
The Use of Serum Proteins in the Laboratory Diagnosis of Health Disorders in Ruminants

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Abstract

Although hundreds of proteins exist in blood serum, little is known about the precise composition and entire set of serum proteins in different ruminant species. Under physiological conditions, the production of serum proteins is closely regulated, but alterations in the serum protein pattern may occur in a wide range of diseases and health disorders. During the last several years, substantial progress was seen in the application of serum protein analyses for diagnostic purposes. The serum protein profile is mostly evaluated by serum protein electrophoresis, which allows the identification of protein fractions, each being composed of several individual proteins with similar electrophoretic mobility. Many disease processes can cause changes in the concentrations of serum proteins. Therefore, the determination of their concentrations and the evaluation of changes in their concentrations during the disease process may provide important diagnostic information for assessing the health state. Despite this usefulness, the evaluation of serum protein pattern is still relatively a less frequently used laboratory diagnostic technique in ruminant medicine. Thus, the usefulness of serum proteins in the diagnosis of health disorders and the possible clinical application of the results of the electrophoretic separation of serum proteins in ruminants will be reviewed in this chapter.

Keywords: acute-phase proteins, blood proteins, dysproteinemia, electrophoresis, laboratory diagnostics, ruminants

1. Introduction: serum proteins

Proteins are the main and most abundant constituents of the blood serum or plasma, having many essential physiological functions. The most of proteins present in the blood are biochemically not pure; usually, they are a mixture of simple proteins combined with other

substances: glycoproteins, lipoproteins, and other conjugated proteins [1]. Proteins have a specific intramolecular structure and amphoteric nature, containing the balanced portions of hydrophilic and hydrophobic groups [2]. They are macromolecules built from one or more unbranched chains of amino acids linked by peptide bonds. The chemical properties of the amino acids determine the biological activity of the protein [3].

Proteins play a central role in biological processes; some of them are involved in structural support of connective tissues, while others play important roles in biochemical reactions. Proteins also serve as buffers, helping in maintaining the acid-base balance and colloid osmotic structure. Some of them act as carriers of lipids, hormones, vitamins, and minerals in the circulatory system, and are involved in the regulation of cellular activity and immune system [4]. Other blood proteins play important roles as enzymes, complement components, or protease inhibitors. Certain blood proteins are essential for hemostasis and have important functions in platelet adhesion and aggregation, as well as coagulation [5].

Hepatocytes play the major role in the synthesis and secretion of blood proteins [6]. The major exceptions are the immunoglobulins that are produced by the immune system consisting of the reticuloendothelial tissues, lymphoid cells, activated B cells, and plasma cells in response to exposure to antigens [7, 8]. Further studies showed that nonhepatic tissues, including the intestine, lung, adipose tissue, and mammary gland, also have the capability to synthesize some serum proteins for specific functions [9, 10].

The protein constituents of the blood serum are qualitatively different from that of plasma, in which fibrinogen has been removed by conversion into a fibrin clot together with some other coagulation factors [11, 12]. Although serum and plasma are considered suitable samples for many chemistry tests, including serum total proteins, differences in the results obtained between these 2 sample types have been reported by some authors [13, 14]. The principal advantage of plasma over serum is the smaller amount of blood specimen that can be collected from some small animal species. In these species, heparinized plasma is the preferred sample for clinical chemistry, including protein analyses [15]. However, according to some authors, plasma and serum are not equally suitable samples for protein analyses due to noticeable differences in the electrophoretic pattern of proteins in serum and plasma. It should be taken into consideration that fibrinogen, which migrates at the end of the β region on the electrophoretogram, may influence the correct separation and identification of protein fractions [16] (**Figure 1**). Errico et al. [17] concluded also that electrophoretic analysis of proteins in plasma may provide inaccurate results unless plasma is defibrinated, caused predominantly by the possible overestimation of the β -globulin fraction.

The concentrations of proteins in serum are tightly controlled to balance their physiological functions in areas of immunity, coagulation, small molecule transport, and inflammation. Any dysfunction and out-of-balance in their concentrations can cause or result from disease processes [18]. Blood serum contains many different proteins. Some of them are present in the blood serum in concentrations higher than mg/ml, including albumin, immunoglobulins, haptoglobin, transferrin, and lipoproteins [19]. In addition to these major constituents, blood serum also contains many other proteins that are secreted by cells, and tissues in very low concentrations (measured in ng/ml or pg/ml) and in veterinary clinical biochemistry are relatively underutilized [20, 21].

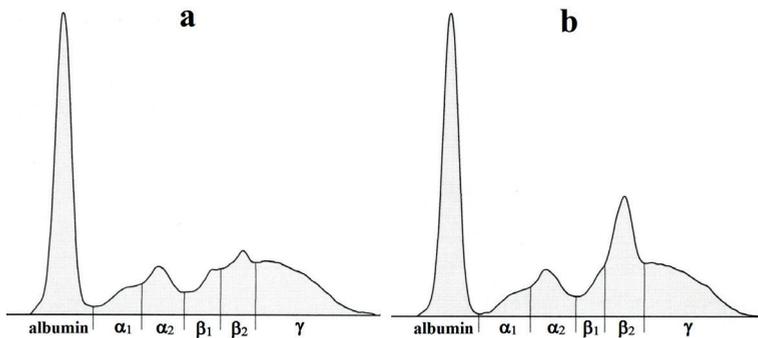


Figure 1. Normal serum (a) and plasma (b) protein electrophoretogram in a clinically healthy cattle.

2. Analyses of serum proteins

Blood proteins are an important indicator of health state and their evaluation represents a basis in general biochemistry [22]. The first step in the analysis of protein pattern is the quantification of total serum or plasma protein concentrations. Several methods have been developed for their determination, which are based on different analytical methods [23]. Several techniques, including chemical and physical methodologies, may be applied to analyze the concentrations of total proteins in blood and other biological fluids. Chemical methods belong to the more commonly used procedures in clinical biochemistry, since they may be to adapt to automated analysers [7].

2.1. Chemical methods

In biochemical laboratories, the most widely used analytical technique to assess the concentrations of total proteins is the biuret method. This method is based on colorimetric principle, in which the copper ions from the biuret reagent react with the amide groups from the proteins at strong alkaline pH, creating a violet color [24, 25]. However, this method is not sensitive enough to measure lower protein concentrations found, for example, in cerebrospinal fluid [26]. Despite of this disadvantage, the biuret assay is still frequently used because of simple analytical procedure, easy preparation of reagents, and when compared with other copper-based assays, this method is less susceptible to chemical interference [27]. Many of the total protein assay kits developed for the automated use in wet biochemical analyzers, as well as dry chemistry analyzers, are based on this principle. This technique is very cheap and this favored its wide application in veterinary medicine.

The biuret method was modified by using the Folin phenol reagent (Folin-Ciocalteu), which is more sensitive and thus more appropriate to measure low concentrations of proteins [28]. In this method, the phenolic groups of tyrosine and tryptophan in proteins react with the Folin-Ciocalteu reagent producing a blue-purple colored complex [29]. The disadvantages of the Lowry method are the sensitivity to the amino acid composition of the protein and the interference with a range of substances, including buffers, drugs, and nucleic acids [30].

Another method for the determination of protein concentrations is the Bradford assay, which is based on the binding of the Coomassie brilliant blue dye to the proteins in an acidic solution to form a complex with increased molar absorbance [31]. This assay is rapid, practical, and suitable for simple quantification of proteins in cell lysates, cellular fractions, and recombinant protein samples [32]. It may be performed also in microtiter plates using micro volumes, but its application area is mainly restricted to research laboratories [33]. Unfortunately, the Bradford assay is linear over a short range (to 2000 $\mu\text{g/ml}$) and shows a curvature over this range of protein concentration, which necessitates the dilution of samples before further analysis [34, 35].

2.2. Physical methods

The concentrations of serum or plasma proteins may be measured also by physical methods. Refractometers are used by many veterinary practitioners, because of their ability to measure the protein concentrations in various biological fluids rapidly. Generally, the refractometric technique is based on the determination of the extent, how light is refracted when it passes from one medium to another of different densities (usually from air into the sample) [36]. The angle of refraction is proportional to the concentration of solute in solution. Seeing that proteins are the most important solute dissolved in serum, the refractive index indicates the concentration of proteins in the sample [37]. A good correlation between refractometry and the biuret method was found in human serum samples [38], but the results for veterinary samples are less consistent. Indeed, whether some authors have reported a good correlation of results for domestic mammals (biuret methods vs. refractometry), others showed either higher or lower values for refractometry compared to the biuret method [39, 40]. The differences between the methods were of 6 g/l and 2 g/l in dogs and cats, respectively [36]. However, the most marked differences between the biuret and refractometric methods were observed in avian samples due to the interference by high concentrations of other light-refractive non-protein components of the blood, such as glucose, cholesterol, or lipids [37, 41]. These variations might be caused by differences in the design of various refractometers assigned by the manufacturers, variation in the biuret reagent mixture, as well as assay [42]. Vandeputte et al. [43] evaluated four different refractometers for measuring serum total protein concentrations in beef calves in comparison with the results obtained by the biuret method. In this study, the refractometric measurements were highly correlated with those obtained by the biuret method indicating similar accuracy for measuring serum total protein values. Calloway et al. [44] and Wallace et al. [45] identified a similar ability to detect failure of passive transfer in calves with refractometers. As the index of refraction is influenced by the temperature of the solute, Automatic Temperature Compensation (ATC) refractometers were commercialized to avoid the impact of potential temperature variations on the results [43]. Recently, digital refractometers have been introduced also into the veterinary medicine, where they demonstrated excellent precision with good sensitivity and specificity [43, 45]. However, according to Hunsaker et al. [46], they did not introduce benefits in accuracy over manual refractometry in regards to potential interference due to non-protein solutes.

3. Evaluation of protein fractions and individual proteins

The identification and quantification of individual serum proteins or groups of proteins are possible only if they are separated. In the protein analyses, the most important method available to measure independent proteins or protein groups is the fractionation technique. Blood serum consists of a large number of proteins; thus, the whole protein complex is not possible to analyze in a simple step by currently available separation technologies [47].

The two major types of proteins in the blood are albumin and globulins. Currently, the bromocresol green (BCG) and bromocresol purple (BCP) methods are the basis for the determination of serum albumin [48]. The BCG method is a dye-binding technique characterized by an ionic interaction between positively charged albumin and negatively charged dye molecules at acidic pH [49]. The bromocresol green binds quantitatively with albumin forming an intense blue-green complex, and the intensity of the color produced is directly proportional to the albumin concentration in the sample [50]. This method is easy to perform, rapid, and cheap, but less sensitive and selective compared to immunoassays [51]. Factors such as optimal pH, ionic strength of buffer, sample preparation, dilution rate, incubation time, and interfering proteins may affect the accuracy of this technique [52, 53]. The reaction between serum and BCG is not specific for albumin; therefore, the BCG method often overestimates the concentrations of serum albumin, but its specificity can be improved by minimizing the contact time with the serum sample [54]. The BCG method is often used to determine the serum albumin concentrations also in animals, including ruminant species [55]. However, albumin methodologies in chemistry analyzers are optimized and designed to measure human albumin. Furthermore, bromocresol green can bind animal globulins with extended reaction times [56, 57]. Therefore, protein electrophoresis may be a better method to provide more accurate albumin quantification [58].

Bromocresol purple is another related dye that may be used for the determination of albumin concentrations, giving more accurate results and thus has better diagnostic utility [54, 59]. Bromocresol purple is an albumin selective dye, which minimizes globulin interference that occurs with bromocresol green by long incubation (more than 30 seconds) [60, 61]. Good correlation was observed between the serum albumin values obtained by the BCP method and immunoassay [61, 62]. Discrepancies may be observed between the serum and plasma albumin values determined by the BCP method. Plasma albumin concentrations may be falsely increased by turbidity due to the precipitation of fibrinogen when plasma is diluted into the BCP reagent [63, 64].

A number of methods have been developed to measure the concentration of globulins. One type of these techniques is based on the precipitation of globulins using solutions of metal salts, e.g., sodium sulfite or zinc sulfate [65, 66]. The addition of salts causes turbidity, which may be visually evaluated or measured by spectrophotometer as units of turbidity. This method may be used as a field test for the evaluation of suckling efficiency or failure of passive transfer of maternal immunity via colostrum in calves and foals [67–69]. However, protein electrophoresis is recommended to accurately determine globulin distribution, allowing to efficiently and precisely detect, as well as quantify several globulin fractions (α -, β -, and γ -globulins) [70].

3.1. Protein fractionation

Electrophoresis is the current standard and most widely used fractionation technique of serum proteins in clinical biochemistry and molecular biology [71]. Several fractionation techniques have been developed to separate and consequently quantify the proteins in serum [72]. The most of them depend on the initial determination of total serum proteins, and then the concentrations of the main fractions can be calculated from the total protein values. Electrophoresis is based on the movement of charged particles through a buffered medium when subjected to an electrical field [73]. Serum proteins have a negative charge, so in the electrophoretic chamber, they migrate toward the positive pole in an electrical field and are separated from each other in different bands according to their sizes [74]. The speed of their movement depends on the characteristics of the protein undergoing separation, including its electrical charge, size and shape, as well as on the strength of the electrical field, type of medium used for the separation, and temperature [75]. After separation, the protein fractions are fixed in an acid solution to denature the proteins and immobilize them on the support medium [3]. The proteins are then stained and quantified by density measuring, providing also graphical data for computer analysis according to the used electrophoretic system [76].

The separation of proteins in an electric field was introduced by Tiselius in the 1930s [77]. The application of serum protein electrophoresis in clinical biochemistry laboratories started in 1950s using paper strips [78], were replaced a few years later by microporous acetate membranes [79]. In the 1970s, agarose gel as a support medium was introduced in the electrophoretic separation of proteins [80], and became a most commonly used supporting substance in veterinary medicine. There are great differences between the electrophoretic methods, which are usually caused by the material of support medium used for the fractionation of proteins [81]. Luraschi et al. [82] stated that the electrophoretic patterns of proteins and the numbers of identified peaks are dependent on the used support material: cellulose acetate vs. agarose gel electrophoresis. The standard agarose gel electrophoresis is a labor-intensive method, but the introduction of prepackaged gels and the development of new equipments allowed the automatization of this procedure [81, 83]. However, the correction of electrophoretograms by visual inspection of an experienced interpreter is very important. Furthermore, agarose gel electrophoresis has several advantages compared to cellulose acetate. Indeed, agarose gel as a support medium provides better resolution, higher reproducibility of results, and greater clarity of the electrophoretic bands [84].

In past few years, capillary zone electrophoresis (CZE) is being used also in veterinary laboratories [85]. In CZE, the separation of protein fractions occurs in a free liquid medium created by the low viscosity buffer, in which the application of high voltage generates an electro-osmotic flow causing rapid movement of proteins toward the cathode [86]. This allows better separation of proteins with similar physicochemical characteristics, thus generating multiple subpeaks or narrower peaks [87]. The higher resolution of CZE can often result in abnormal electrophoretic profiles caused by the aforementioned multiple subpeaks of unknown significance, which is the disadvantage of this method. Recently, laser densitometry was introduced for a precise tracing of the electrophoretic separation [88].

Electrophoresis in ruminant species is normally used in serum, but plasma or other body fluids (urine, cerebrospinal fluid) may also be processed. Serum is the best material for protein electrophoresis, as it does not contain fibrinogen. Electrophoretic technique may be used also for the analysis of urinary proteins, which is a fundamental step in the early diagnosis and subsequent monitoring of renal diseases [89]. It was found that polyacrylamide gel electrophoresis may localize the origin of urinary proteins based on their molecular weight, providing a diagnostic sensitivity comparable to results obtained by kidney biopsy [90]. It is considered a very sensitive method to discriminate between glomerular, tubular, or mixed proteinuria [91]. Agarose gel electrophoresis may be applicable also to separate the main protein fractions in cerebrospinal fluid (CSF) samples. Evaluation of proteins in CSF may provide important information about the production of immunoglobulins within the central nervous system, as well as possible disturbances in the blood-brain barrier [92]. Cerebrospinal fluid contains only a small amount of proteins when compared with serum. Therefore, the proteins in CSF should be concentrated to increase the sensitivity of CSF protein detection by electrophoresis [93].

3.2. Analysis of individual serum proteins

Recently, several techniques, including high resolution electrophoresis, two-dimensional electrophoresis, and proteomic assays were developed for the separation of proteins. These methods allow simultaneous identification of many individual proteins and localize specific proteins within several subfractions. On the other hand, the agarose gel electrophoresis is able to separate serum proteins only into five or six fractions [7]. Changes in proteins with very low concentrations may not be detected by electrophoresis [22]. Immunoassays are another type of methods that may be used for the determination of specific serum proteins. These procedures require a specific antibody against the analyzed serum protein. In biomedical research, enzyme immunoassay (EIA) and enzyme-linked immunosorbent assay (ELISA) belong to the most common analytical methods, which may be used for the identification and quantification of specific proteins, antigens or antibodies [94]. ELISA is based on the concept of an antigen/protein binding to its specific antibody, which allows to detect very small concentrations of antigen/protein [95]. Various types of ELISAs have been developed, while the basic step is the direct or indirect detection of antigen by adhering or immobilizing the antigen or antigen-specific capture antibody onto the well surface [96]. Direct ELISA is considered to be the simplest format of immunoenzymatic assays that determine an antigen immobilized to the plate using an antibody directly conjugated to an enzyme [97]. The indirect ELISA technique requires a secondary antibody to detect the presence of antigen, which is "sandwiched" between the capture antibody coated on the plate and an enzyme-labeled conjugate. Furthermore, the determination of some serum proteins is possible based on their biological activities. For example, the high affinity of haptoglobin for hemoglobin may be used to assess its concentrations. Subsequently, the peroxidase activity of the bound hemoglobin is maintained at low pH [98], the intensity of which is directly proportional to the concentration of Hp in the sample. This colorimetric reaction is not species specific and may be used in several animal species, including ruminants. On the other hand, ceruloplasmin has endogenous oxidase activities, which can be applied to measure its concentrations [99]. However, for the quantitative determination of the most of serum proteins in animals, species-specific assays should still be developed.

4. Physiologic serum protein pattern in large and small ruminants

Following electrophoresis, serum proteins can be separated into four basic fractions including albumin, alpha(α)-, beta(β)-, and gamma(γ)-globulins [100]. Each band consisted of many individual proteins having various metabolic activities. The electrophoretic pattern of serum proteins and its interpretation are related to differences observed among various animal species, as well as among different groups of animals. Great species-specific variations in the type and size of serum protein fractions were observed by many researchers [101, 102]. The number, shape, and size of fractions and subfractions change a lot with the animal species and breed [103]: the most important differences are inside β -globulins and even γ -globulins. Differences in the electrophoretic mobility of serum proteins have been observed also between ruminant species (**Figure 2**).

Nagy et al. [104], by using agarose gel electrophoresis, described six fractions in bovine serum comprising albumin, α_1 - and α_2 -, β_1 - and β_2 -, and γ -globulins. Whereas, Alberghina et al. [105] and Piccione et al. [106] separated the bovine serum proteins into five fractions, comprising albumin, α_1 -, α_2 -, β -, and γ -globulins. The number of protein fractions in sheep and goat serum varied between various authors. Nagy et al. [104] and Esmaelinejad et al. [74] in sheep serum recorded albumin, α_1 -, α_2 -, β -, γ_1 -, and γ_2 -globulins, while the goat serum proteins showed albumin, α_1 -, α_2 -, β -, and γ -globulin fractions [104, 107]. In contrast, Cyrillo et al. [108], Fernandez et al. [109], and Alberghina et al. [102] determined only one α -globulin and two β -globulin fractions in goat serum.

4.1. Prealbumin (transthyretin)

Prealbumin (transthyretin, TTR) is the most rapidly migrating protein fraction in serum visible as a band anodic to the main albumin fraction on the electrophoretic gels [79]. According to Hamilton and Benson [110], this property is attributed to human prealbumin, not to bovine. Kaneko [22] stated also that prealbumin is not always visualized in electrophoretograms and may not exist in all animal species, including ruminant species. Therefore, in these animals, species-specific ELISA assays should be used for the detection and quantification of transthyretin.

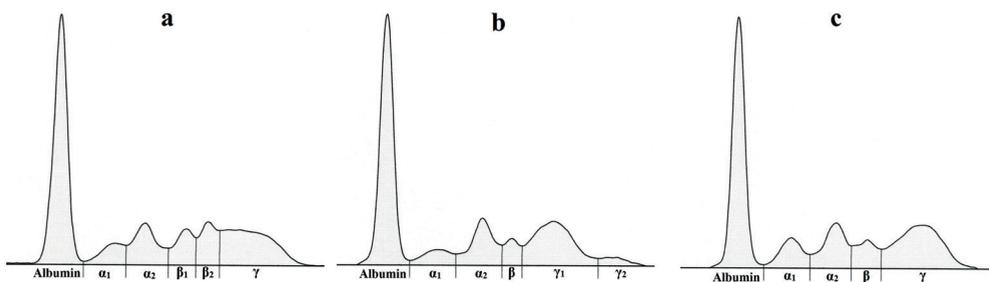


Figure 2. Representative agar gel electrophoretogram in a cow (a), sheep (b), and goat (c) [104].

Transthyretin is a small globular non-glycosylated tryptophan-rich protein of a homotetrameric structure, composed of four identical subunits with two thyroxine binding sites per tetramer [111]. The main physiological functions of TTR include the carriage of thyroid hormones [112]. Another important function of TTR is the transport of retinol (vitamin A) through its association with retinol-binding protein (RBP) from its main storage site in the liver to target cells [113]. From this reason, in the 1980s, the name prealbumin was changed to transthyretin (TTR) describing its ability to bind both thyroid hormones, and retinol binding protein (RBP) [114]. Furthermore, transthyretin acts as a negative acute-phase reactant, serum concentrations of which fall due to decreased synthesis in inflammation, trauma, tissue injury, or stress [115]. It is synthesized mainly by hepatic parenchymal cells and in the choroid plexus of the brain, which has the highest concentration of TTR in the body [116, 117]. In cerebrospinal fluid, it is the second most abundant protein [118]. The major sites of transthyretin degradation are the liver, muscles, and skin [119]. It has a half-life in blood serum of approximately 2 days, which is much shorter than that of albumin [120]. Transthyretin is, therefore, more sensitive to changes in protein-energy status, and thus may be used as an indicator of malnutrition [121].

The concentrations of TTR in blood serum may be affected by many factors, including age, gender, as well as blood-drawing methods. A marked increase of TTR values from 72.9 to 251.4 mg/l was observed by Tóthová et al. [122] in calves 1 day after colostrum intake with a consecutive gradual decrease till the end of the third month of life. Rona [123] described that bovine colostrum contains, among other bioactive molecules, a small amount of prealbumin (transthyretin). Thus, the increase of serum TTR concentrations observed in calves after colostrum intake may reflect the adequate nutrition, as well as its hepatic synthesis due to adequate protein and energy intake [112]. The effect of hormonal changes during pregnancy on the concentrations of TTR in animals has not been reported. Our findings suggest no significant changes in TTR concentrations during the last week of pregnancy and early stages of lactation in dairy cows (unpublished data). The usefulness of prealbumin in the clinical and laboratory diagnosis of diseases was evaluated in dogs with nonthyroidal illness (including neoplasia, allergy, cardiac disease, gastrointestinal disease, parasitism, and hepatic disease) and in pigs with *Streptococcus suis* type 2 infection, showing its lower concentrations compared with healthy ones [124, 125]. In cattle, there are very little published reports about the use of prealbumin in the diagnosis of diseases. Our preliminary results suggest lower concentrations of TTR in diarrheic calves at the age of 1 month compared with healthy animals at the same age. Similarly, *Mycobacterium avium paratuberculosis* seropositive cows showed lower TTR values than those obtained in healthy cattle (unpublished data).

4.2. Albumin

Albumin is the most abundant protein found in blood plasma or serum, and essential part of the biochemistry profile. It is a homogenous protein fraction and is visible as a discrete zone on the electrophoretogram. In animals, 35–50% of the total serum protein concentration is made up from albumin [22]. The shape and size of albumin fraction are very similar in all ruminant species, which are related to its high serum concentration, homogenous electric charge, and high staining affinity. However, there are great differences in its relative

concentrations between different animal species [126]. Albumin can be seen on the left side of the electrophoretogram closest to the anode, where forms a large peak [76].

Albumin is small size protein with a molecular weight of 69 kDa. The main functions of albumin are the maintenance of homeostasis and transportation of substances, and it also acts as a free-radical scavenger [127]. It is responsible for about 75% of the osmotic pressure of plasma and is a major source of amino acids that can be utilized by the animal's body when necessary [128]. It also serves as a carrier protein for many insoluble organic substances (e.g., unconjugated bilirubin). Serum albumin is the major negative acute-phase protein. The synthesis of positive acute-phase proteins is markedly increased during the acute inflammatory processes. These reactions require a great amount of amino acids. Thus, albumin synthesis is downregulated and amino acids are used mainly for the synthesis of the positive acute-phase proteins [129]. Catabolism of albumin occurs in various tissues, where it enters cells by pinocytosis and is then degraded by proteases [130]. The major sites of these catabolic processes are muscle, liver, and kidney. There are major species-specific differences in the turnover of albumin, reflecting the body size. The half-time for clearance of albumin varies from 1.9 days in the mouse to 14–16 days in ruminants, and because of this, it may serve as a marker of chronic nutritional status [131]. Furthermore, many studies have established albumin as an indicator of morbidity and mortality [132].

4.3. Globulins

The globulin fractions may be found on the right side of the electrophoretogram. These peaks include a very heterogenous group of proteins, and depending on the species, there may normally be one or two α , one or two β , and one or two γ fractions [22].

4.3.1. The α -globulins

The α fraction is the most rapidly migrating protein of all the globulins, and in most species, it migrates as α_1 (fast) and an α_2 (slow) fraction. Many diagnostically important acute-phase proteins migrate in this fraction. Alpha₁-antitrypsin, α_1 -acid glycoprotein, α_1 -antichymotrypsin, α_1 -fetoprotein, serum amyloid A, and α_1 -lipoprotein have been identified in the α_1 -globulin fraction, while haptoglobin, α_2 -microglobulin, α_2 -macroglobulin, ceruloplasmin, α_2 -antiplasmin and α_2 -lipoprotein in the α_2 -globulin fraction [100, 133]. Acute-phase proteins are a large and varied group of serum proteins, with numerous differences in their concentrations between different animal species [134]. Their concentrations change in response to any alterations in homeostasis or tissue injury. They have specific functions in the regulation of inflammatory processes, predominantly at the site of inflammatory lesions, but they may act also systemically [115]. In general, the main function of the acute-phase proteins is to defend the host against pathological damage, remove the causative agents of disturbances, assist in the restoration of the homeostasis and in the regulation of different stages of inflammation [135, 136]. Moreover, some proteins from these fractions may act as inhibitors of enzymes, as digest proteins, as compounds of the blood coagulation system or as carrier of copper [71].

4.3.1.1. *Alpha-1 antitrypsin*

Alpha-1 antitrypsin (AAT) is the major inhibitor of serine proteases (serpin) such as neutrophil elastase and proteinase-3 in the blood [137]. It is also an acute-phase protein. In some acute-phase inflammatory reactions, the concentrations of AAT may increase in order to limit the damage caused by activated neutrophil granulocytes and their enzyme elastase, thus limiting the tissue injury caused by proteases at the site of inflammation [138]. The clinical importance of AAT is underlined in patient with AAT deficiency, a hereditary disorder that can lead to severe tissue breakdown during inflammation [139]. Consequently, pulmonary emphysema, chronic obstructive lung disease, liver diseases, as well as liver cirrhosis may occur in these patients. In addition, liver cells produce an abnormal protein, which may accumulate in the body, leading to inflammation and/or cirrhosis of the liver [140]. From animal species, Sevelius et al. [141] measured the concentrations of alpha-1 antitrypsin in dogs and evaluated whether AAT aggregates could initiate liver disease. In cattle, little is known about the diagnostic utility of alpha-1 antitrypsin.

4.3.1.2. *Alpha-1 acid glycoprotein*

Alpha-1 acid glycoprotein (AGP) or orosomuroid is a highly glycosylated protein of which about 45% is carbohydrate and the composition of the glycan residues is known to alter during an acute-phase response [142]. AGP is considered as a natural anti-inflammatory and immunomodulatory agent. It has also been suggested that AGP is required to maintain capillary permeability [142]. Furthermore, AGP is one of the most important drug-binding proteins in plasma that can have important pharmacokinetic implications [143]. It has a moderate acute-phase response in most animal species and is more likely associated with chronic conditions. The serum concentration of AGP may be a valuable differential diagnostic analyte in the identification of feline infectious peritonitis [144].

In ruminant species, the concentrations of AGP were evaluated by Tóthová et al. [145] in calves during the first month of life. In this study, the AGP values were roughly uniform shortly after birth with an increase of values from the day 2 of life till the end of the first month of age, probably related to the normal process of growth, exposure of animals to changing environmental conditions, and nutritional factors. Similar findings were demonstrated by Rocha et al. [146]. On the other hand, the highest concentrations of AGP in the plasma were found by Itoh et al. [147] in calves immediately after birth (1368 µg/ml), gradually decreasing to 249 ± 100 µg/ml during the first 3 days of life, which are comparable to physiological values in adult bovine. Similarly, high plasma concentrations of AGP were observed by Orro et al. [148] in calves after birth, which was followed by a decrease during the first 3 weeks of life to adult values. The very high concentrations of AGP in the fetal stages may be related to synthesis of AGP in the embryonic liver [147]. These studies indicate that the production of AGP in the neonatal period is fetally regulated and its high serum concentrations after birth are not necessarily a sign of the activation of the acute-phase response by external stimuli.

4.3.1.3. *Alpha₁-fetoprotein*

Alpha₁-fetoprotein (AFP) is the predominant serum protein in the bovine fetus, which is mainly produced by the yolk sac and at the later period by the fetal liver [149]. Because of its biochemical similarity to albumin, AFP could be a carrier protein, or even take part in the metabolism of bilirubin [150, 151].

Smith et al. [152] have demonstrated that the concentrations of AFP in fetal bovine plasma reach the highest values in the 3–4th of fetal period, which is followed by significant decrease until birth. A decrease of the values of AFP in the first hours of life was obtained by Bader et al. [149] that may represent the physiological effect on the fetal tissues changing from an intrauterine to an extrauterine environment, with decrease in the production of fetal proteins in the liver. Lee et al. [153] concluded that following the initial decrease of the values of AFP within hours after birth, the concentrations of AFP tend to stabilize during the rest of the first week, and then decrease rapidly. On the other hand, Tóthová et al. [122] found a marked increase of AFP concentrations 1 day after colostrum intake, with following gradual decrease of values up to day 30 of life. The relatively higher values of AFP after birth may be associated with its synthesis (not ceasing entirely at birth) by fetal hepatocytes that continue during the early postnatal period [154]. Furthermore, colostrum contains many non-nutrient substances and immune factors, including alpha₁-fetoprotein, which may be responsible for the increased concentrations of AFP in calves 1 day after colostrum intake [155].

Alpha₁-fetoprotein may be used as a tumor marker to help detect and diagnose cancers of the liver, testicles, and ovaries. Sturgeon et al. [156] reported increased concentrations of AFP in approximately 70% of patients with hepatocellular carcinoma. Increased AFP concentrations were found also in 50–70% of patients with nonseminomatous testicular tumors. In veterinary medicine, the possible use of AFP as disease marker was not yet evaluated.

4.3.1.4. *Serum amyloid A*

Serum amyloid A (SAA) is a small hydrophobic protein that belongs to the family of apolipoproteins associated with high density lipoprotein [157]. Different isoforms of SAA are expressed constitutively at different levels in response to inflammatory stimuli [158]. During inflammation, SAA1 and SAA2 are expressed principally in the liver, whereas SAA3 is induced in many distinct tissues, including the mammary gland [159]. The fourth isoform, SAA4, does not respond to external stimuli [160]. The main functions of SAA are the reverse transport of cholesterol from tissue to hepatocytes, opsonization, inhibition of phagocyte oxidative burst, and platelet activation [136]. The M-SAA3 isoform found in colostrum stimulates the production of mucin from intestinal cells and thus helps to prevent bacterial colonization [161].

In ruminants, SAA belongs to major acute-phase proteins which increases more in acute rather than in chronic conditions [162]. Intense changes in the concentrations of SAA were reported in dairy cows with various inflammatory diseases, including cows with endometritis, mastitis, as well as in lame cows [163–165]. It was raised also in cattle experimentally infected with *Mannheimia haemolytica* and bovine respiratory syncytial virus, or with bovine viral diarrhoea virus [166, 167].

Eckersall et al. [168] found significantly elevated SAA concentrations in sheep with experimental caseous lymphadenitis induced by *Corynebacterium pseudotuberculosis*. Chalmeh et al. [169] observed in sheep, a rapid increase of SAA values during experimentally induced endotoxaemia by lipopolysaccharide from *Escherichia coli*. Another study conducted by El-Deeb [170] showed an increase in the concentrations of SAA in ewes with pregnancy toxemia. Marked increase of SAA concentrations was recorded also in sheep following experimental infestation with *Psoroptes ovis* [171]. After treatment, the SAA values decreased rapidly within 3 days and returned to the pre-infestation values for 10–14 days. The alterations in the acute-phase protein production during experimental caprine coccidiosis were evaluated by Hashemnia et al. [172]. They found markedly higher concentrations of SAA at day 7 after inoculation. Furthermore, the magnitude and duration of the acute-phase responses are correlated well with the severity of the clinical signs and diarrhea in goat kids.

4.3.1.5. Haptoglobin

Haptoglobin (Hp) is a glycoprotein that consists of two α and two β chains, connected by disulfide bridges [173]. In the circulation, Hp is highly polymerized, having a molecular weight of approximately 1000–2000 kDa, and exists also as a polymer associated with albumin [174]. The primary function of Hp is to bind free hemoglobin released from erythrocytes and thereby inhibits its oxidative activity [175]. The Hp-hemoglobin binding also reduces the availability of the heme residue from bacterial growth [176].

Many studies have indicated the significance of Hp as a clinically useful parameter for measuring the occurrence and severity of inflammatory responses in cattle with various diseases, including mastitis, enteritis, peritonitis, pneumonia, as well as endocarditis [136, 177]. Higher concentrations of Hp were found also by Sheldon et al. [178] in cows with uterine bacterial contamination. In addition, Hp was detected in ewes as prognostic indicator of ovine dystocia [179]. Gonzalez et al. [180] studied the possible use of acute-phase proteins as markers of subacute ruminal acidosis in goats. They found a moderate increase of Hp concentrations during the induction period, while SAA did not change. In a further study, Gonzalez et al. [181] determined the effect of fasting-induced pregnancy toxemia on the concentrations of acute-phase proteins in goats. They found a significant increase only in the concentrations of Hp, but not in other acute-phase proteins. The changes of some inflammatory markers were evaluated also in goats around kidding [182]. Their results suggest that an increase of inflammatory indicators (mainly Hp) before kidding may be related to the changes in the energy balance status around parturition.

4.3.1.6. Ceruloplasmin

Ceruloplasmin (Cp) is a ferroxidase enzyme that is the major copper-carrying protein in the blood, and plays a role in iron metabolism [183]. Ceruloplasmin carries 70–95% of the total copper in plasma, and thus might play a role in Cu and iron homeostasis [184]. Furthermore, ceruloplasmin is involved in cellular prooxidant and antioxidant processes, and has antibacterial activities [185]. It is produced by the liver as apoceruloplasmin, an unstable non-copper-bound form, which subsequently reacts with seven copper atoms forming holoceruloplasmin, a functional and more stable product [186].

Ceruloplasmin has been evaluated as a marker of animal health and welfare [187]. Several studies in cattle indicate its diagnostic use with applications in many disease conditions, including uterine bacterial contamination, as well as clinical and subclinical mastitis [177, 188]. Hussein et al. [189] evaluated ceruloplasmin activity in dairy cows in different lactation stages, showing higher values in fresh-lactation stage. López-Alonso et al. [190] measured ceruloplasmin as a potential marker of hepatic copper accumulation in cattle. Studies in young animals have shown that the concentrations of ceruloplasmin in the serum increases during induced pneumonic pasteurellosis, with the highest concentration observed 2 and 4 hours after the inoculation [191].

4.3.2. *The β -globulins*

The β -globulins belong to group of globular proteins that migrate faster than γ -globulins in electrically charged solutions, but more slowly than α -globulins. The main components of the β -globulin fraction are transferrin and complement, which may correspond to the 2 subfractions (β_1 and β_2) identified in some animal species [81]. Other important proteins belonging to this fraction are: β_2 -microglobulin, C-reactive protein, ferritin, hemopexin, plasminogen, and angiotatin. Furthermore, in response to the stimulation by different antigens, some IgM immunoglobulins may migrate in the β region, while the IgA and IgE immunoglobulins in the β - γ interzone, which may also correspond to the β_2 subfraction, identified in some animal species [5].

4.3.2.1. *Transferrin*

Transferrin (Tf), the iron-binding protein of serum has been described as a negative acute-phase protein. It is a strong chelator that is able to bind iron tightly but reversibly. The transferrin molecule has high affinity to bind two atoms of ferric iron (Fe^{3+}), being higher in the extracellular pH of 7.4 and decreases in the acidified endosomes, allowing the dissociation of Fe^{3+} [192]. The primary role of transferrin is to transport iron safely around the body to supply growing cells [193]. Essentially, all iron circulating in the blood normally is bound to transferrin. It renders iron soluble under physiologic conditions, prevents iron-mediated free radical toxicity, and facilitates transport into cells [194]. Similar to lactoferrin, transferrin inhibits multiplication and growth of certain viral, bacterial, and fungal organisms by iron inhibition.

Moser et al. [195] evaluated the concentrations of transferrin in cattle in various physiological states, in energy-deficient (ketotic) cows, in cases of several acute and chronic infections, as well as after the administration of endotoxins. The values of transferrin in healthy animals ranged from 2.0 to 6.6 g/l. While in animals with acute infections and ketosis, the values were in the range of 1.5 and 8.5 g/l, chronic infectious diseases (such as paratuberculosis) were associated with relatively low values (below 2 g/l). The evaluation of the effect of age on transferrin concentrations showed its lower values in adult animals compared to young animals [195]. Tóthová et al. [122] presented a marked increase of transferrin concentrations from day 7 of life, reflecting acceptable rate of protein synthesis and good nutritional status. Furthermore, the concentrations of transferrin increased in veal calves with iron deficiency above 8 g/l, resulting in negative correlation between hemoglobin and transferrin [195].

4.3.2.2. Lactoferrin

Lactoferrin (Lf), also known as lactotransferrin, is a multifunctional protein of the transferrin proteins capable of binding and transferring Fe^{3+} ions. Lactoferrin is a globular glycoprotein with a molecular weight of about 80 kDa, which shows high affinity for iron [196]. Although the overall structure of lactoferrin is very similar to that of transferrin, they differ in their relative affinities for Fe and the propensity for release of Fe [197]. The capability of lactoferrin to bind iron is two times higher than that of transferrin [198]. This bound is very strong and can resist pH values of as low as 4 [199]. The ability to keep iron bound even at low pH is important, especially at sites of infection and inflammation where, due to metabolic activity of bacteria, the pH may fall under 4.5 [200]. The most of bacterial pathogens necessitate Fe for metabolic activities, growth, and proliferation. Since lactoferrin has Fe-binding capacity, it reduces the growth of Fe-requiring pathogenic bacteria including enteropathogenic *E. coli* [201]. Lactoferrin is a major component of the innate immune system of mammals and represents one of the first defense systems against microbial agents, which invaded the organism mostly by mucosal tissues [202]. It affects the growth and proliferation of many infectious agents including both Gram-positive and Gram-negative bacteria, viruses, protozoa, and fungi [203].

Lactoferrin is expressed in most biological fluids, including milk, saliva, and nasal secretions. It is present in blood, plasma, or serum in relatively low concentrations, but its concentrations increase during infection, inflammation, excessive intake of iron, or tumor growth [204]. Higher concentrations of lactoferrin were observed in bovine and human milk, or colostrum. The lactoferrin values in milk of healthy cows are quite variable and may range from 1.15 to 485.63 $\mu\text{g/ml}$. On the other hand, sub-clinical and clinical mastitis may lead to rapid increase of its concentrations positively correlating with SCC, stage of lactation, and milk yield [205, 206]. The concentrations of lactoferrin are higher in colostrum (varying between 1 and 5 mg/ml), during drying-off and early mammary involution period than during lactation [207, 208].

Lactoferrin plays a key role in the defense mechanisms of the mammary gland, contributing to the prevention of microbiological infection diseases [209]. Therefore, the concentrations of lactoferrin in milk are markedly influenced by the health status of the cows. Harmon et al. [210] induced *E. coli* infection in bovine mammary gland. In these cows, they found a 30-fold increase of lactoferrin values in the mammary secretion 90 h after the inoculation. Furthermore, they concluded that acute mastitis is associated with 30-fold increase of the concentrations of lactoferrin in the milk with the greatest production in the infected quarter.

4.3.2.3. C-reactive protein

C-reactive protein (CRP) was the first identified acute-phase protein, which was named according to its ability to bind to C-polysaccharide of Gram-positive bacteria [211]. It is a non-glycosylated protein from the group of pentraxins, and is composed of 5 subunits that firmly bind to C-polysaccharides [212]. Following bacterial infection, CRP binds to pathogen and activates the classical complement pathway leading to the opsonization of the bacteria

[213]. It also plays a role in the destruction of the infectious agent through the interaction with specific receptors on phagocytes, which may help in the reduction of tissue damage, and contribute to the tissue repair and regeneration [162].

There are considerable species differences in the magnitude and duration of changes in CRP concentrations during health disorders. In humans, dogs, and pigs, CRP is the major acute-phase protein with approximately 1000-fold increase in serum concentrations during acute inflammatory states [214]. In cattle, CRP has been reported to be a constitutive protein with only a minor increase during disease processes [7]. Despite this disadvantage, Schrodler et al. [215] evaluated the CRP concentrations in cows with mastitis, and found approximately 10-fold higher values in these cows (1083 ± 93 ng/ml) compared with healthy ones (82 ± 66 ng/ml). The data recorded by Lee et al. [216] showed a correlation between serum CRP concentrations and the health condition of dairy cattle.

4.3.2.4. Fibrinogen (in plasma)

Fibrinogen (Fbg), a precursor of fibrin, is also an acute-phase protein, which in coagulation cascade is the final substrate in the formation of a clot being converted to its insoluble fibrin form [217]. Fibrinogen belongs to the group of β -globulins and is present in the plasma. It is composed of three polypeptide chains linked by disulfide bridges and a glycoprotein [218]. Fibrinogen plays an important role in homeostatic processes, providing a substrate for fibrin formation. It is also involved in tissue repair, and provides a matrix for the migration of inflammatory-related cells [219]. During an inflammatory reaction, fibrinogen can increase 2–3 folds, which may significantly increase blood viscosity and cause red cell aggregation, as well as may contribute to the growth of atherosclerotic plaques [220]. In human, studies showed an association between fibrinogen concentrations and subsequent cardiovascular disease risk, atherosclerosis, and acute thrombosis [221]. In cattle, fibrinogen has been used for many years to evaluate inflammatory and traumatic diseases, and is characterized by markedly increased synthesis in response to infection [222].

4.3.3. The γ -globulins

The γ -globulin fraction is predominantly composed of immunoglobulins (Ig) of various classes (IgG, IgA, IgM, IgD, and IgE). While in some animal species (cattle and goats) the γ -globulins constitute one overall fraction, in sheep, they may be visualized as two subpeaks: the γ_1 and γ_2 subfractions [104]. According to Kaneko [22], immunoglobulins from the γ fraction may migrate as fast or slow, which may be seen in these two subfractions. On the other hand, Vavricka et al. [76] indicated that some classes of immunoglobulins may migrate into the β - γ zone or β -region. Immunoglobulins (or antibodies) have major roles in the immune responses of the body, especially in response to foreign molecules, the so-called antigens. Their primary function is the protection of the host due to specific binding of one or a few closely related antigens in order to mediate their neutralization and elimination [223]. Immunoglobulins are produced by cells of the adaptive immune system, activated B cells and plasma cells, in response to the exposure to antigens [8].

The immunoglobulins are glycoproteins composed of two heavy (H) and two light (L) chains linked by disulfide bridges [223]. According to the structure of the H chain, immunoglobulins are classified into the following classes: IgG, IgM, IgA, IgE, and IgD. The L chain consists of either kappa (κ) or lambda (λ) chain, which indicates the type of immunoglobulins. Based on the structural variations in the variable regions of H or L chains, immunoglobulins can be further divided into subtypes and subclasses. For example, two subclasses of IgG have been identified in cattle (IgG1 and IgG2) [7].

Most viral, bacterial, and toxin antibodies are of the IgG type and are present in all animals. It is the predominant type of immunoglobulins found in the body and has the longest serum half-life. IgE is involved in allergic and anaphylactic reactions, whereas IgA can be found in the secretions of the respiratory, genitourinary, and gastrointestinal tracts [7]. IgM functions be opsonizing antigens for destruction and fixing complement, and usually are associated with the first line of defense [224]. IgD is found in very low concentrations in the serum and has a short half-life. The functions of circulating IgD are not well understood [225].

5. Changes in the serum protein electrophoretic pattern

Several factors, including non-pathological and pathological conditions, may influence the concentrations of proteins in the serum, thus the entire profile of serum proteins [226]. Many disease processes are associated with abnormal serum protein profiles. Changes in the protein profile commonly occur as secondary symptoms in numerous diseases, but may be also the primary symptom of some specific disease conditions [227]. Thus, the results of the electrophoretic analyses of serum or plasma proteins may provide a basis for the establishment of further specific diagnostic procedures and may be helpful by the differential diagnosis of several disease processes. However, abnormalities in the serum protein profile must be interpreted with regards to many influences that are not associated with pathological processes.

5.1. Serum protein pattern variations related to non-pathological conditions

Variations in the serum protein profile and shifts in albumin and globulin concentrations may occur not only under pathological, but also under physiological conditions [102]. Animal age is one of these important factors that may affect the concentrations of the different serum protein fractions or their electrophoretic pattern [103]. It has been shown in young and adult cattle [228], where the most important age-related differences were observed in the α - and γ -globulin fractions. While the values of α_1 -globulins were higher in calves, the adult animals had higher γ -globulin concentrations. In particular, it has been stated that the most important changes occur in the first month of the life of calves, and are associated with the changes in nutrition and adaptation processes during the neonatal period [229]. The total serum proteins and γ -globulin concentrations increase rapidly 1 day after the intake of colostrum, and then decrease gradually till the end of the first month of age. According to Hammon et al. [230], the concentrations of total proteins in the serum are very low at birth, due to the minimal quantities of immunoglobulins but, it increases during the first 24 hours of life as a result

of the intestinal absorption of proteins (particularly immunoglobulins) from colostrum. On the other hand, the concentrations of albumin decrease 1 day after colostrum intake, with a subsequent gradual increase from day 2 till the end of the first month of life. At birth, calves' α_1 -globulins comprise almost 30% of total proteins, but their concentrations decreased approximately by 50% at 1 day after birth, with a further decrease up to day 30 of life [229]. In the absolute concentrations of α_1 -globulins, a temporary slight increase after birth has been observed with a subsequent gradual decrease. The delivery is surely a stressful situation for the offspring and it could typically be expressed by higher concentrations of acute-phase proteins at birth, which migrate into this fraction [148]. The acute-phase response may be then substituted by the following increase of the IgG concentrations from the colostrum. Acute-phase proteins are produced mainly by the liver, which is less mature in newborn than in young or adult animals. Thus, the most of the acute-phase proteins have lower concentrations at birth than in the next days [231]. Similarly, large amounts of α -globulins were observed in lambs during the first month of life [232].

Pregnancy and lactation are further factors that may influence the concentrations of albumin and globulin fractions. Variations in the serum protein profile were found in ewes during the pregnancy and lactation, as well as in periparturient goats [233–235]. Changes in the concentrations of protein fractions during the last phase of pregnancy and early *post-partum* were recorded also in dairy cows [236]. Lower concentrations of total serum proteins were found by Grünberg et al. [237] in cows around parturition than outside the parturient period and in the following stages of lactation. These changes may be associated with the transfer of immunoglobulins from the bloodstream to the mammary gland for the synthesis of colostrum [238]. The results of Piccione et al. [235, 239] showed increasing values of α -globulins in dairy cows and ewes *post-partum*, which were probably related to the higher concentrations of the acute-phase proteins in response to the processes occurring around the time of parturition.

The concentrations of serum proteins may be influenced also by hormonal changes and stress. Stress may cause a decrease of serum protein and albumin concentrations, but often may be accompanied by an increase of the α_2 -globulin fraction associated with the acute-phase response [7].

5.2. Pathological serum protein pattern: dysproteinemias

A wide variety of diseases can cause changes in the serum protein pattern [240]. The serum protein electrophoresis is a very important technique for the evaluation of these abnormalities and the nature of the hyperproteinemia or hyperglobulinemia [241]. The protein electrophoresis may be very useful when routine investigations are not effective for making medical decisions, providing the basis for further specific laboratory analyses [22, 242].

5.2.1. Changes in the albumin fraction

The decrease of the concentrations of albumin is one of the most frequently occurring types of dysproteinemias. Hypoalbuminemia can be caused by decreased production due to liver diseases such as chronic hepatitis, cirrhosis, or liver failure [243]. Hypoalbuminemia may be

also present in renal diseases and nephrotic syndrome, in which there is an increased loss of this protein in urine caused by glomerular damage [244]. Moreover, low albumin concentrations may indicate chronic malnutrition, inadequate protein intake, or being associated with gastrointestinal diseases, internal parasitism and protein losing enteropathy [245]. On the other hand, serum albumin is the major negative acute-phase protein and its synthesis may be markedly reduced during the acute-phase response [246].

Rarely, a serum protein anomaly called bisalbuminemia may be observed on the electrophoretogram. Bisalbuminemia is characterized by the occurrence of a bicuspid electrophoretic pattern in the albumin fraction, where albumin produces two heads (equally staining bands or bands of unequal intensity) [247]. In this abnormality, albumin may either have increased (fast type variants) or decreased electrophoretic mobility (slow type variants) [248]. In humans, the presence of bisalbuminemia have been described in some pathological conditions, including chronic renal diseases, nephrotic syndrome, diabetes mellitus, pancreatic disease or Alzheimer's disease [249]. In ruminants, bisalbuminemia was not yet found. According to Vavricka et al. [76], the presence of bisalbuminemia may be caused by increased mobility of albumin due to its binding to bilirubin, non-esterified fatty acids, penicillin or acetylsalicylic acid.

The increased concentration of albumin in the serum is called hyperalbuminemia, which may be observed in cases of severe dehydration. However, hyperalbuminemia was recorded also in dogs with hepatocellular carcinoma [250].

5.2.2. Changes in the globulin fractions

Increases in the globulin fractions may be frequently seen on serum protein electrophoretograms. Since many acute-phase proteins belong to the alpha-globulin fraction, increase in the α_1 - and α_2 -zones may be typical for many acute, as well as chronic inflammatory diseases caused by the activation of the host inflammatory responses [71]. Increased α -globulins (predominantly α_1 -globulins) were found in sheep naturally infected with *Babesia ovis*, as well as in calves affected by respiratory diseases [251, 252]. The α_2 -globulin fraction typically increases in patients with nephrotic syndrome as a result of the increased synthesis of α_2 -macroglobulin that migrates in this fraction. Because of its size, the α_2 -macroglobulin is unable to pass through glomeruli and therefore it remains in the bloodstream [253]. Decreases in the α_1 -globulin fraction may be detected in the α_1 -antitrypsin deficiency, a rare genetic disorder in humans and even more rare in animals, but in ruminants, it was not yet detected [254]. Similarly, the α_2 -globulin zone may be typically decreased in hemolytic anemia, when haptoglobin from this fraction binds with the free hemoglobin released from the destroyed red blood cells, forming haptoglobin-hemoglobin complexes that are rapidly removed by phagocytes [76]. On the other hand, the inflammatory conditions that develop in association with hemolytic anemia leads to an increase of haptoglobin concentration that may induce an increase of α_2 -globulins [255].

Some acute-phase proteins migrate into the β -region. Thus, several inflammatory diseases and infections may be accompanied also by increases in the β -fraction as a result of the elevated

production of these proteins. Kaneko [22] stated that increases solely in the β -globulin fraction are not frequent and may be typical for active hepatitis. Chronic persistent liver disease, liver cirrhosis, as well as nephrotic syndrome may be associated with elevations in the β -region due to the increase of the concentrations of β_2 -microglobulin in these conditions [256]. High β -globulin concentrations may be associated also with hypercholesterolemia, which is caused by increased concentrations of β -lipoproteins in this fraction [257]. Furthermore, increased β -globulins are typical for iron deficiency anemia associated with higher values of transferrin [258]. The increase of β -globulins in hemolytic anemia may depend on the presence of free hemoglobin that typically migrates in this region. On the other hand, malnutrition is often accompanied with decreased concentrations of β -globulins.

In some conditions, the increase in the β_2 - and γ -globulin fractions may result in a beta-gamma fusion. This phenomenon is called β - γ bridging and is characterized with no clear demarcation between these two fractions. It is caused by an increase of the concentrations of IgM or IgA, which may migrate in the region between the beta and gamma zones [259]. According to some authors, the pattern of β - γ bridging is pathognomonic for chronic liver diseases or hepatic cirrhosis [260]. However, Camus et al. [261] stated that β - γ bridging does not have a strong predictive value for hepatic diseases in some animal species, including dogs, cats, or horses, and may be frequently found in association with infectious diseases, including leishmaniasis or ehrlichiosis [262]. Tóthová et al. [263] observed also a β - γ fusion in cows with severe hoof diseases. Other possible source of the β - γ bridge is the use of plasma instead of serum, caused by the migration of fibrinogen between the β and γ regions [16].

Increases of the γ -globulin fraction (the so-called gammopathies) belong to the frequent serum protein alterations, and are typical for many pathological conditions. Two types of gammopathies were differentiated: monoclonal and polyclonal. Monoclonal gammopathy is characterized by a sharp, homogenous, spike-like peak in the focal region of the γ -globulin zone. This pattern may be caused by the production of excessive amounts of one type of immunoglobulin secreted by a single clone of B lymphocytes, or an immunoglobulin fragment described as paraprotein or M protein [264]. Multiple myeloma is the most common malignant disorder of plasma cells, in which usually IgA and IgG paraproteins can be found [265]. Monoclonal gammopathies in farm animals are not frequent. Some cases were recorded in horses and small animals, which has been associated with plasma cell myeloma, malignant lymphoma, or erythrophagocytic multiple myeloma [264, 266].

Polyclonal gammopathy is associated with the presence of a diffuse hypergammaglobulinemia, in which all immunoglobulin classes may be increased. It is characterized by a diffuse, broad increase in the γ -globulin zone on the electrophoretogram. This swell-like elevation of γ -globulins is mostly caused by inflammatory reactions, and usually indicates a non-malignant condition [71]. The most common causes of polyclonal gammopathies are chronic inflammatory processes (gastrointestinal, respiratory, endocrine, cardiac), severe infections, as well as immune-mediated disorders [76, 267]. The decrease of the concentrations of γ -globulins in the serum is called hypogammaglobulinemia. This pattern is typical for fetal or precolostral sera in some animal species. In calves, precolostral serum normally contains no (agammaglobulinemia) or very low concentrations of γ -globulins, but they start to increase within a few hours

after the intake of colostrum, and the absorption continues for up to 24–36 hours after birth, after which gut permeability ceases [268, 269]. Hypogammaglobulinemia may be commonly seen also in patients with recurrent infections or in cases of immune deficiency, including primary immunodeficiency disorders [1].

The aforementioned shifts in the concentrations of albumin and globulins lead also to changes in the albumin: globulin ratio (A/G). The normal A/G ratio is in the range of 0.6–0.9 in cows, but the relative concentrations of albumin and globulins may be altered in many disease conditions, which results in changes in their proportion [22]. Decreased A/G ratio may be associated with the overproduction of globulins, decreased synthesis of albumin, or with losses of albumin from the circulation. On the other hand, higher A/G ratio is usually caused by the underproduction of globulins. Thus, the interpretation of A/G ratio is very important itself providing information about the changes in pattern of serum proteins, and could help in the classification and identification of dysproteinemias [105].

6. The use of serum protein electrophoresis in bovine clinical practice

The analysis of serum proteins and their electrophoretic separations have been extensively used in human medicine for many years. Serum protein electrophoresis has been studied intensively also in small animal and equine medicine, especially to support a clinical diagnosis of diseases characterized by dysproteinemia (leishmaniasis, ehrlichiosis, feline infectious peritonitis), or to identify the presence of inflammation with increased α -globulins [76]. In bovine clinical practice, serum protein electrophoresis is a rarely used diagnostic tool.

The diagnostic significance of protein electrophoresis in cows with traumatic pericarditis was evaluated by Yoshida [270]. In the affected cows, slight hypoproteinemia, moderate hypoalbuminemia, and a slight increase of the α - and β -globulin concentrations were observed. In cows with purulent pericarditis, they found a tendency of hypergammaglobulinemia, while fibrinous or sero-fibrinous pericarditis was associated with a large indentation between the β - and γ -fractions. The changes in the electrophoretic pattern of serum proteins and immunoglobulin concentrations were studied also in cows with lymphoma [271]. Moderately increased concentrations of α_2 -globulins were found in these cows, while the β_2 -globulin fraction was significantly decreased due to the lower concentration of immunoglobulins. Recently, Tóthová et al. [252] evaluated the effect of chronic bronchopneumonia on the serum protein pattern in calves. These authors found significantly higher concentrations of α_1 -, β_2 -, and γ -globulins in the affected animals compared with healthy ones. Alterations in the electrophoretic pattern of serum proteins were found also in dairy cows with inflammatory diseases [263]. In this study, *post-partum* metritis was associated with significantly lower concentrations of albumin and higher values of α_1 -globulins compared with clinically healthy cows. The cows with clinical mastitis showed higher β_1 - and γ -globulin fractions, while in cows affected by hoof diseases significantly lower concentrations of albumin and higher values of α_1 -, β_1 -, β_2 -, as well as γ -globulins were found. Furthermore, the serum protein electrophoretic pattern of more than

half of the group of cows with hoof diseases showed β - γ bridging [263]. Constantin et al. [272] evaluated the serum protein profile and its changes in cows affected by clinical endometritis, and found lower concentrations of albumin and higher values of α_2 , as well as γ -globulins.

7. The use of serum protein electrophoresis in small ruminants

The usefulness of the electrophoretic separation of serum proteins was studied by Woolf et al. [273] in bighorn sheep with chronic pneumonia attributed to *Mycoplasma*. In this study, diseased sheep had significantly lower albumin, and higher α_1 - and γ -globulins. The alterations of the serum protein electrophoretic profile were investigated also in sheep naturally infected with *Babesia ovis* [251]. In this study, the diseased sheep before treatment had markedly lower concentrations of both total serum proteins and all protein fractions when compared with healthy animals. A significant increase of total serum proteins and globulins (except for the α -globulin fraction) was found 5 days after treatment, but the values were still lower than those obtained in healthy sheep. The aforementioned authors stated that babesiosis may induce intense proteolysis of the circulating proteins probably due to the altered protein synthesis by the liver, which was improved by the eradication of parasites. Similarly, the alterations in the serum protein profile induced by the infection with *Haemonchus contortus* were studied by Diogenes et al. [245] in goats. In the infected goats, severe hypoproteinemia and hypoalbuminemia were observed, while the concentrations of α - and γ_2 -globulins were markedly increased. Experimental infection of goats by *Fasciola hepatica* resulted also in changes in serum protein profile, manifested by decreased concentrations of albumin, increased values of total serum proteins, γ -globulins, and increased proportion of acute-phase proteins from the α - and β -globulin fractions [274].

Changes in the serum proteinogram were found also in sheep with acute ruminal lactic acidosis with the most intense alterations in the α -globulins [275]. Increased concentrations were recorded in the concentrations of haptoglobin, probably due to the death of Gram-negative bacteria caused by decreased ruminal pH, as well as inflammatory processes induced by ruminitis. Acute ruminal acidosis in sheep was accompanied also by the increase in the values of α_1 -antitrypsin, ceruloplasmin, as well as fibrinogen.

8. Conclusions

The obtained data suggest that the analysis of serum protein profile may be a useful diagnostic tool also in ruminants. It may provide important diagnostic information for clinicians in the determination and differentiation of dysproteinemias or paraproteinemias. Changes in serum proteins can be indicative of many health problems and may serve as potential diagnostic markers for some pathological conditions. The abnormal electrophoretic pattern of serum proteins may be characteristic for some disorders or disease conditions, but others may indicate only non-specific pathological processes. Despite of this low specificity in the diagnosis of some diseases, the determination of the serum protein pattern also in ruminants and

the correct interpretation of their results are very useful for clinicians in diagnosing healthy and sick animals, and may provide a basis for further specific laboratory investigations. This review suggests that the analysis of serum proteins in ruminants provides still many areas to study their changes in various health disorders and diseases.

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