

Detection of early pregnancy in domestic ruminants

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Summary. Tests for the detection of pregnancy early after insemination have not yet reached their full potential. Currently, the milk progesterone assay provides the earliest possible test, at an interval of one oestrous cycle after insemination, i.e. 17, 21 and 21 days in sheep, goats and cows respectively. This assay is pregnancy non-specific and rate of detection of pregnant animals is acceptable but less than desirable.

Detection of activity of early pregnancy factor may develop into an excellent early test for many species, but the rosette inhibition test which is currently required has limited development and use. Pregnancy-specific protein B has been developed as a radioimmunoassay and is reliable under laboratory situations for ruminants. It can be used after 24 days of gestation in the cow. Application to field testing awaits development. Other pregnancy-associated or specific substances which are found in maternal body fluids might develop as pregnancy markers. Ultrasonic devices might provide very early detection in cattle but the expense of a test will limit application.

All tests for pregnancy early after insemination have an inherent inaccuracy. Presence of an embryo at the time the test is applied will not assure pregnancy at the time of a confirmatory test, such as birth of live young or rectal examination in cows after 35 days of gestation. Therefore, no matter how early the test, a follow-up examination might be desirable in intensively managed herds or flocks.

The animal industry is on the verge of new biotechnological approaches to reproductive management. The potential seems as great as the imagination.

Introduction

Pregnancy is routinely detected in cows by inserting the hand into the rectum and palpating through the rectal and uterine walls for fetal membranes, the amniotic vesicle or cotyledons within the uterus (Wisnicky & Casida, 1948). This can be accomplished by well-trained technicians by 35 days after insemination but accuracy is considerably improved by waiting until 40–50 days. In small ruminants, older methods have included rectal–abdominal palpation in sheep (Hulet, 1972) and ultrasound detection in sheep and goats (Hulet, 1969). These methods have not been sensitive enough to be accurate much earlier than 60–80 days after insemination.

Proteins produced by the placenta have been used to detect pregnancy for many years in certain species. For example, human chorionic gonadotrophin (hCG) was discovered by Aschheim & Zondek (1928) in urine of pregnant women and can be assayed in urine and blood as early as 8–10 days after conception (Marshall *et al.*, 1968). A host of other pregnancy proteins in the human have been found, some of which reach maternal serum and may be useful as pregnancy markers (Bohn, 1985). Pregnant mare serum gonadotrophin (PMSG) was first reported by Cole & Hart (1930) and measurement in serum was shown to be useful for detection of pregnancy (Cole & Hart, 1942). Other than in equids, reliable pregnancy markers have not been used to detect pregnancy in farm animals.

Several laboratories have reported the presence of pregnancy proteins in cattle and sheep. Rowson & Moor (1967) found that sheep embryos of 13 days of age produced a heat-labile substance which appeared to be responsible for maintenance of the corpus luteum. Similarly, the

embryo of the cow affects luteal maintenance (Northey & French, 1980; Humblot & Dalla Porta, 1984). This effect is probably antiluteolytic in action and is caused, at least in part, by trophoblastin (Martal *et al.*, 1979) or ovine trophoblast protein-1 (OTP-1) or other conceptus secretory proteins in sheep (Godkin *et al.*, 1984). Uterine flushings from ewes more than 14 days pregnant contained three proteins not found in serum (Roberts *et al.*, 1976). A heat-labile protein with a molecular weight greater than 8000 was found in serum of pregnant ewes (Cerini *et al.*, 1976a, b). These studies were extended by Staples *et al.* (1978). When first found, this protein showed promise of being useful for pregnancy detection.

In the cow, Roberts & Parker (1976) detected three proteins which appeared in uterine flushings beginning on Day 7 of gestation. Laster (1977) found a pregnancy-specific protein with a molecular weight of 50 000–60 000 in the cow uterus at Day 15. Butler *et al.* (1982) reported a protein of similar molecular weight which could be measured in sera of pregnant cattle (Sasser *et al.*, 1986). This protein is termed pregnancy-specific protein B (PSPB). Several proteins, including bovine trophoblast protein 1 produced by cow conceptuses in culture, have also been reported (Bartol *et al.*, 1984; Knickerbocker *et al.*, 1984) and are known to extend luteal life-span, probably through an antiluteolytic role, when infused into the uterus of cyclic cows. Reviews on these proteins have been published (Bazer *et al.*, 1985; Thatcher *et al.*, 1985). Future work may show that several of these secretory products can enter the circulation of the mother and can be measured in body fluids, thus permitting the detection of pregnancy.

For a test of pregnancy to be useful in livestock management it should detect pregnancy before the time of next expected oestrus after insemination. Unfortunately, the classical established techniques that were mentioned above for cattle and sheep cannot be used that soon after insemination. The only test in practice that approaches this is the assay for progesterone in milk or serum. This can be used to confirm the absence of a corpus luteum and therefore non-pregnancy near the time of next expected oestrus. Other tests such as real-time ultrasound, early pregnancy factor (EPF) in serum (Morton *et al.*, 1979a) and pregnancy-specific protein B (Sasser *et al.*, 1986) are not routinely used in practice but may develop into appropriate early tests. Measurement of oestrone sulphate can probably provide a convenient and reliable test later in pregnancy (Holdsworth *et al.*, 1982). This review will consider only the ultrasonic and more recently described biochemical tests for early pregnancy detection.

Ultrasonic detection of pregnancy

Ultrasonic detection of pregnancy has been used with various degrees of success depending on the type of instruments that are used. A-mode is a one-dimensional display of echo amplitude versus distance. B-mode produces a two-dimensional image of soft-tissue cross-section. Real-time ultrasound scanning is a modification of B-mode which utilizes a number of crystals within the transducer head. The result is a moving two-dimensional image that can be displayed on a video monitor (Reeves *et al.*, 1984). This method was tested by Memon & Ott (1980) for accuracy in ewes and goats. In general, externally applied transducers that did not employ real-time were able to detect pregnancy accurately in the last half of gestation. Intrarectal probes permitted slightly earlier detection but were more labour intensive.

The image provided by real-time scanning allows for a more precise and accurate decision by the operator as to the state of pregnancy. This has resulted in an overall accuracy of 97.1% (5370 correct scans/5530 total scans) in sheep. Ewes that were less than 40 days pregnant were too early to diagnose and were not included in the analysis (Fowler & Wilkins, 1984). Earlier detection has been reported (Tainturier *et al.*, 1983) but more time per animal is required and numbers of animals in the study were limited.

Probes for transrectal use have been applied in pregnancy detection in horses as early as 14 days (Chevalier & Palmer, 1982; Simpson *et al.*, 1982; Rantanen *et al.*, 1982). The same instrument that

was used by Rantanen *et al.* (1982) was used by Reeves *et al.* (1984) for transrectal probing for pregnancy in cattle. Pregnancy was detected as early as 28 days after insemination and they attributed the difference in time of detection to a difference in anatomy of the reproductive tracts. Pierson & Ginther (1984) were able to detect the bovine embryonic vesicle by 12–14 days, and the fetus and fetal heartbeat at 26–29 days. In 32 cows scanned at regular intervals between 20 and 140 days of gestation, pregnancy was detected with confidence at 30 days (White *et al.*, 1985).

The use of real-time scanning in practical farm situations is limited by the cost of the instrument. It is likely that less expensive equipment will become available for use in cows, sheep and goats.

Biochemical detection of pregnancy

Biochemical detection of pregnancy relies upon detection of substances that arise in the maternal system when an embryo is present in the uterus. Pregnancy-specific substances are produced by the conceptus. A qualitative assay in body fluids may be sufficient for pregnancy detection. Pregnancy-associated substances are produced by the maternal system but generally are increased in quantity when a conceptus is present. In this case a quantitative assay may be more important. The pregnancy-specific substances would include hCG and PMSG and a pregnancy-associated substance would be milk or serum progesterone. Several such proteins have been listed by Chard (1985) for humans.

Biochemical markers for pregnancy in farm animals are either steroidal or proteinaceous. Those which may be used effectively are progesterone and oestrone sulphate in the former category and early pregnancy factor (EPF) and pregnancy specific protein B (PSPB) in the latter. These will be discussed in more detail.

Steroids

Progesterone. Detection of pregnancy by analysis of plasma (Shemesh *et al.*, 1968) or milk (Laing & Heap, 1971; Lamming & Bulman, 1976; Shemesh *et al.*, 1978) for progesterone content relies upon the decline in concentration at the time of the next expected oestrus if conception does not occur after insemination. A low progesterone concentration suggests that luteolysis has occurred (non-pregnant) and a high content suggests that luteal maintenance had occurred in response to stimuli of the conceptus. Low progesterone concentrations predict oestrus or non-pregnancy (Booth, 1979; Shemesh *et al.*, 1978) with a high degree of accuracy. However, high progesterone concentration is not as reliable a predictor of pregnancy since luteal life-span can vary and samples may be collected too early or too late with respect to luteolysis in non-pregnant animals. Early embryonic death after the sample is collected and before rectal examination at about 50 days after insemination also contribute up to 8% error in the test (Cavestany & Foote, 1985).

Radioimmunoassay (RIA) for milk progesterone for pregnancy detection has been reported several times. Such assays are in use today but more recently enzyme immunoassays have been developed. The accuracy of certain tests in cattle and goats are presented in Table 1. The day of milk sampling for cattle ranged from 20 to 25 days after insemination and is 21 days for the goat. In general the optimum time is at 23, 24 or 25 days for the cow. Two studies (Cavestany & Foote, 1985; Marcus & Hockett, 1986) showed improvement in accuracy of predicting pregnancy by sampling milk at the time of insemination and again in 3 weeks; both values are then used in the evaluation of pregnancy status.

All assays have been adequate in detecting non-pregnancy. Most values approached 100% and so low milk progesterone concentration is a good indicator that luteolysis has occurred and that the conceptus was absent. Detection of pregnant cows is generally less than 85% accurate and ranges from 60 to 100%. The exceptional RIA with 100% accuracy (Shemesh *et al.*, 1981) utilized progestagen-impregnated vaginal sponges for the purpose of reducing variability in time of return

Table 1. Accuracy of the milk progesterone test (radioimmunoassay (RIA) or enzyme immunoassay (EIA)) for pregnancy detection

Reference	Type of assay	No. of samples	Day of sampling after insemination	Detected pregnant correctly (%)	Detected not pregnant correctly (%)
Hoffman <i>et al.</i> (1974)	RIA	168	20	77	100
Heap <i>et al.</i> (1976)	RIA	176	24	85	100
Pennington <i>et al.</i> (1976)	RIA	116	21	73	98
Shemesh <i>et al.</i> (1981)	RIA				
Group A		96	21-24	79	100
Group B*		50	21-24	100	100
Montigny <i>et al.</i> (1982)	RIA	275 (goats)	21	91	97
Nakao <i>et al.</i> (1982)	EIA	268	20, 21 or 22	60	100
Chang & Estergreen (1983)	EIA	115	22	79	98
Cavestany & Foote (1985)	RIA				
Group A		313	23 or 25	73	99
Group B		313	0 & 23 or 25†	84	100
Marcus & Hockett (1986)	EIA	152	0 & 21†	96	90
Sauer <i>et al.</i> (1986)	EIA	110	24	94	100

*Cows were treated with progestagen-impregnated vaginal sponges from 6 to 17 days after insemination.

†Assays at insemination and at an interval of one oestrous cycle later.

to oestrus and so if a conceptus was absent low milk progesterone would be more likely on the day of milk sampling.

The enzyme immunoassays (EIA) have been as successful as RIAs with the exception of one recent assay by Sauer *et al.* (1986) which detected 93.5% of the pregnant animals. The other EIA, which reported 96% accuracy, required two samplings, one at time of insemination and the other 21 days later (Marcus & Hockett, 1986). A value was not reported for success based upon only the 21-day sample but the authors did not believe it was adequate.

Accuracy of overall detection of pregnancy by RIA of progesterone in milkfat of goats was 90.5% while that on whole milk was 85.4%. Concentration of progesterone in whole milk and in milkfat of pregnant goats was 5 times and over 10 times greater respectively than that of non-pregnant goats on Day 21 after insemination. Milkfat is the preferred medium for analysis in goats (Montigny *et al.*, 1982). Similar success in detecting pregnancy in sheep with the serum progesterone test has been reported (Shemesh *et al.*, 1973). An improvement of accuracy was obtained with RIA for serum progesterone by taking multiple samples in animals without known breeding dates (Döbeli & Schwander, 1985). Three samples were collected at 6-day intervals beginning 9 days after the removal of rams from the flock. Accuracy in predicting pregnancy or non-pregnancy was 98.8% and 100% respectively.

The milk or serum progesterone assay has come into greater use with the availability of reliable commercial assay kits. EIAs permit safe, simple-to-conduct, laboratory or on-the-farm evaluation of milk progesterone for pregnancy detection. Disadvantages are that insemination dates must be known to obtain results from a single sample and, since progesterone is not pregnancy-specific, incorrect positive detection can occur.

Oestrone sulphate. This substance is primarily produced by the fetal-placental unit and enters the circulation of the mother. Oestrone sulphate in sera of pregnant pigs was elevated over that of non-pregnant pigs between 20 and 29 days of gestation (Robertson & King, 1974) and can provide an early test for pregnancy. However, in sheep the increase in serum does not occur until 70-90 days of gestation and can be measured at 100 days as a late test for pregnancy (Thimonier *et al.*,

1977). It has been used for late pregnancy detection in goats after 60 days of gestation and was 96% accurate in pregnant (N=260) and 98% accurate in non-pregnant (N=60) does (P. Humblot, unpublished). In the pregnant cow, concentrations of oestrone sulphate in milk were increased above non-pregnancy levels by 100 days of gestation and remained high throughout the remainder of gestation (Holdsworth *et al.*, 1982). An EIA in microtitre plates has been developed for rapid solid-phase measurement of oestrone sulphate in bovine milk without extraction. The assay was 99 and 86% accurate in predicting pregnant and non-pregnant animals respectively (Power *et al.*, 1985). Assay of milk oestrone sulphate is of significance and is mentioned herein since it would provide an easy, late, reliable test for confirmation of pregnancy. A serum test might be particularly advantageous for small ruminants for which insemination dates are not known.

Proteins

Early pregnancy factors. EPF was first identified in pregnant mice by its ability to inhibit rosette formation between T lymphocytes and heterologous red blood cells. A rosette inhibition test is a useful assessment of lymphocyte activity (Morton *et al.*, 1976). When antilymphocyte serum is added to lymphocytes before the addition of red blood cells, the inhibition of rosette formation can reach 25%. The titre (rosette inhibition titre, RIT) at which an antilymphocyte serum can inhibit rosette formation does not vary significantly when using lymphocytes from normal animals, but if lymphocytes are subjected to in-vivo immunosuppressive agents the formation of rosettes occurs at a much higher dilution of antilymphocyte sera (Shaw & Morton, 1980).

The RIT has been used to detect early pregnancy in sheep (Morton *et al.*, 1979a, b) and cows (Nancarrow *et al.*, 1981). A positive test was achieved for sera of sheep from 72 h after insemination to 16 weeks of gestation and then it was no longer detectable (Morton *et al.*, 1979a). Only in pigs has EPF activity been found throughout pregnancy (Morton *et al.*, 1983).

An excellent review of use of EPF in detecting pregnancy in farm animals has been presented by Koch & Ellendorff (1985). In this detailed study to evaluate the RIT assay for sera of pigs, they showed that it was possible to detect non-pregnancy in 91.4% and pregnancy in 55.6% of pigs. Using data of three tests on the same samples of sera, the respective values were 91.4% for non-pregnancy and 87.8% for pregnancy detection.

The RIT test is time consuming and difficult to maintain. Development of a RIA or EIA for EPF would probably provide a more reliable test. This will first require chemical isolation of EPF. Progress on this has been reported (Wilson *et al.*, 1984; Clark & Wilson, 1985). Until the assay is improved one must withhold judgement as to the potential use of EPF in detection of pregnancy in farm animals (see Ellendorff & Koch, 1985).

Pregnancy-specific protein B. This protein was isolated and partly purified by us at the University of Idaho (Butler *et al.*, 1982). The protein was found after immunization of a rabbit with homogenates of placenta, collection of rabbit antisera and adsorption of antisera with somatic tissues to remove antibodies to proteins not specific to the placenta. Remaining antibodies were against alpha-fetoprotein and PSPB of the placenta. Immunoelectrophoresis and immunodiffusion techniques using the adsorbed antisera were used as markers in chemical isolation of PSPB. Placental extracts were subjected to ion-exchange and gel-filtration chromatography and isoelectric focussing. A preparation of PSPB (R-37) was used to immunize rabbits to obtain an antiserum for development of a double-antibody radioimmunoassay (Sasser *et al.*, 1986). It was established that the assay was specific for bovine PSPB with minimal cross-reactivity (<0.05%) with bovine LH and ovine FSH and partial cross-reactivity with ovine placental lactogen (<0.05%). There was no cross-reactivity with hCG, PMSG, bovine PRL, bovine TSH or bovine growth hormone (Fig. 1). Pools of sera from rams, wethers and steers and non-pregnant and pregnant ewes were assayed in

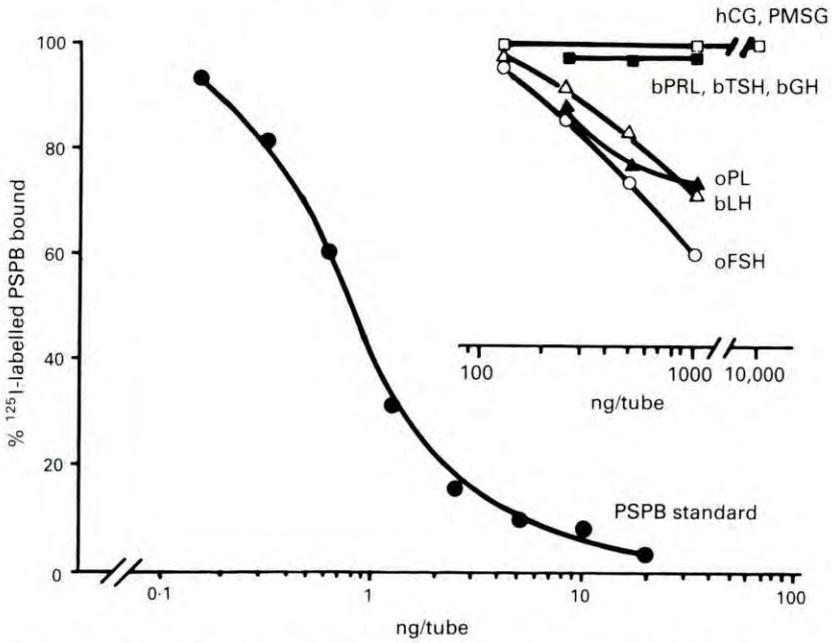


Fig. 1. Cross-reactivity of various pituitary and placental hormones with antiserum to the PSPB standard. The amount of radiolabelled PSPB bound to antiserum in tubes containing no PSPB was designated as 100% ¹²⁵I bound. Note the location of the continued x axis. (From Sasser *et al.*, 1986.)

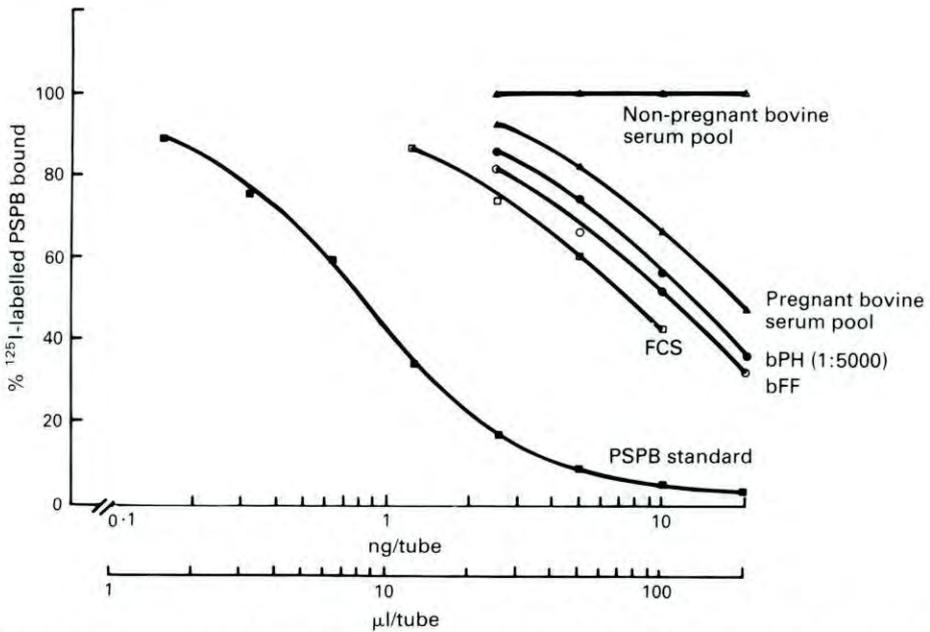


Fig. 2. Cross-reactivity of fetal calf serum (FCS), bovine fetal fluids (bFF), bovine placental homogenate (bPH, diluted 1:5000) and pools of sera with antiserum to the PSPB standard. (From Sasser *et al.*, 1986.)

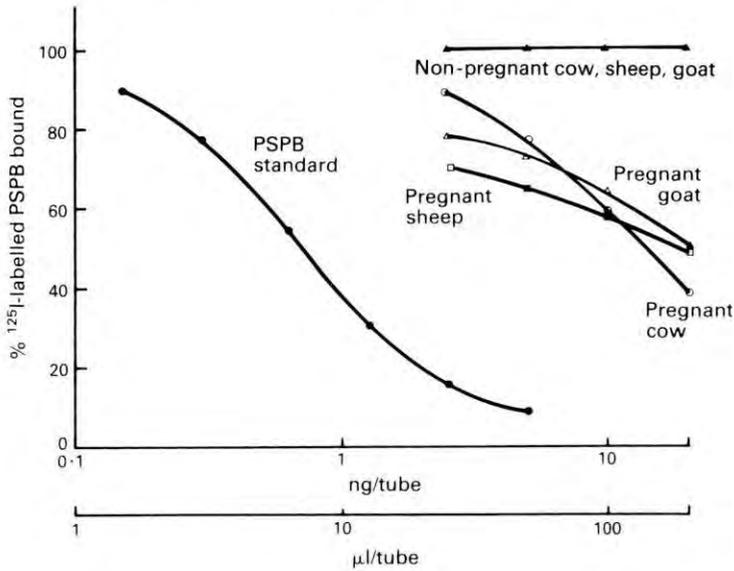


Fig. 3. Cross-reactivity of sheep and goat sera with antiserum to the PSPB standard.

the PSPB RIA. There was no PSPB in ram, wether, steer or non-pregnant ewe sera. Pools of sera from pregnant cows inhibited binding in a manner parallel to the standard curve, whereas serum pools from non-pregnant cows did not. Also, bovine fetal fluids, supernatant from placental homogenate and fetal calf serum resulted in an inhibition curve that was parallel to that of the standard. This indicated that the isolation procedure resulted in a protein similar to the native protein (Fig. 2). Pregnant ewe and goat sera contained cross-reacting antigens but the inhibition curve was not parallel to the standard curve (Fig. 3). These data suggest that not only could PSPB be measured in serum of pregnant cows, but that it was pregnancy-specific and, therefore, the RIA might be useful for pregnancy detection.

For PSPB to be considered as a pregnancy marker it would be desirable that it be present throughout pregnancy. To determine this, 5 dairy cows were bled twice weekly from mating until 21 days *post partum*. PSPB was detectable from about 24 days after insemination until 21 days *post partum* (Fig. 4). To confirm the time that PSPB first appeared in maternal serum, 21 cows were bled daily from 15 to 30 days after insemination. PSPB was present in sera of 3 cows at 15 days and by 24 days 12 had PSPB in the sera; 3 more acquired PSPB by Day 28 but were non-pregnant by rectal palpation at 45 days after insemination and had long interoestrous intervals while the former 12 cows were confirmed pregnant. PSPB could not be detected in the serum of the remaining cows and these animals were confirmed as non-pregnant at 45 days after insemination.

We have found that PSPB remains in maternal serum for several weeks after parturition. To determine whether there was continued secretion of PSPB after parturition (Ruder & Sasser, 1986), 5 cows were hysterectomized on Day 21 after parturition and 5 other cows served as intact controls. Blood was collected from parturition until Day 53 *post partum*. Half-life for PSPB in control and hysterectomized cows was 8.4 days and 7.3 days ($P=0.04$) respectively from Days 21 to 53 *post partum*. These results showed that PSPB has a long half-life and is released from the uterus after parturition or, on the other hand, the uterus could alter metabolism of PSPB resulting in an increase in half-life. Continued secretion is not likely since immunocytochemical studies have shown that PSPB is localized in (Eckblad *et al.*, 1985) and secreted from (Reimers *et al.*, 1985) the binucleated cells of the trophoblastic ectoderm.

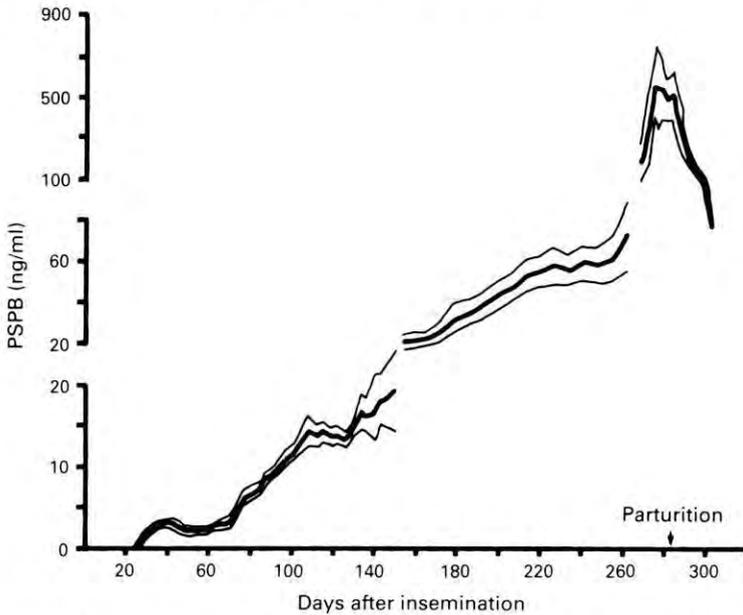


Fig. 4. Concentration (mean \pm s.e.; heavy and light lines, respectively), of PSPB in serum of 5 dairy cows from insemination until 21 days after parturition. Note the three scales of the y axis. (From Sasser *et al.*, 1986.)

The long half-life could pose a major limitation on this method of pregnancy detection if cows are to be examined before 80 days *post partum*. This would not usually be the case since cows are rarely inseminated before 55 days *post partum* and PSPB would not be detectable until 24 days later for a total of 79 days *post partum*. A major advantage of this method is that insemination dates are not required because PSPB is pregnancy-specific and is present continually after 24 days of gestation.

Pregnancy detection by RIA of PSPB in sera was highly accurate in a commercial herd of beef cows. At first examination 99 cows were detected pregnant by PSPB assay; 98 calved and 3 of 4 non-pregnant ones were accurately detected. Serum samples were collected on the 27th day after the end of a 75-day breeding period. There was an exceptionally high number of pregnant animals in this study and it was impossible to evaluate accuracy in non-pregnant cows. However, we have shown that RIA of serum for PSPB detected 177 of 187 non-pregnant cows from which uteri were examined for presence of a conceptus at slaughter. In detection of pregnant animals the RIA predicted 189 of 191 cows. Cattle had been inseminated 25–61 days before slaughter (Maurer *et al.*, 1985).

We have shown that assay for PSPB is a reliable serological test for pregnancy in sheep. It was as accurate as abdominal real-time scanning at 35 days of gestation. Real-time ultrasound predicted pregnancy in 163 and non-pregnancy in 17 ewes while RIA predicted 161 and 19 respectively; 159 ewes gave birth and 21 did not (Ruder *et al.*, 1984). Although there are cross-reacting antigens in sera of pregnant goats, we have not conducted a study of the accuracy of assay for PSPB in pregnancy detection for these animals.

These are the first experiments to demonstrate a simple, accurate and specific serological test for pregnancy in cattle and sheep. In cattle pregnancy can be detected as early as 24 days after insemination until parturition. Insemination dates are not needed as they are for measurements of progesterone in milk or serum or EPF in serum. The assay can be applied to other ruminants as

well (Sasser *et al.*, 1985; Wood *et al.*, 1986) and it will probably be as versatile as the pregnancy-specific chorionic gonadotrophin tests in humans (Marshall *et al.*, 1968).

Development of an animal-side test

Recent developments in diagnostic testing have made it possible to have reliable, sensitive and accurate assays that do not require sophisticated equipment. Approaches to use of non-radioactive markers in immunological assays are presented by Ngo & Lenhoff (1980). These assays utilize enzymic reactions resulting in development of a colour. Colour is monitored with an instrument (spectrophotometer) or visually. Enzyme immunoassays are of two types. The heterogeneous ones require physical separation of the unbound antigen while homogeneous assays do not (Rubenstein *et al.*, 1972). In the latter, total activity can be measured without prior separation and exploits the ability of the sample antigen to modulate an immunospecific signal.

Enzyme immunoassays can be immobilized on an inert support (Zuk *et al.*, 1985) for testing in less technical laboratories or in the field. Such assays are currently available for analysis of progesterone in milk or serum. Any substance that becomes a pregnancy marker could easily be assayed by this method. In addition, quantitative assay on test-strips (Zuk *et al.*, 1985) may provide an added advantage in predicting the stage of pregnancy if the antigen in question varies considerably throughout pregnancy.

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