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Evaluation of an oestrone sulphate dipstick immunoassay for pregnancy testing mares

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ABSTRACT

Serum samples from 213 mares were received for routine pregnancy testing and analysed by a conventional oestrone sulphate (OS) enzymeimmunoassay (EIA) and a new OS dipstick immunoassay. OS concentrations, measured by EIA, in 122 of the samples were <10 ng/ml, indicating the mares were not pregnant. The same 122 samples also returned a 'not pregnant' diagnosis when analysed by the dipstick immunoassay. The remaining 91 samples returned diagnoses of 'pregnant' by the OS EIA, i.e., OS concentrations were >20 ng/ml. Of these, 89 also returned a 'pregnant' diagnosis by the dipstick assay, but 2 returned a 'not pregnant' diagnosis. Both these mares were found to be pregnant on follow up, indicating that the dipstick test had returned a false negative diagnosis in each instance. These results show that there is 100% agreement between the conventional OS EIA and the new dipstick assay in diagnosing 'non pregnancy'. However, the dipstick assay may return a small proportion (2.2%) of false negative diagnoses relative to the EIA. Overall, the new dipstick immunoassay offers a practical alternative to the OS EIA for diagnosing pregnancy status in mares.

Keywords: Oestrone sulphate; horse; serum; pregnancy; dipstick immunoassay.

INTRODUCTION

In horses, the average length of pregnancy is 335 to 343 days (Gordon, 1997), and it is not uncommon for owners to want to confirm the pregnancy status of their mare(s) during this long period. As pregnancy progresses in horses, the foetal-placental unit produces increasing amounts of oestrogen, and after approximately 70 days of gestation it is the major source of maternal circulating oestrogen. This is reflected in elevated concentrations of serum oestrone sulphate (OS) with all pregnant mares at least 100 days post-mating having concentrations above 20 ng/ml, and with most exceeding 50 ng/ml. In contrast, serum OS concentrations of non-pregnant mares are generally <5 ng/ml, although occasionally concentrations of up to 10 ng/ml may be found at oestrus. Measurement of serum OS concentrations from 100 days after mating thus provides a convenient means of monitoring the pregnancy status of mares for more than 200 days of their gestation period. Mares with serum OS concentrations above 20 ng/ml can be confirmed pregnant. Those with levels below 10 ng/ml, and which are at least 100 days post-mating, can be diagnosed as not pregnant.

The Reproductive Technologies Group, at the AgResearch Wallaceville campus in Upper Hutt, has been providing a diagnostic pregnancy testing service to horse owners for several years that is based on measuring OS concentrations in a blood sample using an enzymeimmunoassay (EIA) procedure. While EIA is a robust and accurate technique, it is complex and can take several hours to generate a result. Recently, we have described a competitive dipstick immunoassay that allows serum OS concentrations to be estimated within 20 minutes (Henderson & Stewart, 2000).

To evaluate the practical potential of the OS dipstick test, samples submitted to this laboratory for routine pregnancy testing have been analysed by both the usual OS EIA and the new dipstick test. Here we report the level of agreement between the 2 assays in diagnosing mare pregnancy status.

MATERIALS AND METHODS

Blood samples

Blood samples from 213 mares were submitted for pregnancy testing by veterinarians throughout New Zealand. The samples were collected from mares that were between at least 100 days post mating and one month of expected foaling, as determined by information provided by the owners. Serum was prepared from the blood samples and kept at 4°C for up to 3 days and at -20°C for longer periods. Aliquots of the serum samples were assayed in duplicate by both the OS EIA and the dipstick immunoassay. A serum OS concentration of ≤10 ng/ml was interpreted as indicating a mare was not pregnant if blood was sampled at least 100 days after breeding. A serum OS concentration of >20 ng/ml was interpreted as indicating a mare was pregnant.

OS immunoassays

The EIA to measure OS concentrations in horse serum has been described in detail previously (Henderson *et al.*, 1998). Briefly, the assay was performed as follows. Wells of microtitre plates (Nunc, Maxisorp C12) were coated with a monoclonal antibody to OS by overnight incubation. Following blocking with a 0.5% gelatin buffer solution and washing, the antibody-coated wells received a 10 µl aliquot of horse serum, or an aliquot of OS standard, plus an aliquot of oestrone glucuronide-horseradish peroxidase conjugate in a total volume of 0.2 µl. The wells were then incubated overnight at 4°C. After emptying and washing the wells, 0.1 ml 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 OS in the assayed horse serum samples was calculated by interpolation from a standard curve. The working range of the standard curve was from 2 to 500 pg/well. The intra- and inter-assay coefficients of variation of the assay were <9%.

The dipstick immunoassay for OS was also performed as described in detail previously (Henderson & Stewart,

2000). Briefly, 6-ketoestrone 6-carboxymethyloxime conjugated to bovine serum albumin (OCMO-BSA) was 'dotted' 25 mm from the bottom edge of 45x5 mm strips of 3µm PE-supported cellulose nitrate membrane. The strips were blocked, dried and a 15x5 mm cellulose absorbent sink attached 10 mm from the top of each strip. The manufactured dipsticks were stored with desiccant at room temperature. A monoclonal antibody to OS (the same one as used in the EIA) was coated onto uniform blue-dyed polystyrene microspheres (mean diameter 0.31µm) by adsorption. After blocking, several washes and resuspension by sonication, the antibody-coated microspheres were stored at 4°C. The concentrations of OCMO-BSA dotted onto the dipsticks, and OS antibody coated onto the microspheres, were optimised to produce a test that allowed maximum discrimination between the concentrations of OS found in serum of pregnant mares relative to those found in non-pregnant mares. To perform the dipstick test, 30 µl of carrier buffer (0.05% Tween 20 in saline), 10 µl of OS antibody-coated microspheres and 10 µl of serum sample were pipetted into a tube and mixed. A dipstick was placed in the solution. All the liquid migrated up the dipstick into the absorbent sink within 15 to 20 minutes leaving a blue dot where the OCMO-BSA had been placed. The intensity of colour of the blue dot was inversely related to the concentration of OS in the serum sample. A serum OS concentration <5 ng/ml produced a deep blue dot, 20 ng/ml produced a light blue dot and a concentration >50 ng/ml produced a very faint blue dot, or none at all.

RESULTS AND DISCUSSION

Table 1 shows the distribution of OS concentrations, measured by EIA, in the 213 serum samples submitted for pregnancy testing. In 122 of the samples, OS concentrations were below 10 ng/ml, indicating that the mares were not pregnant if blood sampled at least 100 days after breeding. The remaining 91 samples all had OS concentrations >20 ng/ml indicating that the mares were pregnant.

TABLE 1: Distribution of oestrone sulphate (OS) concentrations measured by EIA in 213 horse serum samples submitted for pregnancy testing.

	Serum OS conc. (ng/ml)	Number of samples
Values indicative of non-pregnancy	<5	116
	6 to 10	6
	11 to 20	0
Values indicative of pregnancy	21 to 60	4
	61 to 100	9
	>100	78

Table 2 shows a comparison of the OS EIA and the dipstick test in determining pregnancy status. The 122 samples returning a 'not pregnant' diagnosis by the OS EIA also returned a 'not pregnant' diagnosis when assayed by the dipstick immunoassay. Of the 91 samples returning a 'pregnant' diagnosis by the OS EIA, 89 also returned a 'pregnant' diagnosis by the dipstick immunoassay. However, the remaining 2 samples returned a 'not pregnant' diagnosis by the dipstick assay, generating test dots with a

TABLE 2: Comparison of the number of mares diagnosed as pregnant (P) or not pregnant (NP) by the OS EIA and dipstick tests.

Nos. of NP and P mares as determined by OS EIA	Nos. of mares diagnosed as NP or P by the dipstick test	
	NP	P
122	122	0
P		
91	2	89

colour intensity indicative of an OS concentration of <10 ng/ml. These 2 particular serum samples had OS concentrations of 58 and 74 ng/ml, when measured by the OS EIA, which are relatively low for mares supposedly over 100 days pregnant, as most have concentrations >100 ng/ml (Table 1). Follow up of these 2 mares indicated that both were over 100 days pregnant at the time of sampling. Thus, the OS EIA had returned the correct diagnosis for both mares while the dipstick immunoassay had returned a 'false negative' diagnosis in each case.

Why 2 serum samples returned OS concentrations of <10 ng/ml by the dipstick immunoassay when the real concentrations were >50 ng/ml as determined by the EIA is unknown. The most likely explanation is that some component in these 2 serum samples interfered in the assay, causing the samples to behave as if they had much lower concentrations of OS than they actually had. Interference such as this is commonly classified as a 'matrix effect', the manifestations of which have been described in detail by Wood, 1991.

Overall, the results of this small trial indicate that the dipstick test may offer a practical alternative to a conventional EIA for measuring serum OS concentrations to diagnose pregnancy status in mares. For the 213 mares analysed in this study, the 'specificity' of the dipstick test (the probability that the test returns a negative result for non pregnant mares) was 100%. The 'sensitivity' of the dipstick test (the probability that the test returns a positive result for pregnant mares) was 97.8%. The positive predictive value of the test was 1, and the negative predictive value 0.98. At present the dipstick test is not quite as accurate as the OS EIA for diagnosing pregnancy status, because of the 2.2% incidence of false negative results returned by the dipstick test. However, the simplicity of the dipstick test and the speed at which the test can be performed might give it some appeal, despite the small incidence of false negative results. Nevertheless, alternative ways of formatting the dipstick test are currently being investigated to try and further reduce or eliminate the discrepancy between the 2 assays.

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