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Non-invasive pregnancy determination in mares by enzymeimmunoassay of estrone sulphate concentrations in faeces

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ABSTRACT

Measurement of faecal estrone sulphate by enzymeimmunoassay was evaluated as a non-invasive means of monitoring pregnancy status in mares. Faecal samples from non-pregnant mares had a mean \pm s.e.m. estrone sulphate concentration of 23 ± 0.9 ng/g (N=242). In pregnant mares, mean faecal estrone sulphate concentrations increased progressively during the first half of gestation. The values rose from a mean \pm s.e.m. of 49 ± 4 ng/g (N=121) for samples collected <80 days post-mating, to 122 ± 9 ng/g (N=30) for samples collected 161 to 180 days post-mating. Thereafter, faecal estrone sulphate concentrations remained relatively constant for the remainder of pregnancy. From 150 days post-mating onwards, <2% of faecal samples from pregnant mares had an estrone sulphate concentration below 66 ng/g which was the value 3 standard deviations above the mean value found for non-pregnant mares. Following foaling or foetal death, faecal estrone sulphate concentrations returned quickly to non-pregnant levels. Using this estrone sulphate enzymeimmunoassay, mares may be confirmed pregnant once faecal estrone sulphate concentrations rise above the 66 ng/g cut-off value. Those mares in which faecal estrone sulphate concentrations have not risen above 66 ng/g by 150 days post-mating may be considered to be non-pregnant. Measurement of faecal estrone sulphate thus offers a convenient, non-invasive means of monitoring pregnancy status.

Keywords: estrone sulphate; horse; faeces; pregnancy; enzymeimmunoassay.

INTRODUCTION

The foetal-placental unit produces increasing amounts of estrone sulphate as pregnancy progresses in mares. The estrone sulphate enters the maternal circulation and is excreted in the faeces. Measurement of the concentration of estrone sulphate in blood is an established method of determining the pregnancy status of mares (Sist *et al.*, 1987a). However, the measurement of estrone sulphate in faeces would provide a non-invasive alternative to this. Estrone sulphate is generally measured by radioimmunoassay, which restricts the assay to relatively sophisticated laboratories having the facilities to handle radioactive materials. Recently, a technically straightforward enzymeimmunoassay has been developed for measuring estrone sulphate concentrations in cows' milk, to provide a non-invasive means of determining pregnancy status in dairy cows (Henderson *et al.*, 1994). The purpose of the present study was to determine the suitability of this enzymeimmunoassay for measuring the concentration of estrone sulphate in faecal samples from mares, and so provide a non-invasive means of determining pregnancy status in this species.

MATERIALS AND METHODS

Freshly passed faecal samples were obtained throughout gestation from 37 Thoroughbred mares known to be pregnant, and from 74 non-pregnant mares over the same time period. After collection, the faecal samples were

stored frozen until assayed for estrone sulphate. At the time of assay, the samples were thawed, and estrone sulphate was extracted from 1g aliquots of the faecal samples by shaking in methanol (10ml) for 2h. Debris was pelleted by centrifugation, and 1 to 10 μ l aliquots of the methanolic supernatant assayed directly for estrone sulphate using the antibody-coated enzymeimmunoassay developed for assaying cows' milk, as described in detail previously (Henderson *et al.*, 1994). This assay is now available in a test-kit form and is marketed as Confirm™ by Immuno-Chemical Products Ltd, Auckland, New Zealand. Briefly, the assay was performed as follows. Wells of microtitre plates (Nunc, Maxisorp C12) were coated with a monoclonal antibody to estrone sulphate by overnight incubation. Following blocking with a 0.5% gelatin buffer solution and washing, the antibody-coated wells received a 1 to 10 μ l aliquot of faecal extract, or an aliquot of estrone sulphate standard, plus an aliquot of estrone glucuronide-horseradish peroxidase conjugate in a total volume of 0.2 ml. The wells were then incubated overnight at 4°C. After emptying and washing the wells, 0.1ml of o-phenylenediamine substrate-chromogen solution was added and colour allowed to develop for 20 to 30 minutes before the reaction was stopped with 2M H₂SO₄ (0.05 ml/well) and colour intensity read at 490 nm. The concentration of estrone sulphate in the assayed faecal extracts was calculated by interpolation from a standard curve and the values normalised to faecal weight. The working range of the standard curve was from 5 to 200 pg/well. The intra- and inter-assay

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coefficients of variation of the assay were <11%. Dose-response lines of the estrone sulphate standards and 1 to 10 µl volumes of the faecal extracts, containing different concentrations of estrone sulphate, ran parallel.

RESULTS

The mean ± s.e.m. concentration of estrone sulphate found in 242 faecal samples collected from 74 non-pregnant mares over a 12 month period was 23 ± 0.9 ng/g faeces. The faecal estrone sulphate concentrations were not influenced by the time of year that the samples were collected. In the 37 pregnant mares studied, mean faecal estrone sulphate concentrations increased as pregnancy progressed (Fig. 1). From 121 days of pregnancy, the values of the lower 95% confidence interval of the means were higher than the value 3 standard deviations above the mean non-pregnant value. There was considerable variation between individual mares in the pattern of faecal estrone sulphate concentrations observed throughout gestation. At one extreme, faecal estrone sulphate concentrations gradually rose out of the non-pregnant range (ie. rose above the value 3 standard deviations greater than the mean non-pregnant value) with peak values occurring relatively late in gestation (Fig. 2a). At the other extreme, faecal estrone sulphate levels had risen outside the non-pregnant range by 50 days post-mating, and peak values occurred relatively early in gestation (Fig. 2b). Following foaling, faecal estrone sulphate concentrations rapidly returned to values typical of non-pregnant animals (Fig. 2a & 2b). The usefulness of faecal estrone sulphate levels as a means of monitoring pregnancy status was also demonstrated. One mated mare, which was initially diagnosed as pregnant by ultrasound scan, later unfortunately lost her foetus. Measurement of estrone sulphate concentrations in faecal samples from this mare showed levels outside the

FIGURE 1: Estrone sulphate concentrations in faecal samples from non-pregnant and pregnant mares.

Values are means and 95% confidence intervals. Mean values with different letter superscripts differ significantly (P < 0.05, analysis of variance in conjunction with Newman-Keuls multiple range test). The horizontal dotted line indicates the value 3 standard deviations above the mean non-pregnant value.

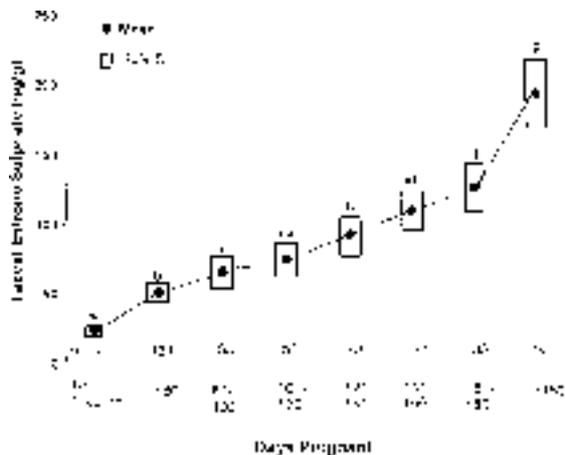
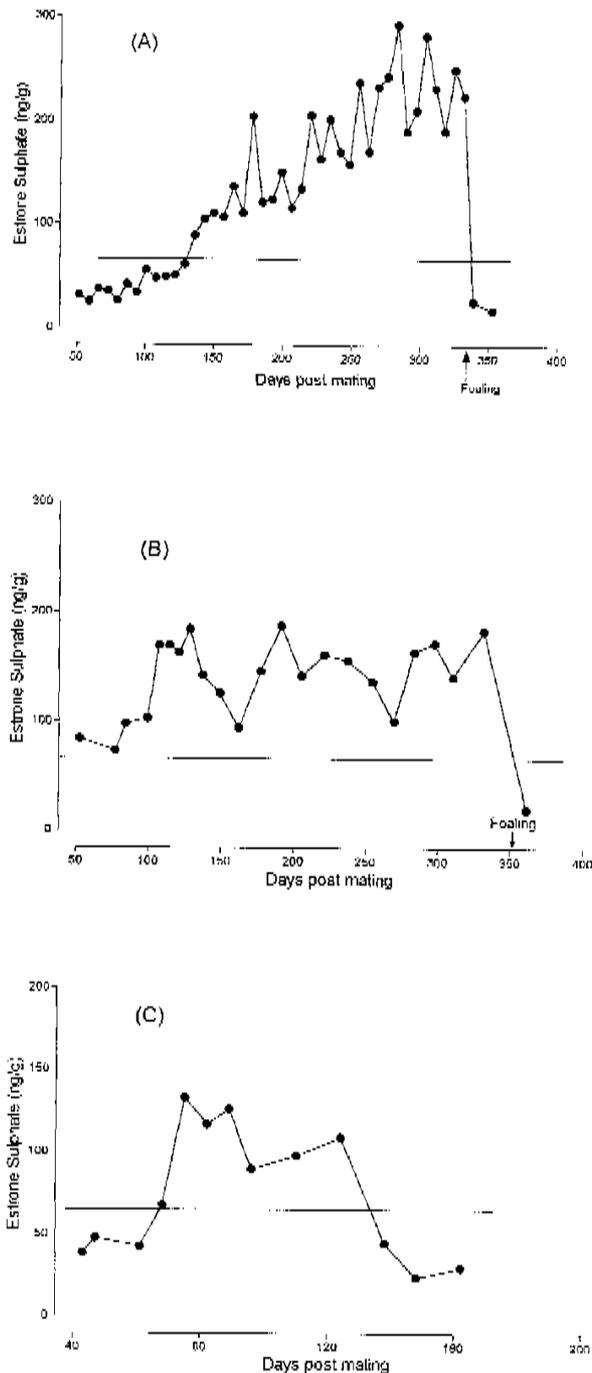


FIGURE 2: Faecal estrone sulphate concentrations during pregnancy for 3 individual mares. Two mares (A and B) had normal pregnancies which went full term, while the 3rd mare (C) lost her foetus in the 5th month of gestation.

The horizontal line indicates the value 3 standard deviations above the mean non-pregnant value.



non-pregnant range from days 68 to 124, indicative of the presence of a live foetus (Fig. 2c). However, by day 138 estrone sulphate levels had plummeted into the non-pregnant range indicating that foetal death had occurred which was confirmed by veterinary examination.

Table 1 shows the numbers of faecal samples, collected at different stages of pregnancy, with estrone sulphate concentrations above or below 66 ng/g, ie. higher or

TABLE 1: Numbers of faecal samples from pregnant mares with estrone sulphate concentrations above and below 66 ng/g.

Faecal estrone sulphate conc.	Days Pregnant				
	<60	61-100	101-140	141-180	>180
<66ng/g	60	58	47	3	0
>66 ng/g	14 (19%)	42 (42%)	56 (54%)	59 (95%)	92 (100%)

66 ng/g is the value 3 standard deviations above the mean non-pregnant value

lower than the value 3 standard deviations above the mean non-pregnant value. For samples collected before 100 days of pregnancy, most had values <66 ng/g. However, after 100 days most values were >66 ng/g, with the proportion having a concentration below 66 ng/g decreasing as pregnancy progressed. Indeed, <2% of samples collected after 140 days of pregnancy had a value <66 ng/g, and none had a concentration <51 ng/g, the value 2 standard deviations above the mean non-pregnant value.

DISCUSSION

The results of the present study demonstrate that measuring faecal estrone sulphate concentrations by enzymeimmunoassay provides a non-invasive means of monitoring pregnancy status in mares, and so provides an alternative to the more usual technique of measuring estrone sulphate in serum. When estrone sulphate is to be measured in serum to determine pregnancy status, it is generally recommended that the blood sample be taken at least 100 days post-mating, as by this time serum estrone sulphate concentrations in pregnant mares are very high relative to non-pregnant mares (Sist *et al.*, 1987a). If faecal estrone sulphate is to be measured, delaying sampling to 140 to 150 days post-mating is advisable to allow accurate identification of non-pregnant mares. Pregnant mares can be identified as soon as the faecal estrone sulphate concentrations rise outside the non-pregnant range, and for some mares this may occur by 50 to 60 days post-mating (Fig. 2b, Table 1). However, for some pregnant mares this rise might not occur until approximately 140 to 150 days post-mating (Fig. 2a). Thus non-pregnancy cannot be confirmed until a value in the non-pregnant range is measured in a sample collected at least 150 days post-mating. Measurement of faecal estrone sulphate should best be regarded as a means of verifying pregnancy status rather than diagnosing pregnancy status, as this is usually done earlier by other methods such as ultrasound, or measurement of blood PMSG. The gestation period in mares at approximately 342 days is lengthy, and pregnancy is not visually apparent until relatively late in gestation. For many own-

ers, being able to confirm an initial early diagnosis of pregnancy with a non-invasive test later in the gestation period would hold considerable appeal.

Measurement of estrone sulphate, whether it be in a blood or faecal sample, has the advantage that it provides an indication of whether a foetus is alive or not. Should foetal death occur, then estrone sulphate levels plummet back into the non-pregnant range of values (Fig. 2c). In contrast, while measurement of PMSG in blood allows earlier detection of pregnancy (approximately 40 to 100 days post-mating) false positives can result from the maintenance of the PMSG secreting endometrial cups after foetal death (Daels *et al.*, 1991).

Measurement of faecal estrone sulphate concentrations for determining pregnancy status has some advantages. Collecting faecal samples is non-invasive and straightforward, and enables pregnancy status to be monitored easily on multiple occasions throughout gestation if required. Estrone sulphate is a stable steroid, and so faecal samples can be submitted to a testing laboratory by mail or by courier from anywhere in the country. The assay itself is sensitive and requires only 1 gram of faeces for analysis, and so only a small container of faeces needs to be submitted for testing. Although this study has focussed on samples from Thoroughbred mares, preliminary studies with ponies and miniature horses indicate that this estrone sulphate assay will be applicable for these also, and this is supported by studies elsewhere which indicates the appropriateness of estrone sulphate analysis for a wide variety of horse breeds and types (Sist *et al.*, 1987b).

The estrone sulphate assay used in this study was originally developed for measuring estrone sulphate in cows' milk, and is now marketed for this purpose in a test-kit form as Confirm™ by Immuno-Chemical Products Ltd, Auckland, New Zealand. The results of this study indicate that Confirm™ will also have potential as a test for monitoring pregnancy status in mares.

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