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Research Article
Interpretation of Protein Electrophoresis on Avian Samples

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Abstract

Background and Objective: Protein electrophoresis has been used in avian medicine as a diagnostic tool for many years, mainly in the diagnosis of inflammatory phenomena. The objective of this study was to compare protein profiles of normal plasma, hemolyzed plasma, normal serum and pathological serum of adult broiler chickens in order to provide a comprehensive review of plasma and serum protein electrophoresis in healthy and diseased chickens. **Materials and Methods :** The study involved 28 adult broilers from Isa 15 strain. Four mililitres of blood were drawn from the jugular vein. Half of the blood was collected in heparinized tubes and immediately separated by centrifugation. The rest of the whole blood was collected in dry tubes, incubated at 30°C for 2 h and then centrifugated. Hemolyzed plasmas were previously frozen-thawed 3 times to induce hemolysis and subsequently centrifuged. Pathological sera of chickens with New Castle disease confirmed with the hemagglutination-inhibition test. Agarose gel electrophoresis of freezed undiluted plasma and serum was used and the biuret method for total protein concentration was used for all samples. **Results:** Electrophoresis revealed the following protein fractions in plasma and serum samples: albumin, α_1-globulins, α_2-globulins, pre β-globulins, β_1-globulins, β_2-globulins and γ-globulins. The result is expressed by a curve with 7 peaks. Densitometric quantification of plasma protein fractions revealed a very significant increase in total proteins, β_1-globulins fraction, γ-globulins fraction and a significant increase in β_2-globulins fraction. The ratio A/G was significantly lower in plasma when compared to serum samples. Hemolysis resulted in a significant increase in plasma α_2-globulins fraction and pre β-globulins fraction. Densitometric quantification of serum protein fractions from diseased chickens revealed a significant decrease in albumin and A/G ratio and a very significant increase in total proteins, α_1-globulins, all proteins of beta and gamma fraction. **Conclusion:** This study has shown differences between the electrophoretic patterns of plasma, serum, hemolyzed plasma and abnormal values for protein electrophoresis in birds with New Castle disease were reported. Therefore, the results must be interpreted according to the nature of the sample and the condition of the animal. Protein electrophoresis is the most reliable assessment of avian protein profiles in healthy and diseased patients.

Key words: Agarose gel electrophoresis, plasma, serum, hemolysis, Newcastle disease, protein fraction, broiler chickens

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Avian species are renowned for their ability to conceal clinical evidence of disease until the pathological process is advanced. As in mammalian species, total protein measurement forms part of any routine biochemistry profile, often evaluated in addition with other biochemical variables to assess the health status of the bird. However, significant variation in protein fractions may occur in the absence of alterations in total proteins, creating a need for a reliable method to evaluate the individual fractions[1].

Protein Electrophoresis (EPH) is a diagnostic procedure that currently is routinely applied to pet bird species, mainly psittacines and raptors. It consists of separating blood proteins into different fractions, according to their isoelectric profile. When combined with a measurement of total protein content, EPH allows these fractions to be quantified[2]. First performed on paper support and on cellulose acetate, actually the most used technique in veterinary laboratory diagnostics is the agarose gel electrophoresis, but it tends to be supplanted in humans by capillary electrophoresis[3].

In birds, EPH is mainly used for the diagnosis of inflammatory processes associated with bacterial, viral or parasitic infections[4]. It is a method of choice for the determination of concentrations of albumin and globulins on avian blood samples, however, some authors draw attention to the variations in results between laboratories, especially for fractions of low amplitude[5]. The difficulty comes from the identification of different protein fractions that do not migrate to the same place in different species[6]. Indeed, it appears that many publications are contradictory regarding the number of separated fractions, concentrations of these fractions, or general appearance of the electrophoresis curve. So, there is still a lack of information regarding the electrophoretic profile of avian species. In order to assess its utility, the objective of the present paper was to provide a comprehensive review of plasma and serum protein electrophoresis in healthy and diseased broiler chickens.

The purpose of the present study was to:

- Report the values (median+range) for plasma and serum total proteins, all protein factions and electrophoretograms for clinically healthy adult broiler chickens
- Locate fibrinogen by comparing plasma and serum protein electrophoresis
- Investigate the effect of hemolysis in plasma protein concentration measurements
- Investigate the electrophoretic pattern of pathological serum of chickens with New Castle disease that was diagnosed using serological tests.

MATERIALS AND METHODS

Blood samples: The samples were collected at the chicken's slaughterhouse KHALED located at EL MERIDJ (CONSTANTINE-East of ALGERIA). At 60 days of age, 10 Chickens from Isa 15 strain were sacrificed by decapitation. Four mililitres of blood were drawn from the jugular vein. Half of the blood (2 mL) was collected in heparinized polystyrene tubes and immediately separated by centrifugation at 3,000 rpm for 10 min. The rest of the whole blood (2 mL) was collected in dry tubes, incubated at 30°C for 2 h and then centrifugated at 3,000 rpm for 10 min. Hemolyzed plasmas were obtained from 10 other broiler chickens. The samples were previously frozen-thawed 3 times to induce hemolysis and subsequently centrifuged[7]. Visually hemolysis has conferred a detectable red hue to hemolyzed plasma.

Pathological sera were also used in this study. Eight chickens of 45 days with New Castle disease confirmed with the hemagglutination-inhibition test, were sacrificed by decapitation. Two mililitres of blood for analysis were collected from the jugular vein, harvested into dry tubes, incubated for 2 h and then separated by centrifugation at 3,000 rpm for 10 min. All plasmas and sera were frozen at -20°C for later analysis.

Biochemical analysis: Total protein concentration was determined for each sample using a Randox RX Daytona chemistry analyzer (Randox laboratories-US, Ltd. Kearneysville, West Virginia) by the Biuret method[8,9].

Agarose gel electrophoresis: Agarose gel electrophoresis of freezed undiluted plasma and serum samples was performed in alkaline buffer; tris-barbital (pH 9.2), using HYDRAGEL 7 PROTEIN(E) (Sebia, France), on an automated system HYDRASYS 2 SCAN (Sebia, France). The automated steps included: sample application, electrophoretic migration, drying, staining, destaining and final drying. A volume of 10 µL of undiluted plasma/serum was applied to the gel through the supplied template. The gel was subjected to 22 min of pulsing at 90 V, dried, stained with AMIDOSCHWARZ, soaked in 3 successive baths and then dried. The tracks were evaluated visually for all samples. Reading is done by densitometry that gives a quantitative analysis of each individual fraction. The relative percentage of each protein

fraction was calculated by the densitometer from the area under the curve created by the protein band. The densitometer automatically calculated the absolute value for each fraction by multiplying the total protein of the sample by the corresponding fractional percentage. Albumin to globulin ratios (A/G) were determined according to the following equation: (albumin)/(alpha globulins+beta globulins+gamma globulins).

Statistical analysis: Systat 7.0 software (SPSS Inc., Chicago, Illinois, USA) was used for all analyses. Considering the small size of the population studied, non parametric Wilcoxon paired test was performed to compare the differences between serum and plasma. Non parametric Mann-whitney test was used to describe the statistical difference between normal and hemolyzed plasma and between normal and pathological serum. Results are presented as median and minimum-maximum range. *p<0.05, **p<0.01, ***p<0.001.

RESULTS

Plasma and serum protein fractions: Electrophoresis revealed the following protein fractions: albumin, α_1-globulins, α_2-globulins, pre β-globulins, β_1-globulins, β_2-globulins and γ-globulins. The result is expressed by a curve with 7 peaks (Fig. 1). Median values and range (maximum-minimum values) for all investigated parameters are expressed in (g L^{-1}) and presented in Table 1. Densitometric quantification of plasma protein fractions revealed a very significant increase in total proteins, β_1-globulins fraction, γ-globulins fraction and a significant increase in β_2-globulins fraction. The ratio A/G was significantly lower in plasma when compared to serum samples.

Normal plasma and hemolyzed plasma protein fractions: Hemolysis resulted in a significant increase in plasma α_2-globulins fraction and pre β-globulins fraction.

Protein fractions of pathological sera: Electrophoretogram is expressed by a curve with 6 packs: albumin, α_1-globulins, α_2-globulins, β_1-globulins, β_2-globulins and γ-globulins (Fig. 1). Densitometric quantification of serum protein fractions from diseased chickens revealed a significant decrease in albumin and A/G ratio and a very significant increase in total proteins, α_1-globulins, all proteins of beta and gamma fraction. Only α_2-fraction did not show a statistical difference.

Table 1: Protein electrophoresis fractions values and summary of statistical analysis comparing normal plasma and normal serum, normal plasma and hemolyzed plasma, normal serum and serum with New castle disease. Results are expressed as median+range

	Plasma (median+range)	Serum (median+range)	Plasma vs serum	Hemolyzed plasma (median+range)	Normal plasma vs hemolyzed plasma	Serum with NC disease (median+range)	Normal serum vs serum with NC disease
T. prot (g L^{-1})	35.7 (30.6-38.6)	30.4 (29.1-31.9)	0.008**	33.1 (33.1-33.6)	NS	88.5 (88.3-88.5)	0.0001***
Alb (g L^{-1})	14.5 (12.7-14.7)	14.1 (12.9-15.7)	NS	14.0 (12.1-14.9)	NS	13.0 (12.3-13.8)	0.02*
α_1 glb (g L^{-1})	1.7 (1.5-2.8)	2.1 (1.3-2.2)	NS	1.7 (1.7-1.9)	NS	5.3 (3.7-5.3)	0.0001***
α_2 glb (g L^{-1})	5.7 (5.3-6.3)	5.9 (5.0-6.3)	NS	6.3 (6.0-6.7)	0.03*	9.7 (1.4-9.7)	NS
Pre β glb (g L^{-1})	1.6 (1.0-3.9)	1.5 (1.4-2.2)	NS	2.9 (2.6-4.0)	0.01*		
β_1 glb (g L^{-1})	4.5 (2.7-6.3)	1.0 (0.9-1.4)	0.005**	3.2 (2.6-6.2)	NS	15.8 (8.9-15.8)	0.0001***
β_2 glb (g L^{-1})	3.6 (2.2-4.4)	2.4 (2.0-4.1)	0.01*	3.9 (2.3-4.0)	NS	25.1 (14.8-25.1)	0.0001***
γ glb (g L^{-1})	4.2 (3.3-6.5)	2.9 (2.7-3.3)	0.009**	5.3 (3.2-6.8)	NS	18.8 (18.8-45.0)	0.0001***
A/G ratio	0.56 (0.51-0.74)	0.88 (0.74- 0.97)	0.005**	0.61 (0.55-0.75)	NS	0.18 (0.18-0.20)	0.0001***

NC: New castle disease, NS: Not significant, *p<0.05, **p<0.01, ***p<0.001

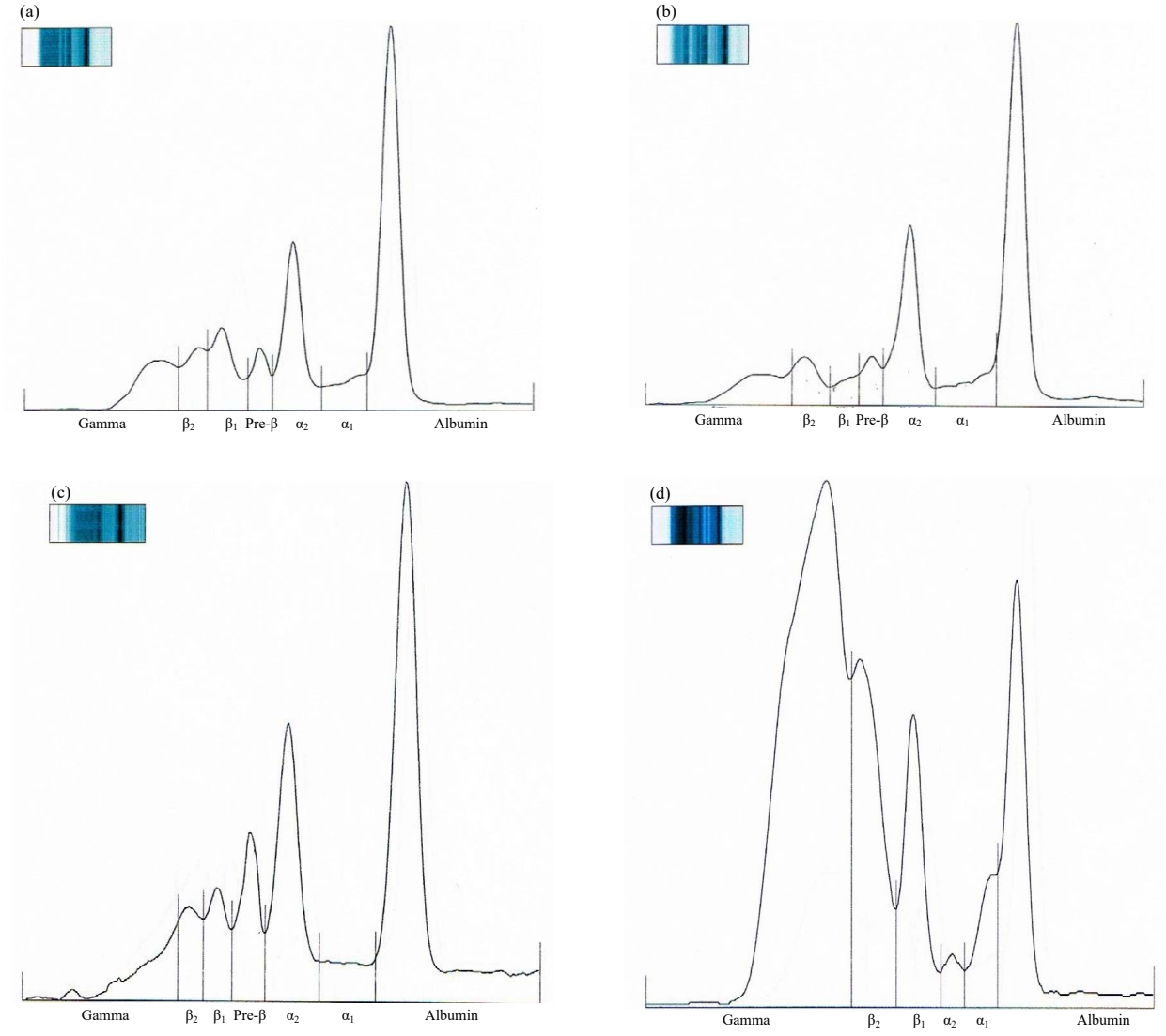

Fig. 1(a-d): Electrophoretic patterns of blood protein fractions from chickens of Isa 15 strain. Protein fractions (from right to left) are albumin, α_1-globulins, α_2-globulins, pre β-globulins, β_1-globulins, β_2-globulins and γ-globulins. (a) Typical pattern from plasma broiler chickens with highest levels of β_1-globulins, β_2-globulins and γ-globulins, (b) Typical pattern from serum broiler chickens, (c) Typical pattern from hemolyzed plasma samples with highest levels of α_2-globulins and pre β-globulins, (d) Typical pattern from serum broiler chickens with New Castle disease with lowest levels of albumin and highest levels of α_1-globulins, β_1-globulins, β_2-globulins and γ-globulins

DISCUSSION

Protein electrophoresis is widely used in human and veterinary medical diagnoses and in avian medicine. Electrophoretic analysis has been shown to play an important role to characterize dysproteinemias associated with different diseases[10].

It has been estimated that 30% of apparently healthy birds will have elevated EPH values without changes on the hematologic and biochemical analyses. Thus, the veterinarian could miss illness in approximately one-third of avian patients if an EPH is not performed in conjunction with a count blood cells and/or biochemical analysis[11].

Avian total proteins consist of albumin and globulins. Total proteins are a common parameter used to assess the health status. The routine measurement of total protein concentration is commonly performed by biuret method which continues to be the most widely used colorimetric

method in avian samples[12]. Total concentration of blood proteins of birds is about the same as half its value in mammals. It is probably due to the extremely high concentration of glucose in bird blood. Glucose is an active osmotic substance that reduces the protein concentration in order to maintain the colloid-osmotic pressure[13].

In healthy animals, albumin is the largest protein electrophoresis peak, it is a tall thin peak and is the most intense anodic fraction. Albumin is synthetized by the liver as are all plasma proteins except for the immuoglobulins. Albumin's main function is the maintenance of colloid oncotic pressure in the intravascular and extravascular spaces. Albumin also functions as a protein carrier to transport a large number of compounds[14].

Different methods have been used for determining plasma albumin concentrations and , in general, dye binding methods are unreliable in avian blood[15].

Globulins are involved in a range of processes including transport of ions, hormones and lipids, acute-phase responses, and, as immunoglobulins immune response.

The α fraction is the most rapidly migrating of all the globulins. In most species, except for ruminants, it migrates as an α_1 (fast) and α_2 (slow) fraction. It is a fraction of very heterogeneous proteins with varying biological roles; α-lipoproteins, α_1 antitrypsin, α_1 acid glycoprotein, α_2-macroglobulin, haptoglobin. α-lipoproteins (HDL) migrate as α_1, pre-β-lipoproteins (VLDL) migrate as α_2[16]. Most of these proteins are positive acute phase proteins[17]. The α_2-macroglobulin, haptoglobin, ceruloplasmin and amyloid A are also important acute phase proteins[16].

Beta fraction is, as the alpha fraction, consisting of very heterogeneous proteins having different biological roles; beta lipoprotein LDL, fibrinogen, transferrin, ferritin, hemopexin, complement (C3, C4) and C-reactive protein (CRP). These proteins are mostly acute-phase proteins. Transferrin; a negative acute-phase protein in mammals is a positive acute-phase protein in birds[17]. The proteins of the beta fraction play their essential role in iron metabolism, particularly the transferrin and ferritin as forms of transport and storage of iron and hemopexin which maintains the heme in soluble form[16].

The gamma fraction consists of immunoglobulins synthetized by B cells, including IgM, IgA, IgE and IGY in birds, but may, in some species contains some proteins of β fraction (beta-lipoprotein)[4,18].

By convention, in birds, the ratio albumin/globulin (A/G) is calculated by dividing the albumin fraction (A) by the sum of globulins (G)[4,18,19]. This ratio is generally close to 1 in healthy birds[20]. In the absence of recognized standard values for each protein fraction, the most relevant parameter for the assessment of a bird's health status seems to be the A/G ratio, because it reflects the global evolution of all globulin fractions[2].

Differences between normal plasma and normal serum protein electrophoresis pattern: Serum is qualitatively different from plasma in that the bulk of the fibrinogen has been removed by conversion into a fibrin clot. Varying amounts of other proteins are removed into the fibrin clot either by specific or non-specific interactions[21]. Fibrinogen is an important indicator of inflammation and is present in plasma[22], which is preferable to serum for nonmammalian blood for electrophoresis because it contains fibrinogen[4,19,23].

Thus, in avian medicine, plasma rather than serum is commonly used to conserve sample volume and because it also can be used for other clinical chemistry analyses[20]. Especially in birds, only small blood specimens can be collected from sick animals[24,25] and electrophoretic analysis of native plasma provides the same diagnostic information as analysis of serum, except for possible overestimation of the beta globulin fraction[25].

In this study, a statistical difference was observed between plasma and serum for total proteins, all proteins of beta fraction and gamma globulins. Lumeij and Maclean[26] found that the mean total protein concentration in pigeon plasma was higher than that of serum. Kaneko *et al.*[12] and Hawkins *et al.*[27] also reported in pigeons a higher concentration of total proteins, that was attributed to fibrinogen removal during the clotting process.

Camus *et al.*[28] reported that in interpreting the electrophoretogram of nonmammalian species, it is important to remember that plasma rather than serum is often utilized to evaluate fibrinogen levels and that fibrinogen migrates in the late beta-globulin region and can bridge the area between the beta and gamma regions, particularly when increased levels of fibrinogen are present, making identification and interpretation difficult. Roman *et al.*[29] affirmed that fibrinogen migrates to a beta location on avian electrophoregrams and as this protein is easy to identify by comparing plasma and serum EPH, its localisation would be considered as a reference for defining protein fractions in avian plasma. It would be expected in the present paper that serum samples would produce different EPH results, especially in the beta fraction, in which fibrinogen normally migrates. The rise of proteins of beta fraction and gamma fraction in plasma may explain lower ratios A/G described in plasma samples. Muller[30] and Filipovic *et al.*[31] reported that the increased levels of alpha,

beta and gamma globulins with age and at the same time, a decrease in the levels of albumin were observed by many authors in several avian species at the end of the rearing period and may explain the low level of minimal values in plasma samples.

Differences between normal plasma and hemolyzed plasma: In vitro hemolysis is common in birds. It has been related to improper specimen handling such as forcing blood through small needles during sampling, long storage of the blood before centrifugation, or excessive agitation when mixing. In humans and birds, hemolysis can lead to artifactual high values for plasma or serum total protein concentration measurements. There is less information for the influence of hemolysis in agarose gel protein electrophoresis. In mammals as in humans, hemolysis has been shown to generate artifacts in serum electrophoresis; hemoglobin-haptoglobin complexes move to the α_2 fraction and free hemoglobin migrates to the beta fraction[32].

In birds, this phenomenon has only been described by Werner and Reavill[18] in a review article and it has been recently investigated in psittacine birds where an increase in the gamma fraction is reported[20]. The purpose of the study reported here was to more thoroughly investigate the effects of hemolysis in plasma protein concentration measurements and electrophoresis patterns.

In an hemolyzed sample, constituents released in the plasma or serum from broken blood cells can interfere with laboratory results. Leakage of these intracellular components leads to increased concentration in plasma or serum. Hemolysis is described to induce artifactually higher total protein values[32].

In this study, electrophoresis patterns of hemolyzed chicken's plasma mainly show a rise in the alpha-2 globulin and pre-beta fraction. These changes differ from those observed in Bar-headed Geese and Black Kites in wich hemoglobin migrates in the gamma fraction, but look like the changes observed in mammals in which hemoglobin-haptoglobin complexes move to the α_2 fraction and free hemoglobin migrates to the β_1 fraction[32]. Martínez-Subiela *et al.*[33] found that free haemoglobin present in haemolytic canine sera migrated slowly in the β_1 region and that free hemoglobin migrated in the β_2 fraction inducing the presence of an interferent peak in this region. Lippi *et al.*[34] affirmed that the reliability of protein electrophoresis might also be influenced by haemolysis in human serums, as haemoglobin-haptoglobin complexes move between the α_2 and beta globulin fractions and free haemoglobin migrates as a diffuse reddish band in the beta globulin fraction. However, the plasma of all mammals has been shown to contain haptoglobin, a protein that binds free hemoglobin with high affinity and inhibits its oxidative activity, but this protein has not been identified in chicken plasma and may be replaced by another hemoglobin-binding protein called PIT 54; it is the only hemoglobin-binding protein in these animals[35]. We suppose that this protein migrates in the same location in mammal's plasma as well as in chicken's plasma. This could explain the rise in α_2 fraction and pre beta fraction in our study.

Differences between normal serum protein electrophoresis pattern and serum with new castle disease: Newcastle disease (ND), is a highly contagious viral disease, that affects domestic poultry and wild birds. It causes devastating economic losses in the global poultry industry. The disease is distributed worldwide and affects a wide range of avian species, causing high mortality and severe clinical symptoms. The lesions severely affect the respiratory, gastrointestinal and nervous system[36-38]. The serum samples were tested to determine the antibodies against New Castle disease virus, using the standard hemagglutination inhibition method. All serum samples were found to be positive for specific immunity against New Castle virus.

Compared with the normal serum EPH profile, protein EPH showed decreased levels of albumin and increased levels of total proteins, α_1-globulins, β_1-globulins, β_2-globulins and γ-globulins in chickens with ND. Only α_2-fraction did not show statistical difference. The increased globulin concentrations resulted in decreased A/G ratios. Increased concentration of beta globulins, decreased concentration of albumin and decreased A/G ratio have been previously described in psittacine birds with asprgillosis[39]. Acute-phase proteins (APPs), such as alpha-lipoprotein and haptoglobin, are said to migrate within the α_1 and α_2-globulin fraction, whereas the APPs fibronectin and transferrin migrate through the beta globulin fractions of the EPH[11]. Increased alpha globulins (predominantly α_1-globulins) were found in sheep naturally infected with *Babesia ovis*, as well as in calves affected by respiratory diseases. Also, selective increases in the α_1-globulins have been observed in psittacine birds with parasitic infection, caused by an increase in α_1-antitrypsin[26]. According to Csilla *et al.*[40]. the alpha globulin fraction includes many of the APPs (ceruloplasmin, haptoglobin, α_1-acid glycoprotein, some lipoproteins), which have a great potential as biomarkers of many economically important diseases. Results of the present study showed that the concentrations of these proteins rise with respiratory diseases and that the increase in the concentration of beta globulins occurs with inflammation. On the other hand, there is a concomitant decrease in albumin as a result of its decreased synthesis.

Decreased albumin levels and increased globulin concentrations is the most common pattern in animals subjected to inflammatory diseases. This shift in the albumin and globulin concentrations resulted in significantly lower A/G ratio suggesting the significance of A/G ratio in the classification of the electrophoretic profile and identification of dysproteinemias.

Results of the present study showed that the changes in the beta fraction seem to be more consistent than those recorded in alpha fractions. The beta fraction contains fibrinogen, which is one of the most abundant APPs in birds. In the case of an inflammatory condition, its increase is fast and massive[2]. An increase of beta globulins has also been described in chickens experimentally inoculated with turpentine and other irritants, demonstrating that most APPs (transferrin, fibrinogen, beta lipoprotein, complement) in avian species migrate in this sector. The transferrin appears to be the major APP in avian species in contrast to what happens in mammals[41]. Although it is not specific to a particular disease, it has an important prognostic value: its short half-life (24-48 h) makes the monitoring of beta globulins an excellent indicator of the effectiveness of a therapy[24].

Higher concentrations of gamma globulins obtained in chickens with ND may reflect the response of the organism to inflammation. The gamma region consists of immunoglobulin molecules. Because of the relatively slow migration of most of these molecules, the term of gamma globulin was at one time synonymous with immunoglobulin. It is now clear that antibody molecules migrate anywhere from the alpha to the slow gamma region. The term gamma globulins reflected the fact that most of the serum antibodies are of the IgG class that primarily migrates in a gamma location[42].

Facon *et al.*[2] affirmed that in the absence of recognized standard values for each protein fraction, the most relevant criterion for the assessment of a bird's health status seems to be the A/G ratio, because it reflects the global evolution of all globulin fractions. The A/G ratio decreases during an inflammatory condition due to an increase in the globulin fractions and a decrease in the albumin fraction.

However, the ratio A/G remains the most reliable parameter in the interpretation of diagnostic significance of the electrophoretogram, since any inflammatory process leads very quickly to a characteristic inversion of this report[19].

CONCLUSION

Protein electrophoresis has been demonstrated to be a valuable technique in veterinary medicine. Once the total protein level has been determined by the biuret method, the electrophoretic fractions are calculated based on the total protein concentration. It is important to be aware of differences between values obtained from serum and plasma samples and of artifacts that may be caused by improper sample handling and condition of animals. Since the evaluation of the concentrations of specific acute phase proteins is limited in avian medicine, electrophoresis remains the main tool in assessing an inflammatory status.

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