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Affinity of Staphylococcal A and Streptococcal G Proteins to West Indian Manatee (*Trichechus manatus manatus*) Immunoglobulins

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ABSTRACT: The West Indian manatee (*Trichechus manatus manatus*), a subspecies that inhabits coastal areas of Central and South America, has been listed as a vulnerable species because of the rapid decline in its population. Commercially available immunologic reagents specific for sirenians are lacking, limiting the development of sensitive immunodiagnostic assays. We observed the affinity of the microbial proteins A and G to *T. m. manatus* immunoglobulins. Manatee serum pools were analyzed using enzyme-linked immunosorbent assay (ELISA) to determine the affinity intensity followed by western blotting to confirm the specific binding of proteins A and G to immunoglobulins. The ELISA demonstrated maximum affinity of both proteins until the serum dilution of 1:12,800, with a similar affinity for both proteins. Because both A and G proteins exhibited affinity to manatee immunoglobulins, they can be used to develop sensitive immunodiagnostic assays for this species, contributing to manatee conversation procedures.

Key words: Antibodies, ELISA, immunodiagnosis, manatees, sirenians.

The manatees are the only herbivorous and exclusively aquatic mammals of the order Sirenia, and the West Indian manatee (*Trichechus manatus manatus*) is a subspecies that can be found in the coastal waters and the inlets of Central America, South America, and the Caribbean (Vianna et al. 2006). Due to the constant decline in populations, the International Union for Conservation of Nature (2017) has classified this species as vulnerable.

Because of the limited availability of diagnostic assays and specific reagents for these species, there is little information available about the influence and impact of diseases occurring in sirenian populations (Marmontel et al. 1997). Unexpected changes occurring in the health of the manatees are commonly a consequence of environmental disorders, based on which these animals have been considered as bioindicators of the surrounding ecosystem (Bossart 2011).

Staphylococcus aureus surface protein A (PrtA) has been implicated in several mechanisms of host immune response evasion, preventing bacterial immune recognition and the consequent effector functions (Falugi et al. 2013). Protein A is composed of five subunits, with each subunit consisting of an immunoglobulin-binding site (Atkins et al. 2008). Protein G (PrtG) is also a cell surface component identified in *Streptococcus* groups C and G (Björck and Kronvall 1984). Both PrtA and PrtG have high binding affinity to the Fc fragment of immunoglobulin G from numerous mammalian species (Akerström and Björck 1986). These proteins have been used as an alternative to the limited commercial availability of specific anti-antibodies for several wild animals as reagents in enzyme-linked immunosorbent assay (ELISA) and western blotting, and the affinity of immunoglobulins of different mammals to these proteins has been reported to be variable (Pelli et al. 2012).

Given the lack of information regarding the affinity of PrtA and PrtG to sirenian immunoglobulins, and the need for more-sensitive and specific immunodiagnostic assays for research and conservation of this species, we analyzed the reactivity of horseradish peroxidase (HRP)-conjugated PrtA and PrtG to *T. m. manatus* antibodies. The manatees that we used were from preservation units located in northeast Brazil. Nine manatees of both sexes were used, aged from 3 yr or older, and identified as healthy by a clinical evaluation

performed by veterinarians who specialized in wild animals. Blood samples were collected from these nine manatees from the interosseous space of the radius and ulna, centrifuged for 10 min at 3,000 × G, and sera were separated and stored at –20 C until use. A serum pool prepared with these samples was used in this study. The collection of the blood samples was approved by the Service of Authorization and Information on Biodiversity of the Brazilian Federal Government, protocol number 55433-1/43406.

The protein concentration of the serum pool was measured using a commercial kit based on the bicinconinic acid methodology (ThermoFisher Scientific®, Waltham, Massachusetts, USA). Pool volumes containing 50 µg/mL of protein were run on a 10% polyacrylamide gel electrophoresis system. After the electrophoretic run, the samples were transferred to nitrocellulose membranes which were blocked with 5% casein in phosphate-buffered saline (PBS; pH 7.4) overnight at 4 C. After two washes with PBS containing 0.05% Tween® 20 (PBST), the membranes were incubated with HRP-conjugated PrtA or PrtG (Sigma-Aldrich®, St. Louis, Missouri, USA), diluted 1:500 in PBS, and incubated for 1 h at 37 C. After four washes with PBST, the enzymatic reaction was developed using a solution of hydrogen peroxide and 4-chloro-1-naphthol. A blank control consisting of the electrophoretic-separated sample without incubation with the conjugate was also used.

The affinity of PrtA and PrtG to the manatee immunoglobulins was evaluated using ELISA as described by Pelli et al. (2012) with some modifications. Briefly, volumes of 100 µL/well of pooled sera diluted from 1:50 to 1:13,107,200 in bicarbonate-carbonate buffer (pH 9.6) were used to sensitize polystyrene 96-well plates (Costar®, Corning, New York, USA) in triplicates for 14 h at 4 C. After two washes with PBST, the plates were blocked with 5% casein in PBS (pH 7.4) for 3 h at 37 C, followed by four washes with PBST. Then, HRP-conjugated PrtA or PrtG at 2.5 µg/mL diluted in PBS containing 1% casein was added to the wells and incubated for 1 h at 37 C. The plates were then washed six times with PBST, and the reaction was developed using a solution of o-phenylenediamine and hydrogen peroxide. The reaction was stopped using 1 N sulfuric acid solution, and then the absorbance was read at 450 nm in a microplate reader (BIO-RAD®, Hercules, California). Serum pools of dogs (*Canis lupus familiaris*) and Blue-and-yellow Macaws (*Ara ararauna*) were respectively used as positive and negative controls at the same dilutions as used for manatee samples. These serum samples were obtained from the serum bank of the Laboratory of Veterinary Infectiology (Salvador, Brazil). These control serum samples were selected based on previous studies that reported the affinity of both PrtA and PrtG to dog immunoglobulins (Ferreira et al. 2013) and the absence of affinity of these bacterial proteins to avian immunoglobulins (Higgins et al. 1995). The affinity results were classified according to the dilution that presented the maximum optical density (OD) readings: low (1:50–1:250), medium (1:1,250–1:156,250), and high (1:781,250–1:3,906,250) as described by Pelli et al. (2012).

The ELISA results were analyzed by the Shapiro-Wilk statistical test to confirm whether the affinity data presented normal distribution. In the absence of normal distribution, the data were analyzed by the Wilcoxon signed-rank test to compare the PrtA and PrtG affinity curves. The curves were considered to be significantly different when $P<0.05$.

The western blotting results showed same-intensity single bands when the serum sample pool was incubated with HRP-conjugated PrtA or PrtG (Fig. 1) with a molecular weight of approximately 150 kDa, which has been described for the immunoglobulin G of other mammals (Pelli et al. 2012). No other band was observed, which indicated that the binding of the two bacterial proteins was specific.

The ELISA readings of the serum pool dilutions incubated with both the HRP-conjugated bacterial proteins (Fig. 2) exhibited a maximum OD up to the dilution of 1:12,800. Both PrtG and PrtA have a medium

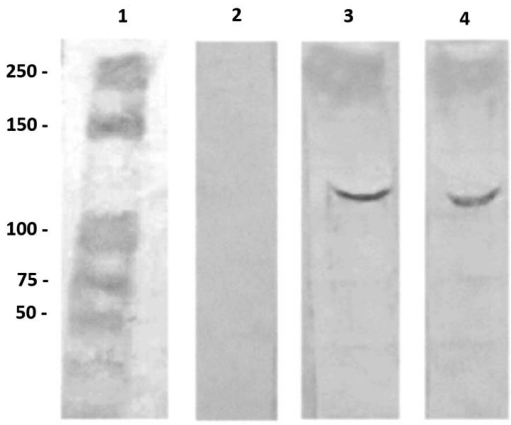

FIGURE 1. Western blot analysis of the horseradish peroxidase (HRP)-conjugated staphylococcal A (PrtA) and streptococcal G (PrtG) proteins binding to West Indian manatee (*Trichechus manatus manatus*) immunoglobulins. A manatee serum pool was subjected to a reducing polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and incubated with HRP-conjugated PrtA (Lane 3) or HRP-conjugated PrtG (Lane 4). Lane 1 represented the molecular weight standard profile, and Lane 2 represented the blank control with no conjugate incubation. Numbers at the left represent the molecular weight in kDa of each standard band.

affinity to the immunoglobulins of *T. m. manatus* (Pelli et al. 2012). Both curves reached a zero reactivity at a dilution of 1:405,600. No statistically significant difference was observed between the two affinity curves when compared using the Wilcoxon signed-rank test (P=0.754). The assay using dog immunoglobulins revealed a maximum reactivity to the 1:25,600 dilution when PrtG was used whereas PrtA presented a maximum OD value to the 1:51,200 dilution, with manatee and dog PrtA affinity curves presenting a significant statistical difference (P=0.030); the avian serum sample pool did not exhibit any reaction in the ELISA.

The affinity of PrtA and PrtG to *T. m. manatus* antibodies that we demonstrated should be considered as a first step toward the improvement of the immunologic knowledge of this species. A routine evaluation of infections and the monitoring of emerging diseases in the manatee populations are essential, considering the vulnerability of these animals and their role as bioindicators (Sulzner et al. 2012). Therefore, owing to the scarcity of specific commercially available kits

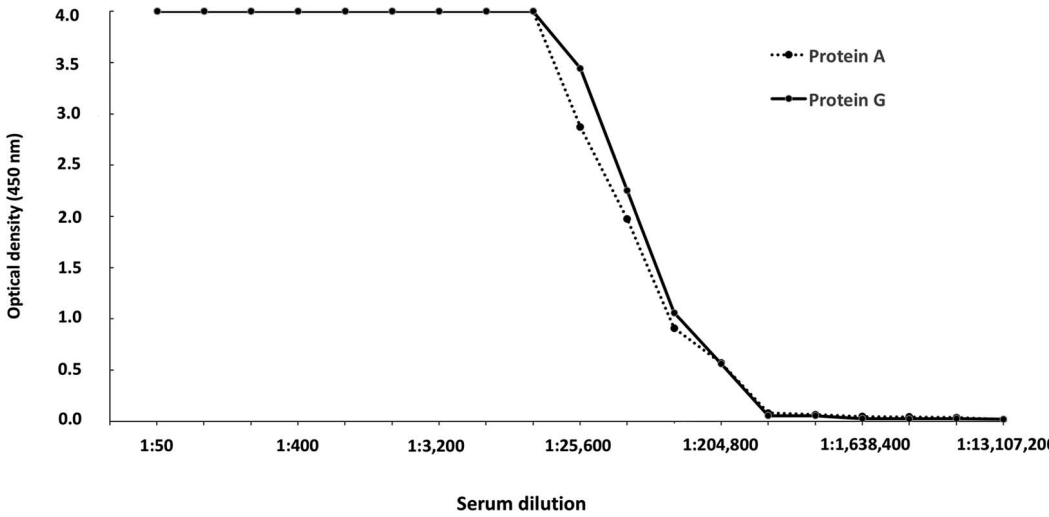

FIGURE 2. Affinity of staphylococcal A (PrtA) and streptococcal G (PrtG) proteins to West Indian manatee (*Trichechus manatus manatus*) immunoglobulins as defined by enzyme-linked immunosorbent assay (ELISA) with serial dilutions of *T. m. manatus* serum pool. The ELISA plates were sensitized with serial dilutions of pooled sera and incubated with horseradish peroxidase-conjugated PrtA or PrtG. The results are expressed as mean values of optical density of each dilution tested in triplicate in two independent experiments. The curves were statistically compared using the Wilcoxon signed-rank test, and no statistical difference was observed between the affinity curves (P<0.754).

and reagents for these animals, there is a need for developing diagnostic methods with high accuracy (Harr et al. 2006). The present study demonstrated a similar pattern of affinity of PrtA and PrtG to manatee immunoglobulins. Other studies have also demonstrated that these proteins have equivalent patterns of affinity to the immunoglobulins of wild animals such as punares (*Thrichomys apereoides*) and opossums (*Didelphis albiventris*) or nonhuman primates (Shearer et al. 1999; Pelli et al. 2012).

The immunologic assays used for diagnosing diseases in manatees, such as agglutination (Sulzner et al. 2012; Attademo et al. 2016), currently are not based on species-specific immunologic reagents. Such methods are characterized by a low sensitivity, which can consequently lead to a significant number of false-negative results. Our study results could contribute to the development of more-accurate and sensitive assays, as both PrtA and PrtG can be conjugated to enzymes, fluorophores, chemiluminescent substances, and radioisotopes.

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