Determination of Haptoglobin in Bovine Serum using Polyclonal and Monoclonal Anti-human Haptoglobin Antibodies

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Abstract

Two ELISA procedures to determine haptoglobin (Hp) in bovine serum were developed. Equine haemoglobin was used as the solid phase. Self-developed goat polyclonal antibody (variant I) and monoclonal antibody (variant II) raised against human Hp were used. The results were compared with the guaiacol method. High correlation was found (r = 0.96 and r = 0.90, respectively) based on the results of 548 bovine serum samples, of which 357 were from clinically healthy cows and 191 from cows and calves monitored during treatment for the most common diseases. The Hp detection limit of ELISA using polyclonal Ab was 0.1 mg/l and using MoAb 0.21 mg/l. The addition of 2% PEG 6000 at the antibody-binding steps enabled major shortening of the incubation periods. The relatively short time, low cost of reagents, and high correlation with the reference method support the use of these ELISA variants in bovine diagnostics.

Cattle, acute-phase protein, enzyme immunoassay, haptoglobin antibody

The acute-phase response (APR) is a local and systemic nonspecific response to inflammatory agents. During APR, acute-phase proteins (APPs) are produced mainly in the liver and released to the blood plasma. The plasma concentrations of the majority of APPs are known to increase during an acute-phase response, but some, including albumin, decrease (Eckersall and Conner 1988; Heinrich et al. 1990; Stefaniak et al. 1995). During the acute-phase response in cattle, the concentrations of many major APPs, such as haptoglobin (Hp), serum amyloid A, and fibrinogen, and minor APPs, such as ceruloplasmin, α_1-antitrypsin, and α_1-acid glycoprotein, show dynamic alterations (Conner et al. 1986; Eckersall and Conner 1988; Horadagoda et al. 1993; Dowling et al. 2002).

In the serum of the *Bovidae* and *Cerviadae* families, Hp is composed of 2 to 20 polymerized forms of $\alpha_2\beta_2$ tetramer and its structure is similar to human Hp type 2-2 (Travis and Sanders 1972). The molecular weight of bovine Hp ranges from 200 to 2000 kDa. The N-terminal amino-acid sequence of the α chain is 56% and of the β chain 90% identical to human Hp (Morimatsu et al. 1991). In contrast to monogastric mammals, about 50% of healthy cattle show an undetectable Hp level in the blood plasma, and in the remaining healthy animals the concentration is below 0.1 g/l. Moreover, the Hp concentration in cattle is not affected by age, sex, pregnancy, or lactation (Richter 1974). A rise in concentration follows injury, inflammation, and infection and is related to the intensity of inflammation (Conner et al. 1988; Deignan et al. 2000; Dowling et al. 2002; Godson et al. 1996; Gruys et al. 1993). About 10 to 24 h after induction of the inflammatory response, the Hp concentration rises rapidly, a high concentration being achieved between days 2-3 with the maximum usually on the 4th day, and decreases to normal levels after about 11 days (Conner et al. 1988).

The determination of Hp concentration in blood serum may be a valuable diagnostic tool for assessing problems occurring in cows during puerperium (Chan et al. 2004) as well

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as for determining fatty liver (Yoshino et al. 1992). Hp is commonly used in monitoring the treatment of limb diseases (Jawor et al. 2008) and the health of the udder (Kováč et al. 2007), detecting diseases of clinical or subclinical course in calves, and in monitoring herd health (Ganheim et al. 2003). The broad availability of Hp in cattle diagnostics has resulted in this APP becoming the most commonly determined in this species. It is especially important in cattle because classical inflammatory indicators (sedimentation rate, leukocytosis) show poor usefulness.

Many methods of Hp determination were designed for cattle. Connell and Smithies (1959) described a method based on the peroxidase activity of the Hp-MetHb complex and Spooner and Miller (1971) developed a method of detecting the Hp-Hb complex in gel. Other methods utilize the differences in electric field migration between free Hb and the Hp-Hb complex using agar gel electrophoresis and zone capillary electrophoresis (Mazur 1980; Pirlot et al. 1999). Methods of immunological determination of Hp using radial immunodiffusion were described by Morimatsu et al. (1992). In that study, a large molecule of bovine Hp was reduced by cysteine to diminish differences in polymerization degree among individuals. The competitive (McNair et al. 1995) and noncompetitive (Sheffiled et al. 1994; Godson et al. 1996) variants of ELISA to determine Hp were described, both based on anti-Hp monoclonal antibodies (MoAbs).

Kątnik et al. (1989) produced and characterized the unique monoclonal antibody clone no. 2.36.71.41 which was able to recognize not only human Hp type 2-2, but also goat, equine, and cow Hp with high affinity as well as the Hp of many wild ruminants of the *Bovidae* and *Cervidae* families (Kątnik et al. 1991; Gospodarek et al. 1997; Gospodarek 1998). The 2.36.71.41 MoAb clone was shown to recognize the epitope in haptoglobin located around the disulphide bond linking subunits of Hp. Monoclonal antibody 2.36.71.41 was used in ELISA for the quantitative determination of Hp in goats (Kątnik et al. 1991).

The aim of the study was to design, standardize, and compare two ELISA variants for the quantitative determination of Hp in cattle serum. The ELISAs are based on the mouse monoclonal antibody (clone 2.36.71.41) described earlier (Kątnik et al. 1989; Kątnik et al. 1991) and a goat polyclonal antibody against human Hp produced by us and showing cross-reactivity with bovine Hp.

Materials and Methods

The study design was approved by the Second Local Ethics Commission for Experiments on Animals at the Agricultural University of Wrocław (no. 91/04 II).

Sampling

There were 548 bovine serum samples examined, of which 357 were from two dairy herds during one year of herd health monitoring and 191 from cattle monitored during the treatment of different diseases (ketosis, mastitis, displacement of the abomasum, limb diseases, and bronchopneumonia and diarrhoea in calves). No haemolysis was observed in the serum samples.

Antibodies

Human serum Hp was isolated according to Kątnik and Jadach (1993) using an affinity column filled with Sepharose 4B coupled with two MoAbs against Hp (clones 2.36.71.41 and 7.60.66.55). The hyperimmune antiserum was produced by immunizing a goat with purified human Hp. Five injections of 100 µg of Hp diluted in 0.5 ml of PBS and emulgated with 0.5 ml of Freund's incomplete adjuvant (Sigma) were administered subcutaneously in 14-day intervals. Blood was taken 12 days after the last injection and the serum was prepared and stored at -20 °C until use. Anti-Hp polyclonal antibody was isolated from the goat antiserum on a column filled with Sepharose 4B coupled with human Hp 2-1. The anti-Hp antibody was conjugated with horseradish peroxidase according to Farr and Nakane (1981) (modified by Stefaniak 1993). Mouse ascites containing MoAb produced by hybridoma clone 2.36.71.41 was obtained from Middle-European Diagnostics, Wroclaw.

Hp concentration measurements

Guaiacol method

Hp concentration was determined using the guaiacol method (Jones and Mould 1984). ELISA for human

haptoglobin (Kątnik and Dobryszycka 1990) was modified for bovine Hp determination. Two variants of ELISA were conducted.

Variant I - Hp-ELISA based on goat polyclonal antibodies

The wells of a microplate (Nunc Maxisorp F) were coated with 100 µl of 0.5% horse haemoglobin (Hb) prepared according to McQuarrie and Beniams (1954). Freshly prepared Hb solution was stored at -20 °C until use. The thawed Hb solution needs to be centrifuged at 1400 × *g* for 15 min before use. The microplate coated with Hb was incubated for 2 h at 37 °C and overnight at 4 °C. The wells were washed 5 times with PBS-T (phosphate-buffered saline with 0.05% Tween-20). Cow serum containing Hp at a concentration of 2.5 g/l was diluted with PBS to obtain Hp concentrations of 5, 2.5, 1.25, 0.63, 0.31, 0.21, and 0.1 mg/l used to construct a standard curve. The haptoglobin concentration was initially converted using the common logarithm.

The serum samples as well as the Hp standard solutions were added (100 µl/well) in triplicate wells. Samples having an Hp concentration < 1 g/l were tested in two dilutions (1:300 and 1:1000 with PBS) and those with concentrations > 1 g/l in three dilutions (1:1000, 1:2000, 1:4000). For serum with an Hp concentration > 3 g/l, the dilution was 1:4500. On each microplate a positive control sample of cow serum containing 1.8 g/l haptoglobin was tested. A blank sample containing PBS without haptoglobin was also included on each plate. Then the plate was incubated for 1 h at 37 °C with gentle stirring (Elpan, Laboratory shaker type 358S). The microplate was then washed five times with cold PBS-T-PEG (PBS-T and 2% polyethyleneglycol 6000).

After washing, the wells were filled with 100 µl of goat anti-human Hp polyclonal antibody conjugated with horseradish peroxidase (HRPO) diluted at 1:200 with PBS-T-PEG. The microplates were incubated for 30 min at 37 °C with gentle stirring and washed again as above. The colour reaction was developed using o-phenylenediamine (o-PDA). The wells were filled with 100 µl of freshly prepared substrate solution (5 mg o-PDA in 10 ml of 0.05 M citrate-phosphate buffer, pH 5.0, and 3 µl H_2O_2) and incubated for 30 min in the dark at room temperature. The reaction was stopped by addition of 30 µl of 2 M H_2SO_4. The absorbance was read at a wavelength of 492 nm (SLT-Spectra microplate reader). The blank value was subtracted and the absorbance at λ = 492 nm was introduced into the formula of the STATISTICA program for calculation. Then the antilog of the measured Hp concentration was multiplied by the dilution. The final Hp concentration was calculated as the mean of the results of two neighbouring dilutions. If the Hp concentration differed in the neighbouring dilutions by more than 15%, the results were disqualified and the sample was tested again.

Variant II - Hp-ELISA based on MoAb

Coating of the microplates and bovine serum incubation were performed as in variant I with the exception that all the reagents were added at 200 µl/well. After washing five times with PBS-T-PEG, 200 µl of ascites containing anti-human Hp MoAb (clone 2.36.71.41) diluted 1:4000 with PBS-T-PEG was added to each well, incubated for 30 min at 37 °C on a laboratory stirrer (Elpan, laboratory shaker type 358S), and washed again in the same manner as in variant I. Then 200 µl of goat anti-mouse FcIgG HRPO-conjugated antibody diluted 1 : 40 000 (Sigma) was added to each well and incubated and washed as in the former step. The colour reaction was developed using o-PDA. To each well, 200 µl of the same substrate solution and reaction conditions as in variant I were applied. The reaction was stopped by addition of 60 µl of 2 M H_2SO_4. Absorbance reading and preparation of the results were conducted as in variant I.

Statistical analysis

Pearson's correlation coefficient was calculated using the statistical package STATISTICA 6.1 for data obtained by the guaiacol method and both ELISA variants.

Results

The coefficients of correlation between the Hp concentrations determined using variants I and II of Hp-ELISA and the guaiacol method were 0.96 and 0.9, respectively. The correlation coefficient between both ELISA variants was 0.88. All the calculated correlations were significant ($p \leq 0.001$). Hp concentrations determined using variant I of the Hp-ELISA (Fig. 1) were in most cases lower than those obtained by the guaiacol method, whereas variant II gave higher results (Fig. 2). The differences between the methods increased when the concentration of Hp exceeded 1 g/l. Figs 3 and 4 show the mean absorbance and SD calculated on the basis of twenty standard curves obtained using the two ELISA variants.

Hp detection limit

The Hp detection limit of ELISA variant I using polyclonal Ab was 0.1 mg/l and of variant II using MoAb 0.21 mg/l. The detection limits were calculated on the basis of 20 blank samples using the formula: GW = x + 3SD, where GW is the detection limit, x the mean, and SD the standard deviation. The mean Hp concentration of 20 repeated measurements

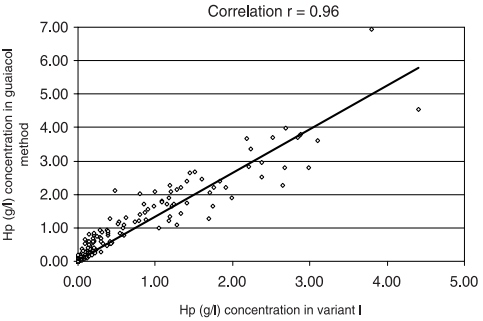

Fig. 1. Comparison of results of ELISA (variant I) and the peroxidase activity-based method

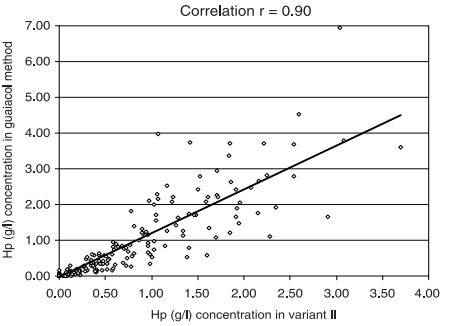

Fig. 2. Comparison of results of ELISA (variant II) and the peroxidase activity-based method

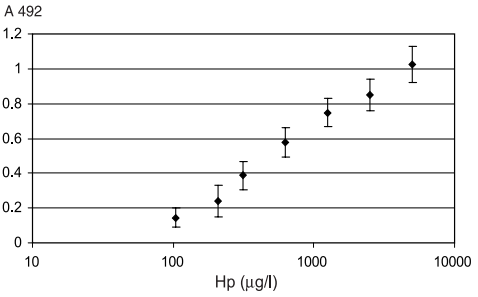

Fig. 3. Mean absorbance and SD of Hp concentration (n = 20) in variant I ELISA

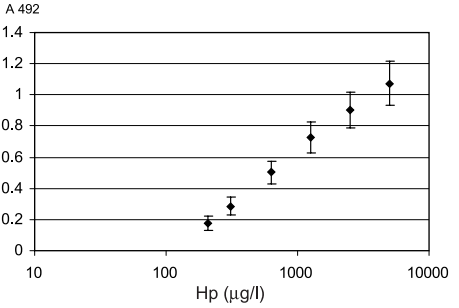

Fig. 4. Mean absorbance and SD of Hp concentration (n = 20) in variant II ELISA

of cow serum containing 1.8 g of Hp/l using the Jones and Mould (1984) method diluted to 1:600 was 1.1 ± 0.23 g/l in variant I and 0.9 ± 0.22 g/l in variant II ELISA.

Discussion

The presence of Hp as a major APP in cattle prompted the development of many Hp determination methods. Different ELISA protocols were proposed in the literature for determining bovine haptoglobin. In some of them the microplate was coated with serum samples (Deignan et al. 2000; Young et al. 1995). A low correlation coefficient (r = 0.72) compared with peroxidase activity-based Hp determination methods was found. Moreover, the results were frequently overestimated (Young et al. 1995). Other procedures, such as competitive ELISA, utilized Hp as the solid phase (McNair et al. 1995; Young et al. 1995; McNair et al. 1997). This procedure showed a significantly higher correlation coefficient compared with the peroxidase activity-based method (r = 0.97 in Young et al. (1995), r = 0.94 in McNair et al. (1997)). A third ELISA protocol used Hp MoAb coated on the microplate (Godson et al. 1996).

An alternative arrangement was first described by Kątnik and Dobryszycka (1990) which utilizes the ability of Hp to bind Hb. These authors developed an ELISA to detect Hp in human biological fluids. Very high correlation between this method and the method based on peroxidase activity was found (r = 0.99). The use of Hp binding to haemoglobin as the solid phase for the determination cattle haptoglobin was later used by Young et al.

(1995) and Saini et al. (1998). The correlations of these protocols with the peroxidase activity-based method were lower (r = 0.93 and r = 0.82, respectively). MoAb was used in the former case and ovine polyclonal Ab in the latter.

Both of the ELISA variants we used showed high correlation coefficients (r = 0.96 and r = 0.90, respectively) compared with the guaiacol method. The slightly lower Hp values obtained by ELISA variant I (polyclonal Hp Ab) than by the Jones and Mould (1984) method may be caused by the fact that the antibodies were directed against human Hp 2-1, whereas bovine Hp is more similar to human Hp 2-2. However, it is possible to detect additional peroxidase activity from other serum proteins, which may cause an overestimated concentration of Hp in the method based on the peroxidase activity of Hp-Hb complexes (Cerda and Oh 1990). The lower correlation between the results of the ELISA variants (r = 0.88) was probably a consequence of divergence between the results obtained by the two methods. Thanks to the stable conditions maintained during the procedures, the standard curves had stable absorbance values in repetitive tests (low variability between tests).

The utilization of MoAb produced by clone 2.36.71.41 in ELISA variant II to measure Hp concentration in bovine serum was possible because of its cross-reactivity. Pupek (1996) demonstrated the cross-reactivity of this antibody with Hp of goat, sheep, cattle, horse, and rabbit using ELISA. Among ruminants, this MoAb reacted strongest with goat Hp and weakest with bovine Hp. The cross-reactivity of this MoAb with the Hp of many species of the *Bovidae*, *Cervidae*, and *Equidae* families was shown by Gospodarek et al. (1997; 1998).

The higher Hp levels obtained by ELISA with MoAb may have resulted from the presentation of epitopes available to Ab independently of the degree of Hp polymerization, whereas polymerization probably limited the availability of methaemoglobin binding sites on the Hp molecule utilized in the guaiacol method. Javid (1965) (cited by Bowman and Kurosky 1982) suggested that steric hindrance may occur in the case of large Hp polymers. Convergent indications result from the study by Morimatsu et al. (1991), who calculated the bovine haemoglobin-binding capacity of Hp in the $\alpha_2\beta_2$ complex as between 1:1.1 and 1:1.4.

It was found that Hp determination using both ELISA variants needs different serum dilutions depending on the Hp content:

1. Serum samples in which the Hp concentration determined by the guaiacol method is < 0.1 g/l (Jones and Mould 1984) should be diluted at 1:300.
2. Serum samples with Hp concentrations between 0.1 and 1 g/l should be diluted at 1:300 and 1:1000
3. Serum samples with Hp concentrations > 1 g/l should be diluted at 1:1000, 1:2000, and 1:4000.

Such a dilution procedure dependent on Hp concentration is due to the observation that serum samples with high Hp concentrations (> 1 g/l) showed lower results when tested at low dilutions. Results comparable with the reference method occurred only at higher dilutions. Moreover, significant differences in Hp molecular dimensions associated with different degrees of polymerization may cause deviations in Hp level in cattle regardless of the method used (Morimatsu et al. 1992; Young et al. 1995).

Because the concentrations of Hp in bovine serum samples are unknown, it is necessary to use all of the proposed serum dilutions simultaneously (1:300, 1:1000, 1:2000, 1:4000) in ELISA. Under field conditions, the use of only the 1:300 and 1:1000 dilutions appears sufficient because exact concentrations may be evaluated at Hp concentrations of 0-1 g/l. A calculated Hp level exceeding 1 g/l indicates a severe generalized inflammatory response. In such cases, Hp determination may be repeated using a higher serum dilution. It should be stated that haemolysed serum samples must be rejected because the presence of free haemoglobin in serum competes with haemoglobin coated on the microplate.

Morimatsu et al. (1992) stated that the cattle Hp detection limit was 10 mg/l using

radial immunodiffusion. McNair et al. (1995) detected 0.344 mg of Hp/l using competitive ELISA. The detection limit estimated in the study by Young et al. (1995) was 80 mg/l. Heegaard et al. (2000) and Nielsen et al. (2004), using sandwich ELISA according to Godson et al. (1996), estimated the detection limit as 18 and 0.05 mg/l, respectively. Both ELISA variants used in the present study allowed us to detect relatively low Hp levels in bovine serum; using the polyclonal antibody it was 0.1 mg/l and using the MoAb 0.21 mg/l. If the sample is diluted 1:300, these detection limits allow the identification of all individuals that have an Hp concentration over 0.1 g/l.

Bovine Hp determination using immunoenzymatic methods needs many hours of incubation; Sheffield et al. (1994) needed 3 h 15 min., Godson et al. (1996) 4 h 30 min, and Deignan et al. (2000) 3 h 5 min. McNair et al. (1997) used a significantly shorter incubation period (1 h 40 min) with competitive ELISA. The ELISA variant I which we developed using goat Hp antibodies conjugated with HRPO requires 2 h and ELISA variant II with MoAb 2 h 30 min because of the additional step. Shortening of the incubation periods was enabled by the addition of 2% PEG 6000 to accelerate Ab-Ag complex production (Kątnik et al. 1987). The presence of PEG 6000 enables shortening the incubation period to 15 min. It would be difficult to maintain proper incubation periods when testing a large number of samples, but this is feasible for screening herd health.

In summary, the developed methods show:

- highly significant correlation with the reference method;
- short incubation time (which could be further shortened for field practice);
- shorter time to examine a higher number of samples compared to other methods (radial immunodiffusion, capillary, and gel electrophoresis);
- comparable or shorter time of the procedure compared to other ELISA protocols in cattle;
- reduced costs due to the use of haemoglobin and only one antibody (in variant I).

These advantages support the use of these ELISA variants in monitoring herd health and cattle treatment.

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