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REVIEW ARTICLE

Application of native agarose gel electrophoresis of serum proteins in veterinary diagnostics

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Abstract

Electrophoretic techniques, used to separate mixtures of electrically charged particles, are widely used in science. One of these techniques, native protein electrophoresis in an agarose gel, is applied in human and veterinary medicine. Changes in the proportions of individual protein fractions correspond to significant changes in the physiology of the body. Although the pattern obtained by electrophoretic separation rarely indicates a specific disease, it provides valuable information for the differential diagnosis. Decades of research on the types of patterns obtained in the case of particular diseases have led to the accumulation of substantial knowledge. The paper presents the available information on this topic. Serum protein electrophoresis is recommended in cases of increased levels of total protein in order to reveal the nature of the process. The basic information which can be obtained from electrophoretic separation includes the immune status of the organism. Both increased antigenic stimulation and immunodeficiency are clearly visible in electropherograms. Moreover, the level of heterogeneity of the corresponding protein fractions can help to distinguish between infectious diseases and cancer – multiple myeloma – the latter producing a homogeneous immunoglobulin fraction. Analysis of other protein fractions helps to detect or confirm an ongoing inflammatory process and provides information regarding liver function. Even when the concentration of total protein is within the reference range, this analysis can be recommended as a basic laboratory test.

Keywords: native electrophoresis, agarose gel, serum proteins, diagnostic utility.

Introduction

Electrophoresis is an analytical technique based on the motion of electrically charged particles in a solution under the influence of an electric field. In clinical chemistry, electrophoretic techniques are most often used to separate serum proteins. A serum sample is placed on a medium which allows molecules to move and conduct electricity. Proteins and other electrically charged particles move in the medium. The speed of their movement depends on the characteristics of the protein undergoing separation, *i.e*. its electrical charge, size, and shape, as well as on the strength of the electric field, type of medium, and temperature. As a result, different proteins migrate at different speeds (45).

Serum is the body fluid most frequently used for electrophoretic analysis, but body cavity fluids, urine, and cerebrospinal fluid are also used in diagnostics (14, 17, 49). In the case of the last two, however, the sample must first be concentrated, as the amount of protein they contain is often insufficient to ensure that fractions will be detected with the necessary sensitivity after separation. This means that after electrophoresis the protein concentration in individual fractions will be too low to determine.

Protein electrophoresis techniques have been known since the 1930s (18). In human diagnostics, characteristic electrophoresis patterns have been described for numerous cases, including acute and chronic inflammation and malignant tumours (16). Protein electrophoresis has been widely used as a diagnostic test in human and veterinary medicine for over 40 years (7). The last 20 years have seen an increase in the application of serum protein electrophoresis as a diagnostic test in veterinary patients (6).

Electrophoretic techniques can be used to separate various kinds of macromolecules, including nucleic

acids (DNA or RNA), proteins, lipids, enzymes, and carbohydrates (6, 18). Depending on the medium in which the mixture is placed for separation, several types of electrophoresis are distinguished: agarose, polyacrylamide, or cellulose acetate gels and capillary electrophoresis. Agarose gel is the most frequently used medium. The latest method, however, ensuring the best separation of individual groups of proteins, is capillary electrophoresis (6, 31, 45).

Native protein electrophoresis is performed under non-denaturing conditions. It enables analysis of protein complexes which would decompose under denaturing conditions. The buffer for electrophoresis and buffer dissolving proteins contain no denaturing substances. The serum sample is untreated, apart from dilution in some cases. This type of method is in opposition to electrophoresis in denaturing conditions. In this case, the serum is treated with a denaturing agent, such as sodium dodecyl sulphate (SDS), urea, or guanidine hydrochloride, resulting in the denaturation of proteins, which leads to the destruction of their secondary and tertiary structure. Electrophoretic mobility of proteins in such a mixture depends exclusively on their molecular weight and is in inverse proportion to it.

Following separation of the sample, protein fractions are identified and their quantities are determined. For this purpose, the sample is stained to reveal characteristic bands whose width and intensity of colour provide information about the amount of protein in a given fraction (49). This is considerably facilitated by computer analysis of the image (16). The result obtained is the percentage share of each fraction in the sample and the output is a characteristic graph enabling visual analysis of the result. Over 200 proteins have been identified in different fractions following electrophoretic separation (7).

Laboratory evaluation of plasma and serum proteins is one of the basic tests used in animal haematology and biochemistry. Changes in the protein profile commonly occur as secondary symptoms in numerous diseases, and may be the primary symptom of certain conditions. Determination of the concentration of serum proteins often provides vital information which can be used to reduce the list of diseases considered, and in some cases may reveal a specific disease (14, 45).

Many factors may influence the concentration of proteins in serum. For this reason, serum protein electrophoresis is particularly advisable in patients with non-specific clinical symptoms, such as depression, fever, weight loss, diarrhoea, abdominal pain, or polyuria. The albumin to globulin (A/G) ratio is particularly important in clinical pathology, as it enables systematic classification of the electrophoretic profile and identification of dysproteinaemia.

Although a final diagnosis of a disease is rarely based exclusively on electrophoresis of proteins, it is an excellent method for detecting acute and chronic inflammation and a stimulated humoral immune response. In veterinary medicine it plays an important auxiliary role in the diagnosis of diseases such as feline infectious peritonitis (FIP), ehrlichiosis, multiple myeloma (7), and other disorders in the protein profile (34).

The authors of the study have observed a growing interest in determining the concentration of individual protein fractions in the blood serum of animals. Therefore there is also an increasing need for studies enabling interpretation of the results obtained. Although the result of electrophoresis rarely indicates a specific disease entity, it is a highly useful tool in differential diagnosis. The aim of the article was to associate abnormal electropherograms with the diseases accompanying them.

Analysis of electropherogram patterns – typical electropherogram

A typical electropherogram is presented in Fig.1.

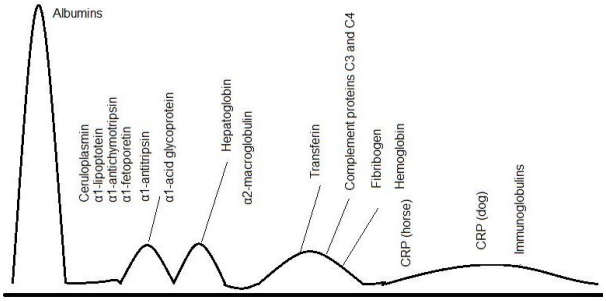

Fig. 1. A typical electropherogram showing proteins present in their respective fractions

During separation the original protein mixture is usually divided into five basic fractions: albumins, α_1-globulins, α_2-globulins, β-globulins, and γ-globulins (7). In some cases the β-fraction is divided into β_1 and β_2, and sometimes the γ-fraction is divided into γ_1 and γ_2 (6, 31). Each of the globulin fractions consists of acute-phase proteins or antibodies, and sometimes both (7).

The α-globulin fraction contains such proteins as α_1-fetoprotein, α_1-acid glycoprotein, α_1-antitrypsin (protease inhibitor), α_1-antichymotrypsin (protease inhibitor), α_1-lipoprotein (HDL, which transports lipids), ceruloplasmin (transports copper), haptoglobin (binds haemoglobin), α_2-macroglobulin (protease inhibitor), and serum amyloid A (6, 42, 45).

The β-globulin fraction contains β_2-lipoprotein (LDL, which transports fats), transferrin (transports iron), ferritin (stores iron), components of the complement system (C3 and C4), and fibrinogen (in plasma, but not in serum) (6, 18, 40, 42, 45). Class IgM and IgA immunoglobulins may migrate in the β-region as well. Free haemoglobin also migrates in the

β-fraction if it is present in the sample (*e.g.* in the case of intravascular haemolysis or *in vitro*, when haemoglobin is released into the serum before being separated from blood cells) (31).

The γ-fraction consists of various classes of immunoglobulins. Antibodies are produced by plasmatic cells in response to antigen stimulation. In dogs, C-reactive protein (CRP) migrates in this fraction as well. In horses it is found in the region between β- and γ-globulins (23, 43, 45, 51).

Both increased and decreased total protein concentrations are frequently observed during laboratory tests. They may be due to changes in the concentration of albumins, globulins, or both fractions, and their interpretation requires knowledge of which fractions of the serum – a mixture of proteins – have an abnormal concentration. An alteration in the concentration of albumins or globulins does not always cause detectable differences in total protein concentration. Therefore, concentrations of total protein and that of albumins and globulins should be evaluated. Causes of changes in the concentration of total protein, albumins, and globulins are presented below.

Decreased total protein concentration may result from a reduced quantity of albumins, globulins or both proteins. Concurrence of hypoalbuminaemia and hypoglobulinaemia may be the result of water intoxication (during fluid therapy or water intake by the animal) or the loss of protein fractions. The latter cause is much more common and occurs in several cases:

1. Loss of blood. All types of proteins are lost. The remaining blood, in order to maintain a suitable volume, is diluted with extracellular fluid, which enters the bloodstream. Hypoproteinaemia resulting from blood loss is caused by external or internal haemorrhaging, and may also be induced by parasites feeding on blood (external or internal) (45).

2. Enteropathy with protein loss. This may be the result of numerous generalised pathological changes, including intestinal inflammation, infectious diseases, neoplasia, prolonged starvation, cachexia, or bleeding into the gastrointestinal tract (29, 38).

3. Severe exudative skin disease or burns lead to protein loss due to increased vascular permeability. However, a concomitant immune response may increase globulin concentration (27).

4. Exudative disease resulting in the accumulation of body fluids with a high protein concentration, which leads to a decrease in the serum concentration of albumins and globulins (42).

Selective hypo-hyper albuminaemia or globulinaemia

Decreased albumin concentration unaccompanied by decreased globulin concentration may be due either to reduced production or increased loss of this protein fraction. If the globulin concentration increases at the same time, the total protein concentration may remain within the reference range. Reduced albumin concentration may occur in the following cases:

1. Liver damage. Albumins are produced in the liver. The reserve capacity of the liver is large enough that only damage at a level of 60%–80% induces a perceptible reduction in the production of this group of proteins.

2. Starvation or cachexia. Severe malnutrition or starvation decreases protein production due to reduced availability of essential amino acids. Under conditions of cachexia associated with cancer or chronic infections, a negative protein balance causes increased protein catabolism in the body (45).

3. Gastrointestinal parasites. These may cause hypoalbuminaemia in at least two ways. The organisms compete with the host for nutrients, and the amount of available amino acids is insufficient for albumin production. In addition, parasites may feed on the host's blood, leading to a reduction in the concentration of albumins and globulins (25). However, parasitic infections of the gastrointestinal tract are rarely serious enough to induce hypoalbuminaemia (45).

4. Malabsorption or digestive disorders. Animals with this condition often have a history of diarrhoea or loose stools.

5. Inflammation. Albumins are negative acute-phase proteins. If there is a simultaneous increase in globulin concentration, the total protein concentration may remain within the reference range.

6. Kidney disease. Albumins, as smaller molecules with a more negative charge than globulins, more easily penetrate a damaged glomerular membrane (20).

Selective hypoglobulinaemia without hypoalbuminaemia is usually due to reduced β- or γ-globulins. This is most often the result of a reduced concentration of immunoglobulins. It may occur in the following cases:

1. Failure of passive absorption of immunoglobulins from the colostrum. Most mammals are born with a very low concentration of immunoglobulins and take them up from milk during the postnatal period. Failure of this transfer is well documented in domesticated animals (8, 47, 48).

2. Innate or acquired immune deficiency associated with B lymphocytes and plasma cells. This leads to decreased concentrations of γ-globulins and sometimes total globulins. It has been described in foals, calves, and puppies (8, 42).

An increased total protein concentration may result from an elevated concentration of albumins, globulins, or both. Changes in these fractions, however, cannot always be detected in the form of increased total protein concentration. In clinical practice, electrophoresis is performed on the proteins of sera in which the globulin concentration is high, but the cause is unknown – inflammation or chronic antigen stimulation (45).

Dehydration is the main cause of hyperalbuminaemia. Water loss from the blood plasma causes a corresponding increase in the concentration of albumins and globulins. The albumin to globulin ratio is not disturbed because both fractions undergo equal concentration. The haematocrit increases as well, provided anaemia is not present at the same time.

The significance of hyperglobulinaemia depends on the magnitude of increase and type of globulins, which can be determined by protein electrophoresis.

An increase in the concentration of α- or β-globulins may be due to acute or chronic inflammation. In the case of acute inflammation, an increase in α- and β-globulins is usually mild. Acute-phase proteins are located in two protein fractions, with the exception of fibrinogen, which is lacking in serum following its removal in the clotting process. The group of acute-phase proteins is numerous and usually the concentration of many of them must be elevated in order to induce hyperglobulinaemia and an increase in the total protein concentration (42). Because albumins are negative acute-phase proteins, their production decreases during inflammation, even by up to 30% (36).

Increased concentration of γ-globulins

The γ-globulin fraction consists of different classes of immunoglobulins. Gammopathy may be mono- or polyclonal, which can be differentiated by observing the electropherogram. Polyclonal gammopathies are characterised by a wide peak, wider than the albumin peak. They contain a heterogeneous immunoglobulin population produced by B lymphocytes, plasma cells, or both. Each of these populations produces its own immunoglobulin specific for the antigen epitope (Fig. 2, Table 1).

Monoclonal gammopathies are characterised by a narrow peak and contain a homogeneous type of antibodies, produced by a single line of B lymphocytes or plasma cells (Fig. 3, Table 2).

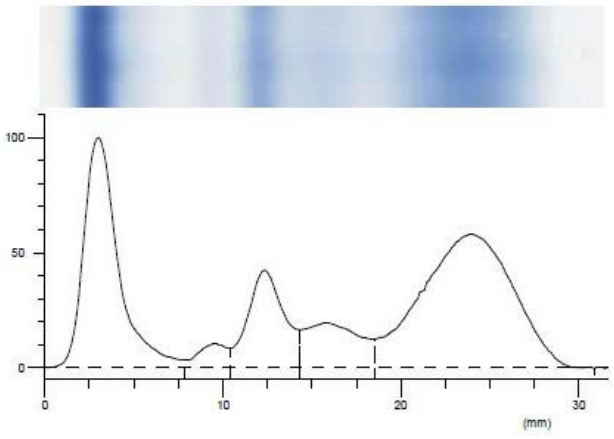

Fig. 2. Electropherogram of cat serum with pronounced polyclonal gammopathy

Table 1. Analysis of electropherogram of cat serum with pronounced polyclonal gammopathy

Fraction	Percentage (%)	Concentration (g/l)
Albumins	29.66	21.95 L
α1	2.6	1.92 L
α2	13.13	9.72 H
β	9.1	6.73
γ	45.52	33.68 H
Total		74.0
A/G ratio	0.42	

H – High – above reference range
L – Low – below reference range

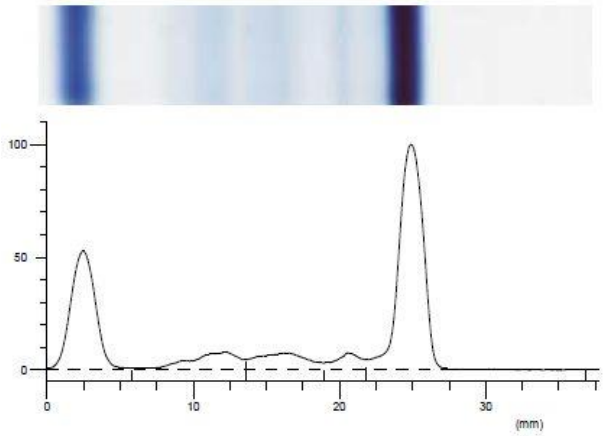

Fig. 3. Electropherogram of cat serum with pronounced monoclonal gammopathy

Table 2. Analysis of electropherogram of cat serum with pronounced monoclonal gammopathy

Fraction	Percentage (%)	Concentration (g/l)
Albumins	27.61	36.99 H
α	8.49	11.37
β1	8.22	11.01 H
β2	4.09	5.48 H
γ	51.6	69.14 H
Total		134.0
A/G ratio	0.38	

H – High – above reference range

Monoclonal peaks may also occur in other fractions. Fig. 4 and Table 3 show an example of the occurrence of such a peak in the β-region.

Another interesting case is the overlap of monoclonal and polyclonal gammopathy, illustrated in Fig. 5 and Table 4. Occasionally, monoclonal gammopathy may be completely obscured by polyclonal gammopathy, making the former very difficult to detect without more advanced electrophoretic techniques.

Sometimes two sharp peaks can be observed. This is known as biclonal gammopathy. The gammopathy occurs when a single line of tumour cells produces molecules which migrate separately. It may be caused by the formation of immunoglobulin dimers, incomplete molecules (light chains), or antibody class switching (39, 50). Sometimes two lines of B lymphocytes or plasma cells multiply, resulting in the production of two homogenous types of antibodies (37). Monoclonal gammopathy may be concealed by

concurrent polyclonal gammopathy. In these cases visual evaluation of the electropherogram may suggest the existence of such a peak. To confirm this, more sensitive and specific methods may be necessary (*e.g.* immunoelectrophoresis or immunofixation) (2, 26).

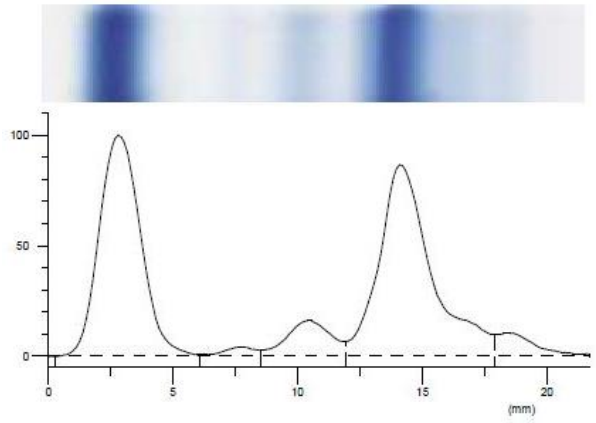

Fig. 4. Electrophoresis of dog serum with a monoclonal peak in the β-region

Table 3. Analysis of electropherogram of dog serum with a monoclonal peak in the β-region

Fraction	Percentage (%)	Concentration (g/l)
Albumins	41.48	33.6
α1	1.35	1.1 L
α2	7.21	5.84
β	45.72	37.03 H
γ	4.24	3.43 L
Total		81.0
A/G ratio	0.71	

H – High – above reference range
L – Low – below reference range

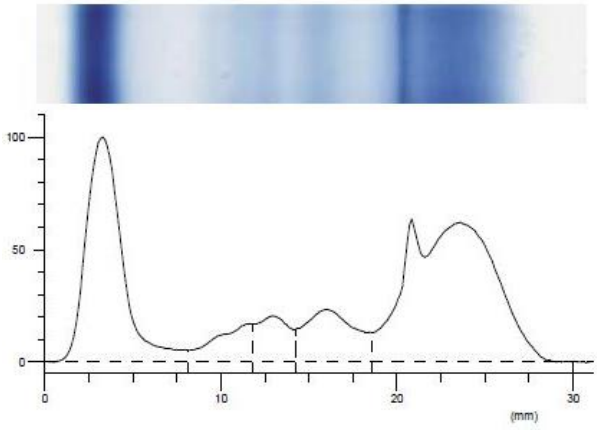

Fig. 5. Example of an electropherogram of cat serum with overlapping monoclonal and polyclonal peaks

Table 4. Analysis of electropherogram of cat serum with overlapping monoclonal and polyclonal peaks

Fraction	Percentage (%)	Concentration (g/l)
Albumins	30.87	35.19
α1	5.35	6.1 H
α2	5.61	6.4 H
β	10.11	11.52 H
γ	48.06	54.79 H
Total		114.0
A/G ratio	0.45	

H – High – above reference range

The conditions usually responsible for polyclonal gammopathy include the following:

1. Chronic inflammation or antigen stimulation. During chronic inflammation, production of immunoglobulins, proteins of the complement system, and acute-phase proteins may increase. Immunoglobulins usually migrate in the γ-globulin region, and some (together with proteins of the complement system) migrate in the β-globulin region. The increase in production of these proteins varies in magnitude but in certain cases it can be considerable (17). Gammopathies underlying chronic inflammation are usually polyclonal (as in the case of canine ehrlichiosis and feline infectious peritonitis). However, there have been some reports of monoclonal gammopathies in dogs with chronic ehrlichiosis, chronic pyoderma, or plasmacytic enteritis (3, 4, 15, 21, 30).

2. Liver disease. Chronic liver disease may lead to increased globulin production, which has been well described in horses but occurs in other species as well (5). These globulins are often antibodies which migrate in the β- and γ-regions, sometimes blurring the boundaries between them; this is known as β-γ bridging. At the same time, albumin production often decreases as a result of reduced synthesis in the liver.

3. Lymphoma and lymphocytic leukaemia. An increase occurs in the production of heterogeneous immunoglobulins by numerous lines of proliferating cancerous lymphoid cells. Although monoclonal gammopathies are common in these diseases, secondary infections may stimulate the production of heterogeneous immunoglobulins (24).

Monoclonal gammopathies occur in the following cases:

1. Multiple myeloma. This is usually the result of proliferation of a single line of plasma cells producing a homogeneous protein called paraprotein or M protein. These proteins are generally situated in the γ- or β-globulin region and rarely in the α-region (19, 35).

2. Extramedullary plasmacytoma – proliferation of plasma cells outside of the bone marrow. These are usually single, benign skin lesions, occurring more often in dogs than in cats. Plasmacytomas in the gastrointestinal tract have a greater potential to become malignant. This type of lesion in cats has been known to lead to multiple myeloma (32).

3. Lymphoma and lymphocytic leukaemia. In this case, monoclonal gammopathy occurs with a frequency from 5% to even 50% (in the case of chronic lymphocytic leukaemia) (28, 41, 44).

Less often, the following diseases may be associated with monoclonal gammopathy:

a. Canine ehrlichiosis. Although polyclonal gammopathy is more common in such cases, monoclonal peaks have been observed as well. Sometimes, as the disease persists, the electropherogram image migrates towards the latter.

Monoclonal peaks disappear after ehrlichiosis treatment is introduced (3).

b. Chronic pyoderma. A case has been described of monoclonal gammopathy that resolved following treatment (4).

c. Plasmacytic enteritis. A case has been described in a dog with monoclonal gammopathy which subsided following treatment and the resolution of inflammation (10).

d. Visceral leishmaniasis. Most dogs with this disease exhibit polyclonal gammopathy. In a few cases one clone of plasma cells proliferated, showing monoclonal gammopathy of IgG antibodies (1, 13).

Finally, monoclonal gammopathy may have an undetermined cause. These animals exhibit no clinical symptoms and may produce a monoclonal protein for a prolonged period (9, 33).

Apparently pathological cases

In some cases, results which appear to be pathological in fact represent a physiological state. This occurs in the following cases:

1. Proteins not present in pure serum samples. If plasma is used for analysis, we may obtain a peak in the β_2-region originating in fibrinogen (which in the form of fibrin is removed from the blood during the clotting process) (11, 40).

2. A high concentration of haemoglobin (released from blood cells during *in vitro* or intravascular haemolysis) or triglycerides may cause an increase in the percentage share of the peak in the β_2- and α_2-regions respectively (31).

3. In young mammals, before they have received colostrum, the amount of protein in the γ-region is below the reference ranges. Thus in very young animals this may be within the normal physiological range, but in adult individuals it may suggest immune deficiencies (22).

4. Variation between breeds within a species. In comparison with other breeds of dog, greyhounds exhibit a lower total protein concentration in the serum, due to the lower concentrations of α- and β-globulins (12).

5. The final, but very important factors that must be taken into account are the duration and conditions of storage of the serum before analysis. If the analysis cannot be performed immediately, it is sufficient to refrigerate the serum at 4°C for three to five days. If a longer delay is anticipated the serum should be frozen. Repeated freezing should be avoided (46, author's experience – unpublished data). Fig. 6 and Table 5 show an electropherogram of serum stored for several days in a refrigerator and at room temperature and then frozen. Note the merging of the bands in the α- and β-regions.

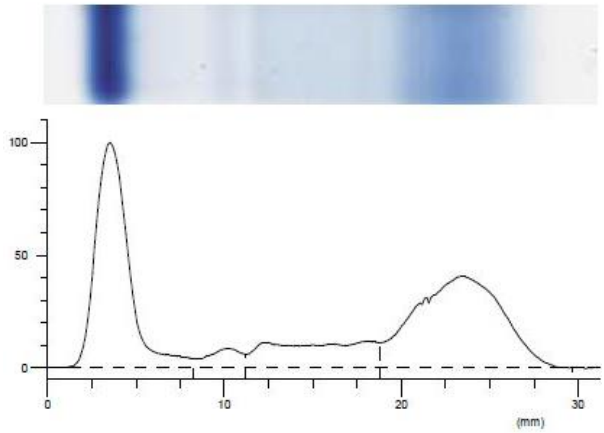

Fig. 6. An example of electrophoresis of improperly stored serum

Table 5. Analysis of electropherogram of improperly stored serum

Fraction	Percentage (%)	Concentration (g/l)
Albumins	39.46	33.93
α	3.51	3.02 L
β	14.18	12.2 H
γ	42.85	36.85 H
Total		86.0
A/G ratio	0.65	

H – High – above reference range
L – Low – below reference range

Conclusions

Native electrophoresis of serum proteins is a useful analysis for determining the nature of increased total protein concentration. It is performed when there is a suspicion of multiple myeloma. Interpretation of the result requires some experience. Apart from knowledge of which species we are investigating, it is important to take into account the means of storage, the quality of the serum (*e.g.* lipaemic or with visible haemolysis), and the age and breed of the animal. Most importantly, as with every laboratory analysis, interpretation takes place in the context of the clinical picture. Also of importance is the development of more advanced techniques (such as immunoelectrophoresis or immunofixation) enabling visualisation of a monoclonal peak covered by a polyclonal peak. Even after decades of use, native electrophoresis of serum proteins is still a highly valuable diagnostic tool.

Conflict of Interests Statement: The authors declare that there is no conflict of interests regarding the publication of this article.

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