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Original Study

Protein Electrophoresis of Serum and Heparinized Plasma in the Common Mynah (*Acridotheres tristis*)

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Abstract: Although serum protein electrophoresis is a diagnostic tool available through many veterinary laboratories, there currently are no reference intervals for protein fractions in healthy common mynahs (*Acridotheres tristis*). Therefore, electrophoretic patterns of proteins in serum and heparinized plasma of the common mynah were evaluated. Blood specimens were collected from 55 healthy adult common mynahs of unknown age (26 males and 29 females). The serum total protein and protein fractions were measured using the biuret method followed by cellulose acetate electrophoresis (CAE). The serum level of albumin was compared with bromocresol green (BCG) dye-binding and CAE methods. Four protein fractions, including albumin and α, β, and γ globulins, were recorded in the electrophoretogram of serum specimens. Sex appeared to have no significant effect on the measured parameters. The serum BCG albumin fraction was significantly higher than the CAE albumin fraction ($P = .01$). Also, the comparison of total protein and protein fractions in serum and plasma specimens of 25 of the 55 birds sampled showed that total protein (Cohen index $d = 0.66$, $P = .03$), gamma globulin ($d = 1.13$, $P = .00$), and total globulin ($d = 0.67$, $P = .00$) in plasma samples were significantly higher than those in serum samples. The results of this study provide the specific reference intervals for total protein and protein fractions in common mynahs, which are essential for proper interpretation of laboratory results and also revealed that the albumin measurement by the BCG method yields unreliable results in common mynahs.

Key words: cellulose acetate electrophoresis, blood proteins, bromocresol green, avian, common mynah, *Acridotheres tristis*

INTRODUCTION

Serum protein electrophoresis (SPE) is a valuable complementary diagnostic tool for hematological, biochemical, and serological assays. The SPE test can provide information that contributes to the antemortem diagnosis and monitoring of the treatment response in birds presenting with clinical disease (eg, mycosis, inflammation, nephritis, hepatitis, avian chlamydiosis).[1–3] By measuring acute phase proteins and immunoglobulins, SPE is an inexpensive but nonspecific technique for staging acute and chronic inflammatory disease processes.[4,5] Since the bromocresol green (BCG) method is not accurate in many avian species,[6,7] SPE is generally considered the preferred method for albumin and globulin quantitation and calculating the albumin : globulin ratio (AGR) in avian species.[8,9] However, since the intricate SPE diagnostic test is not available in every laboratory the BCG method is used routinely as an analytical method to measure avian blood albumin concentration.

Determination of the physiological electrophoretic patterns in different avian species is necessary for the diagnostic application of SPE. Despite the increased use of this diagnostic test in avian medicine, there are no data regarding reference intervals for protein fractions in the healthy common mynah (*Acridotheres tristis*).

Generally, for most animals, a serum specimen is the preferred sample for routine biochemical

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analysis. However, plasma specimens are usually obtained in avian clinical practice because the small size of most birds limits the volume of blood that can be obtained. When the blood volume collected from an avian patient is limited, only 1 heparinized blood sample will be submitted for both hematologic and biochemical tests.[10] Conversely, the presence of fibrinogen in plasma samples may interfere with the interpretation of the β and γ protein fractions. Therefore, heparinized plasma is not the ideal sample to submit for protein electrophoresis testing. Therefore serum samples are recommended for protein electrophoresis testing.[11]

The objectives of the present study were to determine the normal serum total protein as well as protein fractions for common mynahs, compare the concentration of different protein fractions and electrophoretic patterns between males and females, compare electrophoretograms between serum and plasma specimens, and compare plasma albumin levels measured by BCG and cellulose acetate electrophoresis (CAE) methods.

MATERIALS AND METHODS

All animal experiments were performed in strict accordance with the guidelines approved by the Animal Ethics Committee of Ferdowsi University of Mashhad, Iran (IR.UM.REC.1400.077). In December 2016, 64 adult wild common mynahs of unknown age (body weight: 118 ± 11 g) were collected from different geographic locations of Iran (Qazvin, 25 birds; Fars, 24 birds; and Tehran, 15 birds). The birds were housed in individual cages (30 cm × 40 cm × 80 cm) under controlled environmental conditions (humidity 35%–55%, temperature 19–23°C [66.2–73.4°F], 12-hour light cycle) and provided fresh tap water on a daily basis. A commercial concentrate food (Dr. Birds, Mynahplex 70, Mashhad, Razavi Khorasan, Iran) plus vegetables and fruits ad libitum was fed to the subject animals.

The common mynahs were held for 2 weeks in their new environment for conditioning purposes. To evaluate the overall health status of the birds, an external physical examination and diagnostic tests were performed.[12] Fecal flotation was used to evaluate the birds for gastrointestinal parasites while microscope slides prepped with blood and crop swabs were examined for the presence of protozoa. Radiographic images were acquired from each bird using mammography film. Following the external physical examination, and diagnostic imaging testing, 55 apparently healthy individuals were included in the present study, and those with any abnormal findings (n = 9) were excluded.

The first objective of this study was to determine the electrophoretogram pattern in serum samples collected from common mynahs and identify differences in protein fractions between females and males. To obtain serum specimens, blood samples were collected from the right jugular vein of 55 fasted individuals (male = 26, female = 29) using a 25 gauge needle on a 2-mL syringe. The blood samples were then transferred from the syringes into serum tubes with a gel separator (BD Microtainer; Becton Dickinson, Franklin Lakes, NJ, USA). To compare the electrophoretogram patterns between plasma and serum samples, heparinized whole blood samples were also obtained in the manner described previously from 25 of the birds. For the plasma samples, the collected blood was transferred into heparinized blood tubes (BD Microtainer).

The blood samples were immediately transported on ice to the laboratory of Clinical Pathology, Ferdowsi University of Mashhad Veterinary Teaching Hospital. For serum and plasma separation, blood specimens were centrifuged at 3000 rpm for 10 minutes. Plasma and serum samples, which were free of visible lipemia and hemolysis, were placed into 1.5-mL flat-cap microcentrifuge tubes (Eppendorf, Hamburg, Germany) and then stored at –70°C (–94°F) until analyzed.

Serum and plasma levels of total protein and albumin were determined by biuret and BCG dye-binding methods, respectively, using a biochemical autoanalyzer (Minderay, Nanshan, Shenzhen, China) and commercial biochemical kits (Pars Azmoon, Tehran, Iran). Prior to sample evaluation, acceptable analyzer performance was confirmed through quality control (Randox, Crumlin, County Antrim, UK).

Protein electrophoresis (15 μL) was performed on Titan III cellulose acetate strips at 350 V for 25 minutes in barbital buffer (Tris buffer 45%, sodium 5,5 -diethylbarbiturate 1%, pH 8) using an electrophoresis power supply and tank (Paya Pazhouhesh, Mashhad, Khorasan Razavi, Iran). Strips were stained with Ponceau S (Merck, Darmstadt, Hesse, Germany) and destained by soaking 3 times (each time for 10 minutes) in acetic acid 5% and once in methanol solution (100%). Finally, the cellulose acetate strips were soaked in the clearing solution for 4 minutes. After drying the cellulose acetate strips in the oven at 37°C (98.6°F) for 20 minutes, Hewlett-Packard scanner (HP DeskJet 2130, Palo Alto, CA, USA) and PhotoElectrophoresis software (Hooshmand Fanavar, Tehran, Iran) were

Table 1. Coefficients of variation (CV) for serum protein fractions in 55 wild-caught and fasted adult common mynahs (*Acridotheres tristis*) (male = 26, female = 29) using cellulose acetate electrophoresis interassay and intra-assay methods.

Fraction	Interassay CV (%)	Intra-assay CV (%)
Albumin, g/dL	4.5	3.0
α globulin, g/dL	9.7	8.8
β globulin, g/dL	3.6	2.1
γ globulin, g/dL	8.2	7.4

used for quantification of the protein fractions. Fraction demarcation was conducted based on the midpoint of the electrophoretogram pattern that usually lies between the α and β fractions.

The absolute value of each protein fraction (g/dL) was calculated by multiplying the relative concentration of the fraction (%) by the total protein level determined via the biuret dye-binding method. The AGR was calculated by dividing the albumin by the sum of α, β, and γ globulins. For intra-assay variation, 8 aliquots were analyzed by CAE in 5 replicates. For interassay variation, 8 samples were analyzed by CAE for 5 days. Then the coefficient of variations (CV) was determined, with both intra-assay and interassay being less than 10% (Table 1).

Statistical analysis

Statistical analysis was conducted using SPSS (SPSS Inc, Chicago, IL, USA) and MedCalc (MedCalc Software Ltd, Mariakerke, Belgium) software with $P < .05$ determined to be statistically significant. Prior to calculating reference intervals, outliers were identified and eliminated by the use of the Tukey test. The distribution of the obtained data was analyzed by the Shapiro-Wilk test, which revealed all data followed Gaussian distribution. Since the sample size was relatively small ($40 \leq X < 120$), the 95% reference interval with 90% confidence intervals (CI) on reference limits were calculated using a robust approach.[13]

An independent sample *t* test was used for comparison of measured parameters between male and female common mynahs. The concentration of protein fractions in serum and plasma specimens was compared using the paired sample *t* test, and the effect size was estimated by the Cohen *d* index. Based on the *d* index, effect sizes were classified as small ($d = .2$), medium ($d = .5$), and large ($d \geq .8$).[14]

The comparison of serum albumin concentration measured by BCG and CAE methods was conducted using the paired sample *t* test and Pearson correlation. The presence of constant and proportional biases was evaluated using Passing-Bablok regression analysis. Bland-Altman plots were applied to detect bias.[15]

RESULTS

SPE in common mynah

Serum protein fractions in the common mynahs tested with CAE are presented in Table 2. The

Table 2. Reference intervals (95% RI with 90% CI, mean ± SD, minimum and maximum) of relative and absolute concentration of serum protein fractions in 55 wild-caught and fasted adult common mynahs (male = 26, female = 29) (*Acridotheres tristis*) described in Table 1.

			95% RI (90% CI)	
Parameters	Mean ± SD	Min–max	Lower	Upper
Total protein, g/dL	3.25 ± 0.48	2.30–4.60	2.24 (2.05–2.41)	4.20 (3.99–4.40)
Albumin, CAE				
g/dL	1.07 ± 0.16	0.70–1.40	0.73 (0.67–0.81)	1.41 (1.34–1.47)
%	32.06 ± 6.96	15.70–46.60	18.19 (15.49–21.35)	46.80 (44.16–48.98)
α globulin				
g/dL	0.56 ± 0.18	0.20–1.01	0.18 (0.12–0.25)	0.92 (0.84–0.98)
%	19.34 ± 5.47	6.60–28.70	5.65 (3.76–7.75)	28.63 (26.58–30.51)
β globulin				
g/dL	0.73 ± 0.25	0.25–1.20	0.21 (0.13–0.30)	1.23 (1.13–1.32)
%	22.41 ± 7.08	7.50–37.90	7.83 (5.31–10.63)	36.62 (33.63–39.33)
γ globulin				
g/dL	0.87 ± 0.35	0.20–1.70	0.15 (0.03–0.28)	1.56 (1.42–1.69)
%	27.42 ± 9.95	5.90–49.80	7.12 (3.87–11.07)	46.44 (42.75–50.04)
Total globulin				
g/dL	2.19 ± 0.46	1.27–3.20	1.22 (1.08–1.40)	3.11 (2.91–3.27)
AGR	0.51 ± 0.13	0.28–0.87	0.22 (0.17–0.27)	0.78 (0.73–0.84)

Abbreviations: RI indicates reference interval; CI, confidence interval; AGR, albumin : globulin ratio.

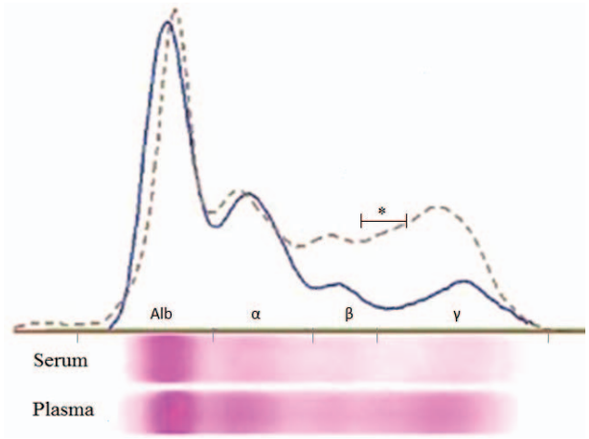

Figure 1. Comparison of the electrophoretogram pattern in heparinized plasma (dashed line) and serum (solid line) specimens obtained from the common mynahs (*Acridotheres tristis*) described in Table 1. * Indicates the location of fibrinogen in the plasma (between β and γ fractions).

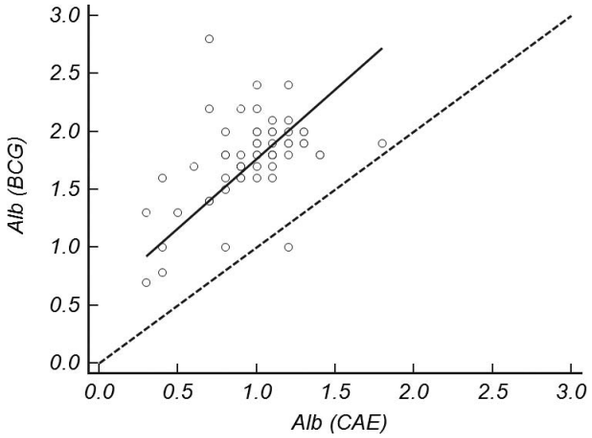

Figure 2. Comparison of bromocresol green (BCG) and cellulose acetate electrophoresis (CAE) methods for measurement of serum albumin (Alb) concentration in the common mynahs (*Acridotheres tristis*) described in Table 1. The dotted line represents the line of identity ($y = x$). The solid line represents the regression line ($y = \alpha + \beta X$; Alb [BCG] $= \alpha + \beta$Alb [CAE]) with intercept = 0.56 (95% CI: 0.15 to 0.75) and slope = 1.20 (95% CI: 1 to 1.60).

CAE of serum specimens revealed 4 protein fractions, including albumin and α, β, and γ globulins. Albumin constituted the predominating fraction followed by α, β, and γ globulins (Fig 1). The prealbumin fraction was observed in just 2 birds, therefore, was not included in the final analysis of this study.

The comparison of total protein, albumin, and electrophoretograms of serum specimens between males and females revealed that sex had no significant effect on the measured parameters, and there were no differences between males (n = 26) and females (n = 29).

Comparison of total protein and protein fractions in serum and plasma samples

The comparison of total protein and different fractions between serum and plasma specimens indicated that concentrations of total protein ($d = 0.66$, $P = .03$), γ globulin ($d = 1.13$, $P = .00$), and total globulin ($d = 0.67$, $P = .00$) in plasma were significantly higher than those in serum (Table 3). Based on the comparison of plasma and serum electrophoretograms, the results indicate that fibrinogen is located between β and γ fractions in the plasma specimen (Fig 1).

Comparison of albumin measurement by CAE and BCG methods

The measurement of albumin by different methods showed that serum albumin values measured by the BCG method (1.76 ± 0.05 g/dL) were significantly ($P < .05$) higher than those measured by the CAE method (0.97 ± 0.04 g/dL). There was a weak positive correlation between albumin measurements with CAE and BCG ($r = .48$, $P = .00$).

The agreement between BCG and CAE methods is presented in Figure 2. In this figure, the data from the BCG (on the y-axis) were plotted against the CAE method (on the x-axis). Setting the line of identity ($y = x$) in the plot indicated that the results of the 2 methods were not equal and there were constant or/and proportional biases. In the Passing-Bablok regression analysis, intercept and slope were 0.56 and 1.20, respectively. Since the 95% CI for the intercept (0.15 to 0.75) did not include 0, it was considered as evidence of constant bias. While the 95% CI for the slope (1 to 1.60) includes 1; the result did not indicate proportional bias.[15] The difference between the BCG and CAE methods was not symmetrically distributed around 0, and 95% of the differences were not within the lines, thus the two methods are not identical within inherent imprecision (Fig 3).

DISCUSSION

Several factors, such as age, sex, environmental conditions, and, most importantly, species, affect the blood concentration of total protein and protein fractions. Therefore, universal protein

Table 3. Comparison of total protein and protein fractions (Mean ± SD, 95% RI with 90% CI) in serum and heparinized plasma specimens of 25 of the common mynahs (*Acridotheres tristis*) described in Table 1.

	Heparinized plasma		
		95% RI (90% CI)	
Parameters	Mean ± SD	Lower	Upper
Total protein, g/dL[a]	3.62 ± 0.88	2.34 (2.06–2.67)	5.60 (4.92–6.39)
Albumin			
g/dL	1.15 ± 0.34	0.39 (0.21–0.62)	1.84 (1.61–2.07)
%	31.18 ± 7.32	15.08 (10.93–19.56)	46.13 (41.03–50.78)
α globulin			
g/dL	0.58 ± 0.17	0.21 (0.11–0.32)	0.96 (0.85–1.06)
%	16.80 ± 5.24	5.61 (3.02–8.85)	27.94 (24.78–30.82)
β globulin			
g/dL	0.55 ± 0.24	0.21 (0.17–0.29)	1.16 (0.93–1.55)
%	16.02 ± 5.20	7.61 (6.32–8.98)	29.72 (23.12–35.72)
γ globulin[a]			
g/dL	1.36 ± 0.46	0.28 (0.05–0.54)	2.30 (1.91–2.61)
%	35.99 ± 5.98	23.02 (19.70–26.88)	48.63 (44.85–51.91)
Total globulin, g/dL[a]	2.48 ± 0.70	1.37 (1.21–1.58)	4.25 (3.49–5.04)
AGR	0.49 ± 0.13	0.15 (0.07–0.23)	0.73 (0.63–0.81)

Abbreviations: RI indicates reference interval; CI, confidence interval; AGR, albumin : globulin ratio.[a] Indicates significant ($P < .05$) difference between serum and plasma specimens.

Table 3. Extended.

	Serum			
		95% RI (90% CI)		Effect size
Parameters	Mean ± SD	Lower	Upper	(Cohen *d* index, *P* value)
Total protein, g/dL[a]	3.15 ± 0.48	2.21 (1.91–2.52)	4.09 (3.79–4.39)	0.66, .03
Albumin				1.13, .00
g/dL	1.10 ± 0.23	0.78 (0.70–0.87)	1.37 (1.26–1.46)	
%	34.98 ± 7.84	15.54 (10.67–20.93)	52.94 (46.53–58.55)	
α globulin				—
g/dL	0.61 ± 0.19	0.17 (0.08–0.28)	1.02 (0.87–1.13)	
%	19.34 ± 5.47	7.62 (4.64–10.88)	30.88 (27.48–33.76)	
β globulin				—
g/dL	0.53 ± 0.23	0.22 (0.18–0.30)	1.44 (1.13–1.81)	
%	18.25 ± 6.17	5.47 (1.75–10.07)	32.05 (28.66–34.86)	
γ globulin[a]				—
g/dL	0.86 ± 0.42	0.10 (0.01–0.20)	2.69 (1.43–2.04)	
%	27.42 ± 9.95	5.48 (0.56–12.22)	47.59 (41.25–53.94)	
Total globulin, g/dL[a]	2.05 ± 0.57	0.82 (0.44–1.20)	3.26 (2.90–3.61)	0.67, .00
AGR	0.54 ± 0.19	0.16 (0.05–0.28)	1.16 (0.80–1.03)	—

reference intervals should not be applied to different avian species.

Based on our results, the serum concentration of total protein in common mynahs is 3.25 ± 0.48 g/dL which is approximately similar to those reported for other avian species.[5,6,16–18] Generally, the range of serum protein levels in birds is 2.5 to 4.5 g/dL, which is lower than in mammals.[10,19]

Researchers have attained different electrophoretograms from dissimilar avian species.[17,20–22] In the present study, 4 fractions, including albumin and α, β, and γ globulins were observed in serum specimens, and similar to other species, albumin was the predominant fraction in the common mynahs in this study. In the present study, prealbumin was measured as a small fraction in only 2 birds, which were not included in the final analysis. The absolute and relative amount of prealbumin in the 2 birds from which measurements were obtained was 0.18 g/dL and 0.30 g/dL

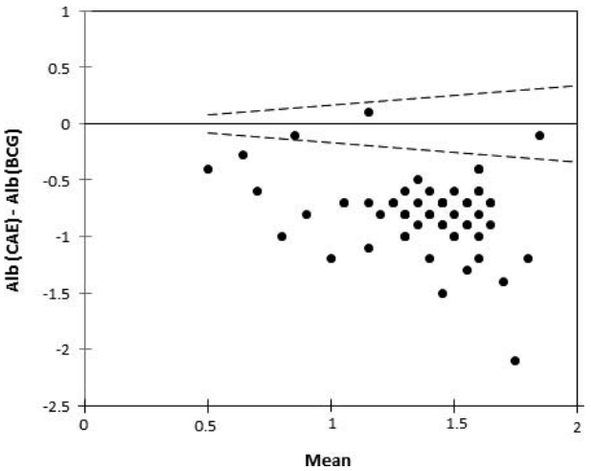

Figure 3. Bland-Altman plot of the difference between serum albumin (Alb) concentrations (g/dL) in the common mynahs (*Acridotheres tristis*) described in Table 1, as measured by bromcresol green (BCG) and cellulose acetate electrophoresis (CAE) methods. The dotted lines represent the outer limits of the inherent imprecision of both respective methods.

and 5.45% and 7.31%, respectively. Prealbumin serves as a carrier protein, and its concentration markedly varies between avian species and seasons, therefore, is considered to have little diagnostic value.[23] In some avian species (eg, monk parakeets [*Myiopsitta monachus*], budgerigars [*Melopsittacus undulatus*], cockatiels [*Nymphicus hollandicus*]) there is a large prealbumin fraction, while there is none or a very small fraction in African grey parrot (*Psittacus erithacus*) electrophoretograms.[20]

In the present study, the AGR ranged from 0.28 to 0.87, which is both similar in some and different in other avian species.[17,21,24–26] Usually, the AGR range in birds is 1.2 to 3.6.[19] It has been found that AGR decreased significantly in patients with certain avian diseases, while the total protein concentration remained within the reference interval.[1] Therefore, the AGR appears to be a useful diagnostic index that, along with protein electrophoresis, helps identify the variations of serum or plasma proteins.

In birds, changes in plasma protein levels are not exclusively related to pathologic conditions. Variation in the plasma protein level of avian patients may also be associated with physiological factors (eg, age, gender, reproductive status, and season).[17,27] However, in the present study, there was no significant difference in the concentration of total protein and protein fractions between the male and female common mynahs. Similar results have been reported in other avian species.[28,29] Changes in the protein concentrations of female birds may occur more often when reproductively active. Immediately before ovulation, the concentration of both total plasma protein and globulin fractions increase due to an elevated blood estrogen level. The total plasma protein level includes vitellogenin and lipoproteins, which are precursors of yolk development.[19,30] Since the present study did not take place when the birds were reproductively active, this may explain the lack of significant differences in protein profile between the sexes.

Another objective of this study was to compare the protein fractions of plasma and serum specimens in the common mynahs. The results obtained showed that the levels of total protein measured by the biuret method in heparinized plasma were significantly higher than those in serum ($P = .03$). The higher level of total protein in plasma can be attributed to fibrinogen and other coagulation factors in plasma that are not present in serum samples.[10,31]

Although the comparison of electrophoretograms showed that the plasma β and γ globulin fractions were higher than those in the serum specimens, this difference was only significant for γ globulin fraction ($d = 1.13$, $P = .00$). Higher levels of γ globulin in the plasma specimens might be related to the presence of fibrinogen, which is placed between the β and γ fractions. Since fibrinogen influences the correct separation and identification of β and γ globulin fractions,[31] it may have resulted in an overestimation of these fractions. Although fibrinogen in the present study was noted between the β and γ globulin fractions, some studies have found that fibrinogen generally migrates in the β fraction of the avian electrophoretogram.[32,33] The different fibrinogen migration patterns have been pointed out in various bird species, and it may be due to differences in the protein's structure and surface charge distributions.[34]

Measurement of albumin by reference methods (eg, electroimmunoassay electrophoresis, rocket immunoelectrophoresis) suggested that the results obtained by the CAE method were more reliable than those measured by the BCG method.[35] The more reliable CAE testing method could be due to BCG reaction with α, β, and γ globulins, which results in an overestimation of the albumin level.[35,36] A weak correlation has also been found between BCG albumin and CAE albumin in pigeons (*Columba livia*), chickens (*Gallus gallus*), ducks (*Anas platyrhynchos*), pheasants (*Phasianus colchicus*), and turkeys (*Meleagris gallopavo*).[37–39]

Similarly, the results of the present study showed that the BCG method overestimated albumin levels in both serum and plasma specimens, which were significantly higher than those measured by the CAE method ($P < .05$). There is a difference in the affinity of albumin binding to the BCG reagent in different avian species.[19] Therefore, the discrepancy between CAE albumin and BCG albumin in the present study may also be associated with the use of a non–species-specific testing standard (bovine) to calibrate the autoanalyzer. Previously, alterations in the albumin levels have been reported in birds when human and bovine testing standards were used to calibrate the autoanalyzer.[38,39] Since it is not possible to use a species-specific albumin standard for all avian species, it seems wise to use electrophoresis as a more reliable method than the BCG method to measure albumin.

The results of this study provide the reference protein values of total protein and protein fractions in common mynahs, which can be used to diagnose and monitor the diseases that affect this avian species. It is clear that the CAE diagnostic testing method has low resolution compared to modern electrophoresis techniques, such as agarose gel or capillary zone electrophoresis. Clinicians should be aware that these reference intervals should not be extrapolated with the results generated by agarose gel or capillary zone electrophoresis.

Due to the small size of common mynahs, collected blood is often placed in tubes containing heparin anticoagulant for hematological and biochemical diagnostic tests. The significant difference for total protein and gamma globulins between serum and plasma samples requires the use of plasma reference intervals for accurate interpretation of the results. As with other avian species, the BCG method is not reliable and protein electrophoresis provides a more accurate measure of albumin in common mynahs.

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