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ARTICLE

The Use of Bacteriophages and Immunological Monitoring for the Treatment of a Case of Chronic Septicemic Cutaneous Ulcerative Disease in a Loggerhead Sea Turtle *Caretta caretta*

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Abstract

In this case study, phage therapy was applied to treat a multidrug-resistant case of septicemic cutaneous ulcerative disease (SCUD) caused by *Citrobacter freundii* in a loggerhead sea turtle *Caretta caretta*. Phages were applied topically, intravenously, into the carapace, and into the exhibit water using various phage cocktails specific to the causative agent over an 8-month period. This was performed in conjunction with antimicrobial therapy. The animal was monitored through weekly cultures, photographs, and complete blood cell counts, as well as immune assays (phagocytosis, plasma lysozyme and superoxide dismutase activity, and plasma electrophoresis profiles). The animal, in comparison to an untreated, unaffected control, had elevated antibody titers to the administered phages, which persisted for at least 35 weeks. Although cultures were clear of *C. freundii* after phage treatment, the infection did return over time and immune assays confirmed deficiencies when compared to a healthy loggerhead sea turtle. Immune parameters with statistically significant changes over the study period included the following: decreased phagocytosis, increased alpha- and gamma-globulin protein components, and an increased albumin : globulin ratio. When *C. freundii* appeared again, the multidrug-resistant status had reverted back to normal susceptibility patterns. Although not completely known whether it was another subspecies of bacteria, the therapy did resolve the multidrug-resistant challenge. Phage therapy in combination with antimicrobial agents may be an effective treatment for sea turtles with normally functioning immune systems or less-severe infections. Additional research is needed to better understand and quantify sea turtle immunology.

We present a case study utilizing bacteriophage therapy and immunological monitoring of a 42-year-old female loggerhead sea turtle *Caretta caretta*. The turtle was diagnosed with septicemic cutaneous ulcerative disease (SCUD) in 2014. Septicemic cutaneous ulcerative disease is a shell disease of aquatic turtles and is commonly caused by *Citrobacter freundii*; however, various other bacteria have been shown to cause SCUD (Henrickson 1972; Köbölkuti et al. 2008; Hossain et al. 2017). This disease can cause ulcerated lesions on the plastron and/or carapace, emaciation, lethargy, petechial hemorrhages, and death (Mitchell and Tully 2016).

Bacteriophages or "phages" are viruses that infect bacteria. They are ubiquitous, highly specific to their bacterial host, and the most abundant living entities on the planet (Bergh et al 1989; Wommack and Colwell 2000; Brussow and Hendrix 2002; Wilhelm et al. 2002; Hendrix 2003; Hambly and Suttle 2005; Suttle 2005; Doss et al. 2017). Phages were discovered in the early 1900s by Félix d'Hérrelle and Fredrick Twort, who immediately recognized their potential use as antimicrobials due to their ability to lyse and kill their bacterial hosts. The first phage therapy was performed by d'Hérrelle in France, utilizing many of his first phage isolates to successfully treat children with severe dysentery (Kutter 2008; Pirnay et al. 2011). Although widely used during the 20th century in Europe, phage therapy was largely abandoned in the United States with the development of antibiotics, but it has gained recent interest due to the emergence of antibiotic-resistant pathogens.

Other advantages of the use of phages are that they are relatively easy to isolate, they kill bacteria very effectively, and they exhibit high specificity against one bacterial strain or closely related strains (Nilsson 2014). Thus, they might not disrupt the normal bacterial flora when applied therapeutically, thereby reducing the risk of secondary infections and problems often associated with antibiotic treatment (Sulakvelidze and Kutter 2005; Doss et al. 2017). Antibiotics commonly are excreted and/or degraded and require consistent dosing, which can be challenging in species such as sea turtles, whereas phages continue to multiply as long as the specific host is present and they are able to go beyond vascular transport mechanisms to other areas within the body.

The case highlighted in this study was a candidate for phage therapy for several reasons. The animal had a severe chronic infection of the shell, with multifocal avascular niduses, and after 4 years of various treatment attempts, the causative agent, *C. freundii*, became multidrug resistant. Given that phage therapy has been used to treat multidrug-resistant infections in humans (Gordillo and Barr 2019; Kortright et al. 2019; Rehman et al 2019; Saha and Mukherjee 2019), the therapy was implemented in the present case along with standard antimicrobial treatment and immunological monitoring.

METHODS

Animal Husbandry

Prior to the initiation of phage therapy, the affected turtle was housed with another 42-year-old female loggerhead sea turtle who never developed clinical signs. The two turtles had been housed together for 24 years. Once the *C. freundii* became multidrug resistant, the affected turtle was moved to a 7,571-L (2,000-gal) circular tank with a closed system and sand filtration. Seawater (~34‰) was disinfected by ozonation prior to

use and was maintained at a constant temperature of 27°C.

When phages were used in the tank water, the exhibit was treated with sodium thiosulfate pentahydrate and circulated to ensure that all chlorine had been removed prior to adding phages. Once phages were added, the water was left for 24 h with the phages before dumping the entire tank contents into a separate holding tank and performing a 100% water change on the animal's exhibit. The isolated phage water was then treated with hydrogen peroxide at a concentration of 7.5 mg/L prior to disposal.

Sample Collection

The sea turtle was monitored daily by animal care staff, and physical exams were performed weekly by veterinary staff to monitor the animal's health and progress. Samples for in-house blood analysis, cultures, and immune assays were collected at varying intervals ranging from weekly to monthly over the course of the 8 months of treatment.

Blood was collected through the cervical sinus while the animal was manually restrained, and the blood was immediately transferred to lithium heparin tubes for in-house blood analysis (complete blood cell counts and chemistry) and immune assays. Cultures were collected from superficial and deep tissue using minimal debridement after cleaning the affected area with betadine scrub, allowing a minimum contact time of 5 min, and then flushing with sterile saline. The carapace was divided into four quadrants to help track progress: (1) cranial left, (2) cranial right, (3) caudal right, and (4) caudal left (Figure 1B). Photographs were taken weekly and consisted of a full dorsal view of the carapace as well as each of the four quadrants.

Culture Processing

Culture swabs were streaked onto plated media: tryptic soy agar with sheep blood agar (Thermo Fisher Scientific, Remel Products, Lenexa, Kansas), MacConkey agar (Remel Products), Columbia colistin nalidixic acid agar with 5% sheep blood (Remel Products), and thioglycollate broth (Remel Products). Bacterial colonies were subcultured for purity, and all organisms were identified using the same methodology. Identification was performed by two methods: (1) API 20E Strips BioMérieux manual panel with the APIWEB database (BioMérieux, Inc., Durham, North Carolina), and (2) Vitek 2 Automated Identification System using the Vitek 2 GN ID Card with Vitek 2 database software (BioMérieux). Susceptibility testing was performed via two methods: (1) the Kirby–Bauer method using Mueller–Hinton agar (Remel Products) and Becton Dickinson Laboratories antibiotic discs, and (2) Vitek 2 Automated Susceptibility testing using the Vitek 2 AST card (BioMérieux) for gram-negative organisms.

The organism *C. freundii* was identified to a probability greater than 95% by using these methods. Upon identification, *C. freundii* was subcultured to tryptic soy agar slants and incubated at 35°C for 18–24 h to confirm growth. Slants were packaged according to UN3373 (category B; PHMSA DOT 2006) guidelines (Transporting Infectious

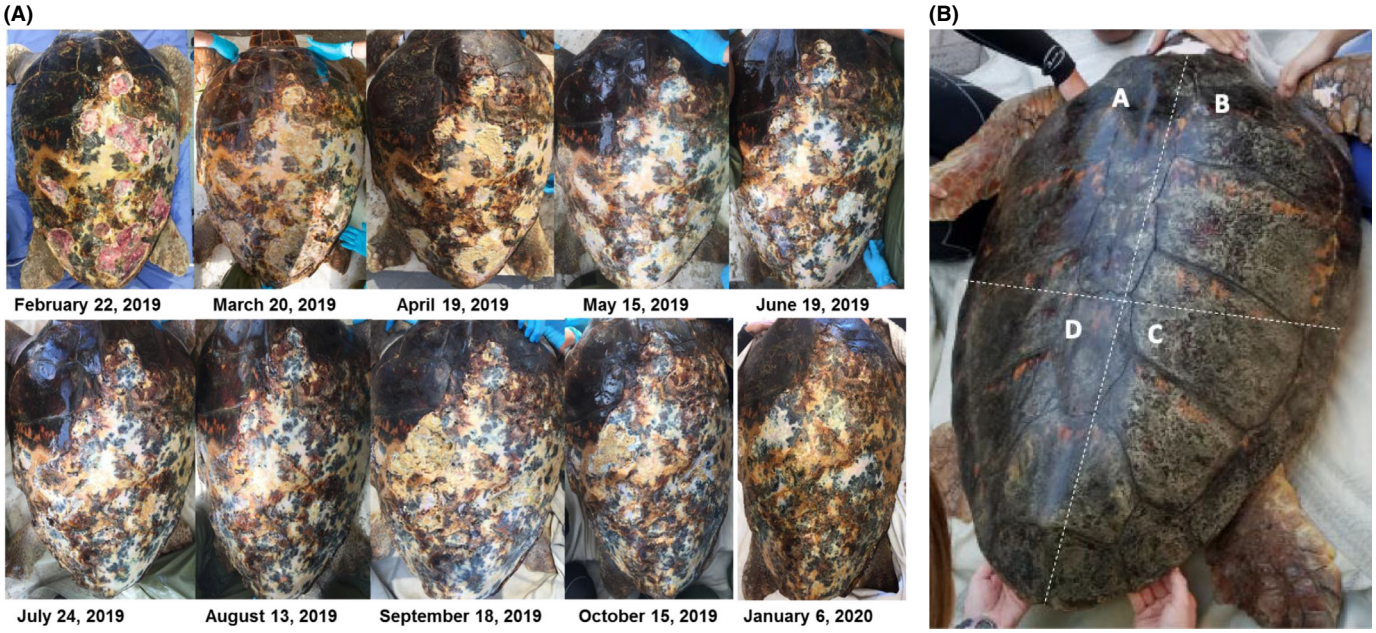

FIGURE 1. **(A)** Carapace healing of the affected loggerhead sea turtle with bacteriophage therapy over time and **(B)** unaffected (control) turtle carapace with markers indicating individual quadrants (A = cranial left; B = cranial right; C = caudal right; D = caudal left). [Color figure can viewed at afsjournals.org.]

Substances Safely, United States Department of Transportation, Pipeline and Hazardous Materials Safety Administration, Federal Register 2006.) and were shipped overnight to the collaborating phage laboratories for bacteriophage development. Cultures were collected after each round of phage administration to track and monitor the presence of *C. freundii* and the respective susceptibility pattern, if found.

Phage Development (Intravenous)

Phage isolation.—Raw sewage (1 mL) collected from multiple locations throughout the United States was incubated with *C. freundii* bacteria (0.5-mL overnight culture in 4 mL of fresh lysogeny broth) for 48 h at 37°C and then centrifuged (>5,000 rotations/min for 20 min) to pellet bacterial debris. The resulting lysate was then used to infect fresh *C. freundii* bacteria and was plated in lysogeny broth top agar. A resulting plaque from each plate was chosen and used to infect fresh bacteria overnight. This plaque purification was repeated three times. The final product was amplified to high titer by using the final lysate from plaque purification to create a lysate from fresh bacteria; it was then concentrated and pooled to create a cocktail of multiple phages at a concentration of 10^{12} PFU/mL. Phages were diluted with phosphate-buffered saline for injection.

Application.—Three milliliters of phage were administered intravenously through the cervical sinus, followed by 20 mL of sterile water to flush the line and ensure that all of the phages were successfully administered over a 5-min period during treatments. The animal was then monitored for a minimum of 30 min before being returned to the tank.

Phage Development (Topical)

Phages were isolated by enrichment from wastewater collected during 2018 in New Haven, Connecticut. Enrichment consisted of combining 6 mL of filtered wastewater (0.22 μm pore size to remove solids and bacteria) with 2 mL of concentrated lysogeny broth (4×) and 10^8 CFU of *C. freundii* and incubating in a shaker incubator at 37°C overnight. The following day, enrichments were centrifuged to pellet bacteria and filtered through 0.22-μm filters. This filtered lysate was then combined in top layer agar with *C. freundii* (10^9 CFU) and incubated overnight. Resulting plaques were purified three times to ensure clonal isolates of phage. Next, phages were amplified to high titer (i.e., $>10^{10}$ PFU/mL) and concentrated via centrifugation through small pore concentrators (Amicon Ultra; Sigma Aldrich, St. Louis, Missouri). The resulting concentrated phages were pooled to create a cocktail with a concentration of 10^{12} PFU/mL. For topical application, the phage cocktail was diluted in phosphate-buffered saline with 10-mM $MgSO_4$ to 10^{10} PFU/mL. Samples were stored at the original concentration at 4°C and were diluted prior to clinical application.

Treatment Administration

Phage cocktails were applied topically, intravenously, directly into the carapace, and at a high concentration directly into exhibit water, but not all methods were used for every treatment. Phage therapy was used 12 times over the course of 8 months on this case, with topical and intravenous treatments used the most frequently. Phage treatment was delivered at weeks 1 (February 22 and 25), 9 (April 19), 10 (April 24), 11 (May 2), 12 (May 9 and 10), 17 (June 13), 18 (June 19), 27 (August 23), 28 (August 27), and 30 (September 11).

Topical administration occurred during anesthetized procedures as well as tank-side, with the animal supported on foam mats and manually restrained by animal care staff. The first phage application occurred under anesthesia, and a drill was used to trephine 25.4-mm deep × 3-mm wide holes throughout the entire carapace on mostly unaffected tissue. The phage cocktail was injected into these holes by using a syringe with an 18-gauge, 38.1-mm (1.5-in) needle (Figure 2). Additional topical applications involved (1) injecting the phage cocktail using a syringe into deep tissue pockets during anesthetized debridement and (2) using an aerosolized sprayer and coating the carapace diffusely. For all topical treatments, a minimum of 20 min of contact time was allotted. Petroleum jelly was applied to increase residual contact time when the animal was returned to the water.

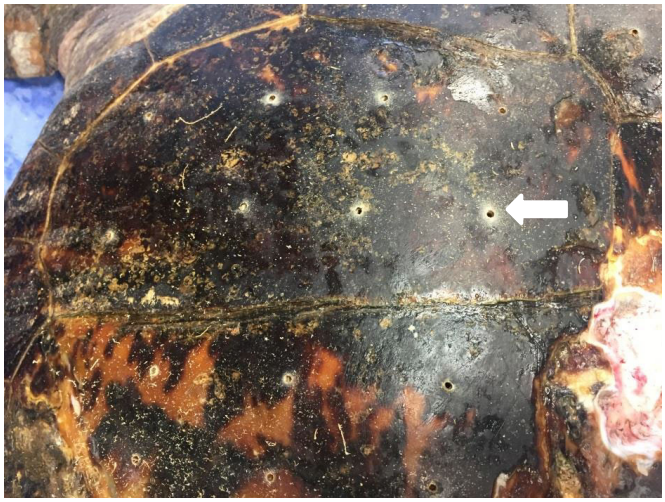

FIGURE 2. Holes trephined into the carapace of the affected loggerhead sea turtle for topical bacteriophage administration (white arrow indicates a single hole). [Color figure can viewed at afsjournals.org.]

Based on culture susceptibility results over the course of treatment, phage treatment was supplemented with 50-mg/kg piperacillin/tazobactam subcutaneous SID (once a day), 10-mg/kg ciprofloxacin PO (oral administration) q48 h (every 48 hours), and 5.4-mg/kg clindamycin PO SID at various times throughout the treatment period. Additionally, levamisole was prescribed at 2.5 mg/kg PO over the long term for immune stimulation.

Clinical Hematology

Blood (6–12 mL) was collected from the cervical sinus and preserved in lithium heparin. Blood films were made and stained using Dif Quik (Siemens, Munich, Germany), and centrifugally spun hematocrit was obtained using a hematocrit tube from the collected sample immediately after collection. Manual white blood cell counts were obtained using Leukopet WBC (Vetlab Supply, Palmetto Bay, Florida). Differentials were calculated after preparing the solution by mixing the collected blood sample with the stain and allowing the mixture to sit for the required amount of time. Heterophils and eosinophils were counted in both chambers of a Neubauer hemacytometer under the 10× objective to determine total leukocyte count. A chemistry panel was run on an Abaxis VetScan VS2 chemistry analyzer (Abaxis, Union City, California) within 1 h after collection using an Avian/Reptilian Profile Plus cartridge (Covetrus, Portland, Maine).

Immune Assays

Phagocytic activity.— Phagocytic activity in peripheral blood leukocytes was determined using a flow cytometry assay kit (pHrodo *Escherichia coli* BioParticles Phagocytosis Kit for Flow Cytometry; Thermo Fisher Scientific, Waltham, Massachusetts) that uses whole blood and determines phagocytic activity based on changes in intracellular pH. Cells were gated on granulocytic populations, and the percentage of phagocytic activity in granulocytic cells was determined.

Plasma lysozyme activity.— Plasma lysozyme activity was measured using a turbidity assay as previously performed with turtle plasma (Walsh et al. 2010, 2019). Hen egg white lysozyme (HEL; Sigma Aldrich; 1-mg/mL stock) was prepared in 0.1-M phosphate buffer (pH 5.9). A standard curve of HEL in concentrations ranging from 0 to 40 μg/mL was prepared by serial dilution. Concentration standards (25 μL/well) were added to three wells, while the fourth well containing plasma received 175 μL of phosphate buffer to serve as a blank. Absorbance (450 nm) was measured using a microplate reader (Synergy H1; BioTek, Winooski, Vermont). Readings were measured at time zero ($T = 0$) and after 10 min ($T = 10$). Absorbance unit (AU) values at $T = 10$ were subtracted from AU values at $T = 0$ to determine the absorbance change. The AU value for the blank sample well was subtracted from the average of triplicate sample wells to compensate for sample hemolysis when present. The resulting AU value was converted to an HEL concentration (μg/mL) by using linear regression of the standard curve.

Plasma protein electrophoresis profiles.— Plasma protein electrophoresis profiles were determined using the QuickGel SPE System (Helena Laboratories, Beaumont, Texas) with agarose (QuickGel Split Beta SPE) gels (Helena Laboratories) stained with acid blue stain (0.5%). Relative density of bands on gels was determined using a gel imager (BioRad ChemiDoc XRS+; BioRad, Hercules, California), and plasma protein profiles were interpreted based on established profiles in loggerhead sea turtles (Gicking et al. 2004). Total solids of plasma were determined using a clinical refractometer (Model S2020; Allied Healthcare, St. Louis, Missouri) in order to estimate total protein.

Superoxide dismutase.— Oxidative stress in plasma was measured by determining the activity of superoxide dismutase (SOD; enzyme number 1.15.1.1, IUBMB 1992) using a commercially available microplate assay (SOD Activity Assay Kit; Cayman Chemical Company, Ann Arbor, Michigan). This assay utilizes a tetrazolium salt for detection of superoxide radicals generated by xanthine oxidase (1.17.3.2) and hypoxanthine. A standard curve was used to determine units of SOD activity per milliliter of plasma (U/mL), and absorbance was measured at 450 nm (Synergy H1; BioTek).

Lymphocyte proliferation.— Cell-mediated immunity was measured by assessing lymphocyte proliferation in response to the T-cell mitogen concanavalin A. Peripheral blood leukocytes isolated from whole blood using Ficoll–Paque (1.077 g/dL) were enumerated using trypan blue to determine viability, and cells were adjusted to a final concentration of 1×10^6 cells/mL. Cells were cultured in RPMI-1640 medium (Thermo Fisher Scientific) supplemented with 10% heat-inactivated fetal bovine serum (FBS), with antibiotics (penicillin, 5,000 IU/mL; streptomycin, 50 IU/mL) and an antifungal (0.1% amphotericin B). Concanavalin A (Sigma Aldrich) was used at 5 μg/mL to initiate lymphocyte proliferation in triplicate wells. Cultures were incubated at 29°C in a humidified, 5% CO_2 atmosphere. To measure proliferation, a thymidine analog, bromodeoxyuridine, was added after 96 h of culture, with cells harvested after an additional 18 h of incubation. Cell proliferation was measured using a bromodeoxyuridine enzyme-linked immunosorbent assay (ELISA; Sigma Aldrich). Stimulation indices were calculated by comparing the absorbance of mitogen-stimulated wells to the absorbance of unstimulated controls.

Enzyme-linked immunosorbent assay phage preparation.— Phages identified as 1A, AEB2, TDE, NHSA, E2, P1, 2DB, E1, AEB1, and E3 were used over the course of treatment, and these same phages were used in an ELISA to determine whether seroconversion

occurred. The coating of the phages on the ELISA plate was optimized to achieve the highest signal-to-background ratio. A phage dilution of 1:10 was chosen for optimal detection. Phages were stored at 4°C until use.

Enzyme-linked immunosorbent assay protocol.— Phages were diluted 1:10 in a sodium bicarbonate coating buffer (15-mM Na_2CO_3, 35-mM $NaHCO_3$). One-hundred microliters of the diluted phage were added to each well of a 96-well enzyme immunoassay plate (Costar 3590; Remel Products), covered with parafilm, and placed at 4°C for 14 h. After incubation, the fluid in the wells was removed and each well was blocked for 2 h at room temperature (21–23°C) with 250 µL/well of casein blocking buffer (50-mM tris, 151-mM NaCl, 1.3-mM EDTA, with technical-grade casein and sucrose [40 and 50 g/L, respectively] added).

While the plates were incubating, serum samples were titrated and preincubated with Tween tris-buffered saline (TTBS) containing 15% heat-inactivated FBS for 1 h at room temperature. Serum samples were assessed at various time points, including pretreatment, five samples during phage treatment, and one sample after phage treatment. Serum from an untreated loggerhead sea turtle was assessed for comparison.

All of the following steps were conducted at room temperature, with each incubation step being 1 h. At the end of the blocking and preincubation steps, the ELISA plates were washed three times in tris-buffered saline, and the plates were subsequently loaded with 100 µL of the titrated serum samples. The titrated serum samples were incubated and washed three times with TTBS, and then 100 µL of a mouse anti-loggerhead immunoglobulin Y monoclonal antibody were added to each well (cell culture supernatant LH9 diluted 1:5 in TTBS + 1% bovine serum albumin [BSA]: Rodgers and Rice 2018). At the end of the incubation, the plates were washed and then 100 µL of a 1-µg/mL solution of goat anti-mouse immunoglobulin G horseradish peroxidase (115-035-062; Jackson ImmunoResearch Laboratories, Inc., West Grove, Pennsylvania) diluted in TTBS + 1% BSA were added to each well. Following this final incubation step, the plates were washed three times and then developed using 2, 2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid). Units of activity for each serum sample were determined as previously described (Arkoosh and Kaattari 1990).

Statistical Analysis

Statistical analysis was performed using multivariate analysis in JMP Pro version 14 (SAS Institute, Inc., Cary, North Carolina) to compare single-sample data with time and obtain Kendall's tau (τ) coefficient and probability of a trend as well as Hoeffding's dependence coefficient (D; see de Siqueria Santos et al. 2014 for a description of these single-sample methods). In addition, ANOVA was used to compare data from the affected turtle with data from the control, or early time points with later time points and the control, using Dunnett analysis of least-squares means. Statistical analysis of control comparisons made throughout this study must be interpreted cautiously due to the low sample number of controls ($n = 2$).

RESULTS

At the beginning of treatment, the majority of the affected region was unpigmented and there were soft spots throughout carapace quadrants 2–4. Upon debridement, the depth of infection was >2.5 cm and multifocal to locally extensive throughout quadrants 2–4. Quadrant 1 appeared unaffected for the duration of treatment; however, the holes that were trephined throughout the carapace, including in quadrant 1, never healed. By the end of the study, the majority of the carapace had healed, with new epithelium and pigmentation, and no soft spots were palpated anywhere on the carapace.

Bloodwork was analyzed throughout the approximately 35-week study, with the majority of bloodwork values being steady throughout the entire treatment period (Supplemental Table 1 [available in the online version of this article]; Figure 3). There was an increase in immature red blood cells as well as white blood cells in weeks 13–15. Two additional increases in white blood cells were seen later in the study at weeks 19 and 28. At each of these periods, there was an absolute increase in total white blood cells, heterophils, lymphocytes, monocytes, and eosinophils (Figure 3). Chemistry results were monitored throughout the treatment period to monitor overall health, and all values were within normal limits for the duration of the study (not reported).

Culture and Antimicrobials

Citrobacter freundii was recovered periodically in cultures throughout the treatment regime (Table 1). Organisms cultured during this study included *C. freundii, Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Vibrio alginolyticus*, *Aeromonas sobria*, *V. parahaemolyticus*, *Enterococcus avium*, and *Bacillus* sp. The *C. freundii* strains cultured prior to starting treatment showed multidrug resistance with susceptibility to amikacin, gentamicin, and piperacillin/tazobactam, while none of the other cultured organisms ever showed multidrug resistance. Thus, *C. freundii* was the target for phage discovery and subsequent therapy. After the initiation of phage therapy, the susceptibility pattern of the isolated *C. freundii* changed repeatedly (Table 1).

The *C. freundii* isolate found after week 35 (in November 2019) was not multidrug resistant and was susceptible to amikacin, trimethoprim/sulfamethoxazole, ceftazidime, ciprofloxacin, enrofloxacin, gentamicin, piperacillin/tazobactam,

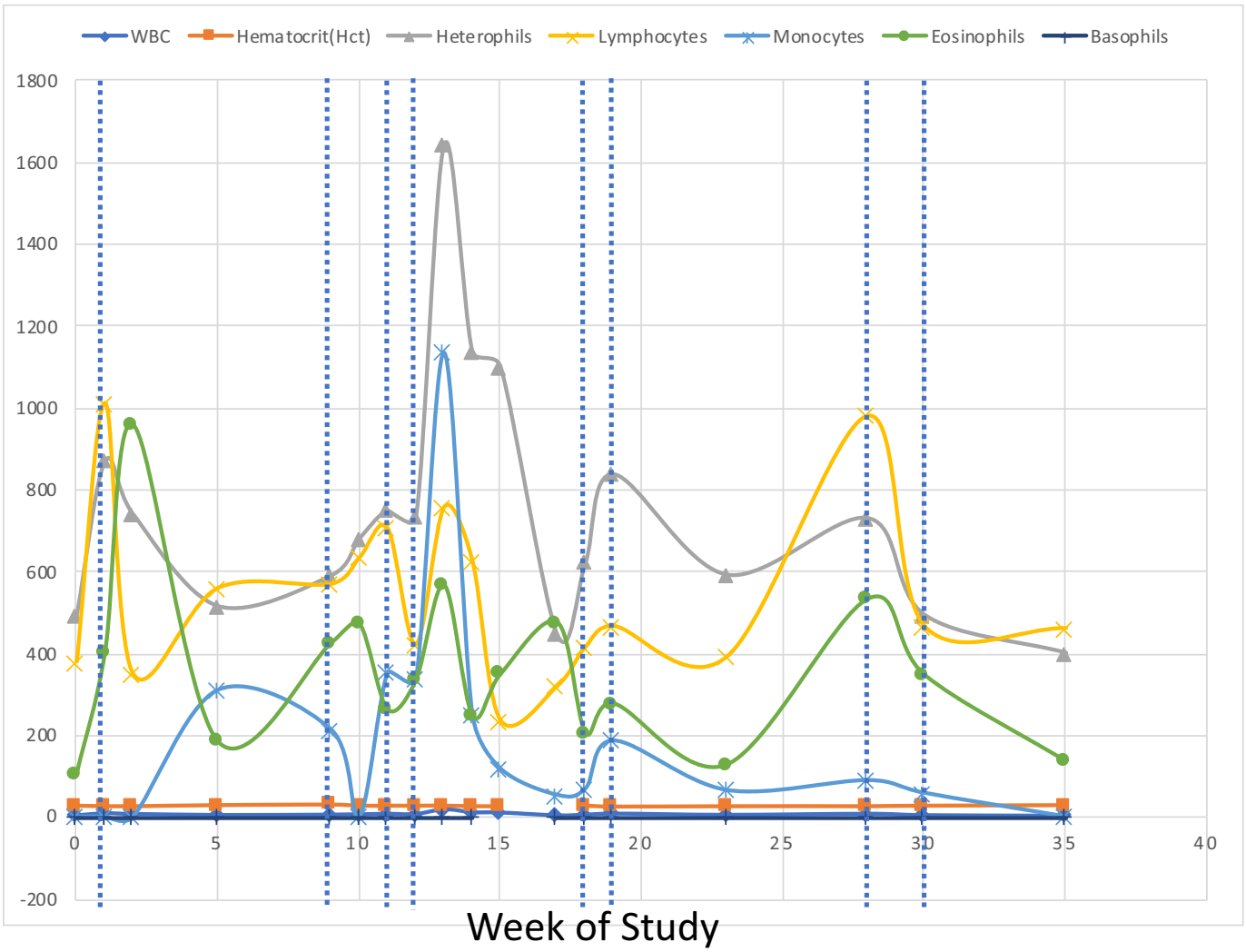

FIGURE 3. Select complete blood cell parameters of the affected loggerhead sea turtle over time in this study, with blue vertical lines indicating days of bacteriophage therapy (WBC = white blood cell count). Heterophils are divided by a factor of 10 for ease in graphic comparison. [Color figure can viewed at afsjournals.org.]

and ampicillin/sulbactam. *Citrobacter freundii* has continued to remain susceptible to a variety of antimicrobials since the conclusion of phage therapy.

Immune Assays

Phagocytic activity.— Percent phagocytic activity in peripheral blood leukocytes (gated on granulocyte populations) is shown in Figure 4. Phagocytic activity was highest near the start of the study, with peaks in phagocytic activity occurring after 3 and 7 weeks. Kendall's τ analysis indicated a significant decrease over time (Kendall's $\tau = -0.4512$, $P = 0.0153$; Supplemental Table 2).

Plasma lysozyme activity.— Lysozyme activity in plasma can be used as a measure of innate immune function. Results from plasma lysozyme assays are shown in Figure 5 and appeared lower (mean = 2.947 μg HEL/mL, $P = 0.0496$) than the values for the control (1.098 μg HEL/mL at week 3 and 1.498 μg HEL/mL at week 8; mean = 1.298 μg HEL/mL) throughout the study, with no significant monotonic trend detected by Kendall's τ (Supplemental Table 2). Lysozyme activity in the affected turtle's plasma appeared to increase during weeks 4, 10, and 13 compared to the control ($P < 0.0001$) or even when compared to the other weeks ($P = 0.0366$), an observation that may reflect an enhanced immune response to phage treatments during the week prior. Note that only two values were available for the control turtle; therefore, statistical analysis of control comparisons throughout this study must be interpreted with caution.

Plasma protein electrophoresis profiles.— Total solids (as an estimate of protein) were measured in each plasma sample by using a refractometer. These results are

TABLE 1. Sensitivity and susceptibility results for *Citrobacter freundii* cultures from the affected loggerhead sea turtle over time (S = susceptible to antibiotic; I = intermediate [borderline susceptible]; R = resistant to antibiotic).

Antibiotic	Before treatment	Week 1	Week 3	Week 5	Week 9	Week 11	Week 13	Week 15	Week 17	Week 19	Week 20	Week 26	Week 28	Week 39
Amikacin	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Amoxicillin/clavulonic acid	R	R	R	R	R	R	R	R	R	R	R	R	R	I
Ampicillin/ Sulbactam	R	R	R	R	R	R	R	R	R	R	R	I	R	S
Aztreonam	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Ceftazidime	R	R	I	S	S	I	R	R	S	R	S	R	R	S
Ciprofloxacin	I	S	S	S	S	S	I	I	S	S	R	I	S	S
Enrofloxacin	R	R	R	R	R	I	I	R	R	R	R	I	R	S
Gentamycin	S	S	S	S	S	S	S	I	S	S	S	S	S	S
Piperacillin/tazobactam	S	S	S	S	S	S	S	I	S	S	S	S	I	S
Trimethoprim/ Sulfamethoxazole	R	R	S	R	R	R	R	R	R	R	R	R	R	S

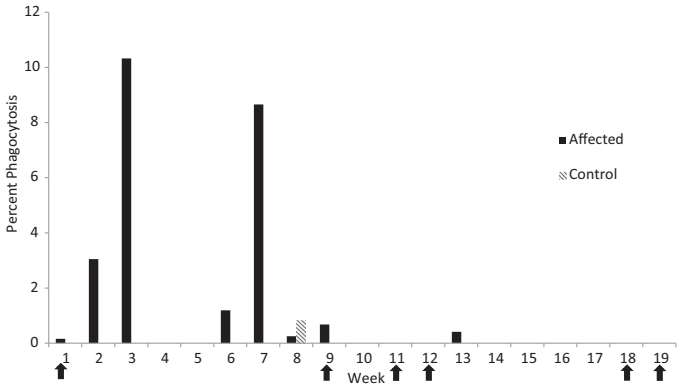

FIGURE 4. Percent phagocytosis in the granulocyte population from affected (solid bars) and control (hatched bars) loggerhead sea turtles over time. Percent phagocytosis was measured using cell flow cytometry and fluorescently labeled *Escherichia coli* (pHrodo *E. coli* BioParticles; Thermo Fisher Scientific). Arrows indicate the dates of bacteriophage therapy.

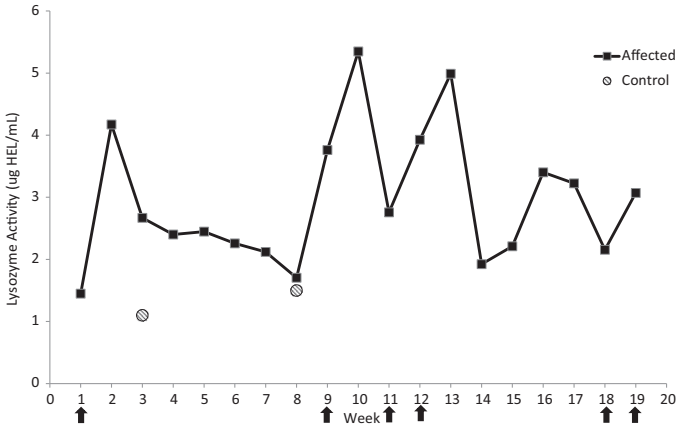

FIGURE 5. Lysozyme activity (μg hen egg white lysozyme [HEL]/mL) in plasma of the affected loggerhead sea turtle (squares) over time. Lysozyme activity was measured using a standard turbidity assay. Circles represent data for the control turtle.

presented in Table 2. Total solids (protein) in the affected turtle ranged from 6.0 to 9.2 g/dL throughout the study period. For the two control samples, total solids were 4.4 and 5.4 g/dL; these values are lower than those detected in the affected turtle's plasma during the study (mean = 4.9 g/dL, $P = 0.0102$). Total solids increased significantly during the study period (Kendall's $\tau = 0.4927$, $P = 0.0039$).

Plasma protein electrophoretic profiles were separated into the following fractions: albumin, alpha (α) globulin, beta (β) globulin, and gamma (γ) globulin (Gicking et al. 2004). The albumin : globulin (A:G) ratio (Table 2; Figure 6A) ranged from 0.29 to 0.44 g/dL (mean = 0.362 g/dL). Only two values were available from the control, providing an average A:G ratio of 0.50 g/dL; both observations were higher than the A:G ratio from the affected animal at any time point ($P = 0.0012$). In the affected turtle, the A:G ratio remained consistent until a decline from week 10 to week 12, followed by an increase in the A:G ratio observed after week 13, and remained between 0.40 and 0.44 g/dL for the remainder of the study, approaching that of the control animal (Figure 6A). The increase in the A:G ratio over time was significant (Kendall's $\tau = 0.3897$, $P = 0.0264$). The increase in A:G ratio appeared to be due to an increase in albumin. Albumin in plasma ranged from 1.59 to 2.97 g/dL (mean = 1.86 g/dL). Control values for albumin were 1.77 and 1.59 g/dL, which fell within the range of albumin values obtained for the affected animal (mean = 1.68 g/dL, $P = 0.5864$). Albumin increased toward the end of the study (Figure 6B), from week 11 on. The association of albumin with study time was significant as determined using Hoeffding's D ($D = 0.1329$; $P = 0.0034$); however, the trending increase was not significant by Kendall's τ, perhaps due to the short time of the increase at the end of the study. Based on ANOVA, values for weeks 14–19 were significantly different from values for weeks 1–13 and from the control values ($P < 0.00001$). In contrast, no significant difference was found between values for weeks 1–13 and control values. Alpha-globulin proteins in plasma ranged from 1.16 to 2.66 g/dL. Control α-globulin proteins, at 1.33 and 0.98 g/dL (mean = 1.19 g/dL), were slightly (~7–16%) lower than the affected turtle's α-globulin protein concentrations (mean = 1.66 g/dL), but this difference did not appear to be significant ($P = 0.4258$; Figure 6C). However, α-globulin proteins also significantly increased in the affected turtle throughout the study, particularly from about week 12 to the end of the study (Kendall's $\tau = 0.4971$, $P = 0.0029$). In contrast, β-globulin proteins stayed within the range of 1.10–1.72 g/dL (mean = 1.38 g/dL). On most dates of the study, the β-globulin proteins in control samples (1.14 and 0.82 g/dL; mean = 0.98 g/dL) were lower (~24–36%) than those of the affected turtle (range = 1.10–1.72 g/dL, mean = 1.38 g/dL; $P = 0.0096$; Figure 6D). The γ-globulin protein concentrations ranged from 1.70 to 2.52 g/dL in the affected turtle (mean = 2.042 g/dL). Gamma-globulin proteins in the control samples were about 40% lower (1.16 and 1.01 g/dL, mean = 1.085 g/dL; $P < 0.0001$) than those in the affected turtle (Figure 6E). Gamma-globulin proteins in the affected turtle increased slightly toward the end of the study, again from approximately week 15 (~2.0 g/dL) to week 20 (~2.5 g/dL; ~25% increase). These increases were significant (Kendall's $\tau = 0.6294$, $P = 0.0029$).

Overall, the biggest difference in plasma protein concentrations occurred in the α-globulin protein fraction, which increased during the middle of the study from 1.4 to 2.7 g/dL, representing an approximately 92% increase. Albumin concentration also increased during this time period, with an increase from about 1.7 to 2.7 g/dL (~59% increase). The β-globulin protein component only

TABLE 2. Total protein (TP), albumin : globulin (A:G) ratio, and protein components from plasma electrophoresis profile results for affected and control loggerhead sea turtles.

Turtle	Week	TP (g/dL)	A:G ratio	Albumin (g/dL)	Alpha-globulin (g/dL)	Beta-globulin (g/dL)	Gamma-globulin (g/dL)
Affected	1	6.8	0.30	1.59	1.88	1.36	1.97
Affected	2	6.4	0.36	1.77	1.41	1.53	1.70
Affected	3	6.8	0.35	1.87	1.43	1.5	1.99
Control	3	5.4	0.44	1.77	1.33	1.14	1.16
Affected	4	6.6	0.35	1.81	1.47	1.72	1.60
Affected	5	6.2	0.35	1.72	1.37	1.23	1.88
Affected	6	6.6	0.35	1.68	1.46	1.13	1.92
Affected	7	6.2	0.35	1.72	1.27	1.19	2.02
Control	8	4.4	0.50	1.59	0.98	0.82	1.01
Affected	8	5.8	0.36	1.65	1.16	1.27	1.72
Affected	9	6.2	0.36	1.74	1.54	1.27	2.03
Affected	10	6.0	0.35	1.69	1.29	1.13	1.83
Affected	11	6.6	0.31	1.74	1.36	1.10	2.01
Affected	12	7.0	0.30	1.79	1.84	1.32	2.01
Affected	13	6.8	0.29	1.76	1.66	1.37	1.97
Affected	15	7.8	0.38	2.32	1.73	1.47	2.28
Affected	16	8.6	0.41	2.71	1.97	1.56	2.36
Affected	17	8.2	0.44	2.55	2.12	1.34	2.18
Affected	18	9.0	0.44	2.97	2.19	1.50	2.34
Affected	19	9.2	0.41	2.81	2.21	1.66	2.52
Affected	20	8.8	0.41	2.66	2.66	1.51	2.47

increased slightly during this time period, from 1.1 to 1.5 g/dL (~36% increase).

Superoxide dismutase.— Activity of SOD in the plasma is shown in Figure 7. The SOD activity in the plasma ranged between 16.75 and 32.92 U/mL for the majority of the study, except for a large jump in activity to 53.56 U/mL in week 17. Early plasma SOD activity did not differ from that of the control ($P = 0.9779$). Plasma SOD activity during weeks 17 and 18 of the study was elevated compared to earlier time points, but this increase was not statistically significant either by comparing early study weeks 1–13 with later weeks 14–19 (as described above) or by Kendall's τ.

Lymphocyte proliferation.— Traditional techniques used for mitogen stimulation (lipopolysaccharide, phytohemaggtlutinin, and concanavalin A) resulted in proliferation values below those of control (unstimulated) wells. This pattern was observed over varying doses of mitogen and at all time points (data not shown).

Enzyme-linked immunosorbent assay.— The ELISA results are presented in Figure 8. In the process of developing the ELISA to assess whether the animal mounted an antibody response to the treatment phages, it was determined that loggerhead sea turtles, especially this affected individual, possessed a robust nonspecific antibody response. Test sera would bind to ELISA wells that were blocked with BSA, casein, or FBS, even in the absence of phage coating, resulting in false positives. The nonspecific response was negated by preincubating the turtle sera with a buffer containing a high level of protein (e.g., TTBS + 15% FBS).

Serum titers for the affected animal as well as the control turtle were assessed for 10 of the phages used during treatment. The control turtle's response to the phages at two time points was equivalent to that of the nonserum controls. The affected animal's antibody titers to the phages were slightly higher than the control before treatment began (pretreatment), changing very little during the treatment period (data not shown). Only upon cessation of treatment, 6 of the 10 phage-specific titers rose greater than 50% above their starting points; however, only two (2DP and E1) showed a positive upward trend during the study (2DP: Kendall's $\tau = 0.7333$, $P = 0.0388$; E1: Kendall's $\tau = 0.7143$, $P = 0.0243$).

DISCUSSION

Phages have been used successfully in humans (Kutter 2008; Pirnay et al. 2011) and other animal species, including a report of phages being used to treat gut-related dysbiosis in green sea turtles *Chelonia mydas* (Ahasan et al. 2020). In the sea turtle study, phage therapy and

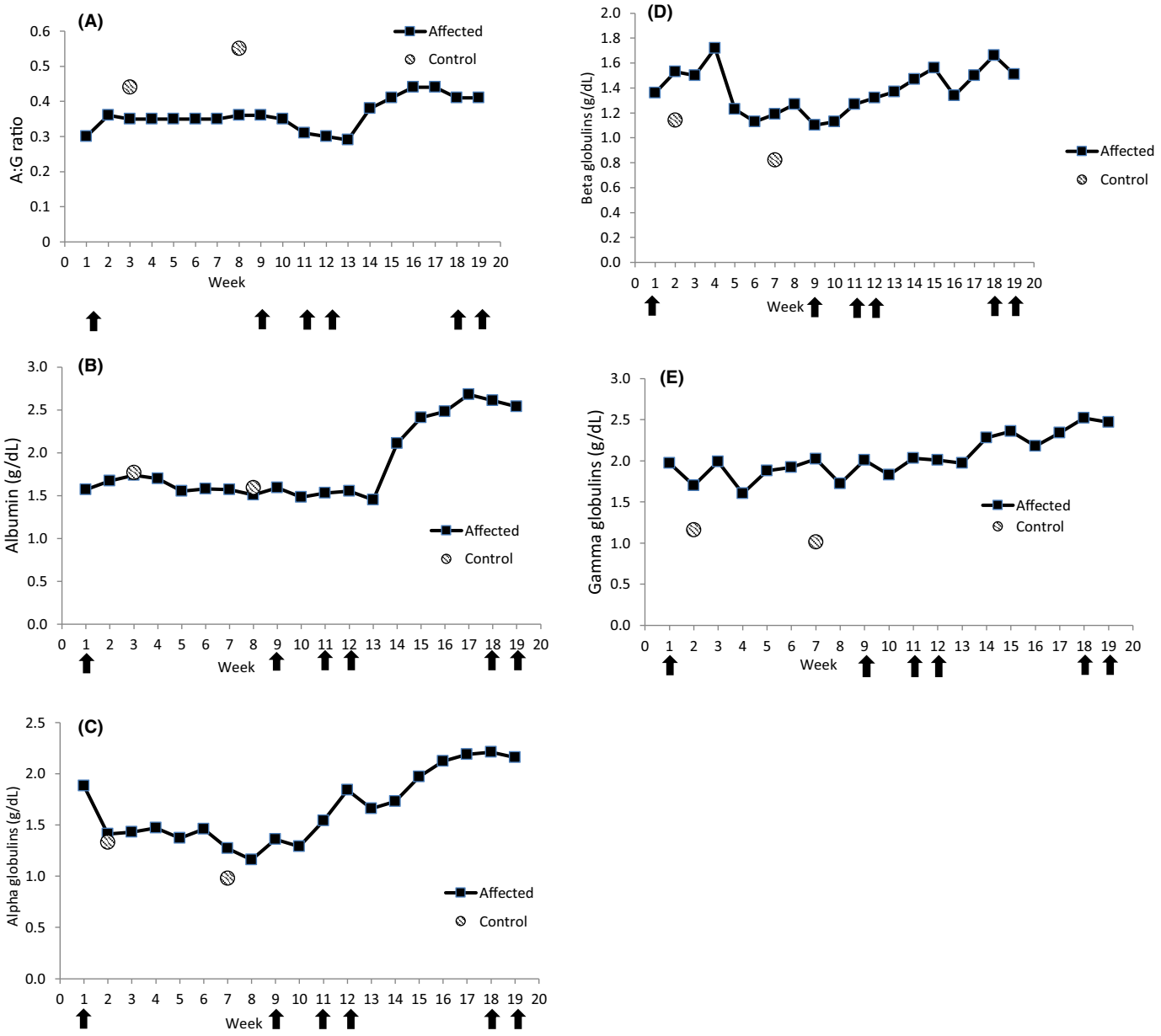

FIGURE 6. Plasma protein electrophoresis profiles to estimate albumin and globulins (alpha, beta, and gamma) in affected (squares) and control (circles) loggerhead sea turtles over time: **(A)** albumin : globulin (A:G) ratio, **(B)** albumin (g/dL), **(C)** alpha-globulin proteins (g/dL), **(D)** beta-globulin proteins (g/dL), and **(E)** gamma-globulin proteins (g/dL). Arrows indicate the dates of bacteriophage treatment. [Color figure can viewed at afsjournals.org.]

antimicrobial treatment with enrofloxacin were compared, resulting in a reduction of the target bacteria population in both treatment groups. There was no change in gut bacterial communities in the phage-treated turtles compared to controls, but there was a significant decrease in the other bacterial communities in turtles treated with enrofloxacin (Ahasan et al. 2020). To our knowledge, the present study is the first report of using phages to treat SCUD in a sea turtle, which healed faster and more completely from this treatment than with previous treatments. Unfortunately, it is not clear whether this was a result of phage therapy alone or other individual or treatment combinations, including antibiotics, levamisole, and/or topical treatments. Other factors, such as environmental or husbandry changes, may have also played a role. However, this seems unlikely given that environmental conditions have been stable and similar antibiotics had been used before without success.

A suite of immune function parameters was analyzed and included phagocytic activity, lysozyme activity, oxidative stress (SOD), plasma protein profiles, lymphocyte

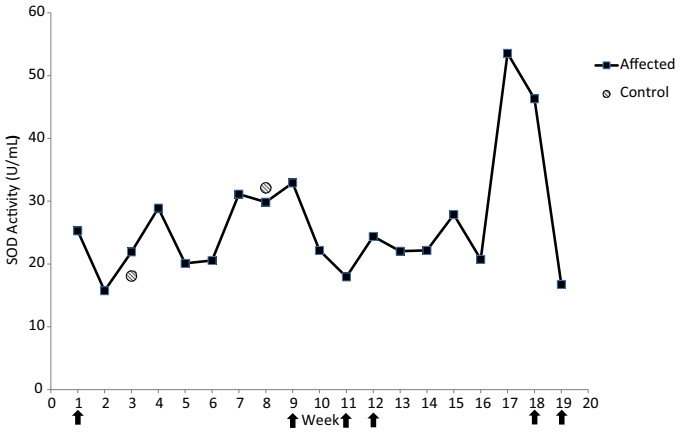

FIGURE 7. Activity of the enzyme superoxide dismutase (SOD) in plasma of affected and control turtles over time. Superoxide dismutase was used as a measure of oxidative stress and was determined using a tetrazolium salt to measure generation of superoxide radicals (Cayman). An increase in plasma SOD activity was evident during weeks 17–18. Arrows indicate time of bacteriophage treatment. Affected = square symbols; Control = circle symbols. [Color figure can viewed at afsjournals.org.]

proliferation, and ELISA. Elevated nonspecific antibody titers have been previously observed following systemic bacterial infections and cancer (Davis et al. 1982; Williamson et al. 1986; Simecka et al. 1991). However, the scant literature available on reptile antibody responses suggests that elevated natural or nonspecific antibody titers may be common in some species (Longenecker and Mosman 1980; Zimmerman et al. 2013). There was a marked difference between the affected turtle and the control, which may represent individual variation or may suggest an underlying immunological disturbance. For example, the underlying bacterial infection involved with SCUD may have led to non-specific activation of the B-cell responses. It is difficult to draw any conclusions from this observation without access to more animals with this condition. More research is needed to determine whether this is a signature of SCUD or just inherent individual variability. Seroconversion was evident after the cessation of treatment, with an increase in phage titers observed at week 30 and later (Figure 8). This may be a result of decreased stress from handlings and a subsequent increase in B-cell response.

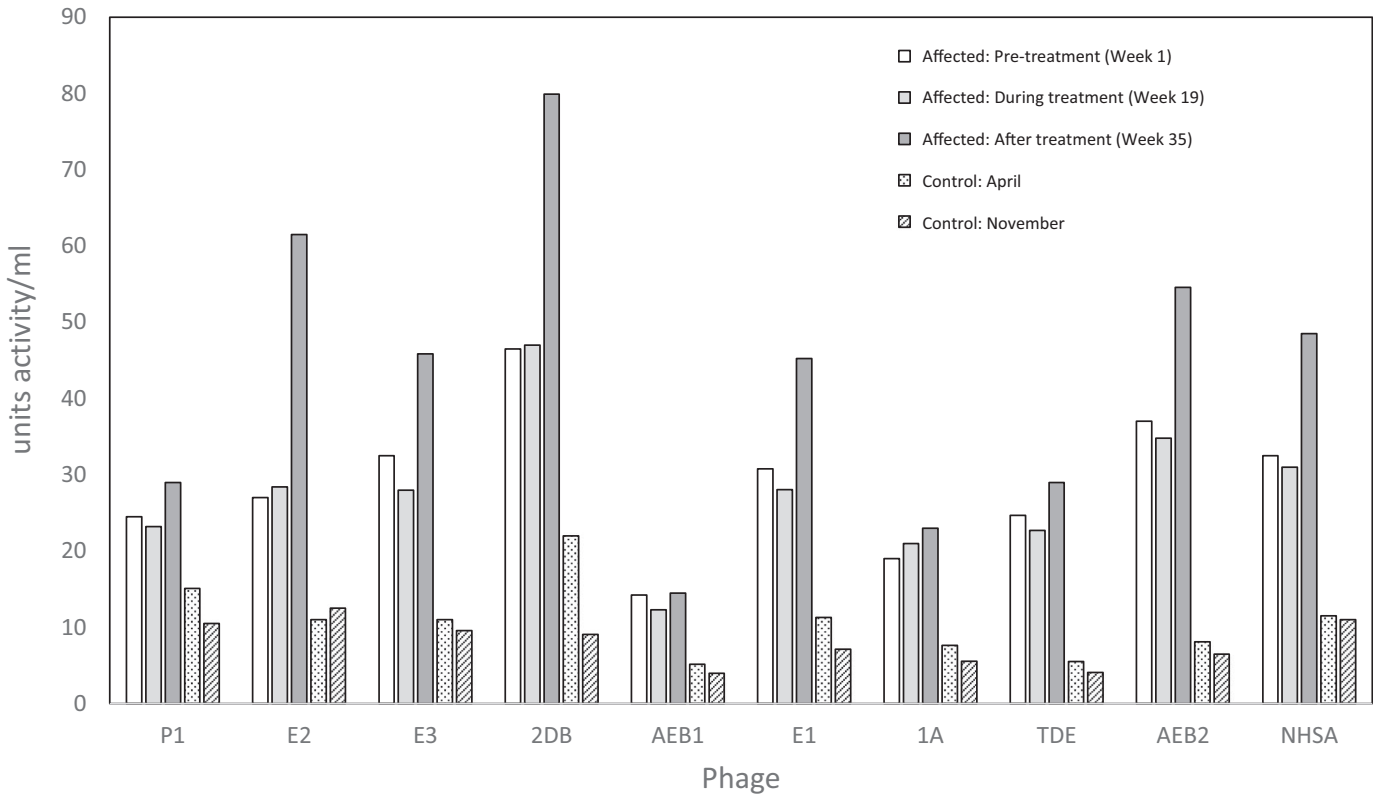

FIGURE 8. Enzyme-linked immunosorbent assay results for antibody titers to 10 phages used over the course of treatment for the affected loggerhead sea turtle. Results for the control turtle are also shown.

Lysozyme activity was similar to levels reported in healthy loggerhead sea turtles in a previous study (Walsh et al. 2010), with values of approximately 4–5 μg HEL/mL in the current study compared to about 5 μg HEL/mL in a healthy individual (Walsh et al. 2010). Lysozyme activity is a component of the innate immune defense system and functions as a naturally occurring antimicrobial agent in many physiological fluids. In the plasma, lysozyme activity functions as a pro-inflammatory molecule and thus initiates inflammation as an immune defense (Weeks et al. 1992; Burton et al. 2002). Fluctuations in the activity of lysozyme provide an indication of changes in the innate immune defense mechanisms of an organism. Based on Kendall's τ trend analysis, there were no significant changes in plasma lysozyme activity over the course of the treatment. Slight increases in plasma lysozyme activity were observed at weeks 4, 10, and 13. This may reflect an activation of innate immune defense in response to phage treatment, as these values increased in plasma 1 week after phage treatment; however, the values were not significant and further study is needed. Total protein (i.e., solids) values reported here (5.8–9.2 g/dL) are higher than other published values (Gicking et al. 2004; Osborne et al. 2010). In the Osborne et al. (2010) study, reference intervals for total protein in plasma of loggerhead sea turtles as measured by the Biuret method ranged from 2.2 to 5.2 g/dL. The values measured in the present study represent total solids, which may partially explain the large variation in protein concentrations. Total solids increased over the course of the study and are likely a reflection of increased antibody production, as indicated by the higher antibody titer. An increase in total solids can also indicate inflammation.

Plasma protein electrophoresis profiles are frequently used as measures of health. The major protein fractions determined using protein electrophoresis include albumin, α-globulin, β-globulin, and γ-globulin. Albumin is the most abundant plasma protein; it serves as a carrier and transport protein and functions in the regulation of blood volume through maintenance of oncotic pressure in body fluids (Kaneko 1997). Acute-phase proteins are included in the α-globulin and β-globulin fractions. The observed increase in the α-globulin fraction may indicate an inflammatory response. Fibrinogen, another acute-phase protein, is a component of the β-globulin fraction. Circulating immunoglobulins and complement comprise the γ-globulin fraction. A substantial increase in the γ-globulin protein fraction was observed and likely represents the higher immunoglobulin titer observed in the affected turtle compared to the control. The A:G ratios changed over the study, but all values fell within reported ranges for loggerhead sea turtles (Gicking et al. 2004; Osborne et al. 2010). An increase in the A:G ratio starting at week 11 may have occurred in response to increased phage treatment during that time period. Overall, the A:G ratio increased over the course of the study, possibly indicating enhanced immune function in response to phage treatment. Similar to mammalian α-globulin, ceruloplasmin and haptoglobulin have been identified in plasma of loggerhead sea turtles (Masquera et al. 1976). Transferrin, a major iron-carrier protein, has been identified in the γ-globulin fraction in loggerhead sea turtles (Masquera et al. 1976), rather than being found in the β-globulin fraction as in mammals (Chang et al. 1999). The immunoglobulin fraction (γ-globulin) increased at the end of the treatment period. The A:G ratio increased toward the end of the study and approached values observed for the control turtle. This may suggest a return to improved health status for the affected animal. The increase in A:G ratio, which reflects changes in some of the protein components, likely indicates a decrease in inflammatory proteins and a restoration of overall health status.

Plasma SOD activity in loggerhead sea turtles has not previously been published. Perrault et al. (2014, 2017) reported SOD activity in the plasma of Kemp's ridley sea turtles *Lepidochelys kempii*; the values reported here are within the range of non-red-tide-exposed turtles. Although SOD activity in the plasma increased slightly during weeks 18 and 19, this increase was not significant and was most likely due to low sample number.

We used one of the same mitogens (concanavalin A) as reported by Keller et al. (2004, 2006) for lymphocyte proliferation assays in loggerhead sea turtles. There was a drop in proliferative responses at week 5, which may indicate a decrease in adaptive immune function at this time period or may be due to a technical issue with using cells from a reptile. This certainly needs to be investigated further on healthy animals so that a robust assay can be used and applied to studying the health of loggerhead sea turtles.

Another interesting observation was that there was no seroconversion to the phages during the treatment period, suggesting that phage treatment could be continued without antibody interference in effectiveness. Another option is that continued handling may have led to a stress response, thereby suppressing B-cell responses. This phenomenon has been reported in the literature (Ellsaesser and Clem 1986; Bhatnagar et al. 1996; Burdick et al. 2011; Martin et al. 2011; Yarahmadi et al. 2016). With all 10 phages examined, the pretreatment titer and the mid-treatment titer remained equivalent (±5%; Figure 8). Upon cessation of treatment, the antibody response to all phages increased; in 60% of the cases, the increase in specific antibody titer was greater than 30%. The rise in titer once treatment ended suggests either that (1) it takes a long time for antiphage antibodies to be made or (2) the handling of the animal during treatment resulted in suppressed B-cell responses. Handling stress and suppressed B-cell responses should be investigated further in healthy turtles.

One of the most significant changes observed over the course of treatment was the alteration to the susceptibility pattern on cultures (Table 1). On initiation of phage therapy, *C. freundii* was multidrug resistant and only susceptible to amikacin, gentamycin, and piperacillin/tazobactam. *Citrobacter freundii* was identified again in November 2019 but was then sensitive to amikacin, trimethoprim/sulfamethoxazole, ceftazidime, ciprofloxacin, enrofloxacin, gentamicin, piperacillin/tazobactam, and ampicillin/sulbactam. Antimicrobials were used in conjunction with phage therapy based on the susceptibility pattern and appear to have had a positive result on the clinical state of the animal as well as the susceptibility pattern. Dual therapy with phages and antibiotics has resulted in significant reductions in bacterial load in other organisms as well (Viertel et al. 2014; Lin et al. 2017; LaVergne et al. 2018).

Conclusions

The results from this study indicate that phage therapy was successful in helping with the treatment of a chronic SCUD case in a loggerhead sea turtle. Throughout the course of treatment, the animal remained in good body condition, continued to eat her normal diet, participated in training and enrichment sessions, and never exhibited abnormal behavior or adverse signs. Additionally, her clinical signs improved overall and the susceptibility pattern of *C. freundii* changed from multidrug resistant to a normal pattern. This study has highlighted multiple areas that are lacking in knowledge regarding sea turtle immunology. Phage therapy may be an effective treatment for multidrug-resistant bacteria or avascular lesions in sea turtles and other aquatic species, but additional research is needed on efficacy, treatment methods, and long-term effects.

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SUPPORTING INFORMATION

Additional supplemental material may be found online in the Supporting Information section at the end of the article.