Dairy Sessions

Moderators: Scott Smith, Chris Malherbe

BioPRYN®, a Measure of Pregnancy-specific Protein B for Detection of Pregnancy in Ruminant Animals

RG Sasser, *PhD;* **J Branen,** *PhD;* **J Howard,** *MS;* **C Passavant,** *PhD;* **D Pals,** *PhD*
BioTracking LLC, Alturas Technology Park, 1150 Alturas Drive, Moscow, ID 83843

Abstract

Pregnancy-specific protein B (PSPB) was discovered in 1979 by immunological methods and was found by molecular cloning to be a sub-group of the aspartic acid protease family of proteins. The original protein isolate had several immunoreactive, molecular weight, and isoelectric variants. Chemical characteristics were studied. Subsequently, similar protein isolates of PSPB of cattle were given a different name. These are pregnancy-associated glycoprotein (PAG) and pregnancy serum protein 60. Precise and accurate radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA) for PSPB have been used to detect pregnancy in ruminant animals. BioPRYN is an ELISA assay, using the PSPB protein isolate, for detection of pregnancy in ruminants. Radioimmunoassay or ELISA (the BioPRYN test), specifically using PSPB as the molecule for standard and antigen for obtaining antibodies, equaled or exceeded transrectal sonography, PAG-1 or palpation in comparative tests for pregnancy detection. Development of tests for molecular variants that appear early in gestation can improve on the technology. BioPRYN is available on a commercial basis for livestock breeders or those managing wildlife species. Several affiliate laboratories across the USA and internationally conduct the BioPRYN test with kits offered by BioTracking LLC, Moscow, Idaho. Over 498,115 cattle tests were sold in 2008.

Résumé

La protéine B spécifique de la gestation (PSPB, en anglais) fut découverte en 1979 par technologie immunologique. Le clonage moléculaire a montré que cette protéine appartenait à un sous-groupe de la famille de la protéase de l'acide aspartique. L'isolat original de cette protéine montrait plusieurs variantes sur le plan de la réaction immunitaire, du poids moléculaire et de la propriété isoélectrique. Ses caractéristiques chimiques ont été étudiées. Par la suite, on a donné des noms différents à des isolats de protéines similaires à la protéine B (PSPB) des bovins; il s'agit de la glycoprotéine associée à la gestation (GAG) et de la protéine 60 du sérum gestationnel. Des tests de dosage radio-immunologique (DRI) et immunoenzymatique (ELISA) d'une grande précision ont servi à détecter la gestation chez les ruminants. À cette fin, il existe notamment le BioPRYN, un test ELISA qui utilise un isolat de protéine B (PSPB). Des tests comparatifs de détection de la gestation ont révélé que le dosage radio-immunologique ou le dosage ELISA (test BioPRYN), en particulier avec la protéine B (PSPB) comme molécule standard et antigénique pour obtenir des anticorps, égalaient ou dépassaient en précision l'échographie transrectale, la glycoprotéine GAG-1 ou la palpation. La mise au point de tests utilisant des variantes moléculaires de ces protéines apparaissant plus tôt dans la gestation pourrait améliorer cette technologie. Le test BioPRYN est vendu commercialement aux éleveurs de bétail ou aux gestionnaires de la faune. Plusieurs laboratoires affiliés de tous les États-Unis et dans le monde effectuent le test BioPRYN, dont la trousse est offerte par BioTracking LLC, de Moscow, en Idaho. Plus de 490 115 tests pour bovins ont été vendus en 2008.

Introduction

Pregnancy-specific protein B (PSPB) was discovered in 1979,[16] more were identified in 1980,[3] and was published in a peer-reviewed publication in 1982.[4] This began a new discipline in reproductive biology[41,46] which led to naming of a new molecule, development of a new method of testing for pregnancy in ungulate animals, and development of a new market through development of BioPRYN. BioPRYN is a system of sampling blood, submitting samples to a laboratory, laboratory analysis of serum for presence of PSPB, and reporting results to the veterinarian or manager of livestock. This paper will describe the molecule, the pregnancy test, and the marketing.

History of PSPB and Subsequently Named Proteins

Pregnancy-specific protein B (PSPB) was discovered over 30 years ago through the research program of R. Garth Sasser in the Department of Animal and Veterinary Science at the University of Idaho. Work began in 1976 and was first published in a Masters thesis by William Hamilton in 1979.[16] This work used immunological methods for detection of antigens in bovine embryonic membranes from embryos of ages 25 to 35 days. Rabbits were immunized with whole homogenates of embryonic membranes, and anti-sera were then screened for antibodies that were specific to the membranes and not embryonic or adult tissues. These antibodies were used by Butler[3] to isolate and identify two antigens (pregnancy-specific protein A and B) that were not in tissues other than membranes. One antigen (pregnancy-specific protein A) was determined to be alpha fetoprotein, a previously identified substance, and the other was novel and unique to the membranes. The research in these two theses, in addition to other research, was published in 1982.[3]

A radioimmunoassay was developed for measurement of PSPB (preparation R37) and was published as a Masters thesis by Kris Ivani[16] and as a peer-reviewed article by Sasser *et al.*[37] These two publications showed for the first time that an antigen of the placenta could be measured in blood of pregnant cattle, and that it provided an excellent specific test for pregnancy.

The Research Connection Between PSPB and Two Other Protein Isolates

Sasser introduced two other groups to PSPB and each group isolated a PSPB preparation. Subsequently, the protein preparation from each group was given an alternative name to PSPB.

One introduction resulted from a sabbatical leave by Sasser at the laboratory of Dr. Jaques Martal in France (1986). Antisera (RGS 38-1) and the RIA of Sasser was used to isolate PSP60 (60 referring to the molecular weight estimate of a variant) by high-performance liquid chromatography. This work resulted in a joint abstract presentation.[5] The other introduction was through research discussions with Dr. Francois Beckers at the above French laboratory and followed by another discussion and presentation at Beckers' laboratory.[34] Protein preparations and antisera were sent to Sasser for testing. Data were subsequently published[54] describing an aspartic acid protease isolate which was newly named pregnancy associated glycoprotein (PAG). These two protein isolates were very similar to PSPB in that they yielded the same N-terminal amino acid sequence,[28,29] although different molecular weights were reported. Sasser cloned a gene from the multi-variate PSPB isolate[28] that contained the identical nucleotide sequence to one cloned by Roberts' laboratory in Missouri[52] using the above preparation of PAG.[55] The identity is not surprising, since methods for obtaining protein isolates were similar for both groups as were the same N-terminal amino acid sequences. The successfully cloned portion from the PSPB preparation was identified as having sequence homology to the aspartic acid protease family of proteins.

Chemical Characteristics of PSPB

Preliminary biological and chemical characteristics of the PSPB isolated by Butler *et al*[4] and which were used for the radioimmunoassay (RIA)[37] were described by Sasser *et al.*[38] The protein preparation was labeled PSPB R37. This publication also describes PSPB R62 (preparation R62) that was extracted from 60-day to 100-day or later bovine cotyledons and purified with an immuno-affinity column containing antisera 38-1 which was obtained using the R37 PSPB as the antigen. Another preparation of PSPB was produced in culture by 90-day bovine cotyledons. All immunological characterizations were done using antiserum 38-1, the same antibody that was used in the RIA pregnancy test.[37] In addition, SDS-PAGE was done under non-reducing conditions for the molecular weight estimate of variants within these preparations of PSPB.[38] Non-reduced and reduced SDS-PAGE resulted in different estimates for molecular weight. For example, non-reduced molecular weight estimates of three major variants in the PSPB R62 were 78,000, 85,000, and 90,000 Daltons, and had respective reduced estimates of 64,000, 70,000, and 78,000 Daltons (Sasser, unpublished), with the 64,000-Dalton variant being the major band in the preparation. The molecular weights for the reduced forms will be used herein. The major band in the PSPB R37 preparation had a molecular weight of 64,000 Daltons (and was the major radio-iodinated band after purification for use in the radioimmunoassay), substantial variants of 70,000 and 78,000 Daltons and unpublished minor variants of 58,000, 45,000, and 32,000 Daltons. The several molecular weight variants were all reactive by Western blotting with the antiserum 38-1. Additionally, 2D gel electrophoresis of the 64,000 Dalton band of PSPB R37 (and of cultured bovine cotyledons, see below) demonstrated that there were seven different isoelectric variants within the isoelectric points (pI) of 4.0 to 4.4. Two variants with pI's of 4.2 to 4.25 were most abundant. Peptide maps of these isomers were similar, indicating that variants are charge isomers rather than different proteins. The 64,000 Dalton band contained ~5% hexose sugars and ~3% sialic acid.

The PSPB in culture media of the cotyledon incubates had molecular weight variants of 78,000, 70,000, 64,000, 53,000, 45,000, and 35,000. A pulse-chase experiment using ovine placentae[53] showed that the larger molecules are formed first, with subsequent cellular processing into lower molecular weight variants. Characterization of an ovine PAG-1 has also been described.[53]

Several Expressed Clones of PSPB

An original PSPB variant was cloned,[28] as was one named PAG-1.[52] Both clones had the exact nucleotide sequence. This has been followed by cloning of several other PAG's of the bovine (exceeding 20 in number) and ovine placenta.[54] Antiserum 38-1 against the original PSPB was used to find that PSPB was localized in the binucleated cells of the trophectoderm of the bovine placenta.[7] Later, Zoli *et al*[56] showed the same for PAG. In addition, Reimers[32] developed enriched fractions of binucleated cells and showed that they secreted PSPB into culture media. Other forms are expressed throughout the trophectoderm.[15] These include bovine PAG-2, -8, -10, and -11. Forms expressed by binucleated cells of the trophectoderm include bovine PAG-1, -6, and -7 after 45 days of age of the placenta. Although one clone[28] in the multivariant PSPB R37 preparation is the same as PAG-1 and may not be expressed abundantly in early placenta,[15] other variants that were expressed throughout pregnancy or at selected other times were present in the PSPB R37. Evidence for this is based upon chemical characterization, as described above, and the ability of the RIA, using antisera against this PSPB preparation, to detect pregnancy as early as 15 days until the end of pregnancy.[37] It has been proposed that there are over 100 trophoblast-expressed genes in the bovine.[53] Recently, a new clone, termed boPAG-22, of the Day 18 bovine conceptus was found.[12] The protein products of boPAG-22, or other identified or unidentified clones, may have been detected by the RIA for PSPB in maternal blood as early as 15 days of gestation.

Summary of Chemistry of PSPB

The PSPB R37 preparation originally isolated[4] and used for the RIA[37] contains several variants of immunoreactive proteins that are specific to ruminant pregnancy. Sasser *et al*[38] showed that the aspartic acid protease (PSPB, PSP60 and/or PAG) contained several molecular weight and isoelectric variants. Heterogeneity of the PSPB protein fraction is likely due to variation in post-translational modifications (i.e. glycosylation) and differences in amino acid sequence depending on the age of conceptus used for isolation of the proteins. Molecular cloning has provided new insight into the various specific gene products that could be isolated in a PSPB fraction during different stages of pregnancy.[15] As a result, differences in isolation approaches and age of tissue used for isolation explain considerable differences in chemical identity of various isolates of PSPB by different groups.

Assay of PSPB

Radioimmunoassay

The first publication of data using the RIA is shown in Table 1. These data are taken from Mauer *et al*.[30] The cows had been bred from 28 to 60 days previously and uteri and blood samples were collected at slaughter. Uteri were examined for presence of a conceptus. Of course, this examination is the most direct means of testing for pregnancy. No test is better for determining pregnancy, not ultrasound or palpation, and this was deemed the true value. The sera and uteri were collected simultaneously. A fault of many studies is that the comparative analysis was done at a different time, often because palpation cannot be done as early as ultrasound or PSPB RIA. The serum was analyzed for presence of PSPB. The sensitivity of the serum test was 99% and the specificity was 94.7%. The test correctly placed most pregnant cows in the pregnant category and called two pregnant cows open, for a sensitivity of 99%. Some with tissue of dead embryos also tested positive for PSPB in

Table 1. Sensitivity and specificity of the blood test by radioimmunoassay for PSPB from 28 to 60 days after breeding in cattle that were blood sampled at the time of visual pregnancy detection at the time of slaughter (From Mauer *et al*[30]).

Cattle examined at slaughter; n = 378			
Uterine		Blood	
Status	Number	Number	% of uterine
Pregnant	191	189	99 (Sensitivity)
Not pregnant	187	177	94.7 (Specificity)

serum, thus reducing the specificity value. These data show that the blood test is highly accurate and is safe to use in management of livestock since pregnant cows are categorized correctly, are rarely called not pregnant, and therefore are not given hormones to induce a new ovulation. Such treatments would induce abortion if cows were pregnant.

In another study,[37] some cattle had PSPB in the circulation as early as 15 days after breeding and by 28 days, 15 of 15 pregnant animals had the protein in the serum. A similar success was presented when 177 cows and heifers were tested.[38] At 24, 26, 30-35, and 70 days after breeding, 86, 88, 90, and 99%, respectively, that tested pregnant by RIA were also pregnant by palpation at 70 days. Similarly, 72, 89, 100, and 100%, respectively, of those detected not pregnant were not pregnant by palpation at 70 days. Humblot *et al*[23] used the RIA and found that by 30-35 days after insemination the test was 100% effective if animals were detected as non-pregnant (n=83) and 90.2% if animals were detected as pregnant (n=92).

The PSPB RIA was compared to an RIA for PAG-1[57] and by ultrasonography for sensitivity and specificity of the tests in Holstein cows.[42] Ultrasonography with a 7.5 MHz transducer was done in two ways: identification of a beating heart (US1) and identification of allantoic fluid (US2). In this study, a test sensitivity of 100% was reached at 53-58, 37-38, 37-38, and 44-45 days after breeding by US1, US2, PSPB, and PAG-1 tests, respectively. Of 135 cows, the respective tests placed 6, 2, 1, and 3 pregnant cows in the open category on days 33-34 after artificial insemination (AI). With use of data comparing each day in the study, the PSPB test was equal to or exceeded US2 and was superior to US1 and PAG-1 in sensitivity and specificity; this was contrary to another interpretation of the data.[8] The PSPB and US2 are safe tests when breeders want to treat cows that are detected not pregnant with drugs to induce a new ovulation. Specificity of the tests for US1 and US2 were 100% at 33-34 and 39-42 days, respectively, after AI. Specificity for the two protein tests did not reach 100% during the test period. The PSPB test increased from 82.6% to 91.9% and the PAG-1 test increased from 56.7 to 79%; a significant difference ($P<0.05$) with PAG-1 being less effective. Not reaching 100% for the protein tests occurred because 31 of the 138 cows were bred before 70 days postpartum, of which some (12 of 31 for PSPB and 21 of 31 for PAG) tested pregnant falsely. This again points out the need to test for pregnancy after 90 days postpartum when using the PSPB test, and perhaps even longer for the PAG test. However, the PSPB antiserum appears to detect different epitopes on the pregnancy protein than does the PAG antiserum, as it detected pregnancy earlier and the postpartum value declined more rapidly. This difference is likely due to differences in the preparation of antigen used for making the antibodies, the specific time after immunization of rabbits, and the individual rabbit making the antibody (unpublished observations; Branen and Passavant, BioTracking LLC). Age in gestation of placental tissue and the approach to isolation of antigens from the tissue will change the content of variants in the preparation. The character of the antigen preparation will dictate the character of the polyclonal antiserum that is raised for RIA development. Work of Ayad *et al*[2] in which five different RIAs were used and in which PAGs for making antibodies were from bovine,[57] ovine and caprine, also suggests that recognition of epitopes varies with the preparation of protein used to produce antibodies. They also show that various polyclonal antibodies may be useful in developing very early tests for pregnancy in cattle.

The PSPB assay has not been compared to the PSP60 assay. However, since this protein is also an aspartic acid protease and has the same N-terminal amino acid sequence, similarities are expected but subtle differences, as shown above, would not be surprising.

The serum profile of PSPB during gestation in five Holstein cows was described.[37] All had detectable levels of PSPB in serum at 24 days and had increased to 3 ng/mL serum by day 42. Values remained near this concentration until 75 days, and then began to increase to reach 72 ng/mL serum by 262 days of gestation. A sharp increase to 495 ng/mL occurred at parturition, and then it declined to 78 ng/mL of serum by 21 days postpartum.

Postpartum levels were examined by Kirakofe *et al*.[27] The PSPB had a seven-day half-life in serum of the postpartum cow, and a positive test would occur in some non-pregnant cows if tested before 90 days postpartum. Thus, if the cow were inseminated at 60 days after calving and serum was taken at the earliest post-insemination time at 30 days later, the cow would be at 90 days. She would be safe for testing without worry of a false positive test. If cows were bred before 60 days, for example at 55 days, breeders would delay sampling, for example at 35 days, after insemination. Few dairy cows would need testing before 90 days, since the average postpartum conception day is approximately 145 days in the USA. The voluntary waiting period in most dairies is 60 days and Ovsynch programs time breeding to after 70 days. Waiting until 90 days postpartum has been easily managed and accomplished by dairy breeders throughout the USA when using the commercially available BioPRYN test for pregnancy (unpublished experience, Sasser at BioTracking, LLC).

The bovine RIA for PSPB was used to detect cross-reacting PSPB in sera of several wild ruminant animals including mountain goats,[19] red deer,[17] musk oxen,[31] elk,[48] moose,[18] mule deer,[51] fallow deer,[49,50] and other domestic ruminants, including goats[36] and sheep.[33,36] Mooreover, specific radioimmunoassays were developed

to measure PSPB in serum of goats,[13,25] sheep,[47] and moose and elk.[21,22]

Enzyme-linked Immunosorbent Assay (ELISA)

An ELISA test has advantages because isotopes are not required, providing for a less expensive test and testing facility. The test is also more mobile and can be placed in other laboratories. BioTracking LLC (Moscow, Idaho) licensed the assay technology in 1993 from the University of Idaho (Moscow, ID) and developed one ELISA for PSPB in blood of cattle and another for PSPB in goats and sheep.[35] The ELISA has been offered on a commercial basis since 2003 under the registered trademark of BioPRYN®. Livestock breeders in the USA, Canada, Hungary, Germany, The Netherlands, and Australia are clients. The test is effective if samples are collected 30 days or later after breeding and 90 days after calving.

Another group has developed an ELISA for detection of PAG-1[9] while using the above described RIA for PAG[57] as a confirmation in development. The ELISA assay was useful beginning 30 days after breeding; however, there was a 7% false-negative result. This is often a problem under current management systems since animals testing false negative would be given drugs to initiate a new cycle, and would therefore abort because of treatment.

Monoclonal antibodies against PAG-1 and other variants of PSPB have also been developed[15] for use in pregnancy detection. It was presumed that monoclonal antibodies that targeted PSPB of early pregnancy may provide a test of early pregnancy without detecting PSPB in late postpartum. Three monoclonal antibodies were identified and pooled for use in development of an ELISA test. The assay was effective in detecting pregnancy in 42 cows and heifers by day 28 of gestation. Forty of these cows were bled weekly until 10 weeks (70 days) postpartum. By week 6, 8, and 10 all but 18, 2, and 1 cows, respectively, had cleared detectable PSPB from the circulation. Data suggest that monoclonal antibodies against specific epitopes of PSPB can shorten the time of the postpartum interval to reach a non-detectable value in the assay.

This same assay was field-tested and compared to transrectal ultrasonography (TU) by Silva *et al.*[40] The TU was used as the gold standard. There were 882, 478, and 313 analyses at 27 days following a first, second or third timed artificial insemination (TAI). The study found that TU at 27 days after AI had an overall sensitivity and specificity of 96.5% and 93.4%, respectively. Similarly, PAG testing had 95.4% and 94.2%. The tests were equivalent in effectiveness; however, a sensitivity of 96.5% for TU and 95.4% ELISA would result in abortion if cows were resynchronized. This would be 35/1000 tests for TU and 46/1000 tests for PAG ELISA, an excessive loss. This also points out that a monoclonal antibody designed to detect earlier pregnancy did not do so. It is likely that waiting to 30 days or more after AI would be better for both TU and PAG ELISA. The polyclonal antibody assays for PSPB wait until 30 days with excellent results.[23,37]

The BioPRYN® ELISA Test for PSPB

Various studies have been conducted to prove efficacy of the RIA in detection of PSPB in blood of cattle and the efficacy of measure in detection of pregnancy.[1,23,37,42] As reviewed above, the RIA specific to the protein preparation named PSPB has proven to be the most accurate test available. Conversion of this assay to an ELISA test allows the same quality of assay in a new format. The RIA was converted to an ELISA to provide an easier, less hazardous, and less expensive test for pregnancy. The test is commercially available under the name of BioPRYN.[35,37]

BioPRYN is a system consisting of blood collection, submission to a laboratory, laboratory assay of the sample, and reporting the results. The test is affordable to run in the laboratory, simple to apply to livestock management, and convenient for the producer. Special expertise at the farm is not needed because of the simple procedure of drawing blood and mailing it to a laboratory. The blood sampling is rapidly done, is easily learned, and saves time in cow lockup and labor applied to pregnancy testing.

Methods in testing

The ELISA methods were validated using the RIA containing antiserum 38-1 and the PSPB R37 standard.[37] This assay was used to help assure a similarly high quality test. Another PSPB protein preparation (from placenta of cows that were less than 100 days in gestation) was used for an antigen and the standard and from which antiserum, B5, was obtained. These were used in continuation of the assay.[20]

Antibodies and protein

The PSPB was isolated after the methods of Butler *et al*[4] from placenta of cows that were less than 100 days in gestation and was used to immunize a New Zealand White rabbit.[37] Rabbit anti-bovine PSPB serum (R38-1 or B5) was adsorbed to 96-well microtiter plates (Maxisorp, Nunc Inc.). Horseradish peroxidase (HRP) was conjugated to immunoglobulin G (IgG) from rabbit anti-PSPB serum by maleimide activation to form the IgG HRP.

BioPRYN assay

The BioPRYN assay is a typical sandwich ELISA. Rabbit anti-PSPB serum was coated to 96-well microti-

ter plates and was used to capture PSPB if it was in a serum sample. The IgG-HRP was used to bind to the PSPB that was captured. The development of color occurred with the addition of 3,3',5,5',-tetramethylbenzidine, the substrate for HRP. Sulfuric acid was added to stop the reaction, and optical density (OD) for each well was obtained from a plate reader (VersaMax, Molecular Devices, Inc). The cutoff OD for the assay plate was determined from the mean OD (triplicate wells) of each of two PSPB standards. The cutoff for determining pregnancy or non-pregnancy status was equivalent to that of the RIA for testing pregnancy in cattle.[1,23,37]

Application of BioPRYN in cattle

Three experiments were presented by Howard *et al.*[20] In one experiment, 336 dairy cows were tested from 30 to 36 days after AI. One week later from 37 to 43 days after AI, a follow-up test was done by transrectal ultrasonography (TU) by an experienced clinician. There were 172 pregnant and 164 non-pregnant cows from the TU test. The BioPRYN test correctly categorized all pregnant cows that were pregnant a week later by TU for a sensitivity of 100%; the test correctly categorized all but 20 non-pregnant cows for a specificity of 87.8%. The 100% sensitivity in this study shows that pregnant cows were safe from being placed in the non-pregnant category, in which case they may be aborted by treatment with re-synchronizing drugs. The specificity of 87.8% is low, but would be expected since some cows detected pregnant by BioPRYN were not pregnant one week later by TU. Embryonic loss is high during this time of pregnancy, and had the BioPRYN test been applied again at the time of TU fewer were also expected to have been pregnant with the second BioPRYN test.

In a second experiment, 191 samples were collected from replacement beef heifers which were to be synchronized to estrus. To assure they were not pregnant before the synchrony treatment, they were palpated per rectum and blood sampled for BioPRYN. All were expected to be not pregnant and were detected open by palpation; they were treated with synchronizing drugs based upon these results. The BioPRYN test detected 188 as not pregnant. One animal that was assigned pregnant showed evidence of abortion after being given prostagladin F2 alpha. The two others that tested pregnant were not observed to have aborted; one was detected "pregnant but repeat," meaning that the quantity of PSPB in the circulation was above but near the cutoff and a repeat test at a later time was necessary to confirm her status. The other heifer had an OD near that of those early in pregnancy. If prostagladin F2 alpha had affected pregnancy at this age, it is likely abortion signs would not be observed and that resorption occurred. For data analysis, these two animals were assigned as tested pregnant incorrectly. Based on the observed abortion, rectal palpation erred by calling one pregnant heifer as not pregnant and was correct by calling 190 other heifers not pregnant. The two of the 190 that were called open by palpation and pregnant by BioPRYN may have been too early in pregnancy to be detected by palpation. Calculation of sensitivity and specificity using palpation and evidence of abortion as the "true value" may not be valid. However, for BioPRYN, these values were 100 and 98.9% and the negative predictive value was 100%.

Whisnant *et al*[45] examined the use of BioPRYN in the North Carolina State University beef cow herd. The assay was compared by TU analysis and calving data. Samples were collected and TU was done after synchronized breeding. Table 1 shows the results of a portion of the study at the North Carolina State University herd located at the Upper Piedmont Beef Research Unit, Reidsville, NC.[50] Pregnancy status was assigned on day 34, 69, and 109 based upon whether the cow delivered or did not deliver a calf after a normal gestation period of 283 days from the time of the first AI.

On day 34, the BioPRYN test detected all 58 that were pregnant and ultrasound detected 57 of them. For those that were not pregnant, BioPRYN detected 17 correctly and 11 (placed into the pregnant category) incorrectly, and ultrasound detected 18 and 10, respectively. There were five animals in which the two tests disagreed. Four were tested again on day 69 by BioPRYN and rectal palpation. Reconciled values are listed in the footnote on Table 1.

On day 109, BioPRYN detected correctly all 107 that were pregnant based upon calving data, and rectal palpation detected 106 and assigned one as not pregnant. For those that were open, BioPRYN found 12 and incorrectly detected seven as pregnant. Rectal palpation found exactly the same status for the animals, providing evidence that the fetus was lost. Sensitivity and specificity of the BioPRYN test was 100% and 60.6%, respectively, on day 34 and 100% and 63.2%, respectively, on day 109. Similarly, values for ultrasound on day 34 were 98.3% and 64.3%, and for palpation on day 109 were 99.1% and 63.2%. The specificity for BioPRYN (as it was for ultrasound or palpation) at 34 days or 109 days was lower than other measures of specificity for pregnancy detection by radioimmunoassays for PSPB.[1,30,42]

One reason for the discrepancy is that the reproductive status at 34 or 109 days in the current study was based upon the calving event that happened 250 or 185 days later, respectively. Animals lose fetuses or embryos during the course of a pregnancy and this will lower the specificity since "true value" is not discerned at time of test application. Had one been able to observe the uterus at time of sampling it would be higher, as was the case in a slaughter study[30] when specificity was 94.7% by RIA test. Likewise, Szenci *et al*[42] used ultrasound at 53 or 58 days after conception as a confirmation

of pregnancy status when RIA samples for measure of PSPB were taken at 33 or 34 days, and got a higher specificity of 86.6%. Of course this was 20 to 25 days after test sampling and embryo loss was shown to be high, at 5.4%, during this time of gestation.[1] It is also evident from current data and that of Silva *et al*[40] that ultrasound is not necessarily a good standard against which to evaluate the BioPRYN test. For example, the negative predictive value was higher for BioPRYN than for ultrasound at 34 days of gestation, even though the positive predictive values were near the same.

Embryonic loss can be monitored by analysis of PSPB and progesterone[24,39,43] by RIA, and by BioPRYN and progesterone ELISA tests.[10,11] A review of PSPB related to embryonic wastage was presented by Whitlock and Maxwell.[46]

The Market for BioPRYN®

The first commercial offering of PSPB testing with the RIA was in 1993. Wildlife biologists or breeders of special livestock took advantage of this test, but the high price of testing by RIA limited use by those who raised commercial livestock. The BioPRYN (an ELISA) test that is more affordable has been used on a commercial basis since 2003 for cattle and 2004 for sheep and goats. Those seeking testing send whole blood samples, without ice, by US Post Office, Federal Express, United Parcel Service, or other carriers to the laboratory. Overnight shipping is not necessary, but timely delivery is often required by animal managers. Test results are available as early as 27 hours after receipt of samples (depending upon the schedule of the participating laboratory). Thus, a report can be available to the manager or veterinarian the day after receipt of samples. A limitation of a laboratory test is the time interval from sample collection until arrival in the laboratory.[8] Certain users of BioPRYN find that it fits into their management scheme.[6] Some, using re-synchronization of non-pregnant cows, find it is possible to give the GnRH injection to all cows at time of blood sampling, obtain the laboratory results, and give the prostaglandin F2 alpha injection one week later to cows assured to be not pregnant. No time is lost in the treatment protocol. The expense of GnRH to all is recovered by reducing costs of days open. A variation of this scheme is to give GnRH after laboratory results are available. Laboratory results are available the day after sampling and the manager gives GnRH to only the non-pregnant cows at one to two days after sampling.

Samples must be collected at 30 days or more (until time of parturition) after breeding in cows and heifers and 90 days after parturition in cows. Similarly, samples from embryo transfer cattle should be taken at 32 days or more of embryo age. Sheep and goats can be tested by 28 days after breeding. Most samples are collected in red-topped vacuum tubes to provide serum for testing; however, the types of tubes that provide plasma can be used. Blood vessels of the tail are a most convenient site for sampling in cows, although blood of the jugular vein or other vessels can be used. Jugular vein blood is most easily obtained in sheep and goats. The 90-day postpartum period before sampling is not a concern of current users of the BioPRYN test. Management programs provide lists with this criterion. Few cows require testing before this time and require only a few days' extra wait.

BioTracking LLC (Moscow, Idaho) now sells test kits for BioPRYN to affiliated laboratories throughout the world. This facilitates testing by providing facilities close to farms and reduces costs of and time in shipping. Table 2 shows the number of cattle tests sold by BioTracking LLC directly to producers and to affiliate laboratories over a three-year period. Rate of change has been 140% from 2006 to 2008. Affiliate laboratories sell a high percentage of the tests. Methods of testing for pregnancy in dairy herds was reported by the USDA[44] for the year 2007. For dairy operations, 85.7% used rectal palpation, 27.4% used ultrasound, and 4.1% use the BioPRYN test. The increase in sales of BioPRYN from 2007 to 2008 was 65.6%, which would predict that 6.8% used BioPRYN in 2008.

References

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Table 2. Detection of pregnancy in the North Carolina State University herd of beef cattle by ultrasonography and BioPRYN on day 34 and by palpation and BioPRYN on days 69[a] and 109 following the day of timed artificial insemination. The True Value was assigned based upon parturition or no parturition following the ensuing calving season, and the True Value was reconciled with test results[a] (Adapted from Whisnant *et al*[45]).

Grouping and Evaluation Day 34: by calving, 58 pregnant, 28 open Day 109: by calving, 107 pregnant, 19 open	Day 34; n = 86		Day 109; n = 126	
	Ultrasound	BioPRYN	Palpation	BioPRYN
a. Pregnant detected correctly as Pregnant	57	58	106	107
b. Pregnant detected incorrectly as Open	1	0	1	0
c. Open detected correctly as Open	18	17	12	12
d. Open detected incorrectly as Pregnant	10	11	7	7
Sensitivity = 100 X a/a + b	98.3	100.0	99.1	100.0
Specificity = 100 X c/c + d	64.3	60.6	63.2	63.2
Positive predictive value = 100 X a/a + d	85.1	84.1	92.8	93.9
Negative predictive value = 100 X c/c + b	94.7	100.0	92.3	100.0

[a] - the results for five cows were different on day 34 by the two tests. On day 69, four were evaluated again by palpation and BioPRYN.
Cow 1002: First test: was open by ultrasound and pregnant by BioPRYN; calved on 11-27-04 on time after 1st AI. Follow-up on day 69, she was pregnant by palpation and BioPRYN.
Cow 1031: First test: was ultrasound open, BioPRYN pregnant; calved on 1-21-05; thus open on first test. Follow-up on day 69, palpation open and BioPRYN pregnant.
Cow 2002: First test: ultrasound pregnant and BioPRYN open; follow-up on day 69, open on both tests. No calf was born.
Cow 9095: First test: ultrasound open and BioPRYN pregnant; calved on 2-5-05; thus open on first test; follow up on day 69, palpation was ~ 40 day fetus, BioPRYN open.
Cow 7017: First test: ultrasound pregnant and BioPRYN open; sold before day 69.

Table 3. Number of test wells sold by BioTracking and affiliate laboratories over three years as a demonstration of increased acceptance of the BioPRYN test for pregnancy in cattle.

	Number of cattle sample wells sold each year		
	2006	2007	2008
BioTracking Laboratory	86,815	130,019	152,979
Affiliate laboratories	120,284	206,712	345,136
Total	207,099	336,731	498,115

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