnant (Table 1). On comparing individual mare wise ELISA results with actual foaling data, it was clear that out of 75, only 74 mares actually foaled, indicating specificity of assay as 99.00 % while sensitivity of the sELISA was cent percent. All the 100 samples observed to be from non-pregnant mares were cross checked to be from non-pregnant mares only which indicated that non-pregnancy predictions were 100% while predictive value for pregnancy was 98.67% by ELISA. One sample which initially indicated pregnancy in mares in terms of eCG contents, was a fetal loss case.

Table 1. Sensitivity and specificity of sELISA

Parameters	Percent (175 serum samples)
Sensitivity[a](%)	100 (74/74)*
Specificity[b] (%)	99.00 (100/101)*
Predictive value/pregnancy[c]	98.67
Predictive value/ non-pregnancy[d]	100

[a] Probability of accuracy identifying the true positive (pregnant) mares out of 74

pregnant mares; [b] Probability of accuracy identifying the true negative (non pregnant) mares out of 101 pregnant mares; [c] Probability that sELISA confirmed pregnant mares are actually pregnant;[d] Probability that sELISA confirmed non pregnant mares are actually non pregnant. * Figures in parenthesis indicates number of serum samples of pregnant mares/ total numbers of serum tested.

Sensitivity of sELISA**,** in terms of minimum detectable eCG content, indicated that this assay was quite sensitive as it could detect even 5mIU of eCG which is very low concentration of this hormone (Fig. 1). Optical density value at this concentration was quite higher even than the values obtained after subtracting 3 times of SD values.

c) *Cross reactivity of sELISA :* sELISA did not show any cross reactivity with any of the serum samples (non pregnant mares, pregnant donkey mare, mule, goat, sheep, buffaloes and cattle) used for testing as the optical densities with all the samples were at par with blank values indicating absence of eCG or eCG like activity in them.

Comparison of sELISA with commercial kit: Equine chorionic gonadotropin contents were estimated in all the 66 serum samples by both commercial kit and sELISA (supplementary Table 1). Non pregnancy status of all the 30 mares was confirmed by both kit and sELISA also, as eCG content was nil in them. Among rest of the 36 samples from pregnant mares, eCG content varied from 20 to >238 IU/ml serum by sELISA while with commercial kit, it ranged between >50 to >100IU/ml serum in 27 samples only. In rest of the 9 pregnant mare serum samples, also declared pregnant by sELISA, eCG contents ranged between 20 to 45 IU/ml which was quite less than the detectable limits of kit.

Equine chorionic gonadotrophin quantification for pregnancy confirmation and fetus viability: In serum

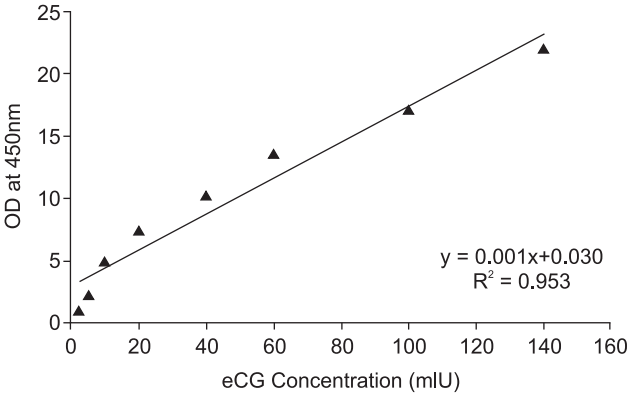

Fig 1. Standard cum Sensitivity curve at different eCG concentrations.

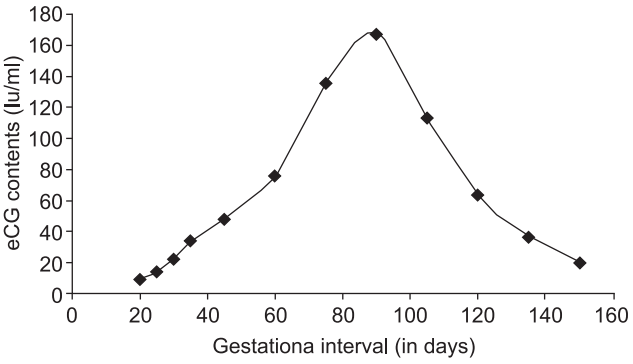

Fig 2. The eCG contents in pregnant mares at different gestation intervals.

samples of all the non-pregnant mares, fillies, pregnant and non pregnant jennies, mule and other animal species, eCG like or its activity was not detected. Equine chorionic gonadotrophin content evaluated in 500 serial serum samples, collected from 25 confirmed pregnant mares at different gestation intervals, revealed a continuous increase in eCG content with gestation period (Fig. 2). In pregnant mares, average eCG content increased significantly from 9.0 IU/ml (20 days) to 167.0 IU/ml (90 days of gestation) and thereafter it slowly decreased forming a bell shaped curve. At 150 days of gestation, eCG content was only 20.60 IU/ml. At early gestation intervals (20–30 days), eCG content varied from 9.0 to 23.0 IU/ml but only about 70% pregnant mares had eCG contents in them while in rest of the mares, eCG content was detected after 30 days of gestation. On the basis of eCG contents, about 2600 mares out of 4000 were confirmed to be non pregnant. In pregnant mares, eCG contents varied from 12.5 to 240 IU/ml between 20 to 150 days of gestation. Variation in eCG content among pregnant mares at same gestation interval was quite high but presence of even low levels of eCG level at early gestation intervals was good enough to confirm that mare has conceived.

In problematic mares (110), initially eCG content was observed to be >10 IU/ml eCG at 30 days of gestation and these were declared as pregnant mares. However these mares showed heat symptom again. Quantification of eCG in serum samples of these mares at regular intervals up to 90 days of gestation, indicated that eCG content did not increase in these mares like pregnant mares (Fig. 3). Maximum eCG content detected was less than 40 IU/ml which decreased continuously and by 90 days of gestation, it was present in trace only. This eCG quantification helped in confirming fetus viability. For confirmation of fetus viability of field sample after early conception confirmation, second serum samples between 40 to 90 days of gestation was again tested and it was observed that all pregnant mares having viable fetus had eCG contents more than 40 IU/ml in them. Therefore, this assay was used for early pregnancy diagnosis and fetus viability both.

During standardization of sELISA, different checker board indirect ELISAs helped in assessing the suitable

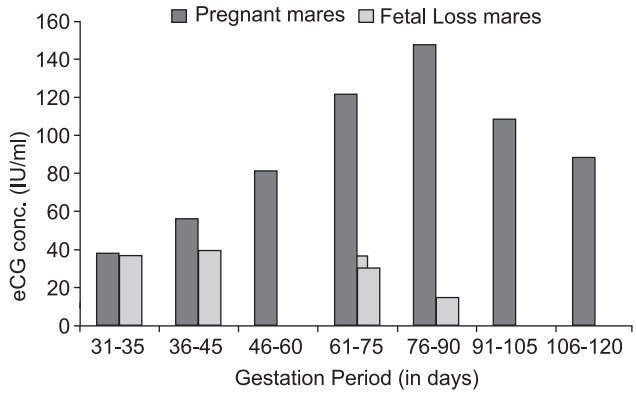

Fig 3. The eCG contents in pregnant and fetal loss mares.

dilutions of different components including antibodies raised against eCG in both small animals, anti rabbit HRPO conjugate, serum dilutions etc. Use of two different polyclonal antibodies increased the sensitivity and specificity of sELISA as compared to simple ELISA for pregnancy diagnosis. Since eCG or eCG like activity was not detected in non-pregnant mare serum, therefore, lower dilutions of this serum (1:250 or 1:500) were successfully used as control as well as diluents for preparing different eCG standards in sELISA to avoid interference of other serum proteins.

Precision of sELISA: This test is quite important to validate and assess the manual error or differences in performing the test due to the nature of the material used as well as its handling by different performers. In sELISA, both inter and intra precision tests were within recommended/ normal limits as percent coefficients of variability were less than 15 and 10 %, respectively (http://www.salimetrics.com retrieved on 28.12.2014). In our assay, these indicated its reproducibility and accuracy for eCG measurement.

Sensitivity and specificity of sELISA: Sensitivity of any assay is generally evaluated by diluting the known positive samples sequentially and determining the dilutions at which the reaction is lost (http://www.poultry-health.com/library/serodiss/assayqc.htm retrieved on 11.12.2014). In present study, sELISA was observed to be very sensitive as it could detect even 5 mIU eCG (5 IU/ml) concentration. However, for better accuracy (sensitivity and specificity) of this test, any eCG content higher than 10 IU/ml serum was taken as cut off value for confirming pregnancy.

Specificity of sELISA was also observed to be very high (99%) with pregnant serum samples while with non-pregnant mares, it was cent percent which directly indicates its ability to assess unequivocally the target in presence of components which may be expected to be present. Further, predictive values of this test for non-pregnant mares was also cent percent with no chance of false result. However, predictive values for pregnant mares was a little lower which was possibly be due to early fetus resorption or abortion after conception. In such cases, paired sera sample testing helped in confirming fetus viability also.

Cross reactivity in sELISA: sELISA did not show any cross reactivity with different serovars including equine specific and non-specific serum of different animal species which could possibly be due to high specificity of both the antibodies for eCG only.

Comparison of sELISA with commercial kit: Comparative analysis of serum samples by sELISA and equivalent serological test (Commercial kit) clearly indicated better sensitivity and specificity of this assay. At higher eCG levels (i.e >50IU/ml), both the assays had similar specificity and sensitivity but at low eCG content (<50 IU/ml), sELISA was much better than commercial kit. Use of non-pregnant serum as control or blank in sELISA helped in accurate and efficient detection of eCG even at as low 10 mIU eCG concentration for pregnancy

diagnosis. Beside this, sELISA results were also validated with actual foaling records.

eCG quantification in serum samples for pregnancy diagnosis and fetus viability: It is well documented that eCG is released in blood stream around 35 to 40 days of gestation (Allen and Moor 1972, Papkoff 1974) while in present study, eCG content was detected even between 20 to 35 days of gestation which indicated high sensitivity of our sELISA. In pregnant mares, continuous increase in eCG contents till 90 days of gestation clearly indicated that this hormone supports the pregnancy for at least during first quarter of gestation which is in concordance with previous findings (Clegg *et al*. 1954, Allen and Moor 1972, Virmani *et al*. 2006). Further, mare to mare variations in eCG content between 35 to 150 days of gestation at the same gestation interval was also appreciable which can be attributed to season, parity (Richter 1963), genetic factors with both paternal (Manning *et al*. 1987) and maternal influences (Martinuk *et al*. 1990). Commercially available pregnancy diagnostic kits are useful for confirming pregnancy only if a particular level of eCG is present in mare's serum but are silent about fetus viability.

Quantification of eCG by sELISA helped not only in pregnancy diagnosis but also in confirming fetus viability. Presence of eCG content in serum of aborted mares due to its secretion from endometrial cup, was taken into account by fixing as cut of value for early pregnancy diagnosis and fetus viability (>40 IU/ml). Fetus viability was however, confirmed using a paired serum sample from the same mare between 40 to 60 days of gestation after early pregnancy confirmation. If eCG levels remain constant or decrease as compared to initial eCG level than it clearly indicates that fetus is not viable. In our sELISA, it is quite important that date of covering and sample collection from pregnant mare should be clearly known. In all the problematic mares which were observed to be empty after initial pregnancy confirmation, their eCG levels remained static for a short period but subsequently decreased. Such a decrease in eCG contents in mares which had lost fetus during early pregnancy had been reported earlier also (Roberts 1986). Presence of small quantity of eCG contents between 35 to 60 days of gestation only indicates that mare had conceived after covering but does not assure fetus viability. Presence of eCG contents in serum in detectable limits of such mares was due to the presence of eCG producing cells which are of fetal origin (Allen 1975, Allen 1978, Rathwell *et al*. 1987). In present study, fetus viability was confirmed on the basis of eCG content (> 40 IU/ml) present in serum. Only 2.75% cases of fetal loss due to fetus abortion or early embryonic deaths were recorded in present study. Assessment of fetus viability is very important as embryonic death rate ranging from 5 to 24% had been reported (Ginther *et al.* 1985, Papa *et al.* 1998). Further this sELISA is also useful as it does not involve rectal invasion as the later may result in fetal loss (Alexander *et al* 1995, Van Niekerk and Morgenthal 1982). Confirmation of fetus viability also helped in re-servicing of empty mares which may improve equine production also.

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