Identification of Coliphages in the Aviary at Brookgreen Gardens and the Factors that Might Influence Coliphage Population Dynamics in this Cypress Swamp Environment

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It has been understood that the presence of coliphages (bacteriophages specific for *E. coli*) in water is an indirect measurement of fecal contamination. Bacteriophages have been suggested to hold an important role in regulating the bacterial population. This pilot study analyzes the relationship between the presence of coliphages in the water in the aviary at Brookgreen Gardens and how environmental factors such as temperature, pH, and turbidity affect the presence of these viruses over the time period of three months (June 2017 through August 2017). Coliphages found in volary water sources were speculated to be deposited directly by birds that live in the Brookgreen Gardens aviary. These birds remain in an enclosed area, thus making the location a reservoir potentially rich in bacteriophage number and diversity. Plaque assays were conducted to look for the presence of the coliphages. Polymerase chain reaction (PCR) analysis was conducted on all samples collected in order to characterize any possible coliphages identified via plaque assays. The results gathered from both microbial (plaque assay) and molecular (PCR) methods varied immensely, with numerous positive results found at each site for each week of analysis over the three-month period. All PCR tests conducted on samples were deemed negative for coliphage presence, indicating that these coliphages are especially unique or may be RNA-based. These findings confirm that there are indeed bacteriophages present in the Brookgreen Gardens aviary, and that pH, turbidity, and temperature did not appear to play a prominent role in the presence of the coliphages. The location in the aviary which yielded the most positive results (Site 1) was the site at which the birds were most frequently observed defecating, providing indirect evidence that the fecal matter of the birds is the most important factor in bacteriophage presence.

Introduction

Bacteriophages are viruses that specifically infect and lyse bacterial cells [1]. Also known as phages, these bacterial viruses are arguably the most abundant biological entities in the biosphere, constituting between 10^{30} and 10^{32} particles on Earth [2,3,4]. As the most ubiquitous semi-autonomous genetic agents on the planet, phages play a prominent regulatory role in the microbial ecology of every ecosystem thus far explored on Earth. Additionally, as an entity of immense host specificity, bacteriophages are often utilized as indicators of distinct bacterial host species in a given environment due to the fact that phages are known to emanate only from bacteria that consequently lyse as a function of phage self-proliferation. Thus, methods involving detection of specific bacteriophages can be conducted as a means to detect respective bacterial hosts.

Since the 1890s, it was generally accepted that the monitoring of public waterways for specific pathogens is incongruent in efficacy, as pathogen diversity in water sources is considerable whilst pathogen concentration remains small and difficult to detect [5]. Total coliforms, fecal coliforms, *Enterococci* spp., and *Escherichia* spp. have thus since become the primary diagnostic tools used to detect fecal pollution. Members of the *Escherichia* genus such as *E. coli* are particularly pragmatic candidates for fecal pollution due to their ubiquity in animal feces in conjunction with their poor capacity to multiply outside the host's intestinal tract and mucus surfaces [6,7].

Coliphages are a specific type of bacteriophage that infects coliform bacteria of the species, *Escherichia coli*. As bacterial viruses, coliphages are more resistant to treatment processes than *E. coli* indicator bacteria [8], suggesting that utilizing bacteriophages may prove more effective in fecal matter detection than utilizing bacterial fecal indicators themselves. In conjunction with higher persistence in water sources relative to bacterial indicators, bacteriophages may provide a pragmatic assessment of fecal pollution in a given environment.

This pilot study investigated fecal pollution in an aviary situated at Brookgreen Gardens in Murrells Inlet, South Carolina, from June 2017 through August 2017. With an approximate circumference of 180 meters, this cypress swamp aviary serves as an enclosure containing a number of native bird species such as the great blue heron (*Ardea herodias*), great egret (*Ardea alba*), American white ibis (*Eudocimus albus*), and black crowned night heron (*Nycticorax nycticorax*), amongst others. The aviary sits adjacent to the Waccamaw River and is thus characterized as a tidal marsh that directly drains into this water system. The aviary is therefore positioned in a dynamic swamp environment in which water influx can be observed infiltrating the aviary from the immediate marsh setting, whilst water efflux can be seen transported from the aviary towards the Waccamaw River.

Previous studies have confirmed *Escherichia coli* presence in the fecal matter of various bird species [9,10]. As the *E. coli* population is directly related to the bacteriophage population, coliphages as a product of avian fecal pollution can be monitored in this restrained enclosure. Therefore, this pilot study seeks to collect water samples from five designated areas inside the Brookgreen Gardens aviary as a means to gain insight into bacteriophage populations found in areas of water influx, water stagnation, and water efflux. Water samples collected from designated locations were tested via both microbial and molecular tests in order to study bacteriophage activity.

This pilot study aimed to address the following questions about bacteriophages: are there coliphages in the tidal swamp of the Brookgreen Gardens aviary and, if so, can these coliphages be characterized using molecular techniques? Additionally, do environmental conditions have any influence on the coliphage population in this ecosystem? Thus, the main purpose of this initial investigation was to establish a basic understanding of coliphage population dynamics as a direct result of fecal pollution introduction from birds in a restrained environment and the effect that environmental condition may or may not have on the dynamics of this population.

Methods

Collection

Five sites within the Brookgreen Gardens aviary were selected for collection of water samples based on site properties, as configured in Figure 1. Fifty milliliter (50 mL) centrifuge tubes (VWR) were used to collect water from each site. These centrifuge tubes were stabilized via a clamp attached to the extremity of a painting stick and served to efficiently obtain water sources from seemingly impervious swamp locations. Samples were placed in an ice chest after collection for roughly 1-2 hours before being processed at Coastal Carolina University. Turbidity of the water was measured concurrently with collection and in the lab. Both the pH and temperature of the water were measured at the site of collection, if environmental conditions allowed.

Figure 1: The red numbers show the collection sites in the Brookgreen Gardens aviary. Site 1 was an open area where the birds were observed to frequent the most. This site was almost fully shaded and fairly shallow for the birds to walk around. Site 2 was similar to Site 1, as it was an open area of water, albeit the water in Site 2 was deeper and perpetually exposed to sunlight throughout the day. Site 3 served as the location of water influx from the external environment directly into the aviary. Site 4 and Site 5 were occasionally connected to the main body of water in the aviary, albeit were mainly observed to exhibit secluded puddle-like formations, depending on tidal formation and local precipitation.

Filter sterilization and amplification of bacteriophages
Crude water samples were filtered through a 0.45 µM PTFE membrane (VWR International) attached to a 25 mL syringe with Luer-Lok Tip (BD) to remove debris and bacteria. Five culture tubes (Thermo Fisher) were prepared with 5 mL of 2x LB Miller broth (Sigma-Aldrich) and 250 µL of commercially-supplied *Escherichia coli* B (Carolina Biological Supply Company) culture at log-phase. After the addition of *Escherichia coli* B to a sterile culture tube, 100 µL of the filtered water sample was added to amplify any bacteriophages present. Each tube was vortexed, placed in an incubator at 37°C overnight, and refrigerated the following day for future use in plaque assay tests and PCR analyses.

Bacterial counting
A bacterial population count from each water sample at each site was conducted for quantitative determination of bacterial colonies. Serial dilutions of up to 10^{-2} utilizing phosphate buffered saline (PBS Tablets, Calbiochem) as dilution buffer was set up to quantify the viable bacterial count found at each designated site. The samples were then spread on an LB agar plate and incubated at 37°C overnight. Quantitative analyses were then performed the following day via Quebec dark field apparatus (Reichert technologies).

Plaque Assay
Five LB agar plates (Sigma-Aldrich) were dried in the incubator at 37°C for 20 minutes and then labeled according to sample number and date collected. Seventy-five microliters (75 µL) of vortexed *Escherichia coli* B culture was spread onto the plate and allowed to dry in the incubator for 30 minutes at 37°C. Four microliters (4 µL) of the amplified samples were spotted onto experimental zones on each plate; an aliquot of 4 µL of PBS served as negative control. Plates were dried and then incubated overnight at 37°C. Analyses of plaque assays were conducted the following day using the Quebec dark field apparatus.

Polymerase Chain Reaction
One-hundred microliters (100 µL) of amplified sample was transferred to 1.5 mL microfuge tubes (VWR) and centrifuged for 5 minutes at 14,800 rpm. The resulting supernatant was transferred to a sterile 1.5 mL microfuge tube. Five µL (5 µL) of Proteinase K (ThermoFisher) was added to sterile microfuge tubes, of which were incubated on a shaking table at room temperature for 60 minutes. Samples were then placed in a heating block at 95°C for 10 minutes to denature proteases in the sample and further release nucleic acids from viral capsids. PCR samples were set up by adding 25 µL of GoTaq Green Master Mix (Promega Corporation), 21 µL of template (treated sample), and 4 µL of a respective primer set (See Table 1) to a 1.5 mL microfuge tube (VWR International). Proteinase-K treated commercial T4 phage (Carolina Biological Supply Company) served as a positive control whilst PBS functioned as a negative control for the PCR reactions. PCR amplification was performed using a Bio-Rad Laboratories, Inc., MJ Mini Thermal Cycler program called T4B. This thermocycler program comprised of an initial 4-minute DNA unwinding step at 95°C, followed by 39 cycles of 30 seconds at 94°C for DNA denaturation, 1 minute at 55°C for primer annealing, and 72°C for 2 minutes for DNA extension purposes. The final PCR product was then held at 4°C for temporary storage until run on a gel.

Gel Electrophoresis
To image the samples, a 2% agarose gel was prepared using Agarose I (VWR) and 1x TAE buffer. Ethidium bromide served as the intercalating fluorescent tag. The gel was covered in 1x TAE buffer, plugged into a Mini 300 Power Supply model from VWR, and run at 60 volts for 90 minutes. The agarose gel was imaged using the UV light mode of the Molecular Imager ChemiDoc XRS+ Imaging System from Bio-Rad Laboratories, Inc. A 100bp ladder (Promega Corporation) was used as a standard to determine band sizes for all PCR reactions. Expected band sizes for identification of bacteriophages are listed in Table 1.

Primers
Individual primers such as K1F, 933, Micro, T4, HK, Mu, N4, JK, and Lambda that comprise coliphage primer sets CPA and CPB, as delineated in Table 1, were extracted from the 2009 UNC Chapel dissertation by Hee Suk Lee [12]. Primers ORF23 and ORF43 that constitute primer set CPO were developed by Dr. Paul E. Richardson's lab at Coastal Carolina University. The 4 µL individual reaction for coliphage primer set CPA consisted of 0.2 µL HK Forward ("For"), 0.2 µL HK Reverse ("Rev"), 0.2 µL 933For, 0.2 µL 933Rev, 0.2 µL T4For, 0.2 µL T4Rev, 0.2 µL MicroFor, 0.2 µL MicroRev, 0.2 µL K1FFor, 0.2 µL K1FRev, and 2 µL of nuclease-free water (Promega Corporation). The 4 µL individual reaction for coliphage primer set CPB consisted of 0.22 µL MuFor, 0.22 µL MuRev, 0.22 µL Lambda For, 0.22 µL Lambda Rev, 0.22 µL JKFor, 0.22 µL JKRev, 0.22 µL N4For, 0.22 µL N4Rev, and 2.22 µL of nuclease-free water. The 4 µL individual reaction for coliphage primer set CPO consisted of 0.67 µL ORF23 For, 0.67 µL ORF23 Rev, 0.67 µL ORF43 For, 0.67 µL ORF43 Rev, and 1.33 µL of nuclease-free water.

Results

Acidity
The acidity of the sites could be broken down into two clusters of data based on the characteristic of the site, as delineated in Table 2. Sites 1 through 4 constitute a highly connected single body of water; average pH readings of these sites were found to be 6.3, or slightly acidic. Site 5 represented a secluded body of water that remained wholly segregated from Sites 1 through 4, and was found to have a slightly alkaline pH of 7.7.

Table 1: Summary of primer sets utilized in this study. Primers comprising primer sets CPA and CPB were derived from the work of Hee Suk Lee [12], whilst primer set CPO was developed by Dr. Paul E. Richardson, at the biochemistry research labs at Coastal Carolina University.

Primer Set	Target Family/ Organism	Gene Target	PCR Fragment Length (bp)	Primer Name
CPA	*Podoviridae*	CKV1F, gp34	2110	K1FFor
				K1FRev
	Podoviridae	933Wp09, *hkaG* gene	488	933For
				933Rev
	Microviridae	Hypothetical protein	1039	MicroFor
				MicroRev
	Myoviridae	Major head protein (gene 23)	704	T4setFor
				T4setRev
	Siphoviridae	cII protein	177	HKsetFor
				HKsetRev
CPB	*Myoviridae*	Tail fiber gene (MUP49)	171	MusetFor
				MusetRev
	Podoviridae	Hypothetical protein	2285	N4For
				N4Rev
	Siphoviridae	Tail fiber protein	878	JKsetFor
				JKsetRev
	Siphoviridae	B gene	307	LambdaFor
				LambdaRev
CPO	Coliphage T2/T4	ORF 23 (Major capsid protein)	405	ORF23For
				ORF23Rev
	Coliphage T2/T4	ORF 43 (DNA polymerase)	198	ORF43For
				ORF43Rev

Table 2. Summary of average acidity, turbidity, temperature, and bacterial counts measured at each site at Brookgreen Gardens aviary from June 2017 through August 2017. Standard deviations (SD) of each parameter measured depict variation from mean value. Data for plaque assays represents percentage of all positive microbial tests analyzed over six-week study period for each site. *pH measurements were limited to one collection cycle only at Site 4 and Site 5.

Parameter	Unit	Site 1	Site 2	Site 3	Site 4	Site 5
Acidity	**pH**	6.2	6.3	6.3	6.2	7.7
	(SD)	(0.1)	(0.1)	(0.3)	(N/A*)	(N/A*)
Turbidity	**ppm**	50.8	59.6	48.6	46.0	45.3
	(SD)	(3.3)	(5.9)	(3.9)	(2.9)	(5.1)
Water Temperature	**°C**	27.3	26.6	28.3	28.1	26.8
	(SD)	(0.9)	(1.0)	(1.0)	(0.9)	(0.8)
Bacterial Counts	**Raw #**	3763.3	3701.7	4415.4	10332.3	17300
	(SD)	(3372.9)	(315)	(2107.2)	(9823.5)	(7790.0)
% Positive Plaque Assays		66.7%	33.3%	33.3%	33.3%	50.0%

Turbidity
The particulate matter in the water varied slightly between collection sites with a range of 59.6 (Site 2) to 45.3 (Site 5), as delineated in Table 2. These values are similar to other swamp water values (unpublished data from previous South Carolina Academy of Science posters).

Temperature
Temperature of the cypress swamp water had a range of 28.3°C to 26.6° C (Table 2). The factor that was directly related to this fluctuation was the depth of the water. Sites with deeper water (Site 2) had lower temperature even though they were most often directly exposed to more sunlight than shaded, shallower sites (Site 3).

Bacterial count
Bacterial count (bacterial organisms per mL) had a high degree of variance based on the property of the collection site at the time collection (Table 2). Any collection site that had the water disturbed during collection had a much higher count than those that lacked similar disruption. If the collection site was also reduced to the size of a puddle (Site 5 and Site 4 partially constituted puddle-like formations while conducting sample collections), they had much higher bacterial counts and their respective standard deviations were sufficiently higher than the other sites. Larger water sources, as is epitomized by Sites 1 through 3, had much lower bacterial counts.

Plaque assay
Each site had the water checked for the presence of bacteriophage after an amplification phase, as depicted in Table 2. Every site during the six-week study contained positive samples at least once during the conduction of this research. Site 1 had the highest presence of bacteriophage with 66.7% of all samples testing positive during the study. Site 2 and 3 were the least likely to be positive and only detected bacteriophages in 33.3% of water samples for each.

Discussion

Taking into account pH, turbidity, temperature, and bacterial count, the data did not indicate that any of these environmental factors had any influence on the presence of coliphages in the aviary. This study was conducted over a six week period of time during the summer months (June – August), so no definitive conclusions can be drawn based on the small data size. Efforts are being made to make this a yearlong project so a better data set can be collected and analyzed in the future. Analysis of the weekly data did have a few interesting observations on coliphage presence unrelated to environmental conditions.

Out of all of the samples that were collected from the aviary, Site 1 samples were the most frequently positive for coliphages, with 66.67% of samples testing positive for lytic coliphage via plaque assays. One possible explanation for this is that these coliphages may originate directly from avian fecal matter, and therefore the birds. This speculation relies upon the observation that Site 1 was the area which birds were observed to frequent the most. This was a qualitative observation based on the time spent in the aviary.

Coliphages are indirect indicators of fecal matter pollution; this, in turn, leads to the conjecture that these coliphages may have originated from avian feces. Consequently, samples of bird fecal matter deposited in the water and on the walkway were tested for the presence of bacteriophage. These tests did come back positive in many samples, but were not included in this study, as there was no means to differentiate whether these coliphages were extracted from the avian fecal matter or were previously present in the cypress swamp. Future development of microbial source tracking (MST) techniques using bacteriophages remains possible, as discussed by Rachel T. Noble, et al. [10], and may be explored in the future.

Site 3, the site representing the location of water influx into the aviary, was found to be positive for coliphages 33.33% of the time. Although this site was positive for coliphages rather infrequently, if Site 3 was positive, every site in the aviary was found to be positive for coliphages. This could possibly be attributed to the fact that Site 3 is the site of water influx into the aviary, leading to the speculation that perhaps the coliphages in the aviary originated from a location external to the cypress swamp aviary, and not internally from the aviary's fixed bird population. Extension of site selection may be expanded in the future to encompass external water sources that directly drain into the aviary along with water sources immediately leaving the aviary's proximity that lead to the Waccamaw River site.

The final observation from this study was the inability of the primer sets to characterize any coliphages in this ecosystem. This might suggest that the phages in this aviary are unique and that these viruses represent novel phages that have yet to be characterized and classified. Another speculation may be that these coliphages are RNA-based viruses, as many studies suggested that bacteriophages such as F+ RNA coliphages constitute the vast majority of bacteriophages recovered from surface waters [12]. Techniques have been developed to help distinguish between these two possibilities and will be carried out to help elucidate this quandary.

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