Comparison of Commercial ELISA Blood Tests for Early Pregnancy Detection in Dairy Cows

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Abstract. The objective of the present study was to compare two commercially available blood-based pregnancy tests, namely BioPRYN, an ELISA for pregnancy-specific protein B (PSPB), and an ELISA for pregnancy-associated glycoprotein (PAG), for early pregnancy diagnosis in dairy cattle using transrectal ultrasonography as a gold standard. Transrectal ultrasonography was conducted 26–58 days after artificial insemination (AI) in 197 cattle from 19 farms. Concurrently, a blood sample was collected for determination of serum PSPB and PAG. Transrectal palpation was performed approximately 120 days after AI to verify that pregnancy was maintained. For PSPB and PAG, there were no significant differences (P>0.05) in sensitivity (98.0 and 97.8%), specificity (97.1 and 91.2%), positive predictive values (99.3 and 97.8%), negative predictive values (91.9 and 91.2%) and accuracy (97.8 and 96.4%). In conclusion, the two blood pregnancy assays were equally efficacious and were highly accurate (based on transrectal ultrasonography as the gold standard).

Key words: Dairy cattle, Pregnancy-associated glycoprotein (PAG), Pregnancy-specific protein-B (PSPB)

Pregnancy-associated glycoproteins (PAGs) belong to a large family of aspartic peptidases, of which pregnancy-specific protein B (PSPB) was the first member to be discovered [1]. These glycoproteins are produced exclusively by specialized trophoblastic giant cells in the ruminant placenta [2], which migrate from the trophoblast to fuse with maternal uterine epithelial cells, and release their granular content (containing PSPB and PAG) into the maternal circulation [3]. The PAGs consist of several isoelectric and molecular weight protein variants that can be partially purified from other placental proteins. Antibodies made against the purified proteins can be used to detect the presence of the protein in the peripheral circulation of cattle. Because these proteins are specific to the placental tissue, it is possible to use detection of PAGs in the maternal circulation as an indicator of pregnancy [4].

Transrectal palpation, B-mode ultrasonography and measurement of blood PSPB and PAG concentrations are commonly used for early pregnancy diagnosis. Serum PSPB and PAG concentrations increase progressively from 30 days of pregnancy, peak 1–5 days before calving and decline thereafter [5, 6]. The first blood-based pregnancy-specific assay for ruminants was described by Sasser \textit{et al.} on the basis of radioimmunoassay for PSPB [5, 7]. Pregnancy associated glycoproteins such as PSPB [8, 9] and other PAGs [6, 10, 11] have been used for early detection of pregnancy in dairy cattle, starting as early as 21 days after AI. Since the concentration of pregnancy proteins increases progressively during pregnancy, the sensitivity of positive pregnancy diagnosis increases with days post AI [7]. As a result, standard application of blood-based detection of PSPB or PAGs is not performed until later than 30 days post breeding under field conditions.

Blood concentrations of PAGs were initially measured by radioimmunoassay [6, 7, 12, 13], and the results have been compared with those of ultrasonography [14]. More recently, ELISA assays for PSPB [4] and PAG [10] became commercially available. The PSPB ELISA is available under the trade name BioPRYN and provides a qualitative pregnancy classification based on measurement of PSPB in the serum of pregnant ruminants. The assay has been commercially available since 2003 in the United States. However, these commercial assays have apparently not been compared directly in the same study under field conditions. Therefore, the objective of the present study was to compare two commercial blood (ELISA) pregnancy tests for PSPB and PAG for early pregnancy determination in dairy cattle using transrectal ultrasonography as a reference.

Materials and Methods

\textit{Animals, pregnancy diagnosis and sample collection}

The study population was comprised of 197 primi- and pluriparous Holstein Friesian cattle from 19 farms in Germany. This research was approved by the Ethics Committee on Animal Rights Protection (Oldenburg, Germany) in accordance with German legislation on animal rights and welfare (reference number: 07A 527).

One blood sample was collected from each cow (from coccygeal...
vessels) into vacuum serum tubes (Sarstedt, Nümbrecht, Germany) for PSPB, PAG and progesterone determination. At time of blood sampling, the cows were between 26–58 days after artificial insemination (<30 days, n=24; 30–33 days, n=63; 34–37 days, n= 58; 38–41 days, n=43; > 41 days, n=10 cows).

Blood samples were centrifuged (2000 × g, 20 min) within 4 h after collection, and serum was removed and frozen at –20 °C until assayed. Concurrently, pregnancy was confirmed by transrectal ultrasonography with a 5.0/7.5-MHz linear array transducer (Tringa 50L, Esaote Pie Medical, Köln, Germany). Confirmation of the presence of an embryo/fetus with a beating heart was the criterion for a positive pregnancy diagnosis. Transrectal palpation was done approximately 120 days after AI to verify that pregnancy was maintained. Ultrasonographic pregnancy confirmation was performed by the same experienced veterinarian in all animals. A second veterinarian was responsible for all rectal palpations.

**PAG and PSPB determination**

Serum PAG concentrations were determined at the Department of Animal Science in Göttingen with a competitive two-step immunometric assay, which used polyclonal anti-bPAG1-IgG rabbit antiserum for specific binding of PAG as previously described [3]. To distinguish between pregnant and non pregnant animals, a cutoff concentration for PAG of <1.5 ng/ml was chosen. Concentrations from 1.0 to 1.5 ng/ml PAG were within a repeat category (resampling recommended at least 1 week later), whereas cows with serum concentrations >1.5 ng/ml were defined as pregnant. The cut-off point selected in the PAG-ELISA was chosen due to an examination of 400 inseminated cows [10]. The mean intra- and interassay coefficients of variation (CVs%) were <10%, and the lower detection limit was 0.4 ng/ml.

Serum PSPB values were measured at the University of Veterinary Medicine Hannover with the commercially available BioPRYN enzyme-linked immunosorbent assay (ELISA; BioTracking LLC, Moscow, ID, USA) according to the instructions of the manufacturer. The BioPRYN assay provides a qualitative pregnancy classification based on comparison of a sample optical density with three threshold optical density values (high, low and cut-off) calculated for each plate. The threshold values were calculated from the optical density (OD, wavelength 450 nm) values of triplicate test wells for two PSPB standards (high and low). The actual concentrations of the standards and threshold values are trade secrets but have been validated in previous work [4, 8, 9]. If the OD exceeded the high threshold the animal was categorized as pregnant. An OD less than the low threshold, was designated as not pregnant. OD results between the high threshold and cut-off were categorized as pregnant repeat, and an OD below the low threshold and cut-off was categorized as not pregnant repeat.

**Progesterone determination**

Serum progesterone concentrations were determined with an enzyme immunoassay previously validated for cows [15]. The intra- and interassay coefficients of variation (CVs%) were <10%, and the lower detection limit was 0.4 ng/ml.

**Calculations and statistical analyses**

Sensitivity, specificity, positive and negative predictive values (PPV and NPV, respectively) and accuracy were determined as described by Kastelic (2006) [16]. These calculations were performed without considering samples within the repeat categories of PSPB and PAG assay. For each assay, a Chi square test was used to compare the pregnancy rates with those obtained by ultrasonography. For all statistical analyses, a P value <0.05 was considered significant.

**Results**

Table 1 shows the interrelation between the numbers of cows defined as pregnant (p), pregnant repeat (pr), non pregnant repeat (npr) and non pregnant (np) using the BioPRYN (PSPB) or pregnancy-associated glycoprotein (PAG) assay in 197 dairy cows.

<table>
<thead>
<tr>
<th></th>
<th>PAG</th>
<th>PSPB</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>p</td>
<td>pr</td>
<td>npr</td>
<td>np</td>
<td>Total</td>
<td></td>
</tr>
<tr>
<td>p</td>
<td>124 (123)</td>
<td>8 (8)</td>
<td>0 (0)</td>
<td>3 (1)</td>
<td>135 (132)</td>
<td></td>
</tr>
<tr>
<td>pr</td>
<td>22 (22)</td>
<td>3 (3)</td>
<td>1 (1)</td>
<td>2 (1)</td>
<td>28 (27)</td>
<td></td>
</tr>
<tr>
<td>np</td>
<td>3 (3)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>31 (1)</td>
<td>34 (4)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>149 (148)</td>
<td>11 (11)</td>
<td>1 (1)</td>
<td>36 (3)</td>
<td>197 (163)</td>
<td></td>
</tr>
</tbody>
</table>

The number of cows diagnosed pregnant by transrectal ultrasonography is indicated in parentheses.
rized as pregnant using the PSPB assay and ultrasonography.

Based on ultrasonography, 82.7% (163/197) of the cows were diagnosed as pregnant between 26 and 58 days after AI, with 160 cows confirmed pregnant (based on rectal palpation) approximately 120 days after AI; therefore, 3 of the 163 pregnancies appeared to be lost. In the three cows with pregnancy loss, the serum progesterone concentrations on days 33, 34 and 44 were 6.3, 5.0 and 8.0 ng/ml, respectively. The mean progesterone concentration in the 160 pregnant cows without pregnancy loss was 7.0 ± 2.7 ng/ml.

One cow (33 days after AI) was diagnosed as non-pregnant with the PSPB assay; the other two were designated pregnant with the PSPB assay on days 34 and 44, respectively. All three cows were designated pregnant with the PAG assay. However, the PAG concentrations were relatively low (1.5, 1.9 and 2.2 ng/ml on days 33, 34 and 44, respectively). The mean PAG concentration in the 160 pregnant cows in which the pregnancy was maintained was 2.7 ± 1.5 ng/ml.

For PSPB (n=185) and PAG (n=169) (after removing repeat animals from the data set), the sensitivities were 98.0% (148/151) and 97.8% (132/135), specificities were 97.1% (33/34) and 91.2% (31/34), positive predictive values (PPV) were 99.3% (149/149) and 97.8% (135/135), negative predictive values (NPV) were 91.7% (33/36) and 92.2% (31/34) and accuracies were 97.8% (181/185) and 96.4% (163/169; Table 2). There were no significant differences between the two ELISA tests for PSPB and PAG in terms of sensitivity, specificity, PPV and NPV and accuracy. The mean progesterone and PAG concentrations (mean ± SD) at different days after AI from the pregnant animals were 7.2 ± 2.2 ng/ml and 2.2 ± 1.0 ng/ml between days 26 and 29 (n=24), 6.6 ± 2.8 ng/ml and 2.7 ± 1.4 ng/ml between days 30 and 33 (n=63), 6.6 ± 2.4 ng/ml and 2.9 ± 1.9 ng/ml between days 34 and 37 (n=58), 7.8 ± 3.0 ng/ml and 2.7 ± 1.2 ng/ml between days 38 and 41 (n=43) and 6.0 ± 3.4 ng/ml and 2.3 ± 0.7 g/ml between days 42 and 58 (n=9), respectively.

**Discussion**

Pregnancy diagnosis for these blood-based pregnancy tests was in close agreement with pregnancy diagnosis by transrectal ultrasonography, which was chosen as the gold standard because it is highly accurate (88–98%) for identifying pregnant cows under field conditions [17]. Furthermore, the accuracy of sonographic pregnancy diagnoses was 100% under controlled experimental conditions [14]. Humblot et al. [12] reported that the accuracy for pregnancy diagnosis with PSPB radioimmunoassay between days 30–35 after AI was 94.8%. In our study, a higher accuracy (97.8%) was found. This could due to the fact that a larger number of cows >35 days after AI were diagnosed in the present study and accuracy increases with the progression of pregnancy [7]. One limitation of the present study was the fact that 12 and 28 cows were placed in the repeat categories of the PSPB and PAG assays, respectively, and could not be used for determination of accuracy parameters because no follow-up testing was completed. For the 197 cows, the incidence of false positive and false negative diagnoses was one and three, respectively, for the PSPB assay and three for the PAG assay. In individual cows, low concentrations of PAGs could be due to either a delay in increase or perhaps a decline due to early embryonic loss. Consequently, cows designated to a repeat category should be retested with a sample drawn three to seven days after the initial sample.

The present results seemed consistent with those reported by Szenci et al. in 1998 [13]; in their study, there was no significant difference in the accuracy of diagnosis of pregnant cows between a PSPB radioimmunoassay and a PAG ELISA, although the former had significantly fewer false positives. In another study, there was also good agreement between pregnancy diagnoses based on PSPB ELISA and transrectal palpation performed 35 to 60 days after AI, especially at longer intervals [8]. It was noteworthy that there was a disagreement for a lot of cows regarding the repeat categories of both assay methods. This could due to the fact that the antibodies used recognized different PAG (i.e., PSPB vs. PAG) molecules with different affinities. The placentation production of both PAG molecules (bPAG-1 vs. PSPB) seems to be different in individuals and may lead to these different repeat classifications of samples. Similar conclusions were made from comparison of three different PAG radioimmunoassays [11].

Only three of the 163 pregnancies were lost; in these cows, interpretation was not improved by concurrently considering the concentrations of PAG and progesterone or by pregnancy classification with PSPB. The pregnancy losses observed in the present

**Table 2.** Contingency table comparing pregnancy detection using the PSPB (A) or PAG assay (B) vs. transrectal ultrasound (US) for cows diagnosed as pregnant or non-pregnant out of the 197 total cows tested (repeat samples excluded from analysis).

<table>
<thead>
<tr>
<th></th>
<th>Pregnant US</th>
<th>Nonpregnant US</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A.</strong> Pregnant PSPB</td>
<td>148</td>
<td>1</td>
<td>149</td>
</tr>
<tr>
<td>Nonpregnant PSPB</td>
<td>3</td>
<td>33</td>
<td>36</td>
</tr>
<tr>
<td>Total</td>
<td>151</td>
<td>34</td>
<td>185</td>
</tr>
<tr>
<td><strong>B.</strong> Pregnant PAG</td>
<td>132</td>
<td>3</td>
<td>135</td>
</tr>
<tr>
<td>Nonpregnant PAG</td>
<td>3</td>
<td>31</td>
<td>34</td>
</tr>
<tr>
<td>Total</td>
<td>135</td>
<td>34</td>
<td>169</td>
</tr>
</tbody>
</table>

a PSPB=pregnancy-specific protein B. b PAG=pregnancy-associated glycoprotein.
study could be classified as late embryonic losses. The percentage of losses in the present study was lower compared with studies focussed on late embryonic death in which the percentage ranged between 3.2–42.7% [18]. This could be explained by good estrus monitoring of the 19 farms. Furthermore, none of the animals were synchronized. It has been suggested that embryonic losses are rare if AI takes place after a spontaneous estrus [18]. In the three cows that experienced pregnancy loss, the PAG concentrations were relatively low (<2.5 ng/ml). Similarly, a previous report showed that the plasma concentrations of PAG and progesterone were not significantly different throughout gestation between cows with or without fetal losses. In a previous study, the risk of pregnancy loss was 10 times more likely in cows with low versus medium (<2.5 versus 2.5 to 4.0 ng/ml) PAG concentrations [19]. However, in the present study, the mean PAG concentration (2.7 ng/ml) of all the pregnant cows was also relatively low, and in 29 of the 163 cows confirmed pregnant by transrectal ultrasonography, the PAG concentration was >2.0 and <2.5 ng/ml.

In the present study, the BioPRYN assay defined one cow with one late embryonic loss (LEL) as non-pregnant. Transrectal ultrasonography identified an embryo in this cow 33 days after AI, but pregnancy was not maintained. According to Gabor et al. (2007) [4], 710 cows with late pregnancy losses had lower serum PSPB concentrations. The PSPB concentration has previously been shown to decrease significantly in cases of late embryonic death and was described as useful in prediction of some pregnancy losses [20]. However, in the present study, the mean PAG concentration (2.7 ng/ml) of all the pregnant cows was relatively low, and in 29 of the 163 cows confirmed pregnant by transrectal ultrasonography, the PAG concentration was >2.0 and <2.5 ng/ml.

In summary, the commercial assays (based on ELISA) for PSPB and PAG had extremely high and similar sensitivities and specificities for early pregnancy diagnosis in dairy cows compared with ultrasound examination.

Acknowledgments

Test plates for BioPRYN were provided by BioTracking, LLC, Moscow, ID, USA. We thank A Jordan, M Baumgarten and K Koslowski for technical assistance with the BioPRYN test for PSPB and Prof H Meyer (Institute of Physiology, Technical University of Munich, Freising, Germany) for antibodies used in the progesterone assay.

References