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The influence of haptoglobin phenotype on differential leukocyte count in neonatal calves

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Abstract: This research was conducted to study the content and phenotypes of haptoglobin and their correlation to the differential leukocyte count in healthy Red-motley Holstein calves (n = 30). The blood samples from calves were taken through puncture of the jugular vein on days 1, 7, 14, and 28 after birth. Differential leukocyte count was analyzed by manual enumeration. Serum haptoglobin content was determined by spectrophotometry (UV-1700 Shimadzu, Japan). Haptoglobin phenotypes were studied using gel electrophoresis. Serum haptoglobin content in calves on day 1 after birth was 3.8 ± 0.7 g/L, on day 7 was 3.5 ± 0.7 g/L, and on days 14 and 28 was 1.9 ± 1.0 and 3.9 ± 0.3 g/L, respectively. The haptoglobin phenotype Hp2-2 was found in 57.6% of calves, while phenotypes Hp2-1 and Hp1-1(?) were found in 30.0% and 13.3% of the examined animals, respectively. The serum concentrations of haptoglobin in calves with phenotypes Hp2-2, Hp2-1, and Hp1-1(?) did not differ. Calves with phenotype Hp2-2 were characterized by the greatest variability in the indicators of neutrophil content and lymphocyte content and their age-related changes. Animals with phenotype Hp2-1, on the contrary, showed the greatest stability of differential leukocyte count in the neonatal period.

Key words: Calves, haptoglobin phenotype, differential leukocyte count

1. Introduction

Haptoglobin is a glycoprotein of the α_2-globulin fraction of serum proteins found in most vertebrates and in all mammals [1,2]. The primary function of haptoglobin is the binding of free hemoglobin, which neutralizes and limits its oxidative damage to various organs [3,4]. Haptoglobin is one of the evolutionarily conserved sets of proteins of the acute phase, whose hepatic expression is strongly induced by inflammatory mediators [5]. Haptoglobin has high peroxidase activity, inhibits cathepsin B, and modulates the activity and proliferation of leukocytes in the focus of inflammation [4]. Some immunomodulating effects of haptoglobin are associated with the suppression of lymphocyte function [6,7]. It is considered that by binding to monocytic cells and lymphocytes through cellular surface proteins CD11b/CD18, CD163, and CD22, the haptoglobin can control the functions of these cells through one or more signaling pathways [8]. The absence of haptoglobin has been found to cause impaired immune response [9]. Haptoglobin is essential for effective coordination of immune system functions, such as antigen presentation and effector function [9].

Haptoglobin was found in the serum of all mammals, but some authors believe that polymorphism is inherent only in human haptoglobin [10]. In humans, three major (Hp1-1, Hp1-2, and Hp2-2) and about 20 rare haptoglobin types are known [4]. In animals, though, there is no consensus on the number of phenotypes of haptoglobin. Some authors consider that the great majority of mammals have only one band corresponding to human Hp1-1, except for sheep, deer, and cows (Ruminantia), which have only slow bands corresponding to Hp2-2 [11,12]. Other

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authors, however, found that in silver and red foxes as well as chickens two variants of haptoglobin exist, and in fish there are three variants [2,13]. Data on haptoglobin polymorphism in cattle are also contradictory. According to Lai et al. [14], haptoglobin in cows is represented by a single genetically determined Hp2-2 phenotype. Klyuchnikova [15] found that there are three main hemoglobin binding proteins in cows: Hp1-1, Hp2-2, and Hp2-1. Häussler et al. [16] indicated that protein polymorphism may not only be genetically determined, but may also result from various tissue-specific glycosylation variants during the posttranslational modification of α- and β-circuits, which makes it difficult to identify the haptoglobin phenotypes. The concentration of serum haptoglobin has been determined in farm animals as an indicator for infection and cells damage. It was also used as a prognostic indicator for many ailments [17,18].

For humans, the haptoglobin phenotype has been associated with diabetes, cardiovascular pathologies, malignant neoplasms, infectious and neurological diseases, and the overall immunological condition [19]. The effect of haptoglobin phenotype on the reactions of immune cells and the establishment of immune system functions in farm animals during the neonatal period is not well studied.

The purpose of this work is to determine the content and phenotypes of haptoglobin in Red-motley Holstein calves and their relationship to the differential leukocyte count in the first 28 days after birth.

2. Materials and methods

2.1. Animal materials and study design

The research was performed in the winter, during the cattle's dry period. It included 30 calves of the Red-motley Holstein breed, selected by random sampling. The animals were kept in a sanitarium with 5–6 heads per cage for 10–20 days. Then they were transferred to group cages of the calf house (5–8 heads each), where they stayed up to 2–4 months of age. Newborn calves received colostrum from their mothers from nipple drinkers 3 times a day. For 10 days, colostrum (then milk) was fed to the calves in an amount equivalent to 1/10 of the animal's weight, then 1/5 of the animal's weight from the 10th to the 20th day, and from the 21st day they were given a substitute of whole milk or skimmed. From 10–12 days old, calves were taught to eat hay, and from 18–20 days old, they were switched to concentrated feed.

The health status of the calves was assessed daily by determining their body temperature, heart rate and respiratory movements, presence/absence of diarrhea, cough, nasal discharge, eye discharge, behavior changes, sucking reflex activity, and appetite.

Samples of venous blood for laboratory studies in calves were obtained on the 1st, 7th, 14th, and 28th days after birth.

2.2. Collection of samples

Blood samples of all calves were obtained from jugular vein puncture and were collected into sterile vacuum tubes with EDTA and without anticoagulant. After clotting for 1 h at room temperature, blood samples without anticoagulant were centrifuged (UC-1612, ULAB, China) at 4000 × *g* for 10 min at room temperature and sera were carefully harvested and stored at –20 °C until biochemical analysis.

2.3. Determination of haptoglobin

The haptoglobin concentration in the serum was determined by the formation of a haptoglobin–hemoglobin compound with the addition of hemoglobin by using the rivanol method [20,21]. An aqueous solution of 0.2 mL with a 0.5% hemoglobin concentration and 0.3 mL of water were added to 0.5 mL of nonhemolyzed serum; in the control, the serum was replaced with an equal volume of water. Lyophilized powder of horse hemoglobin was used (Sigma-Aldrich, USA). After stirring for 10 min, 2 mL of a 3% aqueous solution of rivanol was added. A significant yellow precipitate formed within 5 min, which was separated by centrifugation for 5 min using a SM-50 microcentrifuge (Elmi, Latvia) at 20,000 × *g*. After the precipitation, a 10% $(NH_4)_2SO_4$ solution was added to the samples and left for 1 h.

A standard solution was prepared by adding 0.2 mL of 0.5% hemoglobin solution and 0.2 mL of 10% ammonium sulfate to 2.8 mL of distilled water. The optical density of the samples was measured at 520 nm (UV-1700, Shimadzu, Japan).

The calculation of the concentration of haptoglobin was performed according to the following formula:

$$X = ((Estd - (Eexp - Econt)) \times 2000 \times 0.003) / Estd,$$

where X is the haptoglobin content in the serum, g/L;
Eexp is the extinction of the experiment sample;
Econt is the extinction of the control sample;
Estd is the extinction of the standard sample;
2000 is the conversion for 1 L of serum; and
0.003 is the hemoglobin content in the standard sample, g/L.

2.4. Analysis of haptoglobin phenotypes

To establish the phenotypes, we performed electrophoresis on the calves' serum samples in 7.5% polyacrylamide gel slabs according to standard methods [22] at 4 °C with subsequent identification of peroxidase activity zones [23]. Before the study, 0.075 mL of serum was mixed with 0.025 mL of glycerin as an anticonvection reagent. A coloring mixture was prepared by mixing 1.0 g of o-dianisidine, 2.5 mL of glacial acetic acid, 0.2 mL of hydrogen peroxide, and 50 mL of water [24]. After the appearance of the

orange stripes on the gel plates, the reaction was stopped by adding 15% acetic acid, followed by rinsing with water. The colored gel had a red-brown background color with blue bands of haptoglobin. The gels were dried on glass plates in a solution of alcohol and glycerin (1:1) and stored in the dark.

2.5. Determination of differential leukocyte count

To determine the differential leukocyte count, a drop of venous blood was thinly spread over a glass slide, air-dried, and stained with Romanowsky stain by the May–Grunewald–Giemsa technique. Two hundred cells were then counted and classified and their percentage ratio was determined [25].

2.6. Statistical analysis

Statistical data processing was performed using the statistical software packages STADIA 7.0 (InCo, Russia) and STATISTICA 8.0 (StatSoft Inc., USA). The comparison of median samples of calves with phenotype Hp1-1 and calves with phenotype Hp2-1 was performed using nonparametric X criteria as per Van der Waerden. The coefficient of variation (Cv) was calculated as the ratio of the standard deviation to the average value. The effect of the factors "type of haptoglobin" and "age of calf when sample was taken" on the indicators of differential leukocyte count were measured using two-factor analysis of variance with a fixed effect. The effects of factors (%) was determined using the Snedecor method. Statistical significance was set at $P < 0.05$. All data were expressed as mean ± standard deviation (SD).

3. Results

The concentration of haptoglobin in the calves' blood serum ranged from 1.9 to 3.9 g/L. On the 1st day after birth, it was 3.8 ± 0.7 g/L, on the 7th day 3.5 ± 0.7 g/L, and on the 14th and 28th days 1.9 ± 1.0 and 3.9 ± 0.3 g/L, respectively. We found no statistically significant differences between samples taken on the 1st, 7th, 14th, and 28th days of life.

In the electrophoretic separation of serum proteins, the haptoglobin Hp2-2 and Hp2-1 phenotypes were identified. Electropherograms of haptoglobins in the blood serum of calves are presented in Figure 1. Haptoglobin type Hp2-2 was defined in 56.7% of the animals (17 individuals) examined and represented by two stripes with low electrophoretic mobility, as shown in Figure 1. Three bands of specific staining, which are typical for Hp2-1, were found in 30.0% (9 individuals) samples of calves. In four individuals (13.3% of the examined animals), no protein fractions with peroxidase activity were detected on the tracks of the polyacrylamide gel. Presumably, it could be the isotype Hp1-1, further highlighted as Hp1-1(?). The age-related dynamics of differential leukocyte count changes in calves with phenotypes Hp2-2 and Hp2-1 are presented in Table 1. A differential analysis of the number of leukocytes in calves revealed neutropenia (band neutrophils 7.7 ± 1.0% and 6.4 ± 1.7%, segmentonuclear neutrophils 29.3 ± 1.9% and 24.5 ± 3.5%, in animals with Hp2-2 and Hp2-1 phenotypes, respectively) and lymphocytosis (59.8 ± 2.3% and 67.3 ± 4.1% in individuals with phenotypes Hp2-2 and Hp2-1, respectively). The number of monocytes, basophils, and eosinophils was determined according to age [26–28]. On the first day of monitoring, the leukogram indicators in the group of calves with the Hp2-1 phenotype were more variable than those with the Hp2-2 phenotype ($Cv(\text{band neutrophils})_{Hp2\text{-}2} = 59.0\%$, $Cv(\text{band neutrophils})_{Hp2\text{-}1} = 79.8\%$; $Cv(\text{segmentonuclear neutrophils})_{Hp2\text{-}2} = 29.6\%$, $Cv(\text{segmentonuclear neutrophils})_{Hp2\text{-}1} = 43.3\%$; $Cv(\text{lymphocytes})_{Hp2\text{-}2} = 17.5\%$, $Cv(\text{lymphocytes})_{Hp2\text{-}1} = 18.5\%$). In both groups, the proportion of lymphocytes in

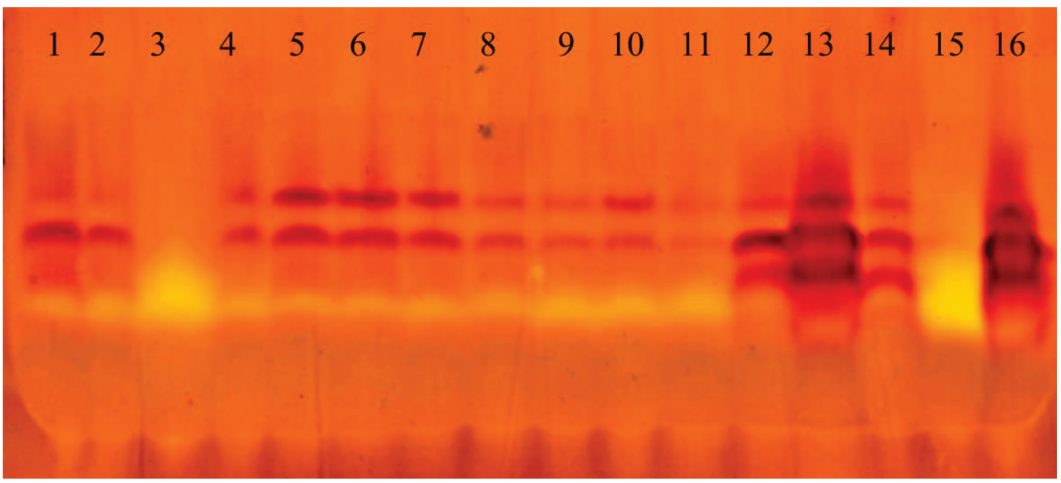

Figure 1. Electropherograms of haptoglobins in the blood serum of calves. Hp2-2: lines 1, 2, 4–11; Hp2-1: lines 12–14 and 16; Hp1-1(?): lines 3 and 15.

Table 1. Differential leukocyte count in calves with haptoglobin Hp2-2 and Hp2-1 phenotypes on the 1st, 7th, 14th, and 28th days after birth (mean ± SD, Cv).

Parameter	Age, days after birth			
	1	7	14	28
Band neutrophils, %	7.70 ± 1.02 (Cv = 59.0%) 6.35 ± 1.69 (Cv = 79.8%)	3.47 ± 0.60[b] (Cv = 77.9%) 3.51 ± 0.81 (Cv = 68.9%)	3.42 ± 0.66[b] (Cv =78.3%) 4.09 ± 2.57 (Cv = 63.2%)	3.58 ± 0.53[b] (Cv =66.0%) 3.42 ± 0.63 (Cv = 51.7%)
Segmented neutrophils, %	29.26 ± 1.94 (Cv = 29.6%) 24.50 ± 3.54 (Cv = 43.3%)	16.54 ± 1.81[c] (Cv = 49.0%) 15.31 ± 1.78[a] (Cv = 34.8%)	13.7 ± 2.01[c] (Cv = 65.7%) 14.39 ± 2.57[a] (Cv = 53.6%)	15.10 ± 1.74[c] (Cv = 51.6%) 13.53 ± 1.95[a] (Cv = 40.7%)
Lymphocytes, %	59.76 ± 2.34 (Cv = 17.5%) 67.28 ± 4.14 (Cv = 18.5%)	77.39 ± 2.02[c] (Cv = 11.7%) 73.33 ± 5.45 (Cv = 22.3%)	80.92 ± 2.58[c] (Cv = 14.2%) 78.70 ± 3.70[a] (Cv = 14.2%)	79.28 ± 2.10[c] (Cv = 11.8%) 82.34 ± 2.80[a] (Cv = 10.2%)
Monocytes, %	1.04 ± 0.31 (Cv = 132.9%) 1.07 ± 0.58 (Cv = 163.9%)	1.73 ± 0.43 (Cv = 111.2%) 3.77 ± 1.60 (Cv = 127.6%)	1.60 ± 0.28 (Cv = 77.7%) 1.87 ± 0.71 (Cv = 115.1%)	1.09 ± 0.29 (Cv = 116.7%) 0.63 ± 0.63 (Cv = 282.9%)
Eosinophils, %	1.88 ± 0.66 (Cv = 157.3%) 1.43 ± 0.84 (Cv = 177.3%)	0.71 ± 0.30 (Cv = 188.1%) 2.8 ± 1.70 (Cv = 181.6%)	0.19 ± 0.13 (Cv = 319.1%) 1.07 ± 0.55 (Cv = 153.7%)	1.89 ± 0.77 (Cv = 181.9%) 1.63 ± 0.75 (Cv = 130.3%)
Basophils, %	0.21 ± 0.14 (Cv = 310.0%) 0.32 ± 0.32 (Cv = 300.0%)	0.17 ± 0.17 (Cv = 447.2%) 0 –	0 – 0 –	0.10 ± 0.10 (Cv = 447.2%) 0 –

Hp2-2 phenotype above the line, Hp2-1 phenotype below the line. [a]P < 0.05, [b]P < 0.01, [c]P< 0.001 in comparison to values from the 1st day of life.

the blood of the examined animals increased significantly by days 7–28, and the number of segmented neutrophils decreased. There were no statistically significant differences detected among differential leukocyte counts in calves with phenotypes Hp2-2 and Hp2-1. However, differential leukocyte counts in the animals with the Hp2-2 phenotype were mainly characterized by higher coefficients of variation compared to the calves with the Hp2-1 phenotype on the 7th, 14th, and 28th days of life, as shown in Figure 2. The more pronounced dynamics of changes in the relative number of neutrophils and lymphocytes were demonstrated in calves with the Hp2-2 phenotype, as identified during the observation period (from the 1st to the 28th day of life), than that in the animals with the Hp2-1 phenotype (Table 1). Two-factor analysis of variance revealed the impact of haptoglobin phenotype and animal age on the differential leukocyte count in calves (Table 2). The influence effect of the "haptoglobin phenotype" factor was 9.7%–9.8%; the effect of factor "age of the animal" was 8.0%–8.5%; interfactor influence was 9.3%–9.7%.

4. Discussion

As previously indicated, haptoglobin is not found in the blood plasma of healthy cows [29–32] and it is detected only when there is an inflammatory process [32]. Such conclusions were probably drawn because insufficiently sensitive analysis methods were used. However, immunoassay studies (western blot and ELISA) showed that haptoglobin is present in cattle plasma in concentrations of 8.4 ± 3.4 g/L [33]. In our study, haptoglobin was detected in the blood serum of healthy calves during the entire monitoring period in concentrations of 1.9–3.9 g/L. This does not contradict the data of other authors [33–35]. Thus, the spectrophotometric method demonstrates sufficient sensitivity and can be used to determine the concentration of haptoglobin in the blood plasma, along

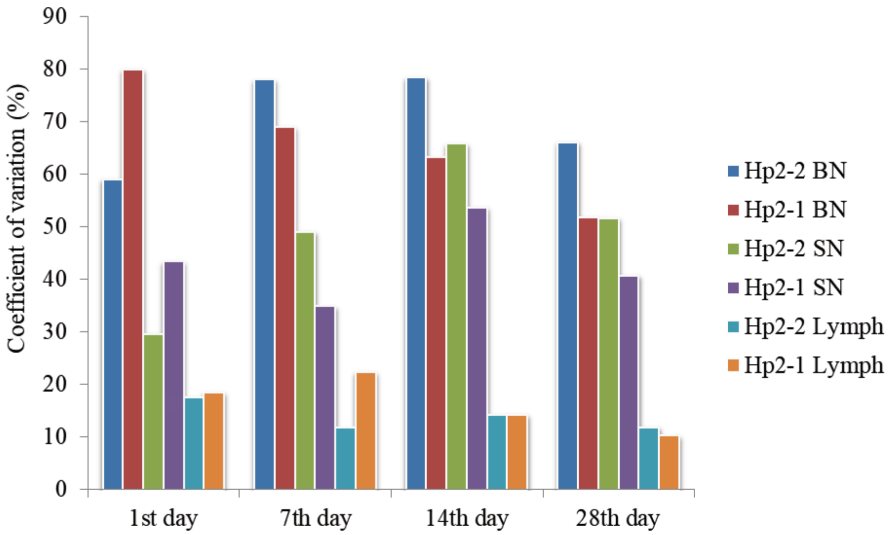

Figure 2. Changes in the variation coefficients of differential leukocyte count of calves with phenotypes Hp2-2 and Hp2-1. Hp2-2 BN and Hp2-1 BN are the variation coefficients of the "band neutrophils" index in calves with haptoglobin phenotypes Hp2-2 and Hp2-1, respectively; Hp2-2 SN and Hp2-1 SN are the variation coefficients of the indicator "segmented neutrophils" in calves with haptoglobin phenotypes Hp2-2 and Hp2-1, respectively; Hp2-2 Lymph and Hp2-1 Lymph are the variation coefficients for lymphocytes in calves with haptoglobin phenotypes Hp2-2 and Hp2-1, respectively.

Table 2. Influence of the factors "phenotype haptoglobin" and "age of calf when sample was taken" on differential leukocyte count in calves.

Factor	Indicator	Degree of influence of factor, %	Significance
Haptoglobin phenotype	Lymphocytes	9.8%	$P < 0.001$
	Segmentonuclear neutrophils	9.7%	$P < 0.001$
	Band neutrophils	9.8%	$P < 0.001$
Age of calf when sample was taken	Lymphocytes	8.5%	$P < 0.001$
	Segmentonuclear neutrophils	8.0%	$P < 0.001$
Multifactor influence	Lymphocytes	9.6%	$P < 0.01$
	Segmentonuclear neutrophils	9.3%	$P < 0.01$
	Band neutrophils	9.7%	$P < 0.05$

with other methods of analysis. Statistically significant changes in the studied indicator on days 7, 14, and 28 compared to the 1st day of life were not detected. The serum haptoglobin content in calves with Hp2-2, Hp2-1, and Hp1-1(?) phenotypes was also the same. Accordingly, in the absence of inflammatory reaction, the studied index was distinguished by high stability.

The haptoglobin polymorphism is due to its α-subunits and the polymerization degree of αβ heterodimers [36,37]. As shown in Figure 3, the Hp1-1 molecule consists of 2 subunits of subtype α_1 (molecular mass about 9 kDa) and 2 β subunits ($(\alpha_1\beta)_2$), and in electrophoretic separation, this protein phenotype in humans is represented by one fraction with fairly high mobility [31,32]. The cyclic polymers of haptoglobin Hp2-2 contain up to 6 subunits of subtype α_2 (molecular mass about 16 kDa) and 6 β subunits ($(\alpha_2\beta)_n$); up to 9 zones of peroxidase activity with low electrophoretic mobility are revealed on electropherograms. The composition of linear polymer Hp2-1 includes subunits α of both subtypes and β subunits ($(\alpha_1\beta)_2$)+($(\alpha_2\beta)_n$)) [38,39]. The haptoglobin of the Hp2-1 phenotype is represented by a large number of fractions;

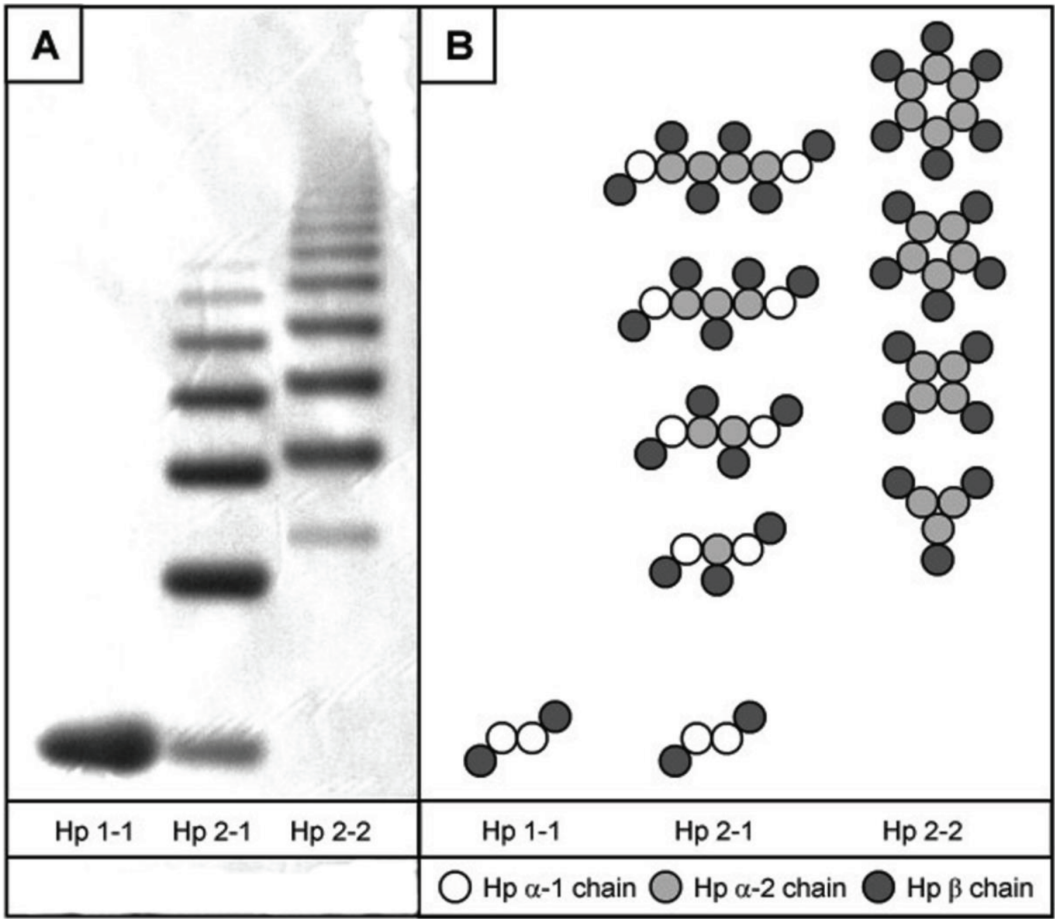

Figure 3. Determination of human haptoglobin phenotypes using one-dimensional polyacrylamide gel electrophoresis [31,32]. A) Electropherograms of haptoglobins Hp1-1, Hp2-1, and Hp2-2; B) structure of haptoglobin molecules of Hp1-1, Hp2-1, and Hp2-2 phenotypes.

however, unlike the Hp2-2 fractions, they are located both in the upper and in the middle parts of the gel. Identification of haptoglobin phenotypes in calves was carried out in the same way as for human haptoglobin [23,38,39]. The most common haptoglobin phenotype in calves was Hp2-2, which some authors [14] consider the only possible genetically determined option in ruminants. Since haptoglobin was detected quantitatively in all serum samples before electrophoresis, the absence of specific staining areas on electropherograms when studying some of the serum samples yielded certain assumptions regarding the fact that some isoforms of haptoglobin (Hp1-1?) may be more sensitive to separation conditions than the others and may lose peroxidase activity during the experiment. There are also cases described in the literature where haptoglobin was not detected in humans or animals at early ages and started to be synthesized as they matured or under the influence of certain specific factors [40]. A small group of calves with an uncertain phenotype (Hp1-1?) was excluded from further research. The association of haptoglobin types and leukogram indices was studied only for animals with haptoglobin phenotypes Hp2-2 and Hp2-1. Changes in the differential leukocyte count affected only neutrophilic granulocytes and lymphocytes. Considering that during the observation period all of the experimental calves remained clinically healthy and we did not observe a significant increase in the concentration of haptoglobin in the serum of their blood, the changes in the differential leukocyte count most likely reflected the processes of cellular immunity formation in calves in the neonatal period [26–28]. The statistically significant effect of the "age of the animal" factor confirms this assumption. However, the impact power of the "haptoglobin phenotype" factor was commensurate with the impact power of the "animal age" factor, underlining the strong correlation between the state of the cellular element of the immune system and the haptoglobin phenotype. The role of haptoglobin as an acute phase protein also suggests its possible immunoregulatory activity. In 1968, Nevo and Stutton [41], and later Langlois and Delanghe [38],

stated that Hp2-2 individuals show greater immunological reactivity (including greater production of antibodies after vaccination) than Hp1-1 and Hp2-1 individuals. Low antioxidant [38,42–45], prostaglandin-inhibitory [46], and antiinflammatory [47,48] activity of Hp2-2 carriers compared to the carriers of other haptoglobin phenotypes may be due to its weak diffusibility because of the high molecular mass. Therefore, lymphocytes may have an increased and neutrophils a reduced sensitivity to the isotype Hp2-2. As a result, imbalance of the granulocyte-agranulocyte ratio in calves with the Hp2-2 phenotype can be more pronounced than in carriers of other phenotypes, which leads to a high heterogeneity of the differential leukocyte count in this group.

In conclusion, the spectrophotometric rivanol method is proved to be sensitive enough to be used to determine haptoglobin in the blood serum of calves, along with enzyme immunoassay, immunoblotting, and polyacrylamide gel electrophoresis. The concentration of haptoglobin in calves within the first month of life is a stable indicator and does not depend on the protein phenotype. The most common, but not the only, haptoglobin phenotype in calves is Hp2-2. The carriers of this phenotype have a high intragroup variability of differential leukocyte counts compared to the carriers of the Hp2-1 phenotype.

In future studies, therefore, there is a need to study in detail the polymorphism of haptoglobin in cattle and its impact on the calves' resistance to stressors and infectious agents. It is likely that animals with the Hp2-2 phenotype, having a broader level of immune response, would demonstrate better adaptation to environmental conditions and better restorative reaction to stressors and infectious agents. On the other hand, genetically determined instability of cellular immunity in calves with the Hp2-2 phenotype may lead to an imbalance of a complex multicomponent immune defense system and cause inadequate reactions to external neutral affects (the occurrence of immunodeficiency states and allergic and autoimmune diseases). From this point of view, calves with the Hp2-1 phenotype, which demonstrate greater stability of leukocyte indicators during the neonatal period, may be preferred for agricultural use.

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