Quantitative Determination of Bovine Serum Haptoglobin and Its Elevation in Some Inflammatory Diseases

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Abstract. A modification of the method of Tarukoski was employed for the determination of bovine serum haptoglobin (Hp). A mixture of serum sample and diluted methemoglobin (Hb') solution (30 mg/100 ml) was incubated with o-dianisidine reagent, pH 4.1, at 37°C for 45 min. Peroxidase activity of free Hb was found almost lost in the mixture, in which HpHb complex activity was well maintained. Relatively reliable values were obtained even in hemolyzed serum samples as long as the contaminating Hb was less than 150 mg/100 ml. The Hp values determined by this method were quite parallel to the results obtained by polyacrylamide gel disc electrophoresis. They were smaller or none in normal cattle, but remarkably large (80-130 mg% HbBC) in cattle suffering from severe inflammatory diseases, such as mastitis, pyometra and traumatic reticulitis.

Haptoglobin (Hp) is a plasma glycoprotein which forms a stable complex specifically with hemoglobin (Hb). Brenner [2] first showed in 1965 that Hp concentration was too low to be demonstrated in cattle, and that local inflammation induced by the injection of turpentine elevated the Hp level greatly. Spooner [7] found that this hemoglobin-reactive protein was detected only in 0.5% of normal cattle, but that it was demonstrated in most cattle suffering from acute inflammation.

The determination of serum Hp levels is expected to be a very useful diagnostic tool in clinical medicine especially in cattle. Only a few papers have been published on clinical work on cattle Hp probably because of the difficulty in quantitative determination by the conventional methods used for human Hp.

For the quantitative determination of bovine serum Hp, the method of Tarukoski [9] was examined precisely and improved with some suitable modifications. The principle of this method is due to the difference in peroxidase activity. Free Hb loses this activity, but the HpHb complex retains the activity in the acidic condition. This paper deals with the examination and its application to diseased cattle.

Materials and Methods

1. Reagents

o-Dianisidine reagent: The reagent was prepared by the method of Tarukoski, in which 0.6 g o-dianisidine (Eastman-Kodak Co., Chicago, U.S.A.), 0.5 g 2Na-EDTA, and 15.6 g NaH₂PO₄·2H₂O were dissolved in one liter of distilled water and stirred overnight. The undissolved materials left were filtered off and the solution was carefully adjusted to pH 4.1 ± 0.05 with 2 to 4 drops of concentrated phosphoric acid. The reagent remained stable for several weeks when kept in a brown bottle at room temperature.

200 mM H₂O₂ solution: The concentration of H₂O₂ was adjusted to 200 mM by the use of a millimolar extinction coefficient of 0.08 at 230 nm.

Stock methemoglobin (Hb') solution: To four volumes of packed bovine erythrocytes previously
Table 1. Outline of procedure of determination of bovine serum haptoglobin

<table>
<thead>
<tr>
<th></th>
<th>Sample</th>
<th>Sample blank</th>
<th>Maximum standard</th>
<th>Standard blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diluted Hb⁺</td>
<td>0.1 ml</td>
<td>0.1 ml</td>
<td>0.1 ml</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>[30 mg/dl]</td>
<td></td>
<td></td>
<td></td>
<td>of standard</td>
</tr>
<tr>
<td>Serum</td>
<td>0.02 ml</td>
<td>—</td>
<td>0.02 ml</td>
<td>—</td>
</tr>
<tr>
<td>H₂O</td>
<td>—</td>
<td>0.02 ml</td>
<td>—</td>
<td>0.02 ml</td>
</tr>
<tr>
<td>Above mixture</td>
<td>0.02 ml</td>
<td>0.02 ml</td>
<td>0.02 ml</td>
<td>0.02 ml</td>
</tr>
<tr>
<td>o-Dianisidine</td>
<td>5 ml</td>
<td>5 ml</td>
<td>5 ml</td>
<td>5 ml</td>
</tr>
<tr>
<td>reagent</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H₂O₂ (200 mM)</td>
<td></td>
<td></td>
<td>Incubation at 37°C for 45 min</td>
<td>0.05 ml</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Allow to stand at room temperature for 60 min</td>
<td>0.05 ml</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Read extinction at 440 nm</td>
<td>0.05 ml</td>
</tr>
</tbody>
</table>

washed five times with physiologic saline, two volumes of distilled water and one volume of toluene were added. The mixture was shaken, centrifuged at 3,500 rpm for 15 min. The clear hemolysate was pipetted off. To 5 ml aliquots of this hemolysate, 3 drops of 10% potassium ferricyanide were added to convert oxyhemoglobin to methemoglobin. After 10 min reaction, the solution was subjected to gel filtration through Sephadex G-25 column equilibrated with 0.15 M NaCl solution. The concentration of Hb⁺ solution was determined by using the standard cyanmethemoglobin method. The solution was diluted with saline so as to contain exactly 3.0 g/100 ml. One ml of each solution was dispensed into small test tubes and stored at −20°C for later use.

Diluted Hb⁺ solution: Before use, an appropriate amount of the Hb⁺ stock solution was diluted with saline so as to contain exactly 30 mg/100 ml.

Maximum standard serum: Standard serum was prepared in the following manner. Approximate levels of serum Hp were checked by using polyacrylamide gel disc electrophoresis in normal and diseased cattle. Hp-rich serum was obtained from a cow suffering from mastitis. It was concentrated about three times so as to contain Hp of more than 150 mg/g% hemoglobin-binding capacity (HbBC). This was used as the standard. In the determination, Hb was added to each assay tube so that the final concentration right be 150 mg/100 ml serum. The maximum standard was made by mixing the standard serum and the diluted Hb⁺ solution to develop a maximum color corresponding to an Hp level of 150 mg/g%. HpHC. Its extinction of optical absorption was used for calculation.

2. Procedure

An aliquot of 0.1 ml of the diluted Hb⁺ solution was mixed with 0.02 ml of the sample serum (equivalent to 150 mg Hb/100 ml serum). Then 0.02 ml of this mixture was transferred to a tube containing 5 ml of o-dianisidine reagent. Each sample was analyzed by using a sample-blank containing all the reagents, except the serum for which distilled water was used instead. Each series of analysis included the maximum standard mentioned above. The maximum standard was obtained against a standard-blank in which the standard serum, instead of diluted Hb⁺ solution was used in the recipe of the sample-blank. The standard-blank was used in order to avoid the effect of Hb which may be slightly present in the standard serum. The content was mixed and incubated in a water bath at 37°C for 45 min. To every tube, 0.05 ml of 200 mM H₂O₂ was added, mixed and allowed to stand at room temperature for one hour. The intensity of the color was read at 440 nm in a spectrophotometer (Hitachi model 139, Tokyo). The outline of the procedure is summarized in Table 1.

A linear line with a range of 0–150 mg/g% HbBC was obtained for the standard curve (Fig. 1). Optical densities at 150, 100, 50 and 25 mg/g% of standard HbBC were obtained with a mixture of standard serum and each diluted Hb⁺ solution containing 30, 20, 10, or 5 mg/100 ml. Because of the linearity of the standard curve, the amount of Hp in a serum sample was calculated by the following formula:

\[
Hp \text{ (mg/g% HbBC)} = 150 \times \frac{\text{Serum sample} \times \text{Ext. 440 nm}}{\text{Maximum standard} \times \text{Ext. 440 nm}}
\]

Unit: The amount of Hp in serum is expressed
DETERMINATION OF BOVINE SERUM HAPTOGLOBIN

Fig. 1. Standard curve for range of 0 to 150 mg/100 ml HbBC.

Fig. 2. Color intensity produced by 1 μg Hb/ml as HpHb complex with 0-dianisidin reagent at varying amounts of H₂O₂.

as its HbBC, i.e., mg of Hb bound by Hb per 100 ml of serum.

3. Polyacrylamide gel disc electrophoresis (PAGE) of serum Hp

This was performed by Davis's method in comparison with and analysis of the present method [3]. Five μl of a mixture of nine volumes of serum and 3 g/100 ml Hb solution was applied to each disc column. After electrophoresis, the gel was stained for HpHb complexes with benzidine-hydrdogen peroxide solution [4].

4. Experimental animals

Adult cows clinically normal and those suffering from various diseases were used in this study. Blood was collected via the jugular vein and allowed to clot. Thus, unhemolyzed serum samples were obtained.

**Results**

1. Hydrogen peroxide concentration

The intensity of the color developed was shown to be highly dependent on the H₂O₂ concentration. The suitable concentration of H₂O₂ to be added was found to be 2 mM in the reagent solution (Fig. 2).

2. Color development by free Hb or HpHb complex

Color development of o-dianisidine was examined in the presence of an equal amount of Hb, either as free Hb or as HpHb complex, in this study. That is, one volume of distilled water or the standard serum was mixed with five volumes of diluted Hb* solution. The former mixture was used as free Hb and the latter as HpHb complex. After the addition of 0.02 ml of each mixture to 5 ml o-dianisidine reagent containing 2 mM H₂O₂, color development by free Hb or HpHb complex was examined at room temperature. The activity of free Hb* was exhausted after a few minutes, while that of HpHb complex proceeded for an appreciable period of time and became a plateau after one hour (Fig. 3).

3. Incubation time and temperature

Incubation time and temperature enough to inactivate the peroxidase activity of free Hb* in acid buffer, phosphate-EDTA, pH 4.1, were examined at 25°C or 37°C (Fig. 4). Incubation at 25°C for 15 min rapidly decreased the peroxidase activity of free Hb*, but no complete inactivation was obtained.
Incubation at 37°C for more than 30 min was needed to inactivate Hb⁺ activity almost completely. In contrast, there was only a slight decrease in peroxidase activity of HpHb complex after incubation at 37°C for 45 min.

4. Effects of Hb and incubation time and temperature on HbBC values

The effect of the amount of Hb dissolved in serum was examined in Hp-poor or Hp-rich serum with incubation at 37°C for 45 min or at 25°C for 15 min (Fig. 5). Less than 150 mg of contaminating Hb per 100 ml of serum had little effect on the value of HbBC when incubated at 37°C for 45 min. When serum was incubated at 25°C for 15 min, it was found that the more Hb was dissolved in serum, the larger the
Table 2. Haptoglobin levels in serum of cattle as assessed by PAGE and the spectrophotometric method proposed

<table>
<thead>
<tr>
<th>Animal No.</th>
<th>Case</th>
<th>Degree of serum Hp by PAGE</th>
<th>HbBC by spectrophotometry (mg/100 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Normal</td>
<td>−</td>
<td>2.7</td>
</tr>
<tr>
<td>2.</td>
<td>Normal</td>
<td>−</td>
<td>0</td>
</tr>
<tr>
<td>3.</td>
<td>Normal</td>
<td>±</td>
<td>5.1</td>
</tr>
<tr>
<td>4.</td>
<td>Mastitis</td>
<td>††</td>
<td>110.5</td>
</tr>
<tr>
<td>5.</td>
<td>Pyometra</td>
<td>††</td>
<td>127.2</td>
</tr>
<tr>
<td>6.</td>
<td>Traumatic reticulitis</td>
<td>††</td>
<td>79.5</td>
</tr>
<tr>
<td>7.</td>
<td>Right-sided abomasal displacement</td>
<td>††</td>
<td>74.0</td>
</tr>
<tr>
<td>8.</td>
<td>Right-sided abomasal displacement</td>
<td>††</td>
<td>30.0</td>
</tr>
<tr>
<td>9.</td>
<td>Left-sided abomasal displacement</td>
<td>††</td>
<td>84.9</td>
</tr>
<tr>
<td>10.</td>
<td>Left-sided abomasal displacement</td>
<td>†</td>
<td>15.6</td>
</tr>
<tr>
<td>11.</td>
<td>Traumatic pericarditis</td>
<td>††</td>
<td>46.4</td>
</tr>
<tr>
<td>12.</td>
<td>Pleurisy</td>
<td>†</td>
<td>18.0</td>
</tr>
<tr>
<td>13.</td>
<td>Bacterial nephritis</td>
<td>†+</td>
<td>17.1</td>
</tr>
<tr>
<td>14.</td>
<td>Leukemia</td>
<td>±</td>
<td>13.0</td>
</tr>
<tr>
<td>15.</td>
<td>3 days after surgical operation</td>
<td>††</td>
<td>75.6</td>
</tr>
</tbody>
</table>

value of HbBC determined.

5. PAGE pattern

HpHb complexes were detected as three bands on the top of the gel by PAGE. The Hp patterns were graded into four degrees, −/±, +, + and †† according to the Hp level. The results obtained are shown in Fig. 6.

6. Hp level in diseased cattle

There was little or no Hp at all in the serum of normal cattle. In contrast, a remarkably large amount of Hp, or a value of more than 80 mg% of HbBC, was found in the serum of cattle suffering from severe inflammatory diseases, such as mastitis, pyometra and traumatic reticulitis. Cattle with mild inflammation showed only a mild increase in the serum Hp level ranging from 10 to 40 mg% HbBC (Table 2).

Discussion

The determination of bovine serum Hp was performed by the modified method of Tarukosi [9]. Methemoglobin (Hb+) was chosen as the Hb to be combined with Hp in the serum, because Hb+ is more stable and easier for acid denaturation than oxyhemoglobin. The Hb+ concentration used was 30 mg/100 ml, instead of 60 mg/100 ml in the original method, which gave a maximum level of serum Hp of 150 mg% HbBC. Serum Hp levels of cattle are likely to be lower than 150 HbBC even in severe inflammatory diseases, as revealed in the present experiment.

Sasazuki et al. [6] reported that bovine Hb+ not bound to Hp was highly resistant to acid denaturation, showing a high peroxidase activity at acidic pH. It was confirmed in this experiment that bovine Hb+ could be inactivated at pH 4.1 by using phosphate-EDTA buffer. It was found, however, that incubation at room temperature for 15 min as that in Tarukosi’s method was not sufficient, and that incubation at 37°C for more than 30 min was needed for the almost complete inactivation of bovine Hb+.

Tarukosi [9] acidified the solution to a final pH value of 2.1 in order to avoid opalescent color development in the solution. No opalescence, however, occurred
even without further acidification in the present method. This is probably because
the final intensity of the maximum color
development was lower at an HpHb amount
of 150 mg% HbBC formed than at an
HpHb amount of 300 mg% HbBC formed
in Tarukoski's method.

The methods of Hp estimation based on
the peroxidase activity of HpHb complex
are excellent in sensitivity, but present un-
reliable values for hemolyzed serum samples.
The present modified method could provide
relatively reliable values even for hemolyzed
serum samples, as long as contaminating
Hb was less than 150 mg/100 ml.

Bovine HpHb complex does not have
an alpha-mobility, although human HpHb
complex does. It reveals a beta-mobility
similar to its Hb by agar gel electrophoresis
[8]. It may, therefore, be impossible to de-
termine bovine serum Hp levels by agar
gel or cellulose acetate membrane electrophoresis. PAGE was then employed for
comparison and analysis of the present
method. It is not a quantitative method,
but is simple and reliable and can be used
to determine approximate levels of bovine
serum Hp.

Bovine serum Hp is usually undetectable
in the serum of normal cattle. Minocceri
[5] found the existence of Hp in only 11
of 106 healthy calves, but that in another
group, all 56 animals suffering from severe
physical stress were positive for serum Hp.
The serum Hp level may reflect the severity
of physical stress, including inflammation

[1, 8]. Determination of bovine serum Hp
is, therefore, suggested to be a valuable aid
in making a clinical diagnosis.

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要約

牛血清ハプトグロビンの定量法と炎症性疾患時における上昇：牧村進・鈴木直義（帯広畜産大学獣医学科家畜病理学教室）—Tarukoshi の方法に適切な改良を加え，牛ハプトグロビン（Hp）の定量法を考案した。本法は，種属メトヘモグロビン（Hb⁺）と血清の混合物を pH 4.1 の o-dianisidine 液に 37℃ 45 分間インキュベートして，HpHb のペルオキシダーゼ活性を保留しつつ，遊離 Hb の活性をほぼ完全に失活させることによりおこなわれた。本法は混入 Hb が 150 mg/100 ml 以下の溶血サンプルでも，比較的簡便で測定値が得られた。さらに本法による測定値は，Polyacrylamide-gel disc electrophoresis による Hp の定量的検出結果とよく一致していた。何例かの疾病牛への本法の適用では，乳房炎，子宮蓄膿症，創傷性第二胃炎等の激しい炎症性疾患牛で，血清 Hp の著増（80-130 mg/100 ml HbBC）を認め，一方正常健康牛の血清中には，Hp はきわめて僅かかまたは全く検出されなかった。