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ORIGINAL ARTICLE

Concentrations of haptoglobin in bovine plasma determined by ELISA or a colorimetric method based on peroxidase activity

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Summary

The objective was to compare different procedures for determination of haptoglobin in bovine plasma. Nine Angus steers were vaccinated against *Mannheimia haemolytica* to stimulate an acute-phase response. Blood samples were collected immediately prior to vaccination (day 0), and on days 1, 3, 5, 7 and 10. Plasma samples were frozen in duplicates at −80 °C. One set of the duplicates was analysed for haptoglobin concentrations using a commercial ELISA kit. A day effect was detected ($p < 0.01$) because haptoglobin peaked on day 3 and returned to baseline on day 7 relative to vaccination. The second duplicate was analysed using a procedure that measures haptoglobin–haemoglobin complexing by estimating differences in peroxidase activity (CPPA) with results expressed as optical density. Further, based on the ELISA results, the plasma sample with the greatest haptoglobin concentration was also serially diluted into a plasma sample with negligible haptoglobin concentration from the same steer (1:1 through 1:1024 dilution). These dilutions were used within the CPPA method to generate a standard curve and estimate plasma haptoglobin concentrations (CPPA + STD). A linear standard curve was generated ($r^2 = 0.99$). A day effect similar to the ELISA method was detected for the CPPA and CPPA + STD methods ($p < 0.01$). Results obtained from CPPA and ELISA methods were positively correlated ($r = 0.97$; $p < 0.01$). The values generated by the CPPA + STD procedure were similar ($p = 0.38$) compared to the values generated by the ELISA method. In conclusion, assessing concentrations of haptoglobin in bovine plasma using the CPPA and CPPA + STD methods generate highly correlated or similar results, respectively, compared to ELISA. Therefore, the CPPA + STD and CPPA methods can be used as a less expensive alternative to ELISA to determine concentrations or monitor changes in plasma haptoglobin in bovine samples.

Introduction

Haptoglobin is a α2-globulin synthesized by the liver during the acute-phase response (Yoshioka et al., 2002). In cattle, circulating concentrations of haptoglobin are negligible during healthy conditions; however, it may increase more than 100-fold during inflammatory and acute-phase reactions (Morimatsu et al., 1992; Nakajima et al., 1993). For this reason, haptoglobin has been used as a marker for infections, diseases and trauma in beef and dairy herds (Horadagoda et al., 1999). Haptoglobin can also be used as an indicator of the bovine acute-phase response induced by stressful management procedures,

such as transportation and feedlot entry (Araujo et al., 2010; Cooke et al., 2011). In fact, plasma concentrations of haptoglobin were negatively associated with performance of overtly healthy cattle (Berry et al., 2004; Cooke et al., 2009), demonstrating the importance of this acute-phase protein for beef production systems.

Our laboratory determines concentrations of haptoglobin in bovine plasma using an economical colorimetric procedure that measures haptoglobin–haemoglobin complexing by estimating differences in peroxidase activity (CPPA; Makimura and Suzuki, 1982). Results are reported as optical density (OD) reading at 450 nm wavelength, given that the CPPA method does not contain a standard curve (Arthington et al., 2008; Araujo et al., 2010; Cooke and Bohnert, 2011). Conversely, commercially available ELISA procedures generate results based on standards with known haptoglobin concentrations (Guzelbektes et al., 2010; Kwon et al., 2011). Therefore, the objective of this study was to determine if the CPPA method generates results compatible with a commercial ELISA kit and is hence appropriate to assess haptoglobin in bovine plasma.

Materials and methods

Animals and sampling

This experiment was conducted in July 2010 at the Oregon State University – Eastern Oregon Agricultural Research Center, Burns. The animals utilized in these experiments were cared for in accordance with acceptable practices as outlined in the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (FASS, 1999).

To obtain plasma samples with a broad range of haptoglobin concentrations, nine Angus × Hereford steers (initial BW 244 ± 8 kg; initial age 440 ± 5 day) were vaccinated against *Mannheimia haemolytica* (2 ml s.c., One Shot®; Pfizer Animal Health, New York, NY, USA) to stimulate an acute-phase response (Stokka et al., 1994). Blood samples were collected prior to vaccination (day 0), and on days 1, 3, 5, 7 and 10, via jugular venipuncture into commercial blood collection tubes (Vacutainer, 10 ml; Becton Dickinson, Franklin Lakes, NJ, USA) containing sodium heparin, and were placed on ice immediately. Samples were centrifuged at 2500 *g* for 30 min for plasma collection and stored in duplicates at −80 °C on the same day of collection. One set of the duplicates was analysed for haptoglobin concentrations using a commercial ELISA kit (#2410-7; Life Diagnostics, West Chester, PA, USA) previously used for bovine samples (Guzelbektes et al., 2010; Kwon et al., 2011). The second set of the duplicates was analysed for haptoglobin using the CPPA procedure.

The ELISA procedure

The ELISA procedure was performed based on the instructions provided by the manufacturer (Life Diagnostics), and read in a microplate reader (VersaMax Tunable Microplate Reader; Molecular Devices, LLC, Sunnyvale, CA, USA) using the included software (SoftMax Pro; Molecular Devices, LLC). All samples were analysed in duplicates and results are reported as μg/ml. The intra-assay and interassay CV were, respectively, 3.3% and 3.5%.

The CPPA procedure

Table 1 describes all materials utilized in the CPPA procedure. Five microlitre of plasma or distillated water (for blank determination) was added to 7.5 ml of O-dianisidine solution (0.6 g/l of O-dianisidine, 0.5 g/l of EDTA and 13.8 g/l of sodium phosphate monobasic in distilled water; pH adjusted to 4.1) in a 16 × 100 mm borosilicate tube. Twenty-five microlitre of a haemoglobin solution (0.3 g/l of bovine haemoglobin in distilled water) was immediately added to each tube. All tubes were incubated in a water bath (#2845; Thermo Fisher Scientific, Waltham, MA, USA) set at 37 °C for 45 min. After incubation, 100 μl of a freshly prepared working concentration of 156 mM hydrogen peroxide solution was added to each tube. All tubes were incubated for 1 h at room temperature. After incubation, 200 μl of each tube was transferred into one well in a 96-well polystyrene flat-bottom microplate, and OD was immediately read at 450 nm in a microplate reader (VersaMax Tunable Microplate Reader; Molecular Devices, LLC) using the included software (SoftMax Pro; Molecular Devices, LLC). The OD from the blank sample was subtracted from the OD of all plasma-containing samples. Results are reported as OD at 450 nm × 100.

Based on the results from the ELISA procedure, the plasma sample with the greatest haptoglobin concentration (steer 238, day 3; 1638 μg/ml) was selected and serially diluted (1:1) with a plasma sample with reduced haptoglobin concentration from the same steer (steer 238, day 0; 0.10 μg/ml) to yield the following standards: 819, 409.5, 204.7, 102.4, 51.2, 25.6, 12.8, 6.4, 3.2 and 1.6 μg/ml. All dilutions were included in the same CPPA assay that analysed plasma samples from the second set of

Table 1 List of materials utilized for determination of haptoglobin concentrations in bovine plasma using the procedure that measures haptoglobin–haemoglobin complexing by estimating differences in peroxidase activity

Material	CAS number*	Provider	Catalogue number
O-dianisidine	119-90-4	Alfa Aesar, Ward Hill, MA, USA	A17150-14
EDTA	60-00-4	Alfa Aesar	A17385-0B
Sodium phosphate monobasic	10049-21-5	EMD Chemicals, Gibbstown, NJ, USA	SX0710-1
16 × 100 mm borosilicate tube	–	VWR, Radnor, PA, USA	47729-576
Bovine haemoglobin	9008-02-0	Sigma-Aldrich, St. Louis, MO, USA	H2500-5g
Hydrogen peroxidase	7722-84-1	J.T. Baker, Phillipsburg, NJ, USA	2186-01
96-well microplate	–	Greiner Bio-One, Monroe, NC, USA	655101

*Chemical identification number assigned by the Chemical Abstracts Service (American Chemical Society, Washington, DC, USA).

duplicates. A standard curve was constructed using the aforementioned dilutions, samples with the highest (1638 μg/ml) and lowest (0.10 μg/ml) haptoglobin concentrations and their respective OD at 450 nm × 100 to generate results expressed as μg/ml (CPPA + STD method).

All samples and dilutions were analysed in duplicates. The intra- and interassay CV for both CPPA and CPPA + STD methods were, respectively, 2.2% and 3.4%.

Statistical analysis

All data were analysed using steer as the experimental unit. Vaccination effects on haptoglobin concentrations within each assay type were analysed with MIXED procedure of SAS (SAS Institute Inc., Cary, NC, USA) and Satterthwaite approximation to determine the denominator df for the tests of fixed effects. The model statement contained the effects of day. Steer was the random variable. The specified term for the repeated statement was time, the subject was steer and the covariance structure utilized was autoregressive, which provided the best fit for these analyses according to the Akaike information criterion. Pearson correlation coefficients were calculated among values produced by the CPPA and ELISA procedures using the CORR procedure of SAS. The GLM procedure was utilized to determine effects of day on correlation coefficients. The MIXED procedure of SAS with Satterthwaite approximation was also used to compare haptoglobin concentrations from plasma samples analysed by the ELISA and the CPPA + STD methods. The model statement contained the effects of assay type, day and the interaction. Steer (assay type) was the random variable. The specified term for the repeated statement was time, the subject was steer (assay type) and the covariance structure utilized was autoregressive based on the Akaike information criterion. Results are reported as least square means and were separated using least square differences. Significance was set at $p \leq 0.05$, and tendencies were determined if $p > 0.05$ and ≤ 0.10. Results are reported according to treatment effects if no interactions were significant, or according to the highest-order interaction detected.

Results

A day effect was detected ($p < 0.01$) for both ELISA and CPPA procedures, with plasma haptoglobin peaking on day 3 and returning to baseline on day 7 relative to vaccination (Table 2). A linear standard curve ($p < 0.01$; $r^2 = 0.99$) was generated by the CPPA + STD method (Fig. 1a). However, the dilutions with concentrations below 25.6 μg/ml and their respective OD (1.0, 1.1, 1.1, 1.2, 1.1 and 1.2 OD at 450 nm × 100 for 0.1, 1.6, 3.2, 6.4, 12.8 and 25.6 μg/ml) did not generate significant linear regression ($p = 0.16$; $r^2 = 0.42$; data not shown) if analysed separately from the remaining standards. Therefore, dilutions with concentrations between 1.6 and 25.6 μg/ml were discarded, and a similar linear standard curve was subsequently generated ($p < 0.01$; $r^2 = 0.99$; Fig. 1b) and used in the CPPA + STD method. Hence, a day effect was also detected ($p < 0.01$) for samples analysed with the CPPA + STD method (Table 2).

Across all sampling days, results yielded by the CPPA and the ELISA method were positively correlated ($p < 0.01$; $r = 0.97$; Fig. 2). However, these correlation coefficient varied among days ($r = 0.95$, 0.96, 0.97, 0.99 and 0.94 for days 1, 3, 5, 7 and 10; data not shown) and results from the CPPA and ELISA methods were not correlated ($p = 0.85$; $r = 0.07$; data not shown) on day 0, resulting in a day interaction in the correlation analysis ($p < 0.01$).

Table 2 Day effects on plasma haptoglobin concentrations, according to three different assay types, of steers vaccinated against *Mannheimia haemolytica* (2 ml s.c., One Shot®; Pfizer Animal Health) after blood collection on day 0*

	Day relative to vaccination							
Assay type	0	1	3	5	7	10	SEM	p-value
CPPA, 450 nm × 100†	0.98[a]	4.09[b]	7.11[c]	3.71[b]	1.31[a]	1.24[a]	0.43	<0.01
ELISA, μg/ml‡	0.11[a]	467.93[b]	1053.25[c]	330.19[b]	24.02[a]	8.59[a]	70.23	<0.01
CPPA + STD, μg/ml§	−10.35[a]	567.86[b]	1131.75[c]	497.96[b]	50.39[a]	37.97[a]	75.37	<0.01

*Within rows, values with different superscripts differ ($p < 0.05$).

†Procedure that measures haptoglobin–haemoglobin complexing by estimating differences in peroxidase activity.

‡Commercial ELISA kit (#2410-7; Life Diagnostics).

§CPPA method including a standard curve based on samples with known haptoglobin concentration analysed with the ELISA procedure.

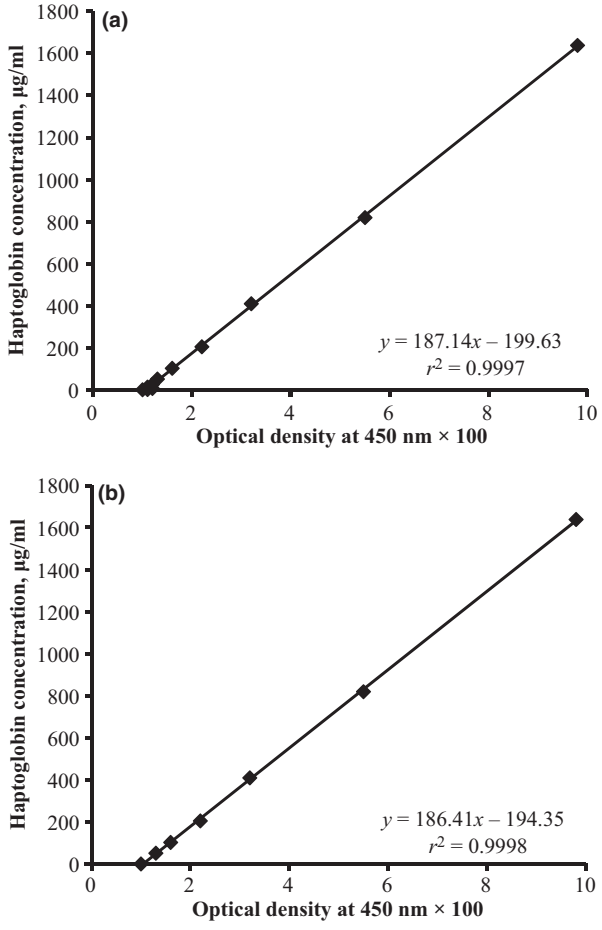

Fig. 1 Standard curves, based on bovine plasma samples with known haptoglobin concentrations, generated using a procedure that measures haptoglobin–haemoglobin complexing by estimating differences in peroxidase activity. Panel (a) reports a standard curve including 12 standards (1638, 819, 409.5, 204.7, 102.4, 51.2, 25.6, 12.8, 6.4, 3.2, 1.6 and 0.1 μg/ml). Panel (b) reports a standard curve including seven standards (1638, 819, 409.5, 204.7, 102.4, 51.2 and 0.1 μg/ml). A linear correlation was detected for both curves ($p < 0.01$; $r = 0.99$).

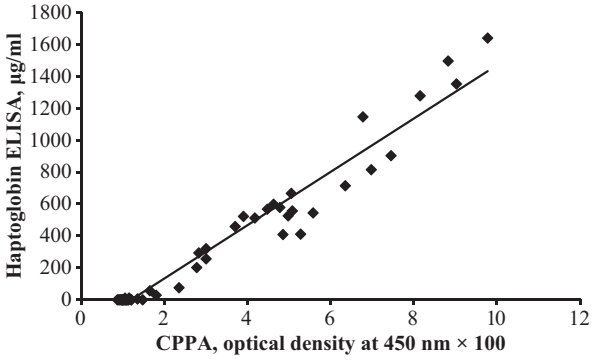

Fig. 2 Correlation between values generated by a commercial ELISA kit for determination of bovine plasma haptoglobin, and a procedure that measures haptoglobin–haemoglobin complexing by estimating differences in peroxidase activity (CPPA). A liner correlation was detected ($p < 0.01$; $r = 0.97$, $n = 54$).

No differences were detected ($p = 0.38$) when results generated by the CPPA + STD method were compared with the ELISA method (379.2 vs. 314.0, respectively; SEM = 48.8), even when assay types are compared with day (Table 2; $p = 0.92$, 0.35, 0.46, 0.12, 0.80 and 0.78 for days 0, 1, 3, 5, 7 and 10, respectively).

Discussion

As expected, plasma haptoglobin concentrations according to the ELISA procedure increased following vaccination, demonstrating that the *M. haemolytica* challenge induced an acute-phase response in steers (Stokka et al., 1994). A similar day effect was detected when samples were analysed using the CPPA assay, suggesting that this procedure can also be utilized to monitor changes in circulating haptoglobin following immune challenges. Supporting

these results, the CPPA method successfully detected increases in circulating haptoglobin following stress challenges (Araujo et al., 2010; Cooke and Bohnert, 2011; Cooke et al., 2011).

The CPPA and ELISA methods generated highly correlated results across all sampling days (Fig. 2). However, correlation coefficients between both procedures differed according to day relative to vaccination. Prior to vaccination (day 0), results generated by the CPPA and ELISA procedures were not correlated. According to the ELISA procedure, plasma concentrations of haptoglobin on day 0 were negligible, concurring with other studies reporting that plasma haptoglobin concentrations in healthy animals are negligible (Morimatsu et al., 1992; Nakajima et al., 1993; Salonen et al., 1996). Nevertheless, results generated by the CPPA and ELISA methods from samples collected after vaccination were highly correlated, demonstrating that both methods are similarly capable of detecting plasma haptoglobin changes in individual animals following an immune challenge.

The standard curves generated by the CPPA + STD method were linear with coefficients of determination >0.99 (Fig. 1), demonstrating that serially diluting spiked plasma samples yielded a strong linear standard curve. However, the dilutions with haptoglobin concentration below 25.6 μg/ml did not generate a significant regression and were thus removed from the standard curve utilized in the CPPA + STD procedure. The haptoglobin concentrations generated by the ELISA and CPPA + STD procedures were similar (Table 2), even when compared within days, suggesting that the CPPA + STD procedure can be used to determine actual plasma concentrations of haptoglobin in bovine plasma. However, the mean haptoglobin concentration detected on day 0 was negligible for the ELISA procedure and negative for the CPPA + STD procedure (Table 2). This outcome, associated with the lack of correlation between OD and known concentrations of dilutions with haptoglobin concentration below 25.6 μg/ml, as well as between results generated by the CPPA and ELISA procedures on day 0, indicates that the CPPA and CPPA + STD methods are not adequate to evaluate plasma samples with low haptoglobin concentrations, and the sensitivity of the CPPA + STD method conducted in the preset study was 25.6 μg/ml. Nevertheless, our results suggest that the CPPA and CPPA + STD methods are appropriate to monitor changes or determine actual concentrations of plasma haptoglobin, respectively, in cattle experiencing an acute-phase response.

Conclusion

Results from this experiment indicate that assessing concentrations of haptoglobin in bovine plasma using the CPPA method yields results highly correlated to ELISA. Inclusion of a standard curve into the CPPA method resulted in haptoglobin values similar to the ELISA method. However, these outcomes were not detected in plasma samples containing negligible plasma haptoglobin concentrations. Therefore, the CPPA and the CPPA + STD methods can be adopted as economical alternatives to evaluate plasma haptoglobin concentrations in cattle exposed to immune or stress challenges, but not to assess small expected differences in healthy animals.

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