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Evaluation of 'CONFIRM' as a method to verify pregnancy in dairy cows

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ABSTRACT

CONFIRM is a recently developed test-kit for verifying pregnancy in dairy cows on the basis of the concentration of oestrone sulphate (OS) measured in a milk sample taken at least 120 days after mating/insemination. In this study, the performance of CONFIRM was evaluated using milk samples from non-pregnant cows, and cows 22-178 days pregnant. 8% of non-pregnant cows returned an incorrect 'confirmed' pregnant diagnosis. The percentage of pregnant cows returning a 'confirmed' pregnant diagnosis increased as pregnancy progressed. While only 42% of cows 22-119 days pregnant returned a 'confirmed' pregnant diagnosis, this value increased to 96% for cows ≥ 120 days pregnant. The diagnoses returned by CONFIRM were highly reproducible for non-pregnant cows, and cows 120 or more days pregnant. Analysis by CONFIRM of a milk sample taken at least 120 days after mating/insemination provides an accurate, non-invasive means of verifying pregnancy in dairy cows.

Keywords: CONFIRM; dairy cows; milk; pregnancy testing; oestrone sulphate; enzymeimmunoassay.

INTRODUCTION

Oestrone sulphate (OS) is a steroid metabolite produced by the foetal-placental unit in increasing amounts as pregnancy progresses in several species including cows, sheep, goats and pigs (Sasser & Ruder, 1987). Several studies have shown that measurement of the OS concentration in a milk sample taken 120 or more days after mating/insemination provides an accurate, non-invasive means of verifying the pregnancy status of dairy cows (Holdsworth *et al.*, 1982; Power *et al.*, 1985; Henderson *et al.*, 1992, 1994a, 1994b). This knowledge has led to the recent development of a diagnostic test-kit, named CONFIRM, for verifying the pregnancy status of dairy cows. The CONFIRM test is based on measurement by enzymeimmunoassay (EIA) of the concentration of OS in a milk sample taken at least 120 days after mating/insemination. If the milk concentration of OS is higher than that of a supplied reference standard, the cow from which the sample was obtained is diagnosed as 'confirmed' pregnant. If the milk OS concentration is less than, or equal to that of the reference standard, the cow is diagnosed as 'not confirmed' pregnant. The low concentration of OS in the milk of cows 'not confirmed' pregnant may be due to either (a) the cow being not pregnant; (b) days since mating/artificial insemination (AI) being less than 120 days; (c) days since fertilization being less than 120 days, i.e. pregnant to a bull later than the date of original AI/mating at which pregnancy was thought to have been established.

The objective of this study was to evaluate the performance of CONFIRM using milk samples from cows of known pregnancy status.

MATERIALS AND METHODS

The CONFIRM diagnostic test-kit

The CONFIRM test-kits were provided by Immuno-Chemical Products Ltd, Auckland, New Zealand. The kits

utilize a competitive EIA for OS and consist of the following: 96-well microtitre plates with the wells coated with an antibody to OS; OS-horseradish peroxidase (HRP) conjugate which serves as the steroid 'tracer'; a reference standard for discriminating between 'confirmed' and 'not confirmed' diagnoses; chromogen and buffers. The test was performed according to the instructions provided with the kit. Briefly, this involved adding 0.05 ml of a representative sample of whole milk or reference standard solution together with 0.1 ml of OS-HRP conjugate to an antibody-coated well. (Milk samples were assayed singly, and the reference standards in quadruplicate). The microtitre plates were then incubated for 2 h at room temperature. The wells were emptied, washed and 0.1 ml of chromogen solution added. The plates were incubated for 15 to 30 min at room temperature in the dark. Further colour development was then stopped by the addition of 0.05 ml/well of 2M H₂SO₄. Colour intensity was quantitated by measuring the optical density (OD) of each well at 490 nm using a microplate autoreader. Milk samples producing a lower OD value than the reference standard (i.e. indicative of a higher OS concentration) denoted a 'confirmed' pregnant diagnosis. Otherwise the diagnosis was 'not confirmed' pregnant.

In order to quantitate the actual concentration of OS in milk samples, a series of OS standards (0-2 ng/ml) was assayed together with the milk samples and reference standard in each microtitre plate. Standard curves were generated from the OD values produced by the standards, and the OS concentrations in the milk samples calculated by interpolation. The working range of the standard curve was from 40 pg/ml to 5ng/ml. Intra- and inter-assay coefficients of variation were <12%.

Milk samples

Milk samples were collected from 422 Jersey and Friesian cows of which 74 were non-pregnant and 348 were from 22 to 178 days pregnant. Pregnancy status was verified by manual palpation of the uterine contents 35 days or more after

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insemination, and eventually by reference to calving records. A single representative sample of whole milk was taken from 19 of the non-pregnant and 160 of the pregnant cows. Two milk samples, taken one week apart, were obtained from 55 of the non-pregnant and 188 of the pregnant cows. The milk samples were stored frozen prior to analysis by CONFIRM.

RESULTS

Figure 1 shows the geometric mean concentrations of OS in the milk samples from non-pregnant and pregnant cows as measured using the CONFIRM kit. The lowest mean value was found in milk from non-pregnant cows, and values then increased progressively as pregnancy advanced reaching a maximum at days 140-159. By 60-79 days of preg-

FIGURE 1: Concentrations of oestrone sulphate in milk from non-pregnant cows and cows 22 to 178 days pregnant measured using CONFIRM

Values are geometric means of 'N' samples with 95% confidence limits indicated by the vertical lines. Geometric mean values with different letter superscripts are significantly different ($P < 0.05$, analysis of variance in conjunction with Newman-Keuls multiple range test).

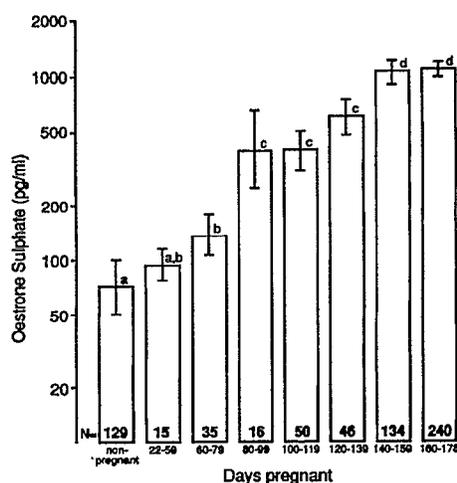
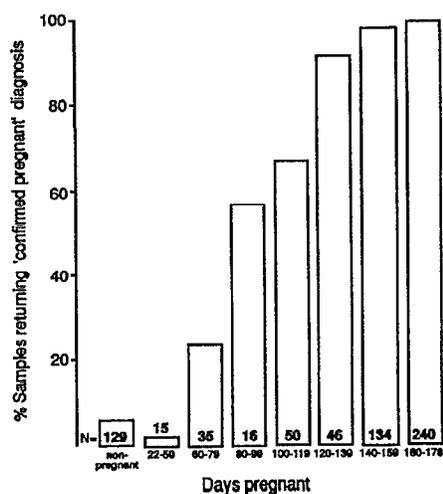


FIGURE 2: Percentage of samples from non-pregnant cows and cows 22 to 178 days pregnant returning a 'confirmed' pregnant diagnosis when analysed by CONFIRM.



nancy, the geometric mean concentration of OS was significantly higher in milk from pregnant cows than in milk from non-pregnant cows.

The percentage/proportion of milk samples returning 'confirmed'/'not confirmed' diagnoses, relative to cow pregnancy status is summarized in Fig. 2 and Table 1. 6% of samples from non-pregnant cows returned an incorrect diagnosis of 'confirmed' pregnant. The proportion of samples from pregnant cows returning a 'confirmed' pregnant diagnosis increased as pregnancy progressed with 97% of samples from cows 120 or more days pregnant returning a 'confirmed' pregnant diagnosis. Overall, 85% of samples from all pregnant cows (i.e. from 22 to 178 days pregnant) returned a diagnosis of 'confirmed' pregnant.

TABLE 1: Proportion of milk samples from non-pregnant and pregnant cows returning a 'confirmed' pregnant or 'not confirmed' diagnosis when analysed by CONFIRM

Actual pregnancy status of cows sampled	Proportion of samples returning a diagnosis of: confirmed pregnant	not confirmed
Non-pregnant	8/129 (6%)	121/129 (94%)
Days pregnant		
22-119	50/116 (43%)	66/116 (57%)
120-178	408/420 (97%)	12/420 (3%)
22-178	458/536 (85%)	78/536 (15%)

Two milk samples were taken from 179 of the 422 cows studied. Both milk samples returned the correct diagnosis in 50/55 of the non-pregnant cows and in 143/145 of the cows ≥ 120 days pregnant that were studied (Table 2). For cows 22 to 119 days pregnant, there was a relatively high percentage of disparate diagnoses (33%). Overall, depending on whether the first or second diagnosis was used for cows which were milk sampled twice, only 5 or 6 of the 74 non-pregnant cows studied were incorrectly diagnosed using CONFIRM. Similarly, of the 275 cows 120 days or more pregnant only 4% (10 or 12 cows) were mis-diagnosed as 'not confirmed'. For the 73 cows 22 to 119 days pregnant, either 40 or 42 cows were diagnosed as 'not confirmed'.

TABLE 2: Reproducibility of CONFIRM diagnoses

Cow pregnancy status	No. of cows in which both samplings diagnose:		Disparate diagnoses
	confirmed pregnant	not confirmed	
Non-pregnant	0	50	5
Days pregnant			
22-119	11	18	14
120-178	143	0	2

Two milk samples were taken 7 days apart from each cow. Disparate diagnoses are those in which one milk sample returned a 'not confirmed' diagnosis and the other a 'confirmed' pregnant diagnosis.

DISCUSSION

The CONFIRM test-kit utilizes a reference standard to discriminate between 'confirmed' and 'not confirmed' pregnant diagnoses. However, by including a series of OS stand-

ards in the assay, the actual concentrations of OS measured in milk by CONFIRM could be quantitated by interpolation off a standard curve. This demonstrated that the concentrations of OS measured by CONFIRM in milk from non-pregnant cows and cows at varying stages of pregnancy (Fig. 1) were very similar to those of several other studies where OS concentrations have been measured in cows' milk by either radioimmunoassay or EIA (Holdsworth *et al.*, 1982; Power *et al.*, 1985; Henderson *et al.*, 1992, 1994a, 1994b).

The CONFIRM test-kit displayed a high degree of accuracy in correctly verifying the pregnancy status of cows. For cows at least 120 days pregnant, 96% were correctly diagnosed as 'confirmed' pregnant while only 8% of non-pregnant cows were incorrectly diagnosed as 'confirmed' pregnant. CONFIRM was less suitable for verifying pregnancy in cows early in pregnancy. Only 43% of milk samples from cows 22 to 119 days pregnant returned a 'confirmed' pregnant diagnosis. However, during this early period of pregnancy it is not uncommon for OS concentrations in milk still to be in the range of values found for non-pregnant cows. Therefore it is recommended that milk samples for analysis by CONFIRM be obtained at least 120 days after mating/insemination. Overall, irrespective of the stage of pregnancy, 85% of pregnant cows returned a 'confirmed' pregnant diagnosis. For large dairy herd owners, using CONFIRM to screen all cows before drying off, irrespective of insemination/mating date, as a means of verifying pregnancy in 80% or more of cows may be advantageous. The remaining cows diagnosed as 'not confirmed' may then either be retested by CONFIRM at a later date, subjected to veterinary manual palpation of the uterine contents per rectum to verify pregnancy status, or managed separately from cows diagnosed as pregnant by CONFIRM.

The CONFIRM test itself is straightforward to perform, and a high throughput is possible. It is estimated that one technician together with an assistant to sort samples should be able to assay up to 4000 milk samples per week. The analytical equipment required to achieve this high throughput would be an automated microplate reader connected to a computer for collecting the data, and a spreadsheet for analysing the data and producing the final diagnostic report.

In summary, the results of this study indicate that analysis by CONFIRM of milk obtained at least 120 days after mating/insemination provides an accurate, non-invasive means of verifying pregnancy in dairy cows.

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