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Protein Electrophoresis of Serum from Healthy Atlantic Bottlenose Dolphins (*Tursiops truncatus*)

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Abstract

Serum protein electrophoresis (SPEP) has been recognized as an important tool in human and veterinary medicine. The present study investigated the use of SPEP in serum samples from healthy Atlantic bottlenose dolphins (*Tursiops truncatus*). Fraction delimitation was defined for the standardization of use by other laboratories. The imprecision of this method was comparable to reports in other species. A significant difference between albumin levels determined by SPEP and the traditional chemistry analyzer method (bromcresol green [BCG]) was observed (BCG = 3.38 ± 0.46 g/dL, and SPEP = 3.74 ± 0.43 g/dL, $p < 0.0001$). Bland-Altman analysis also showed that these two methods were not identical. Notably, several differences were observed between SPEP-derived values using samples from dolphins under human care vs free-ranging dolphins. The total protein was significantly increased in serum from free-ranging dolphins, and the A/G ratio was found to be significantly decreased (under human care: 1.91 ± 0.39 g/dL, free ranging: 1.07 ± 0.39 g/dL, $p \leq 0.05$). The latter change was related to a significantly lower albumin fraction and 2.3-fold increase in gamma globulins. In total, this study provides method standardization and preliminary data toward the generation of reference intervals for this species.

Key Words: serum protein electrophoresis, albumin, bromcresol green, bottlenose dolphin, *Tursiops truncatus*

Introduction

Serum protein electrophoresis (SPEP) has been a proven diagnostic method in human clinical pathology for more than 40 y (Cawley, 1969). Veterinary medicine has utilized this method for over 20 y, with the first applications in the study of serum protein changes in cases of myeloma, feline infectious peritonitis, and ehrlichiosis (Kaneko, 1997). The application of protein electrophoresis in avian species has been extensively studied and has demonstrated that this method can provide complementary information which can aid in diagnostics as well as provide valuable prognostic information (Reidardson & McBain, 1992; Cray et al., 2005, 2009).

Electrophoresis is a method that has grown from basic science investigations into a semi-automated tool to analyze serum proteins with good precision. It is now routinely available at most larger veterinary diagnostic laboratories. SPEP allows for the quantitation of albumin and several fractions of proteins referred to as globulins. These fractions are believed to reflect over 200 proteins which function as part of the acute phase response and are broadly classified as alpha, beta, and gamma globulins (Kaneko, 1997). Fraction migration is quite varied by species, which often makes fraction identification challenging (Kaneko, 1997; Cray et al., 2007; Eckersall, 2008; Gimenez et al., 2010). As SPEP becomes more readily used with animal species, it is important to generate reference intervals as well as to standardize fraction delimitation so that results may be more comparable among different laboratories.

Several studies have compared albumin quantitation by SPEP and by traditional methods routinely employed on automated chemistry analyzers (i.e., bromcresol green [BCG]) (Pemberton & DeJong, 1971; Spencer & Price, 1977; Gentry & Lumsden, 1978; Keay & Doxey, 1983; Evans & Parsons, 1988; Fox, 1989; Barber & Stanhope, 1992; David et al., 1995; Stokol et al., 2001; Muller & Brunnberg, 2009; Cray et al., 2011). Much of this data support disagreement between these methods in samples from several species and indicate there may be multiple etiologies for this disagreement. In many instances, albumin quantitation by SPEP is referred to as the gold standard (Pemberton & DeJong, 1971; Cray et al., 2007).

As a valued technique for clinical diagnostic and research studies, it is important to study the application of SPEP in wildlife species. The main objective of this study was to examine the use of SPEP with samples from bottlenose dolphins. This study used a multifold approach with a standard examination of imprecision with the institution of reproducible fraction delimits. Differences between methods for albumin quantitation were also addressed. Lastly, preliminary reference intervals for samples from dolphins under human care and free-ranging dolphins were generated.

Materials and Methods

Samples

Forty-four serum samples were obtained from two sources. Samples from animals under human care were obtained from the Georgia Aquarium facilities (n = 25, Atlanta, GA, and St. Augustine, FL) and were from individual animals with normal clinical history and physical examination. Routine diagnostic testing, including hematology and serum biochemistry, also showed no abnormalities. Within this group, there were 11 males (age range: 0.5 to 44 y) and 14 females (age range: 2 to 57 y). Samples from free-ranging animals were obtained from the Harbor Branch Oceanographic Institute at Florida Atlantic University (n = 19; Fort Pierce, FL) and collected as part of a health assessment study of dolphins in the Indian River Lagoon. These animals had a normal physical examination, and all routine bloodwork was also within normal limits (Goldstein et al., 2006). Within this group, there were 15 males (estimated age range: 5 to 18 y) and four females (estimated age range: 5 to 16 y). Serum samples were aliquoted and frozen at -80° C until transport to the University of Miami Avian & Wildlife Laboratory (Miami, FL) for analysis.

Protein Electrophoresis

Serum samples were analyzed according to the procedure provided by the *Helena SPIFE 3000* system with the use of Split Beta gels (Helena Laboratories, Inc., Beaumont, TX, USA). Results were produced after gel scanning and analysis by *Helena* software. Percentages for each fraction were determined by this software, and absolute values (g/dL) for each fraction were obtained by multiplying the percentage by the total protein concentration. The A/G ratio was calculated by dividing albumin by the sum of the globulin fractions.

Albumin Quantitation

Albumin was measured by the use of the bromcresol method utilized on the Ortho Vitros 250 automated analyzer (Ortho Clinical Diagnostics, Rochester, NY, USA). This analyzer is maintained by manufacturer's specifications, and quality control samples are run daily.

Precision Study

Within-run analysis was conducted using repeated analysis of a single sample (n = 8). The coefficient of variation (CV) was calculated using *GraphPad Prism 4*, Version 4.00 (GraphPad Software, Inc., La Jolla, CA, USA).

Statistical Analyses

Before analyzing the data, extreme outliers defined by a box and whiskers plot as values above the upper quartile + 3 × inner quartile range or below the lower quartile -3 × inner quartile range were eliminated. By this method, two values for the % alpha 1 globulin fraction were removed. The Kolmogorov-Smirnov test showed all the data were normally distributed. Comparisons between the two groups of dolphins were made with a t-test. Results are reported with mean, median, minimum-maximum, and standard deviation for each group and the two-sided p value of the test. Values of $p < 0.05$ were considered significant. A CV, paired t-test, Pearson correlation, Deming regression analysis, and a Bland-Altman plot were used to compare the differences between the albumin methods as previously recommended (Jensen & Kjelgaard-Hansen, 2006). *SAS* (statistical analysis software), Version 9.3 (SAS Institute Inc., Cary, NC, USA) and *GraphPad Prism 4*, Version 4.00, were used for all data analyses.

Results

Imprecision and Fraction Delimitation

The following coefficient of variation were calculated: albumin – 4.7%, alpha 1 – 15.5% , alpha 2 – 8.8%, beta – 6.3%, and gamma – 4.0%. Fraction delimitation used throughout the study is shown in Figure 1 with representative electrophoretograms from samples from dolphins under human care and free-ranging dolphins.

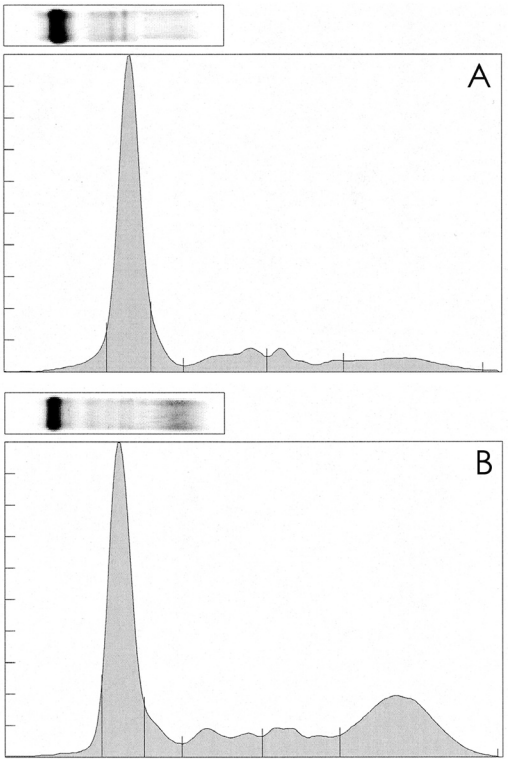

Figure 1. Representative electrophoretograms of serum samples obtained from dolphins under human care (A) and free ranging (B) are shown. Five fractions are represented from left to right: (1) albumin, (2) alpha 1, (3) alpha 2, (4) beta, and (5) gamma globulins.

Albumin Method Comparison

A significant correlation was seen between the BCG and SPEP measures of albumin ($r = 0.75$, $p < 0.0001$); however, a paired *t*-test found significant differences between the mean (± SD) of the two measures (BCG = 3.38 ± 0.46 g/dL, and SPEP = 3.74 ± 0.43 g/dL, $p < 0.0001$). Deming regression analysis indicated neither constant nor proportional error were present. To this point, the 95% CI for the intercept included 0 (-1.78 to 0.39), and the 95% CI for the slope included 1 (0.80 to 1.38). Similar results were obtained using Passing-Bablok regression (data not shown).

Bland-Altman analysis showed a bias, with the albumin determined by SPEP higher than that of the BCG method in nearly all the samples (Figure 2). Imprecision studies were conducted and found a CV of 1.6 and 4.7% for the analyzer and SPEP albumin, respectively. The combined inherent imprecision was 5.0%. In this figure, the two dotted lines represent the expected combined inherent CV based on the two methods—that is, if the two methodologies were identical, all data points should have occurred between these lines. However, as observed here, 22 of the 44 values (50%) were within the combined CV interval lines.

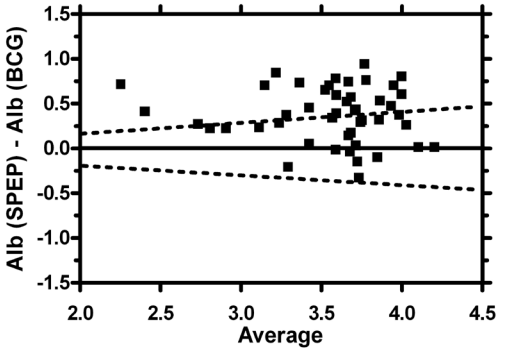

Figure 2. Bland-Altman plot of the difference between serum albumin (Alb) concentrations from dolphins as measured by bromcresol green (BCG) and serum protein electrophoresis (SPEP) methodologies. The dotted lines represent the outer limits of the inherent imprecision of both respective methods. All units are g/dL.

Comparison of SPEP Results from Free-Ranging Dolphins to Those Under Human Care

The total protein of samples from free-ranging dolphins was found to be significantly greater than that of dolphins under human care (Table 1). Serum from free-ranging dolphins was also found to have a significantly lower A/G ratio. This ratio was affected by a decreased albumin and a 2.3-fold increase in gamma globulins. In samples from free-ranging dolphins, mild yet significant increases in alpha 2 and beta globulins were also present.

Discussion

In the current study, the patterns of serum fractionation were examined for bottlenose dolphins and found to be comparable to that reported for other mammals with the ability to define five primary fractions: (1) albumin, (2) alpha 1, (3) alpha 2, (4) beta, and (5) gamma (Baker & Valli, 1986; Cray et al., 2007; Crivellente et al., 2008; Riond et al., 2009). Coefficient of variation data from imprecision studies were found to be good and comparable to previous reports in other species and manufacturer specifications (Cray et al., 2007; Eckersall, 2008). Of the fractions, a higher variation was observed with the alpha 1 and alpha 2 globulin fractions. In clinically normal dolphins, the concentration of alpha 1 globulins appears to be very low. As such, this makes it difficult to assign fraction delimits and results in a higher CV. As the ending (right-hand) fraction delimit of the alpha 1 fraction is subject to variation, this also affects the reproducibility of the alpha 2 globulin fraction. This type of fraction delimit variation has also been observed in the electrophoresis of avian

Table 1. Mean, median, standard deviation, and minimum-maximum are presented for protein electrophoresis values from serum samples from dolphins under human care and from free-ranging bottlenose dolphins. Sample size was $n = 25$ for captive and $n = 19$ for free ranging.

	Under human care		Free ranging	
Analyte	Mean ± SD (median)	Min-Max	Mean ± SD (median)	Min-Max
Total protein, g/dL	6.4 ± 0.8 (6.4)	5.2-8.2	7.2 ± 0.6 (7.2)[b]	6.0-8.4
A/G ratio	1.91 ± 0.39 (1.89)	1.29-3.07	1.07 ± 0.39 (0.99)[b]	0.79-1.51
Albumin, g/dL	3.92 ± 0.52 (4.00)	2.77-5.19	3.44 ± 0.31 (3.43)[b]	2.78-4.11
Albumin, %	61.28 ± 4.39 (62.50)	52.20-71.80	48.27 ± 4.37 (47.70)[b]	41.80-55.90
Alpha 1, g/dL	0.35 ± 0.10 (0.32)	0.24-0.63	0.33 ± 0.06 (0.31)	0.23-0.45
Alpha 1, %	5.20 ± 1.30 (5.10)	3.90-9.10	4.57 ± 0.77 (4.50)	3.40-5.70
Alpha 2, g/dL	0.52 ± 0.11 (0.50)	0.34-0.77	0.60 ± 0.10 (0.60)[b]	0.45-0.80
Alpha 2, %	8.20 ± 1.71 (8.30)[a]	4.20-13.00	8.43 ± 1.46 (8.30)	5.80-11.90
Beta, g/dL	0.54 ± 0.16 (0.50)	0.23-1.10	0.65 ± 0.13 (0.68)[b]	0.38-0.91
Beta, %	8.30 ± 1.78 (8.00)	3.70-13.40	9.04 ± 1.58 (9.10)	6.10-12.60
Gamma, g/dL	0.83 ± 0.26 (0.86)	0.19-1.40	1.93 ± 0.43 (1.89)[b]	1.32-3.17
Gamma, %	12.83 ± 3.58 (13.45)	3.10-20.60	26.78 ± 4.22 (26.70)[b]	21.30-37.70

[a] Sample size: $n = 23$

[b] Values obtained from captive dolphin samples are significantly different than those from free-ranging dolphin samples, $p \leq 0.05$.

samples (Cray et al., 2007). To aid in the standardization of SPEP for dolphin samples among different laboratories, representative electrophoretograms have been presented in Figure 1.

A secondary objective of this study was to compare the quantitation of albumin by SPEP vs traditional and often more routinely utilized methods of albumin quantitation by an automated chemistry analyzer. Methods used to quantitate albumin in human samples have included salt fractionation, immunochemistry, SPEP, and the relatively more recent development of nonspecific dyes such as BCG (Hill, 1985). BCG is found nearly on all automated chemistry analyzers and provides highly reproducible and rapid results. These two characteristics continue to make it the method of choice in clinical pathology even though SPEP is considered the gold standard, and other dyes such as bromcresol purple have been purported to be a better option (Pinnell & Northam, 1978). In humans and other species, BCG has been shown to react with globulins, and the use of heparin has been shown to affect albumin quantitation (Pinnell & Northam, 1978; Hill, 1985; Hallbach et al., 1991; David et al., 1995; Stokol et al., 2001; Cray et al., 2011). Both of these issues result in the overestimation of albumin by BCG. In the present study, with the use of samples from clinically healthy dolphins and the examination of serum (rather than heparinized plasma), the BCG method underestimated albumin concentration. This difference was significant, and Bland Altman analysis indicated that the SPEP and BCG methods are not identical. Our findings may be biased by the use of samples from normal animals. In previous reports in humans and birds where samples from diseased patients were used, a very poor agreement between methods was demonstrated (Slater et al., 1975; Cray et al., 2011). As the present study utilized serum rather than plasma, fibrinogen did not have a role in this difference.

Brief comparisons between albumin methodologies in other species have been conducted, including in manatees (Harvey et al., 2007). In this report, the BCG method resulted in a mean albumin value of 5.1 g/dL whereas SPEP resulted in a mean value of 3.7 g/dL. A previous report using samples from clinically healthy free-ranging dolphins also indicated an apparent higher albumin value by BCG methodology, although a complete statistical analysis of this finding was not presented (Goldstein et al., 2006). A mean value of 4.5 g/dL was found by BCG vs 3.8 g/dL by SPEP. In review of the previous and current study methods, different chemistry analyzers were employed as well as different implementations of the Helena electrophoresis system. Given the discordance between the current results and the previous report in dolphins in which samples were subject to the same preanalytical handling, it is possible that albumin quantitation in this species may be influenced by the automated chemistry analyzer used thereby presenting differences in calibration, reagents, and reaction times. The underestimation of albumin by BCG in the current study may also reflect the binding efficiency of BCG to dolphin albumin. Variable binding ability of this dye has been reported in other species (Pemberton & DeJong, 1971; Spencer & Price, 1977; Evans & Parsons, 1988). Given the current and cumulative data, repeated measures of albumin for clinical or research investigations should be conducted using consistent methods and analyzers; and as methods are changed at the laboratory level, validation studies should occur to ensure continuity of albumin results with samples from nonhuman species.

An interesting finding of the present study was the significantly higher gamma globulin and significantly

lower albumin levels in samples from free-ranging dolphins compared to those under human care. These values affected the A/G ratio, and mild increases in other globulins were also present. These changes are consistent with those reported previously for clinically healthy free-ranging dolphins (Goldstein et al., 2006). It could be proposed that free-ranging dolphins come in contact with greater antigenic exposure, including a wide assortment of infectious agents which may then be reflected in a higher resting level of gamma globulins and a low albumin level (as a negative acute phase protein). To this point, higher levels of gamma globulins had previously been reported in anecdotal fashion (Bossart et al., 2001). More specifically, increased total globulins were reported in dolphins with orogenital papillomas (Bossart et al., 2008). Lower albumin and increased globulins were also observed in dolphins which were seropositive for cetacean morbillivirus (Bossart et al., 2011). Alternatively, although the animals appeared clinically healthy, mild underlying inflammatory or infectious processes may have been present which were not evident in the routine test results. While a lower albumin and A/G ratio was observed in free-ranging vs captive manatees, the pronounced increase in gamma globulins was not reported (Harvey et al., 2007).

It has been observed that clinical signs of ill health are often masked in dolphins; thus, diagnostic testing inclusive of traditional hematologic and biochemistry testing has been important in academic investigations and clinical care (Bossart et al., 2001; McBain, 2001). SPEP has been found to have diagnostic and prognostic value in a variety of species. In the current study, the notable differences between dolphins which were under human care and free ranging also demonstrate the potential utility of this method in studies of this species. Preliminary reference intervals and method standardization provided here should aid in the management of dolphins under human care and the design and interpretation of future studies of free-ranging populations. Future studies using the methods defined in the current study should provide more fully vetted reference intervals, including addressing possible effects of age, sex, and husbandry as well as the effects of environmental factors and infection.

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