

Serum proteins and their diagnostic utility in veterinary medicine: a review

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ABSTRACT: The evaluation of serum proteins and their electrophoretic pattern is a well-established laboratory method in the diagnosis of many diseases in humans, which has replaced the biochemical determination of the concentrations of albumin and the ratio of albumin to globulins. The measurement of serum proteins may be an important diagnostic tool for the detection, diagnosis, and monitoring of various diseases and pathological processes. The results of serum protein electrophoresis can be one of the most useful diagnostic aids in a wide spectrum of diseases, including infectious and inflammatory diseases, renal or gastrointestinal disorders, immunodeficiency states, as well as paraproteinaemias caused by lymphoid or plasma cell neoplasia. Although many studies have been carried out to determine the usefulness of the determination of serum proteins and their electrophoretic pattern in various disease conditions and disorders also in animals, serum protein evaluation is still a relatively little-used diagnostic tool in veterinary medicine. In this article, methods of serum protein determination, their diagnostic utility in animal care practice and their different patterns in dysproteinaemias and paraproteinaemias are reviewed.

Keywords: animals; serum proteins; electrophoresis; diagnosis; dysproteinaemia; paraproteinaemia

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1. Serum proteins – introduction

Proteins, macromolecules built from one or more unbranched chains of amino acids linked by peptide bonds, are the major components of the blood serum or plasma and have many different functions. Most serum proteins are biochemically not pure,

since they are combined with other substances, for example, they can be conjugated to carbohydrate and be present in the circulation as glycoproteins (Stockham and Scott 2002). The chemical properties of the amino acids determine the biological activity of the protein (Tymchak 2010). Proteins are involved in almost all of the reactions occur-

ring in the organism, including the maintenance of colloid osmotic structure, catalysis of biochemical reactions and buffering acid-base balance. 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 the immune system (Anderson and Anderson 2002). Other blood proteins play important roles as enzymes, complement components or protease inhibitors. Certain blood proteins are essential for haemostasis and have important functions in platelet adhesion and aggregation, as well as coagulation (Meyer and Harvey 2004).

Almost all serum proteins are produced and secreted by hepatocytes. The major exceptions are the immunoglobulins that are produced by the immune system consisting of the reticuloendothelial tissues, lymphoid and plasma cells (Eckersall 2008). Further studies have shown that non-hepatic tissues, including the intestine, lung, adipose tissue and mammary gland, also have the capability of synthesising some serum proteins for specific functions (Friedrichs et al. 1995; Vreugdenhil et al. 1999).

Wrotnowski (1998) estimated that up to 10 000 proteins may commonly be found in blood serum. The more heavily represented serum proteins include albumin, immunoglobulins, haptoglobin, transferrin and lipoproteins, and they are found in concentrations that can be measured in g/ml (Burtis and Ashwood 2001). In addition to these major constituents, blood serum also contains many other proteins that are secreted by cells and tissues at very low concentrations (measured in ng/ml or pg/ml) and in veterinary clinical biochemistry are relatively understudied (Kennedy 2001; Schrader and Schulz-Knappe 2001). Protein concentrations in serum are tightly controlled to balance their physiological functions in areas of immunity, coagulation, small molecule transport and inflammation. Any dysfunction or loss of balance in the concentrations of serum proteins can cause or result from disease processes (Pieper et al. 2003).

2. Analysis of serum proteins

2.1. Total protein

The determination of the concentrations of serum proteins and the evaluation of their changes during the disease process is fundamental for their use as

valid biomarkers (Okutucu et al. 2007). The quantification of the total serum protein concentration represents a basic step in general biochemistry and routine clinical laboratory practice. Several methods have been developed for the determination of total protein in serum or plasma, which are based on different analytical methods (Zaia et al. 1998). Chemical, as well as physical methods are available to measure total protein in biological fluids. In clinical biochemistry, chemical methodologies are more frequently used because of the possibility to adapt these techniques to automated analysers (Eckersall 2008).

The biuret assay is one of the oldest and most widely used assays for the determination of total protein concentrations. It is based on a colorimetric (spectrophotometric) technique, in which protein forms a violet-coloured polypeptide chelated with cupric ion, in strong alkaline solution (Gornall et al. 1949; Boyer 2000). However, this method is not sensitive enough to measure lower protein concentrations found, for example, in cerebrospinal fluid (Switzer and Garrity 1999). Despite this disadvantage, the biuret assay is still frequently used because of its simple analytical procedure and the easy preparation of reagents. Further, compared with other copper-based assays this method is less susceptible to chemical interference (Gupta and Stockham 2014). Moreover, many of the assay kits developed for automated use in wet biochemical analysers, as well as dry chemistry analysers, are based on this principle. The technique is very cheap and this has also contributed to its broad 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 (Lowry et al. 1951). In this method, the phenolic groups of tyrosine and tryptophan in proteins react with the Folin-Ciocalteu reagent producing a blue-purple coloured complex (Wilson and Walker 2000). The disadvantages of the Lowry method are the sensitivity to the amino acid composition of the protein and interference by a range of substances, including buffers, drugs, and nucleic acids (Sapan et al. 1999). 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

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(Bradford 1976). This assay is rapid, practical and suitable for simple quantification of proteins in cell lysates, cellular fractions, and recombinant protein samples (Ernst and Zor 2010). It may be performed also in microtitre plates using micro volumes, but its application area is mainly restricted to research laboratories (Da Silva and Zezzi Arruda 2006). Unfortunately, the Bradford assay is only linear over a short range (to 2000 µg/ml) and shows a curvature over this range of protein concentration, which necessitates the dilution of samples before further analysis (Zor and Selinger 1996; Zaia et al. 2005).

Physical methods are also available to measure the protein concentration in serum or plasma. Many veterinary practitioners use refractometry for rapid determination of protein concentrations in plasma or other biological fluids. In general, the refractometer measures the degree to which light bends as it passes through the interface between two substances of different densities (George and O'Neill 2001). The angle of refraction changes proportionally to the concentration of solute in solution. Protein is the most important solute dissolved in serum; thus, the refractive index reflects the concentration of proteins in the sample (George 2001). Results obtained from human serum samples showed a good correlation between refractometry and the biuret method (Lien and Wang 1995), but the results for veterinary samples are less consistent. Indeed, whereas 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 (Green et al. 1982; Thomas and Brown 1992). The differences between the methods were of magnitudes of 6 g/l and 2 g/l in dogs and cats, respectively (George and O'Neill 2001). However, the most marked differences between the biuret and refractometric methods were observed in avian samples (George 2001). These variations may be caused by differences in the design of various refractometers assigned by the manufacturers, variation in the biuret reagent mixture, as well as assay conditions (Doumas et al. 1981).

2.2. Protein fractionation

For identification and quantification of individual serum proteins or groups of proteins, the proteins in

the serum or plasma must either be separated or the individual proteins must be measured independently. Fractionation technologies are among the most important steps in protein analysis, as well as in the discovery of new biomarkers in veterinary medicine. The currently available separation technologies do not allow the analysis of whole protein complexes in a simple step because of the large number of proteins present in serum (Ahmed 2009).

2.2.1. Albumin and globulins

The primary separation of the serum proteins divides complex mixtures into albumin and globulins. Several fractionation techniques have been developed to separate and consequently quantify the proteins in serum (Issaq et al. 2002). Most of them depend on the initial determination of total serum proteins, and then the concentrations of the main fractions are calculated according to the used method. For example, when either the albumin or globulins are determined, the other can be calculated by the subtraction from the total protein concentration (Eckersall 2008). A number of methods have been developed to measure the concentration of globulins. One of these techniques is based on the precipitation of globulins using solutions of metal salts, e.g. sodium sulphite or zinc sulphate (McEwan et al. 1970; Pfeiffer and McGuire 1977). The addition of salts causes turbidity, which may be visually evaluated or measured using a 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 (Sedlinska et al. 2005; Hogan et al. 2015). Currently, the bromocresol green (BCG) and bromocresol purple methods are the basis for the determination of serum albumin (Watanabe et al. 2004). Bromocresol green binds quantitatively with albumin forming an intense blue-green complex, and the intensity of the colour produced is directly proportional to the albumin concentration in the sample (Kessler et al. 1997). This method is easy to perform, rapid, and cheap, but less sensitive and selective compared to immunoassays (Doumas and Peters 1997). The reaction between serum and BCG is not specific for albumin; therefore, the BCG method often overestimates the concentrations of serum albumin. However, its specificity can be improved by

minimising the contact time with the serum sample (Brackeen et al. 1989).

2.2.2. The globulin fractions

The globulins can be subdivided into fractions (α -, β -, and γ -globulins) by electrophoresis, which also allows the quantitative estimation of each fraction from the total protein concentration by proportion. Electrophoresis is the current standard and most widely used fractionation technique for serum proteins in clinical biochemistry and molecular biology (O'Connell et al. 2005). It is based on the movement of charged particles through a support medium soaked in a solution when subjected to an electrical field (Azim et al. 2004). 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 into different bands according to their sizes (Esmailnejad et al. 2014). After separation, the protein fractions are fixed in an acid solution to denature the proteins and immobilise them on the support medium (Tymchak 2010). The proteins are then stained and their quality/quantity is measured by the electrophoretic system which also provides their graphical distribution (Vavricka et al. 2009).

The separation of proteins in an electric field was introduced by Tiselius in the 1930s (Tiselius 1937). The application of serum protein electrophoresis in clinical biochemistry laboratories started in 1950s using paper strips (Turba and Enenkel 1950), which were replaced a few years later by microporous acetate membranes (Kohn 1957). In the 1970s, agarose gel as a support medium was introduced for the electrophoretic separation of proteins (Lehmann et al. 1997), and became the most commonly used supporting substance in veterinary medicine. A major difference between the aforementioned electrophoretic methods is the material of support used for the protein fractionation. According to Luraschi et al. (2003), the protein patterns and the numbers of peaks are dependent on the material used for support, i.e. cellulose acetate vs agarose gel electrophoresis. Standard agarose gel electrophoresis is a labour-intensive method. However, the introduction of pre-packaged gels and the development of new equipment has allowed the automation of this procedure (Karcher and Nuttall 2001; Ceron et al. 2010). Moreover, it 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 (Riond et al. 2009).

In past few years, capillary zone electrophoresis (CZE) is being used also in veterinary laboratories (Crivellente et al. 2008). In CZE, separation of protein fractions occurs in a free liquid medium created by the low viscosity buffer, in which the high voltage applied generates an electro-osmotic flow that forces the proteins to rapidly move toward the cathode (Giordano and Paltrinieri 2010). This allows better separation of proteins with similar physicochemical characteristics, thus generating multiple sub-peaks or narrower peaks (Petersen et al. 2003). The higher resolution of CZE can often result in abnormal electrophoretic profiles caused by the aforementioned multiple sub-peaks of unknown significance, which is the disadvantage of this method.

Electrophoresis is normally used for serum samples, 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. Fibrinogen migrates between the β and γ regions on the electrophoretogram, and can influence the correct separation and identification of these fractions (Rossi et al. 2008). Thus, electrophoretic analysis of plasma may provide inaccurate results unless plasma is defibrinated (Errico et al. 2012). On the other hand, in routine practice plasma is often the only sample available, especially in birds and reptiles, in which only small blood specimens can be collected from sick animals (Briscoe et al. 2010). According to Errico et al. (2012), electrophoretic analysis of native plasma provides the same diagnostic information as analysis of serum, except for possible overestimation of the β -globulin fraction. Electrophoretic techniques may also be used for the analysis of urinary proteins, which is a fundamental step in the early diagnosis and subsequent monitoring of renal diseases (Giori et al. 2011). It was found that polyacrylamide gel electrophoresis may localise the origin of urinary proteins based on their molecular weight, providing a diagnostic sensitivity comparable to results obtained by kidney biopsy (Brown et al. 2010). It is considered a very sensitive method to discriminate between glomerular, tubular, or mixed proteinuria (Zini et al. 2004). 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

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production of immunoglobulins within the central nervous system, as well as possible disturbances in the blood-brain barrier (Chrisman 1992). However, because of the small amount of proteins in CSF compared with serum, prior concentration of the proteins is necessary to increase the sensitivity of CSF protein detection by electrophoresis (Gama et al. 2007).

2.3. Analysis of specific serum proteins

Recently, high resolution electrophoresis, two-dimensional electrophoresis and proteomic techniques were developed for the separation of proteins. These techniques may be used to simultaneously identify hundreds of proteins and localise specific proteins within several subfractions, whereas agarose gel electrophoresis can separate serum proteins only into five or six fractions (Eckersall 2008). Many specific serum proteins can also be measured by immunoassays, which require a specific antibody against the analysed serum protein. Enzyme immunoassay and enzyme-linked immunosorbent assay (ELISA) are the most common analytical tools in biomedical research for the detection and quantification of specific proteins, antigens or antibodies (Gan and Patel 2013). ELISA is based on the concept of an antigen/protein binding to its specific antibody, which allows detection of very low concentrations of antigen/protein (Mariani et al. 1998). Various types of ELISAs have been developed, all based on the basic principle of the direct or indirect detection of antigen by adhering or immobilising the antigen or antigen-specific capture antibody onto the well surface (Dobrovolskaia et al. 2006). Direct ELISA is the simplest format that uses an antibody directly conjugated to an enzyme to detect plate-immobilised antigen (Lin 2015). The indirect detection method uses a secondary antibody to amplify the signal of the primary antibody bound to the antigen. On the other hand, some serum proteins can be measured on the basis of their biological activities. For example, the concentrations of haptoglobin can be rapidly measured using its high affinity for haemoglobin and subsequent preservation of its peroxidase activity at a low pH (Eckersall et al. 1999). The intensity of the peroxidase activity in the bound haemoglobin is directly proportional to the amount of haptoglobin present in the sample. This colorimetric assay is designed to detect haptoglobin in a range of animal species. The concentrations of

ceruloplasmin, another serum protein, can be analysed by measuring its endogenous oxidase activity (Ceron and Martinez-Subiela 2004). However, for the quantitative determination of most serum proteins in animals, species-specific assays still need to be developed.

3. Normal serum protein pattern

Following electrophoresis, serum proteins can be separated into four basic fractions including albumin, α -, β - and γ -globulins (Bossuyt 2006; Figure 1). Each band is made up of a group of individual proteins, each of which is characterised by independent metabolic properties (Table 1). The interpretation of serum protein electrophoretic patterns depends on the variations among different species of animals, as well as among different groups of animals (Figure 2). Indeed, many researchers have noted that serum protein fractions display important differences among all domestic animals (Keay and Doxey 1981; Alberghina et al. 2010). The number, shape, and size of fractions and subfractions vary greatly with the animal species and breed (Fayos et al. 2005): the most important differences are between β -globulins and also γ -globulins.

Protein electrophoresis is also an important laboratory technique for the evaluation of serum protein abnormalities in dogs and cats (McGrotty and Knottenbelt 2002). Using agarose gel electrophoresis Abate et al. (2000) determined six fractions in healthy dogs: albumin, α_1 -, α_2 -, β_1 -, β_2 -, and γ -globulins. Similar findings were reported by Harrus et al. (1996), Fayos et al. (2005) and Maciel et al. (2012). In contrast, Akdogan Kaymaz et al. (1999) separated the serum proteins only into five

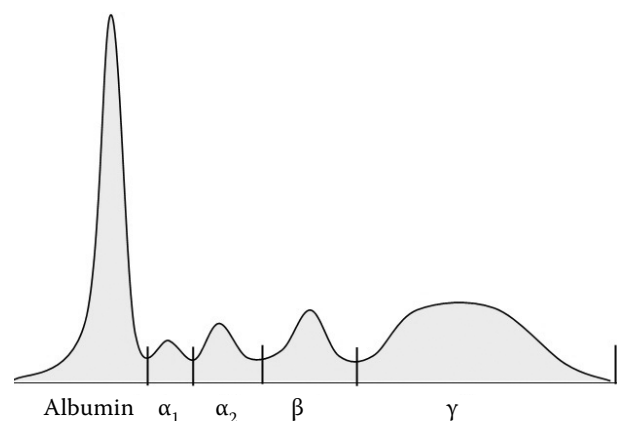


Figure 1. Normal serum protein electrophoretogram

Table 1. Protein components identified by serum protein electrophoresis (adapted from Tymchak 2010)

Protein fraction	Components
Prealbumin	prealbumin (transthyretin)
Albumin	albumin
α_1 -globulins	α_1 -antitrypsin
	α_1 -fetoprotein
	α_1 -acid glycoprotein (orosomuroid)
	α_1 -antichymotrypsin
	α_1 -lipoprotein
α_2 -globulins	haptoglobin
	α_2 -microglobulin (protein HC)
	α_2 -macroglobulin
	ceruloplasmin
	retinol-binding protein
	α_2 -antiplasmin
	α_2 -lipoprotein
	Gc-globulin (group specific component, vitamin D-binding protein)
	transferrin
	ferritin
β -globulins	β_1 -lipoprotein
	haemopexin
	plasminogen
	angiostatin
	pregnancy-specific β_1 -glycoprotein
	β_2 -microglobulin
	C3 complement
	C4 complement
	C-reactive protein
	fibrinogen (in plasma protein electrophoresis)
	γ -globulins
immunoglobulin A	
immunoglobulin M	
immunoglobulin D	
immunoglobulin E	

fractions, consisting of albumin, α_1 -, α_2 -, β -, and γ -globulins. The electrophoretic pattern of serum proteins in cats is poorly characterised. In routine electrophoresis, serum cat proteins are separated into albumin, α_1 -, α_2 -, β_1 -, β_2 -, and γ -globulin fractions (Baker and Valli 1988). However, according to Gerou-Ferriani et al. (2011), the globulin fractions in normal cats may be further divided into α_{1a} -, α_{1b} -, α_{2a} -, α_{2b} -, β_1 - and β_2 -subzones.

In healthy horses, some authors (Kohn 1957; Riond et al. 2009; Gundasheva 2015) clearly separated on the serum protein electrophoretogram six different bands, including albumin, α_1 - and α_2 -globulins, β_1 - and β_2 -globulins, and γ -globulins. In contrast, Matthews (1982) found two γ -globulin zones. The pig electrophoretic serum protein pattern is not well described. Swindle and Sistino (2015) described five swine protein fractions, comprising albumin, α_1 -, α_2 -, β -, and γ -globulins.

Differences in the electrophoretic mobility of serum proteins have been observed also between ruminant species. Using agarose gel electrophoresis Nagy et al. (2015) 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. (2011) and Piccione et al. (2012a) 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. (2015) and Esmailnejad et al. (2014) detected albumin, α_1 -, α_2 -, β -, γ_1 - and γ_2 -globulins in sheep serum, while the goat serum proteins showed albumin, α_1 -, α_2 -, β - and γ -globulin fractions (Perez et al. 2003; Nagy et al. 2015). In contrast, Cyrillo et al. (2006), Fernandez et al. (2006) and Alberghina et al. (2010) determined only one α -globulin and two β -globulin fractions in goat serum.

Serum protein electrophoresis may be a useful diagnostic tool also in avian medicine. It is recommended for many avian species as one of the more reliable techniques to determine the albumin and globulin concentrations, since birds with apparently normal protein values may show abnormalities on the protein electrophoretic pattern (Lumeij et al. 1990). In avian practice, heparinised plasma is the preferred sample for clinical chemistry. Thus, most reports on avian electrophoresis have used plasma instead of serum. There are more variations in protein electrophoretic patterns among avian species than among those of mammals (Werner and Reavill 1999). For example, in avian species β - and γ -globulins migrate in a single fraction with sharp peaks when their concentrations are increased, while in mammals the elevated globulin fractions show broader peaks (Spano et al. 1988). However, many psittacine species have unique protein electrophoretic patterns showing a biped (twin) β -fraction in healthy, as well as diseased individuals (Cray and Tatum 1998). In most avian species, the

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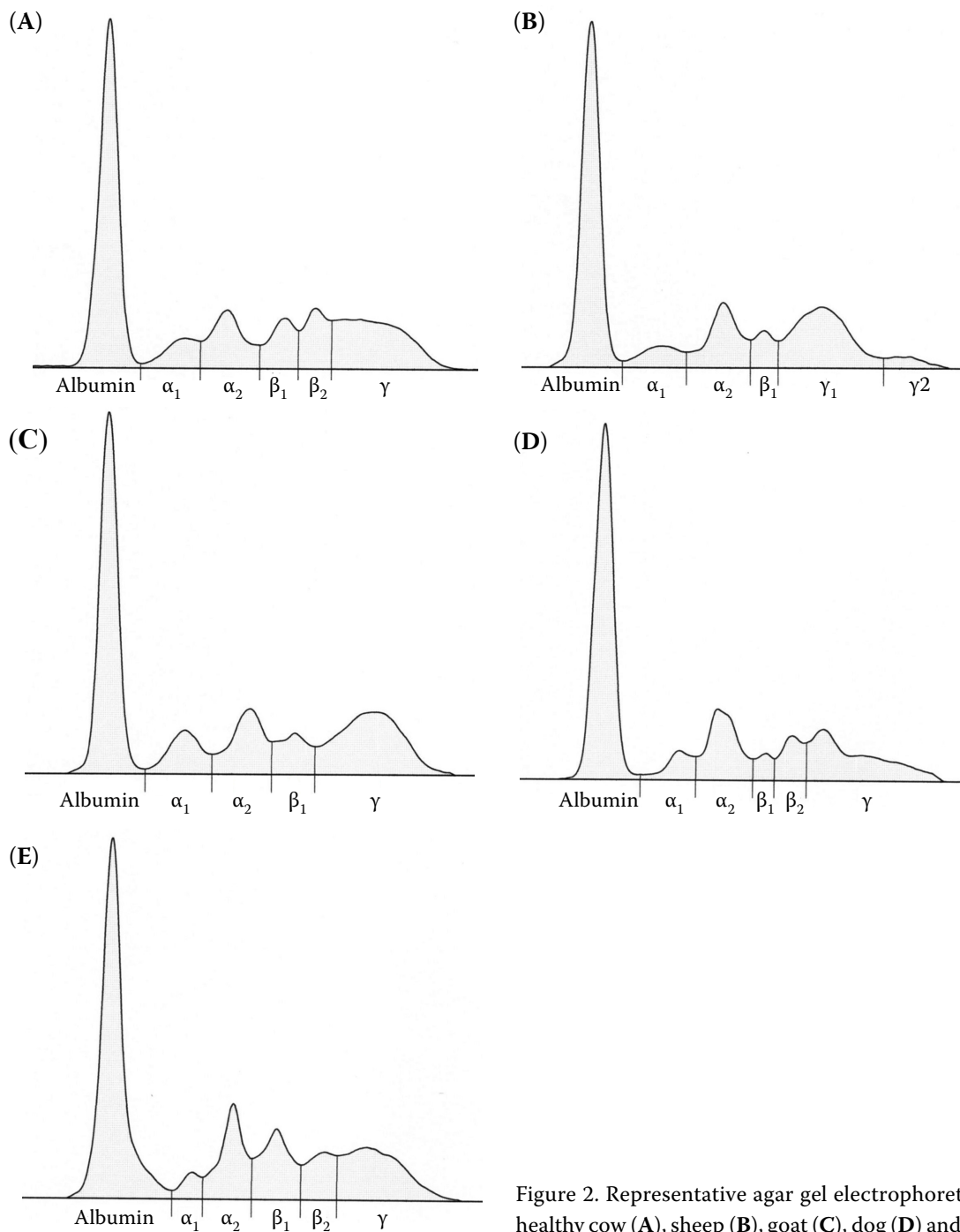


Figure 2. Representative agar gel electrophoretogram in healthy cow (A), sheep (B), goat (C), dog (D) and horse (E)

α_1 -globulin fraction is detectable only in some disease conditions, usually in systemic inflammation. Selective increases in the α_1 -globulins have been observed in psittacine birds with parasitic infection, caused by an increase in α_1 -antitrypsin (Cray 1997). Wide variations exist also in the prealbumin concentrations, which account for as little as 10% of the total albumin concentration in cockatiels (*Nymphicus hollandicus*) and as much as 75% of

the total in budgerigars (*Melopsittacus undulatus*, Cray and Tatum 1998).

3.1. Prealbumin (transthyretin)

Prealbumin is the most rapidly migrating protein fraction in serum and is visible as a band anodic to the main albumin fraction (Harris and Kohn 1974).

However, it is not always visualized in electrophoretograms and may not exist in all animal species (Kaneko 1997). Prealbumin (transthyretin [TTR]), is a negative acute phase protein, serum concentrations of which fall due to decreased synthesis in response to inflammation and stress, as well as in conditions associated with protein malnutrition (Hamilton and Benson 2001; Beck and Rosenthal 2002). This protein is secreted mainly by the liver and is involved in the transport of both thyroid hormones, and is also indirectly implicated in the carriage of vitamin A through the mediation of the retinol-binding protein (Ingenbleek and Young 1994). Serum TTR concentrations are affected by many factors, including age, gender, and blood-drawing methods, as well as factors influencing distribution or rates of synthesis and catabolism. Assaying serum TTR concentration is recommended by some investigators as a screening marker for inflammation, malnutrition, or both (Johnson et al. 2007). In animals, there are only scant literature data about this protein as a biomarker of health state and its use in laboratory diagnostics (Tothova et al. 2016b).

3.2. Albumin

Albumin is often considered as the only discrete and homogenous protein fraction discernible on the electrophoretogram. In animals, it constitutes between 35 and 50% of total serum protein (Kaneko 1997). The shape and size of the albumin fraction is very similar in all ruminant species reflecting its high serum concentration, homogenous electric charge and high staining affinity, but differences may be found in its relative concentrations between different animal species (Keren 2003). It forms the largest peak and lies closest to the anode on the left side of the panel (Vavricka et al. 2009). Albumin plays an important role in maintaining homeostasis, in transport of substances, and acts as a free-radical scavenger (Hankins 2006). Albumin is responsible for about 75% of the osmotic pressure of plasma and is a major source of amino acids that can be utilised by the animal's body when necessary (Mackiewicz 1997). Serum albumin is the major negative acute phase protein. During the acute phase response the amino acid demand for synthesis of the positive acute phase proteins is markedly increased, which necessitates reprioriti-

sation of the hepatic protein synthesis. Thus, albumin synthesis is down-regulated and amino acids are shunted toward the synthesis of the positive acute phase proteins (Aldred and Schreiber 1993). Catabolism of albumin occurs in various tissues, where it enters cells by pinocytosis and is then degraded by proteases (Evans 2002). Muscle, liver, and kidney are the main sites of albumin catabolism. The turnover of albumin differs with species and is related to the body size. The half-time for clearance of albumin varies from 1.9 days in the mouse to 14–16 days in ruminants (Prinsen 2004).

3.3. Globulins

The globulins are a very heterogenous group of proteins forming peaks on the right side of the electrophoretic panel. Depending on the species, there may normally be one or two α , one or two β , and one or two γ fractions (Kaneko 1997).

3.3.1. The α -globulins

The α fraction is the most rapidly migrating protein of all the globulins, and in most species it migrates as an α_1 (fast) and an α_2 (slow) fraction. Many diagnostically important acute phase proteins migrate in this fraction. α_1 -antitrypsin, α_1 -acid glycoprotein, α_1 -antichymotrypsin, α_1 -fetoprotein, 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 have been detected in the α_2 -globulin fraction (Bossuyt 2006; Tothova et al. 2014). Acute phase proteins are a large and varied group of plasma proteins with numerous differences in their concentrations between different animal species (Eckersall and Bell 2010). Their concentrations increase in response to any changes 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 also act systemically (Gabay and Kushner 1999). In general, the main function of the acute phase proteins is to defend the host against pathological damage, assist in the restoration of homeostasis and in the regulation of different stages of inflammation (Petersen et al. 2004). Moreover, some proteins from these fractions may act as inhibitors

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of enzymes, as digest proteins, as compounds of the blood coagulation system or as carriers of copper (O'Connell et al. 2005).

3.3.2. The β -globulins

The β -globulins are a group of globular proteins that are more mobile than γ -globulins in electrically charged solutions, but less mobile than α -globulins. Transferrin and complement are the main proteins in the β -globulin fraction, which may correspond to the two subfractions (β_1 and β_2) identified in some animal species (Ceron et al. 2010). Other important proteins in this fraction are β_2 -microglobulin, C-reactive protein, ferritin, haemopexin, plasminogen, and angiostatin. Moreover, in response to different antigenic stimulations, some IgM immunoglobulins may migrate in the β region, while the IgA and IgE immunoglobulins can be found in the β - γ interzone, which may also correspond to the β_2 subfraction identified in some animal species (Meyer and Harvey 2004).

3.3.3. The γ -globulins

The γ -globulin fraction is predominantly composed of immunoglobulins of various classes (IgG, IgA, IgM, IgD and IgE). While in some animal species (cattle and goats) the γ -globulins migrate as one overall fraction, in sheep they may be separated in two sub-fractions (γ_1 and γ_2 ; Nagy et al. 2015). According to Kaneko (1997), immunoglobulins from the γ fraction may migrate rapidly or slowly, giving rise to these two sub-fractions. On the other hand, Vavricka et al. (2009) stated that some immunoglobulin classes may migrate into the β - γ zone or β -region. Immunoglobulins (or antibodies) function as a part of the body's immune system which responds to stimulation by molecules recognised by the body as non-self, the so-called antigens. They have the ability to specifically bind to one or a few closely related antigens in order to mediate their neutralisation and elimination, which is their primary function in the protection of the host (Schroeder and Cavacini 2010). Immunoglobulins are produced by cells of the adaptive immune system, activated B cells and plasma cells, in response to the exposure to antigens (Jackson and Elswa 2015).

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

Many pathological conditions are associated with slight or more marked alterations in serum protein patterns, such as shifts in albumin and globulin concentrations (Keay and Doxey 1982). On the other hand, variations in the serum protein profile and concentrations may occur also under some physiological conditions (Weaver et al. 2000; Janku et al. 2011).

Animal age is one of these important factors that may affect the concentrations of the different serum protein fractions or their electrophoretic pattern (Fayos et al. 2005). That has been shown both in feline species and horses (Baker and Valli 1988; Paltrinieri et al. 2008) and in young and adult cattle (Tothova et al. 2013a), where the most important age-related differences were related to the concentrations of α - and γ -globulins. Indeed, in the latter the concentrations of α_1 -globulins were higher in calves, whereas the γ -globulins were higher in adult animals. It has been observed that these important changes happen in the first month of calf life, in association with changes in nutrition and because of adaptation processes during the neonatal period (Tothova et al. 2016a). The concentrations of total proteins and γ -globulins increase rapidly one day after the intake of colostrum, and then decrease gradually until the end of the 1st month of age. Hammon et al. (2002) also stated that at birth, the concentration of total serum proteins of most offspring is very low due to the minimal quantities of immunoglobulins but, that this increases during the first 24 h of life thanks to the intestinal absorption of proteins (particularly immunoglobulins) from colostrum. On the other hand, albumin, the most prominent protein fraction at birth, decreases in relative concentration one day after colostrum intake, with a subsequent gradual increase from Day 2 until the end of the first month of life. At birth, the calf's α_1 -globulins comprise almost 30% of total proteins but their concentrations are decreased approximately by 50% at one day after birth, with a further decrease up to Day 30 of life (Tothova et al. 2016a). In the absolute concentrations of α_1 -globulins, a temporary slight increase after birth has been observed with a subsequent gradual decrease. Delivery is surely a stressful situation for the offspring, and manifests as higher concentrations of acute phase proteins

at birth, which migrate into this fraction (Orro et al. 2008). The subsequent increase in the IgG level (due to the colostrum absorption) could substitute for the acute phase response. According to Yuki et al. (2010), most of the acute phase proteins have lower values at birth than in the following days, likely because the liver, which is the main site of production of acute phase proteins, is less mature in new-born than in young or adult animals.

Kaneko (1997) also reported that the large amounts of α -globulins in the blood of new-borns and young animals is due to the higher concentrations of some of the proteins from this fraction (e.g. α_1 -fetoprotein), which function to protect young animals from immunological attacks. Similar findings were observed in lambs during the first month of life (Nagy et al. 2014).

Pregnancy and lactation are further factors that may influence the size of albumin and globulin fractions. Variations in the serum protein profile were found in ewes during pregnancy and lactation, as well as in periparturient goats (Janku et al. 2011; Chiaradia et al. 2012; Piccione et al. 2012b). Changes in the concentrations of protein fractions during the last phase of pregnancy and early postpartum in dairy cows are less well documented. Grunberg et al. (2011) observed lower concentrations of total serum proteins in cows around parturition than outside the parturient period and in the following phases of lactation, which may be associated with the transfer of immunoglobulins from the bloodstream to the mammary gland for the synthesis of colostrum (Roubies et al. 2006). Piccione et al. (2011; Piccione et al. 2012b) found increasing values of α -globulins in dairy cows and ewes post-partum, which may be related to the higher concentrations of the acute phase proteins in response to the processes occurring around the time of parturition.

Hormonal changes and stress may also influence the pattern of serum proteins. Piccione et al. (2009) observed changes in the serum protein profile as a response to exercise in athletic horses. Increased concentrations of total proteins were found in horses during exercise, which may be explained by the redistribution of fluid and electrolytes from the vascular compartment to the extracellular fluid space, accompanied by a decrease in blood volume (Fazio 2011). The extent of these changes in the protein profile is related to the degree of training, as well as to the type and intensity of the exercise (Janicki et al. 2013).

5. Pathological serum protein pattern

A wide variety of diseases can cause changes in the serum protein pattern (Table 2).

Higher concentrations of some acute phase proteins were found in cows with subclinical endometritis, as well as in cows with uterine bacterial contamination (Sheldon et al. 2001; Brodzki et al. 2015). Serum protein electrophoresis is a very important technique to evaluate these abnormalities and to characterise the nature of the hyperproteinaemia or hyperglobulinaemia (Keren et al. 1999). Protein electrophoresis may be very useful when routine investigations are not effective for making medical decisions, providing the basis for further specific laboratory analyses (Kaneko 1997; Mallard et al. 1998).

5.1. Albumin

A decrease in the concentration of albumin is one of the most frequently occurring types of dysproteinaemias. Hypoalbuminaemia can be caused by a decreased hepatic production due to liver diseases such as chronic hepatitis, cirrhosis or liver failure (Lee 2012). Hypoalbuminaemia may also be present in renal diseases and nephrotic syndrome, in which there is an increased loss of this protein in urine caused by glomerular damage (Grauer 2005; Kodner 2009). Moreover, low albumin concentrations may indicate chronic malnutrition, inadequate protein intake, or might be associated with gastrointestinal diseases, internal parasitism and protein-losing enteropathy (Don and Kaysen 2004; Diogenes et al. 2010). 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 (Jain et al. 2011).

Rarely, a serum protein anomaly called bisalbuminaemia may be observed on the electrophoretogram. Bisalbuminaemia is characterised by the occurrence of a bicuspid electrophoretic pattern in the albumin fraction, where albumin produces two heads (bands of equal or unequal intensity; Chhabra et al. 2013). In this abnormality, albumin may either have increased (fast type variants), or decreased electrophoretic mobility (slow type variants; Kobayashi et al. 1995). In humans, bisalbuminaemia has been described in some pathological conditions, including chronic renal diseases, nephrotic syndrome, diabetes mellitus, pancreatic

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Table 2. Abnormalities in serum protein electrophoretic patterns and associated diseases (adapted from Werner and Reavill 1999)

Protein fraction	Alteration	Associated disease condition
Albumin	decrease	liver disease, chronic hepatitis, cirrhosis, liver failure renal diseases, nephrotic syndrome chronic malnutrition gastrointestinal diseases, protein-losing enteropathy, internal parasitism acute inflammation (acute phase response)
	increase	dehydration
α_1 -globulins	decrease	α_1 -antitrypsin deficiency malnutrition, hepatic insufficiency
	increase	active inflammation
α_2 -globulins	decrease	haemolytic anaemia
	increase	inflammatory diseases, trauma nephrotic syndrome
β -globulins	decrease	malnutrition hepatic insufficiency
	increase	inflammatory diseases, infections active hepatitis, chronic liver diseases, liver cirrhosis nephrotic syndrome hypercholesterolemia iron deficiency anaemia
γ -globulins	decrease	foetal or pre-colostral sera recurrent infections, immune deficiency hereditary immune deficiency diseases
	increase	monoclonal – multiple myeloma, chronic lymphatic leukaemia, lymphosarcoma, plasma cell myeloma, malignant lymphoma polyclonal – reactive inflammatory conditions, chronic inflammatory processes, severe infections, immune-mediated disorders

disease or Alzheimer's disease (Ejaz et al. 2004; Shetty et al. 2007). In animals, bisalbuminaemia was found in female bottlenose dolphin (*Turrops truncatus*), but it was not associated with disease (Medway 1979). According to Vavricka et al. (2009), the presence of bisalbuminaemia may be caused by increased mobility of albumin due to its binding to bilirubin, non-esterified fatty acids, penicillin or acetylsalicylic acid. The horses may exhibit a minor post-albumin fraction, which frequently increases in animals with hypoalbuminaemia or acute inflammatory diseases, and it is consistently present in chronic liver diseases (Johns 2015).

An increased concentration of albumin in the serum is called hyperalbuminemia, and may be observed in cases of severe dehydration. However, hyperalbuminemia was recorded also in dogs with hepatocellular carcinoma (Cooper et al. 2009).

5.2. Globulins

Increases in the globulin fractions belong to the common findings on serum protein electrophoretograms. Since many acute phase proteins migrate in the α region, the concentrations of α_1 - and α_2 -globulins may be elevated in acute, as well as chronic inflammatory diseases as a result of the activation of the host inflammatory responses (O'Connell et al. 2005). 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 (Apaydin and Dede 2010; Tothova et al. 2013b). 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 glo-

meruli and therefore it remains in the bloodstream (de Sain-van der Velden et al. 1998). Increases in α_2 -globulins have been reported in small animals affected by various diseases, including leishmaniasis, ehrlichiosis, babesiosis, feline infectious peritonitis, feline cholangitis, haemoplasmosis, as well as any secondary inflammation, especially if caused by opportunistic bacteria (Camacho et al. 2005; Tappin et al. 2011). Decreases in the α_1 -globulin fraction may be detected in α_1 -antitrypsin deficiency, a rare genetic disorder in humans and even more rare in animals (Slev et al. 2008). Similarly, the α_2 -globulin zone may typically be decreased in haemolytic anaemia, when haptoglobin from this fraction binds with the free haemoglobin released from the destroyed red blood cells, forming haptoglobin-haemoglobin complexes that are rapidly removed by phagocytes (Vavricka et al. 2009; Gupta et al. 2011). On the other hand, the inflammatory conditions that develop in association with haemolytic anaemia lead to increased haptoglobin concentrations that may induce an increase in α_2 -globulins (Tecles et al. 2005).

Inflammatory diseases and infections may be accompanied not only by increased concentrations of α -globulins, but also by elevations in the β -fraction as a result of the increased production of some acute phase proteins which migrate into this region. According to Kaneko (1997), 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 cause elevations in the β -region due to the increase of the concentrations of β_2 -microglobulin in these conditions (Revillard 1980; Joven et al. 1997). Higher concentrations of β_1 -globulins were found also in horses with larval cyathostomiasis (Kent 1987; Mair et al. 1993). High β -globulin concentrations may be associated also with hypercholesterolemia, which is caused by an increased concentration of beta-lipoproteins in this fraction (Pagana and Pagana 2006). Moreover, increased levels of β -globulins are typical for iron deficiency anaemia associated with higher values of transferrin (Firkin and Rush 1997; Killip et al. 2007). The increase of β -globulins in haemolytic anaemia may depend on the presence of free haemoglobin 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 produce a β - γ fusion. This phenomenon is called β - γ bridging and is characterised by the lack of a clear demarcation between these two fractions. It is caused by an increase in the concentrations of IgM or IgA, which may migrate in the region between the β and γ zones (Morris and Johnston 2002). According to some authors, the pattern of β - γ bridging is pathognomonic for chronic liver diseases or hepatic cirrhosis (Evans and Duncan 2003). However, Camus et al. (2010) claimed that β - γ bridging does not have a strong predictive value for hepatic diseases in dogs, cats, or horses, and may be frequently found in association with infectious diseases, including leishmaniasis or ehrlichiosis (Romdane et al. 1992). Another possible source of the β - γ bridge is the use of plasma instead of serum, where it is caused by the migration of fibrinogen between the β and γ regions (Rossi et al. 2008).

Increases in γ -globulin production are called gammopathies. They are very frequent and may be found in many pathological conditions. There are two different types of gammopathies: monoclonal and polyclonal gammopathy. Monoclonal gammopathy is characterised by a sharp, homogenous, spike-like peak in the focal region of the γ -globulin zone (Figure 3). This reflects the presence of excessive amounts of one type of immunoglobulin secreted by a single clone of B lymphocytes, or an immunoglobulin fragment referred to as paraprotein or M protein (Edwards et al. 1993). Multiple myeloma is the most common malignant disorder of plasma cells, in which usually IgA and IgG paraproteins can be found (Weiss et al. 2009). However, it is important to differentiate multiple myeloma from monoclonal gammopathy of undetermined significance, which is a premalignant plasma cell dis-

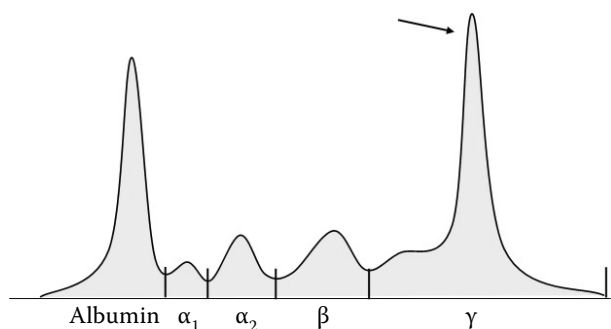


Figure 3. Electrophoretogram of individual with a monoclonal gammopathy. A narrow peak (arrow) is present in the γ -region

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order present in more than 3% of the general human population at the age of 50 years and older (Kyle et al. 2006). Other causes of monoclonal gammopathy are chronic lymphatic leukaemia, lymphosarcoma, or Waldenstrom's macroglobulinaemia, where IgM paraproteins can be found (Kyle 1994). Monoclonal gammopathies in farm animals are not frequent. Some cases were recorded in horses, which has been associated with plasma cell myeloma, malignant lymphoma and idiopathic causes (Kent and Roberts 1990; Edwards et al. 1993). Reports on mono- or oligoclonal gammopathy in small animals are more numerous, and were found in cases with erythrophagocytic multiple myeloma, Waldenstrom's macroglobulinaemia, as well as chronic canine ehrlichiosis with persistently increased antibody titres (Perille and Matus 1991; Webb et al. 2008; Jaillardon and Fournel-Fleury 2011). Biclinal gammopathy has also been described in a cat and in a dog with plasma cell neoplasia and light-chain multiple myeloma (Yamada et al. 2007; Facchini et al. 2010).

Polyclonal gammopathy reflects the presence of a diffuse hypergammaglobulinaemia, in which all immunoglobulin classes may be increased. It is characterised by a diffuse, broad increase in the γ -globulin zone on the electrophoretogram (Figure 4). This elevation of γ -globulins usually indicates a non-malignant condition, and is mostly caused by reactive and inflammatory processes (O'Connell et al. 2005). The most common causes of polyclonal gammopathies are chronic inflammatory processes (gastrointestinal, respiratory, endocrine, cardiac), severe infections, as well as immune-mediated or rheumatological disorders (Dispenzieri et al. 2001; Crisman et al. 2008; Vavricka et al. 2009). Polyclonal gammopathy may be typically described in animals with leishmaniasis, feline infectious peritonitis, feline leukaemia virus, as well as *Ehrlichia canis* infection (Tappin et al. 2011; Baba et al. 2012). A decrease in the concentrations of γ -globulins in the serum is called hypogammaglobulinaemia. This pattern is typical for foetal or pre-colostral sera in some animal species. In calves and foals, pre-colostral serum normally contains no or only very small quantities of γ -globulins, but within a few hours after the intake of colostrum, γ -globulins begin to rise in serum and the absorption continues for up to 36 h after birth, after which gut permeability ceases (Weaver et al. 2000; Blum 2006). Hypogammaglobulinaemia may be commonly seen also in patients with recurrent infections or in cases of immune deficiency (Cunningham-Rundles

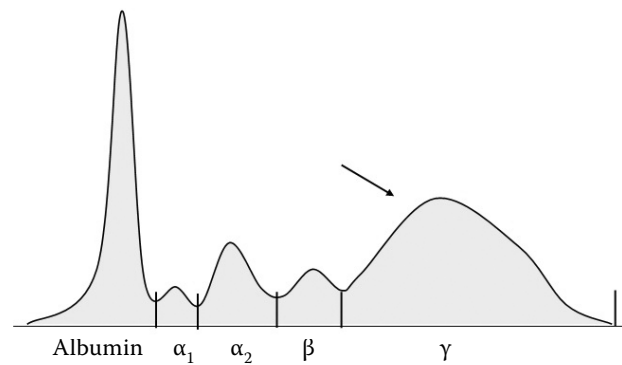


Figure 4. Electrophoretogram of an individual with a polyclonal gammopathy. A broad-based peak (arrow) is present in the γ -region

and Knight 2007). The absence of γ -fraction or very low concentrations of γ -globulins are called agammaglobulinaemia. The condition may be found in people with hereditary immune deficiency diseases, in which the precursor cells that produce gammaglobulins are not sufficiently developed, resulting in low numbers of mature B lymphocytes (B cells; Ochs and Smith 1996). Primary immunodeficiency disorders may be found also in animals, especially in horses (Arabian, Appaloosa) and in some canine breeds, including Basset hounds, Jack Russell terriers, Beagles, German shepherds, Chinese shar-pei, Cardigan Welsh corgi, Doberman pinschers, and Irish setters (Stockham and Scott 2002). Agammaglobulinaemia in adult animals is a rare condition. Primary X-linked agammaglobulinaemia caused by impaired B cells was described in young male horses, and resulted in low immunoglobulin production, marked depletion of the γ -globulin fraction on the electrophoretogram and consequently in clinical manifestation of the disease at the age of 4–5 months (Perryman et al. 1983).

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 ranges from values lower than 1.0 to values just above 1.0 (e.g. 0.6–1.1 in dogs and cats, 0.6–1.4 in horses, 0.6–0.9 in cows; Krimer 2011), but many disease conditions may alter the relative concentrations of albumin and globulins, changing their proportion (Kaneko 1997). A decreased A/G ratio may be caused by the overproduction of globulins, decreased synthesis of albumin, or due to the loss of albumin from the circulation. On the other hand, a higher A/G ratio reflects the underproduction of globulins. Thus, the A/G ratio provides very important information about the changes in the electrophoretic pattern, and

could help in the classification and identification of dysproteinaemias (Alberghina et al. 2011).

6. The usefulness of serum protein electrophoresis in clinical practice

The analysis of serum proteins and their electrophoretic separation has been extensively used in human medicine for many years. In clinical practice, the main area of the application of serum protein electrophoresis is in the identification of patients with multiple myeloma and other disorders of serum proteins (Vavricka et al. 2009). Moreover, the electrophoretic separation of serum proteins is very important in the differentiation of monoclonal gammopathies from polyclonal gammopathies. For gastroenterologists, the method may be of special interest in the diagnosis of chronic liver diseases, hepatic cirrhosis, hepatocellular carcinoma, α_1 -antitrypsin deficiency, inflammatory bowel disease, or amyloidosis, in which clinical signs and symptoms may be seen in the whole gastrointestinal tract (Melmed 2009). In small animals, protein electrophoresis is commonly used to support a clinical diagnosis of diseases characterised by dysproteinaemia (leishmaniasis, ehrlichiosis, feline infectious peritonitis), or to identify the presence of inflammation with increased α -globulins.

Serum protein electrophoresis has been studied intensively also in small animal and equine medicine. Fayos et al. (2005) evaluated the electrophoretic pattern of serum proteins in healthy retired racing Greyhounds in comparison to non-Greyhound dogs of the same age and gender, and they suggested that specific reference intervals should be determined for the parameters related to protein profiles in different breeds of dogs. The serum protein responses, including the concentrations of separated protein fractions were determined in dogs with naturally occurring mild, severe or complicated *Babesia canis* infection (Lobetti et al. 2000). These authors found lower concentrations of total proteins, albumin and α -globulins in dogs with mild and severe babesiosis, while the dogs with complicated babesiosis showed no typical serum protein pattern. Akdogan Kaymaz et al. (1999) evaluated the usefulness of serum protein electrophoresis in the differentiation of gastrointestinal parasitic infections caused by taeniosis, coccidiosis, ancylostomosis, trichuriasis and ascarididosis

in dogs. In animals with coccidiosis significantly lower concentrations of α_1 -globulins were found, while the dogs with ancylostomosis had higher values of α_2 -globulins. On the other hand, the α_2 -globulin fraction decreased significantly in the dogs with ascarididosis. Possible alterations in the serum protein electrophoretic profile after the inoculation of live attenuated vaccine against canine distemper virus and canine parvovirus were studied by Maciel et al. (2012) in puppies. At Day 7 post-vaccination, an increase of the concentrations of α_1 - and α_2 -globulins was observed. An understanding of the pathophysiological mechanisms initiated by the first two vaccinations is very important, because they can be easily confused with diseases in puppies.

Although serum protein electrophoresis provides useful information on the changes in the concentrations of albumin and globulin fractions associated with pathological conditions, in ruminants it is a rarely used diagnostic tool. The usefulness of the electrophoretic separation of serum proteins was studied by Woolf et al. (1973) in bighorn sheep with chronic pneumonia attributed to *Mycoplasma*. In this study, diseased sheep had significantly lower albumin, and higher α_1 - and γ -globulin concentrations. Recently, Tothova et al. (2013b) 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. Yoshida (1991) investigated the diagnostic significance of protein electrophoresis in cows with traumatic pericarditis. In the affected cows slight hypoproteinaemia, moderate hypoalbuminemia, and a slight increase in the concentration of the α - and β -globulin fractions was observed. A tendency toward hypergammaglobulinaemia was found in cows with purulent pericarditis, while a large indentation between the β - and γ -fractions was typical for fibrinous or sero-fibrinous pericarditis. Changes in the serum electrophoretic pattern and immunoglobulin concentrations were examined also in cows with lymphoma (Jacobs et al. 1980). 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. Alterations of the serum protein electrophoretic profile were investigated also in sheep naturally infected with *Babesia ovis* (Apaydin and Dede 2010). The results of this study suggested

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a marked decrease of both total serum proteins and all protein fractions in diseased sheep before treatment compared to healthy animals. The concentrations of total protein and globulins (except for the α -globulin fraction) increased significantly five days after treatment, but the values were still lower than those measured 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, Diogenes et al. (2010) evaluated whether the inflammatory reactions caused by infection with internal parasites like *Haemonchus contortus* may induce alterations in the serum protein profile in goats. The results of this study showed a severe hypoproteinaemia and hypoalbuminemia in infected goats, while the concentrations of α - and γ_2 -globulins were markedly increased.

7. Conclusions

The aforementioned data suggest that serum protein electrophoresis may be a useful diagnostic tool also in veterinary medicine. The electrophoretic technique may provide clinicians with important information to facilitate the differentiation of dysproteinaemias and paraproteinaemias.

Changes in serum proteins can be indicative of non-specific pathological processes or may represent potential diagnostic markers of some pathological conditions. The determination of the physiological electrophoretic patterns in all animal species could be very useful for clinicians in differentiating healthy from sick animals, and may provide a basis for further specific laboratory investigations. Therefore, a main challenge in dealing with serum protein electrophoresis of domestic animals is gaining a clear picture of the differences in the normal physiological pattern between species. Only subsequently can an assessment of the diagnostic significance of the changes in protein fractions be made.

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