Investigating the Lytic *Staphylococcus aureus* Bacteriophage Reservoir Amongst a South Carolina University Population: Discovery, Characterization, and Identification of a Potential Bacteriophage Treatment for Methicillin-Resistant *Staphylococcus aureus*

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Bacteriophages are viruses that only infect bacterial cells and can be used to treat antibiotic resistant bacterial infections. This study focused on the isolation and characterization of bacteriophages lytic to *Staphylococcus aureus* at Coastal Carolina University (CCU) in Conway, South Carolina, as a means to isolate bacteriophages that can potentially be used to treat methicillin-resistant *Staphylococcus aureus* (MRSA), an antibiotic-resistant *S. aureus* variant. From 2014 to 2018, collection of ear and nose samples from 225 randomly selected CCU volunteers was conducted. Filter sterilization, amplification, microbial tests, and PCR analyses were performed in order to identify and characterize bacteriophages. Coliphage populations were also monitored as an indicator of temporal competition and fecal contamination. A pilot study was initiated in 2017 in which 15 CCU volunteers were sampled once a month from October 2017 through March 2018 in order to investigate coliphage and *S. aureus* phage population dynamics. The purpose of this study was to gain insight into the lytic *Staphylococcus aureus* phage repository found in the CCU community, and to explore *S. aureus* phage dynamics amongst the CCU populace. Results indicated that a considerable *S. aureus* and *E. coli* phage reservoir exists amongst the CCU population. Most phages could not be characterized via PCR analysis, suggesting high diversity. The preliminary study indicated that *S. aureus* and *E. coli* hosts potentially exhibit temporal competition, of which might be explanatory of phage population fluctuations.

Introduction

Bacteriophages are viruses that only infect bacterial cells [1]. Also known as phages, these viruses are the most abundant semiautonomous genetic agents in the biosphere, making up between 10^{30} and 10^{32} particles on Earth [2]. As the most ubiquitous living entity on the planet, bacteriophages play a key role in regulating the microbial balance in every ecosystem previously thus far explored [3]. Primarily killing bacteria during lytic cycles, bacteriophages lyse their hosts by inserting their genomic material into the bacterium, hijacking the available host machinery, and using the bacterium's apparatus to synthesize new phage particles. The host cell then lysis and releases new phage particles capable of destroying bacteria of the same strain. Such high specificity in host selection has made phage therapy a popular topic of research in Russia and various Eastern European countries since its co-discovery in 1915 and 1917 by Frederick Twort and Felix d'Herelle, respectively. This geographical seclusion in phage therapy research and application, in turn, can mainly be attributed to the discovery of antibiotic "miracle drugs" such as penicillin in the 1940s, of which became the focal point of antimicrobial research for a number of decades in most of the Western world. Analogously, phage therapy has been ignored in much of the twentieth century by the West due to poor predictability of phage therapy trial results and outcomes attributable to an alluvion of causes, including contamination, phage-bacteria specificity, horizontal toxin transfer amongst temperate phages, antigenicity of the host immune system, and bacterial coevolution [4].

Nevertheless, hundreds of successful phage therapy preclinical studies in both animals and humans continued to be conducted in Eastern Europe and the former Soviet Union. Worldwide phage research became predominantly centered at the Eliava Institute of Bacteriophage, Microbiology, and Virology in Tbilisi, Georgia, and the Hirszfeld Institute of Immunology and Experimental Therapy in Wroclaw, Poland [2]. Numerous phage therapy trials, such as the famous 1960s Georgian study illustrating a 3.8-fold decrease in dysentery incidence in 30,769 children administered a cocktail of bacteriophages over a 109-day period [5], gave rise to a preference of bacteriophage administration to antibiotic treatment in select Eastern European countries, as is epitomized by the numerous commercial phage preparations sold in Russian and Georgian pharmacies and transported to Western European countries as a "last resort" treatment option [2]. Whilst many scientists in the Western sphere deemed Eastern European phage studies to be dubitable in outcome due to limited study control measures, the monumental amount of benefits attributed to the usage of bacteriophage therapy, as excavated and delineated by twentieth-century Eastern European studies, was recently corroborated and ascertained by several experiments in both the United States and United Kingdom, as reviewed by J. Alisky, et al. [6]. Phages are the natural products crafted by billions of years of evolution as highly specialized deliverers of diverse bactericidal agents targeting bacterial cytoplasmic contents. The benefits of bacteriophage therapy can thus be framed in terms of the biochemical properties of the virus [7]. One such benefit of phage therapy circumscribes bactericidal traits, in which a bacterium infected with an obligately lytic phage is incapable of regaining viability [8]. Another benefit of phage therapy is auto-dosing: phages increase in number only where bacterial hosts are located; therefore, phage numbers are specifically dependent on bacterial count and host location, which extemporaneously allows for the propagation of an appropriate number of viral particles in the system predominantly in infected locations only. Furthermore, phages, when correctly purified, are deemed inherently non-toxic for human administration purposes, albeit the release of bacterial toxins upon lysis remains a possibility, as is the case with cell-wall disrupting antibiotic compounds. Moreover, these viruses are known to have minimal disruption of normal flora, a narrower potential for eliciting resistance, along with an efficacy for biofilm clearance not seen in conventional antibiotic treatments. Phage particles are also much more easily discovered, with versatility in application form (e.g., liquids, creams, solids, etc.) representing a pharmacological benefit in phage therapy treatment development, as viral particle administration can even be combined with antibiotic compounds for improved efficacy in treatment success. Additional advantages for phage therapy include single-dose potentials, in which phage amplification is a singularly administered dose serving as a curative agent against a bacterial infection; phage transfer between subjects, when cross-infection may spread to other untreated individuals in a community; and phage administration efficacy, in which a small number of viral particles are needed for bacterial lysis. The implementation and administration of phage therapy thus has an immense amount of conspicuous benefits.

Yet, despite its long and successful Eastern European history, phage therapy remained unauthorized for routine use in human patients in the West until the late twentieth century, when the rise of antibiotic-resistant pathogens such as methicillin-resistant *Staphylococcus aureus* (MRSA) and multidrug-resistant *Pseudomonas aeruginosa* (MDRPA) created a need for phage research and application, as evidenced by the European Union's $5.2 million contribution in 2013 to Phagoburn, the first large, multicentre clinical trial of bacteriophage therapy for human infections [9]. Such recognition of phage therapeutical significance was reinforced

the following year by the United States National Institute of Allergy and Infectious Diseases (NIAID), a center listing phage therapy and phage-derived lysins as a vital constituent in its national approach against bacterial resistance in its 2014 strategic plan [10,11,12].

This study specifically focuses on *Staphylococcus aureus* bacterial hosts and their respective viruses as a means to fight a variant strain of *S. aureus*, methicillin-resistant *Staphylococcus aureus* (MRSA), an antibiotic-resistant bacterial pathogen once again becoming a prominent re-emerging threat to global human health and well-being [13]. The bacterium, known to kill more Americans each year than HIV/AIDS, emphysema, Parkinson's disease, and homicide combined [14,15,16], is a leading cause of nosocomial infections worldwide, causing over 80,000 infections and 11,285 related deaths each year in the United States alone [17]. While invasive MRSA rates have declined in hospital-based settings in the past decade [18], a prominent global emergence of community-associated MRSA (CA-MRSA), a strain characterized by its cutaneous infections in otherwise healthy individuals, has surfaced in non-clinical settings, causing skin and soft tissue infections, osteomyelitis, sepsis, endocarditis, toxic shock syndrome, along with necrotizing pneumonia [19,20,21]. CA-MRSA, a pathogen of epidemic proportions in the United States, has subsequently been non-discriminatory in its selection of hosts, infecting high school, college, and professional athletes; men who have sex with men; military personnel; prison inmates; and children in day care centers [22]. Therefore, no group of individuals can be considered not at risk. Furthermore, a single clone of *Staphylococcus aureus* does not cause CA -MRSA; rather, global CA-MRSA infections are the result of geographically divergent clones in which clonal replacement has been observed in numerous studies [23-28]. This large number of increasingly virulent and transmissible CA-MRSA clones dispersed around the world is a problem that is propagated by the fact that effective treatment for MRSA remains elusive and limited [29]. Thus, despite the recent availability of new antibiotics for *Staphylococcus aureus* [30], new and effective treatment options need to be explored as a means to efficiently treat antibiotic-resistant *S. aureus* strains well into the future [12]. One such naturally occurring treatment option is bacteriophage therapy, a promising solution to the worldwide MRSA epidemic.

Staphylococcus aureus is a common bacterial strain that can be found in the nose, pharynx, perineum, axillae, and skin of humans. Persistent colonization of *S. aureus* and MRSA can be observed in approximately 30% and 1.0%, respectively, of the United States population [31,32]. This commonplace nature of *S. aureus* serves as a foundation for studying *Staphylococcus aureus* and respective *S. aureus* bacteriophages in any local group in the United States. This study thus investigated the population of Coastal Carolina University (CCU), a higher educational institution situated in Conway, South Carolina. When taking into consideration the striking ubiquitous nature of bacteriophages in conjunction to the potential *S. aureus* reservoir amongst the CCU populace, *Staphylococcus aureus* bacteriophages were predicted to be prevalent on randomly sampled volunteers from CCU. In addition, *Escherichia coli* phage, or coliphage, populations were also investigated using the same samples provided by volunteers; the presence of coliphages are indicative of the presence of *E. coli* strains, of which serve as indicators not only of fecal contamination, but temporal competition to the commonly-found *Staphylococcus aureus* bacterial strains expected to colonize students. Thus, coliphage populations were simultaneously studied in conjunction with *S. aureus* phage populations on individual student volunteers.

Investigation of coliphage and *S. aureus* phage populations was conducted from 2014 to 2018. Over this five-year period, 225 randomly selected volunteers from Coastal Carolina University were swabbed behind ears and inside nasal capacities. Samples were filtered and amplified. Microbial tests were conducted in order to detect lytic activity, while molecular techniques were used in order to confirm the presence of specific bacteriophages. The purpose of this study was to address two questions about bacteriophages: does the Coastal Carolina University population contain bacteriophages that are lytic to *Staphylococcus aureus* and, if so, can these bacteriophages be isolated and identified based on polymerase chain reaction (PCR) techniques? Additionally, does the CCU community contain coliphages lytic to *Escherichia coli*, and can these phages be characterized via PCR analysis?

In addition to this sporadic study, a long-term pilot investigation into phage population fluctuations was initiated in 2017. Fifteen volunteers from Coastal Carolina University were randomly selected and sampled once a month from October of 2017 to March of 2018 in order to investigate coliphage and *S. aureus* phage population dynamics. The purpose of this pilot study was to provide a general understanding of bacteriophage population dynamics over an academic year, as fluctuations were informally noted in previous years but never quantified.

Methods and Materials

Collection and Filtration of Sporadic Samples

Ear and nose samples were collected from 225 Coastal Carolina University volunteers over a five-year period from 2014 to 2018. Swabs were taken behind the ear and inside the nasal cavity for each volunteer. Swab samples were then placed in 1.5 mL microcentrifuge tubes (Carolina Biological Supply Company) containing 750 µL of phosphate buffer saline (PBS Tablets, Calbiochem), and were incubated at room temperature (25°C) for 30 minutes. Sterile filtration of PBS samples was performed using a 25 mm syringe filter with 0.2 µM PTFE membrane (VWR International) attached to a 10 mL syringe with Luer-Lok Tip (BD). PBS samples were filtered into sterile 1.5 mL microcentrifuge tubes.

Collection of Long-Term Samples

Fifteen (15) individuals from Coastal Carolina University volunteered samples on a monthly basis from October 2017 to March 2018. Swabs were taken from behind the ear and inside the nasal cavity; processing procedures were identical to those utilized during the sporadic study. Volunteers remained anonymous throughout the study and were given numbers as a means of identification. This pilot investigation was conducted in conjunction with the sporadic study.

Amplification of Viral Particles

Amplification of viral particles was conducted using both *Escherichia coli* B and *Staphylococcus aureus* cultures, and was performed via addition of 100 uL filtered sample to a 15 mL culture tube (Thermo Fisher) containing 3.0 mL of LB Miller broth (Sigma-Aldrich) and 200 uL commercial *Escherichia coli* B (Carolina Biological Supply Company). Such amplification was repeated using commercially-supplied *Staphylococcus aureus* (Carolina Biological Supply Company) as host.

Plaque Assays

Plaque assays were prepared using sterile LB Miller broth and agar (Agar Grade A, BD) cell culture plates. Bacterial lawns were established on individual plates via addition of 75 µL of log-phase *S. aureus* liquid culture, of which was evenly distributed via a sterile spread plate technique. Spread plates were then incubated at 37°C for 30 minutes. Five microliters (5 µL) of amplified viral sample was added to three marked locations on a given plate, whilst 5 µL of PBS served as negative control for a fourth designated location on the plate. Amplified viral aliquots were dried for 10 minutes at 37°C. Plaque assays were then incubated overnight at 37°C. Analysis of plaque formation, if present, was conducted the following day. These plaque assay procedures were repeated using *E. coli* as bacterial host.

DNA Extraction

For viral capsid cleavage, 100 µL of amplified sample was placed into a sterile 1.5 mL microcentrifuge tube containing 10 µL of Proteinase K (ThermoFisher). Samples were vortexed and allowed to incubate for one hour at 37°C. Denaturation of Proteinase K enzymes was induced by placing samples in a heating block for 10 minutes at 95°C. Viral capsid cleavage was conducted for both *S. aureus* and *E. coli* samples.

Polymerase Chain Reaction

Polymerase chain reaction was conducted in a 50 uL reaction

volume consisting of 25 µL of GoTaq Green Master Mix (Promega Corporation), 21 µL of template (treated sample), and 4 uL of a respective primer set (See Table 1) to a 0.2 mL microcentrifuge tube (VWR International). PCR analysis was performed using a Bio-Rad Laboratories, Inc., MJ Mini Thermal Cycler. The 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. The final PCR product was then held at 4°C for temporary storage. PCR analysis was conducted for both *S. aureus* and *E. coli* phage samples. Coliphage primer sets included CPA, CPB, and CPO; *S. aureus* phage primer sets included SPA and SPB.

Gel Electrophoresis and Gel Imaging
Examination of both *S. aureus* and *E. coli* PCR products was then conducted using a 2% agarose gel (Agarose I, VWR) in 1x Tris-acetate-EDTA (TAE) buffer solution. The gel was stained with 5 µL of ethidium bromide. Five (5 µL) of PCR product and 2 µL of 1 kb DNA ladder (Promega Corporation) was loaded to designated wells. The agarose gel was run for 60 minutes at 120 volts and then examined under UV light via the Molecular Imager ChemiDoc XRS+ Imaging System from Bio-Rad Laboratories, Inc.

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 [33]. Primers ORF23 and ORF43 that constitute primer set CPO were developed by Dr. Paul E. Richardson's lab at Coastal Carolina University. Primers such as 3A-Like, Twort-Like, 11-Like, and 77-Like that comprise primer sets SPA and SPB were derived from R. Pantůček, et al. [34]. 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. The 4 µL individual reaction for *S. aureus* primer set SPA consisted of 0.5 µL 3A-Like For, 0.5 µL 3A-Like Reverse, 0.5 µL Twort Forward, 0.5 µL Twort Reverse, and 2.0 µL of nuclease-free water. The 4 µL individual reaction for *S. aureus* primer set SPB consisted of 0.5 µL 11-Like Forward, 0.5 µL 11-Like Reverse, 0.5 µL 77-Like Forward, 0.5 µL 77-Like Reverse, and 2.0 µL of nuclease-free water.

Results

Sporadic Project: S. aureus phages
From 2014 to 2018, an average of 42.9% of all *Staphylococcus aureus* plaque assays were observed to have indications of lytic activity (Table 2). Positive *S. aureus* plaque assays ranged from 23.3% of all 30 samples collected in 2014 to 66.7% of all 60 samples collected in 2017, with a standard deviation of 16.1%. Of the 225 samples collected over the designated five-year period, 21.8% and 45.3% of all *S. aureus* samples tested positive using molecular-based (PCR) and microbial-based techniques, respectively. Positive PCR results ranged from 8.57% of all 35 samples in 2016 to 32.7% of all 30 samples collected in 2014, with a standard deviation of 10.8%. Of the total 102 lytic *S. aureus* plaque assays analyzed, only 48.0% could be characterized via the consensus sequence primers delineated in Table 1.

Sporadic Project: E. coli phages
From 2014 to 2018, an average of 36.8% of all *Escherichia coli* plaque assays were observed to have indications of lytic activity, as delineated in Table 3. Positive *E. coli* plaque assays ranged from 20.0% of all 45 sporadic samples collected in 2018 to 51.7% of all 60 samples collected in 2017, with a standard deviation of 14.4%. Of the 225 samples collected from 2014 to 2018, 12.9% and 36.4% of all *E. coli* samples tested positive using PCR and microbial techniques, respectively. Positive PCR results ranged from 0.0% of all 35 samples in 2016 to 36.7% of all 60 samples collected in 2017. Of the total 82 lytic *E. coli* plaque assays analyzed, only 35.4% could be characterized via consensus sequence primers represented in Table 1.

Long-Term Project Pilot Study
Immense fluctuations in both *S. aureus* and *E. coli* phages were observed between October 2017 and March 2018 (Figures 1-2). The greatest number (N) of positive *S. aureus* phage samples were predominantly observed in the months of October (N_{SE}=13, N_{SN}=14, Figure 1; N_{SE}=5, N_{SN}=4, Figure 2) and November (N_{SE}=5, N_{SN}=6, Figure 1; N_{SE}=1, Figure 2), after which the local *S. aureus* phage population staved off to zero for both nose and ear samples while the local coliphage population upsurged in February (N_{CE}=2, N_{CN}=2, Figure 1; N_{CE}=0, N_{CN}=3, Figure 2) and March (N_{CE}=5, N_{CN}=1, Figure 1; N_{CE}=7, N_{CN}=3, Figure 2).

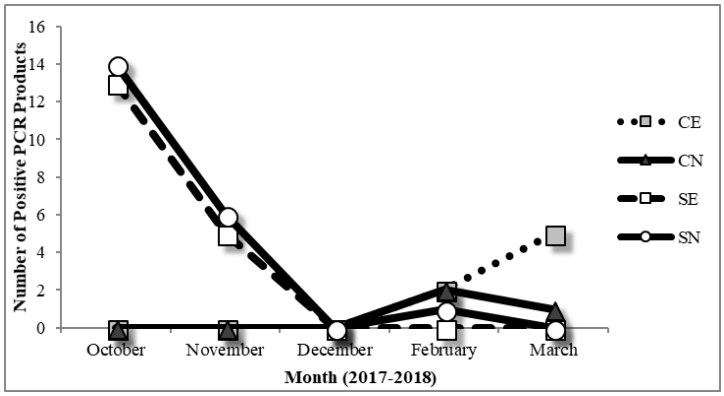

Figure 1. Summary of positive coliphage ear (CE), coliphage nose (CN), *S. aureus* phage ear (SE), and *S. aureus* phage nose (SN) PCR samples from October 2017 to March 2018 in long-term pilot study. No long-term data collected for January 2018.

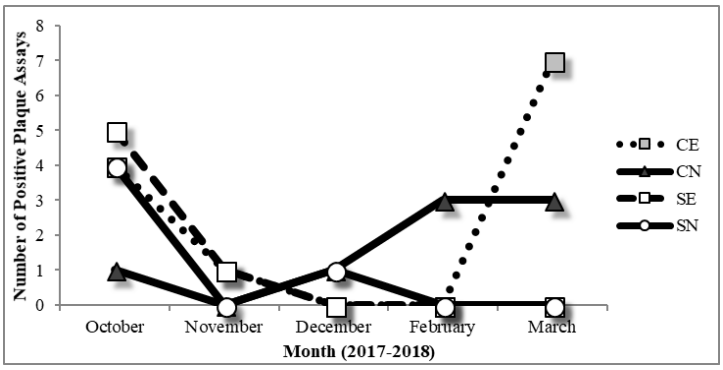

Figure 2. Summary of positive coliphage ear (CE), coliphage nose (CN), *S. aureus* phage ear (SE), and *S. aureus* phage nose (SN) plaque assays from October 2017 to March 2018 in long- term pilot study. No long-term data collected for January 2018.

Discussion

The Coastal Carolina University population was found to contain bacteriophages lytic to *Staphylococcus aureus*, as is indicated by an average of 45.3% of all *S. aureus* plaque assays showing positive lytic activity (Table 2) in plague assays. Yet, of these 102 lytic *S. aureus* assays, only 49 samples (48.0%) were identified via PCR analysis. All 49 samples were classified as 77-Like phages; no other characterizations such as 3A-Like, 11-Like, or Twort-Like were discerned. Such a disparity in molecular phage identification can be attributed to the *S. aureus* primer sets used: primer sets SPA and SPB, as extracted from R.

Table 1. Summary of primer sets used. Primers comprising primer sets SPA and SPB were extracted from R. Pantůček, et al. [34]. Primers comprising primer sets CPA and CPB were derived directly from the work of Hee Suk Lee [33]. Primer set CPO was developed by Dr. Paul E. Richardson, the principal investigator (PI) of this study.

Primer Set	Target Family/ Organism	Gene Target	PCR Fragment Length (bp)	Primer Name	Primer Size (bp)	Primer Sequences (5' to 3')
CPA	*Podoviridae*	CKV1F, gp34	2110	K1FFor	16	TGGAAGCCCGTGAGAC
				K1FRev	18	GCAGCGTCAATCGCTCGG
	Podoviridae	933Wp09, *hkaG* gene	488	933For	18	GCAATACATCAAACGCCG
				933Rev	16	GCGAATGCCAGCGGCG
	Microviridae	Hypothetical protein	1039	MicroFor	25	GCTGCCGTCATTGCTTATTATGTTC
				MicroRev	25	GYTAYCGBMMCATYAAYTAHTCACG
	Myoviridae	Major head protein (gene 23)	704	T4setFor	20	GATATTTGTGGYGTTCAGCC
				T4setRev	24	GTCAAATACACCAGCTTTAGAACC
	Siphoviridae	cII protein	177	HKsetFor	20	CACAGCGAGAAATTGATCGC
				HKsetRev	19	CTAATCGGACTGATGTCTG
CPB	*Myoviridae*	Tail fiber gene (MUP49)	171	MusetFor	21	GAAAACGACTCAATCCTTGCC
				MusetRev	20	TCATCAGGTCTTTTGTTGTGG
	Podoviridae	Hypothetical protein	2285	N4For	20	GCACATGCAGAATAAGGTTG
				N4Rev	20	CCATTAGTAACACCATCTGC
	Siphoviridae	Tail fiber protein	878	JKsetFor	16	GYGAYCAGATGGTTCC
				JKsetRev	16	CAATRTCYTCYTARTTG
	Siphoviridae	B gene	307	LambdaFor	20	TGGGCGTACTTTATGGGGCG
				LambdaRev	20	CGGACCTGCTGGGCAAAAAT
CPO	Coliphage T2/ T4	ORF 23 (Major capsid protein)	405	ORF23For	20	TGGCGCAGTAACTCAGATTG
				ORF23Rev	20	GCACAGCTTCCATTTGTTT
	Coliphage T2/ T4	ORF 43 (DNA polymerase)	198	ORF43For	20	CCCTGCGCCTTTCATAATAA
				ORF43Rev	20	ATCGCAGGAACAGCTCCTAA
SPA	3A-like phages	Tail Fibers	744	3A-like For	20	TATCAGGCGAGAATTAAGGG
				3A-like Rev	23	CTTTGACATGACATCCGCTTGAC
	Twort-like phages	Major capsid protein	331	Twort-like For	20	TGGGCTTCATTCTACGGTGA
				Twort-like Rev	23	GTAATTTAATGAATCCACGAGAT
SPB	11-like phages	Hypothetical tail proteins	405	11-like For	22	ACTTATCCAGGTGGCGTTATTG
				11-like Rev	23	TGTATTTAATTTCGCCGTTAGTG
	77-like phages	Hypothetical tail proteins	155	77-like For	19	CGATGGACGGCTACACAGA
				77-like Rev	23	TTGTTCAGAAACTTCCCAACCTG

Table 2. Sporadic project summary of *S. aureus* microbial (plaque assay) and molecular (PCR) results by year. *Molecular tests were not conducted during the 2014 study year.

Year	# of Samples	# of Positive *S. aureus* Microbial Tests	% of Positive *S. aureus* Microbial Tests	# of *S. aureus* Positive Molecular Tests	% of Positive *S. aureus* Molecular Tests
2014	30	7	23.3%	N/A*	N/A*
2015	55	21	38.2%	18	32.7%
2016	35	17	48.6%	3	8.57%
2017	60	40	66.7%	18	30.0%
2018	45	17	37.8%	10	22.2%
Total	**225**	**102**	**45.3%**	**49**	**21.8%**
Yearly Average	**45**	**20.4**	**42.9%**	**12.25**	**23.4%**

Table 3. Sporadic project summary of *E. coli* microbial (plaque assay) and molecular (PCR) results by year. *Molecular tests were not conducted during the 2014 study year.

Year	# of Samples	# of Positive *E. coli* Microbial Tests	% of Positive *E. coli* Microbial Tests	# of *E. coli* Positive Molecular Tests	% of Positive *E. coli* Molecular Tests
2014	30	12	40.0%	N/A*	N/A*
2015	55	13	23.6%	7	12.7%
2016	35	17	48.6%	0	0.0%
2017	60	31	51.7%	22	36.7%
2018	45	9	20.0%	0	0.0%
Total	**225**	**82**	**36.4%**	**29**	**12.9%**
Yearly Average	**45**	**16.4**	**36.8%**	**7.25**	**12.4%**

Pantůček, et al., consisted of conserved genomic sequences characteristic only of *S. aureus* phage types 3A, 11, 77, and Twort, of which are representative of bacteriophage serogroups A, B, F, L, and D. Serogroups A, B, and F comprise temperate phages that may enter a lytic cycle in favorable environmental conditions, and thus may only prove useful in *S. aureus* phages that make use of both lysogenic and lytic cycles. This study, it must be noted, principally focused on phages lytic to *Staphylococcus aureus*, albeit temperate phages were simultaneously characterized via PCR analysis, as well, as many non-lytic *S. aureus* samples were shown to be positive for lysogenic strains. Furthermore, *S. aureus* primer sets derived from R. Pantůček, et al., only represent five out of the eleven *S. aureus* phage serogroups [35]. Thus, the fact that only 48.0% of all lytic *S. aureus* plaque assay samples could be identified and characterized via PCR analysis may be due to utilizing primer sets unrepresentative of every *Staphylococcus aureus* phage serotype. Another speculation may be that the *S. aureus* phage human reservoir has yet to be thoroughly explored, as only around 250 staphylococcal bacteriophages have ever been described in literature; likewise, *S. aureus* phages that have thus far been characterized, in turn, were determined to have high diversity and mosaicism in genomic constitution resulting from recombination and horizontal transfer of sequences [34]. Thus, the *S. aureus* phage reservoir appears to be not only rich in the number of clandestine lytic constituents, but potentially abundant in a diverse repository of staphylococcal phages yet to be characterized. Exploration of novel primers may improve such molecular phage characterization, and thus provide improved insight into the depth of the *S. aureus* phage cistern and its promising utility against *Staphylococcus aureus* and its antibiotic resistant variants.

As aforementioned, an average of 42.9% of all *S. aureus* plaque assays had indications of lytic activity. Assuming automatic phage presence amongst naturally-occurring *S. aureus* colonies in human populations, this statistic generally coincides with the data published by the American College of Physicians (ACP) of which, using extrapolated data, predicted *Staphylococcus aureus* cultures colonize around 31.6% of individuals in the United States [38]. This study focused on the university population of individuals living in an environment comprised of a stressful atmosphere and a higher population density, of which may contribute to an inflation in the human community phage reservoir. A more pronounced phage population, in turn, can be attributed to stress-related immunosuppression, stress-induced susceptibility to bacterial infections, and therefore elevated phage production and increased phage detection rates.

Analogously, of the 82 lytic *E. coli* plaque assays analyzed, only 35.4% could be identified and characterized via PCR analysis (Table 3). Such a divergence may also be attributed to the primer sets used, as CPA, CPB, and CPO primer sets represent somatic coliphages belonging to the following taxonomic families: *Podoviridae, Microviridae, Myoviridae,* and *Siphoviridae*, along with ICTV type T4, of which represents its own group. The discrepancy in molecular identification of lytic coliphages could therefore be associated with primer sets that appear inadequate in representing the extremely diverse group of somatic coliphages.

A reservoir of coliphages appears to be prominent amongst the Coastal Carolina University human population, as 36.8% of all volunteers sampled between 2014 and 2018 were found to be positive for coliphages (Table 3). Forty-five percent (45%) of all positive PCR products were detected using the CPO primer set, of which includes primers ORF 23 and ORF 43 that encode a major capsid protein and T-even DNA polymerase enzyme, respectively. Fifty-five percent (55%) of all positive coliphage PCR products were identified via CPA and CPB primer sets. Such discrepancy can be described in terms of the specificity of the target genes of primer sets: the CPO primer set targets highly conserved T-even phage consensus sequences encoding the T-even DNA polymerase and a T-even major capsid protein. Primer sets CPA and CPB, originally derived from the work of Hee Suk Lee, were developed for the purpose of optimizing group-specific PCR detection of coliphage families that were predominantly found in fecal contaminated water settings [33]. Local coliphage detection via utilization of primer set CPO has been confirmed by Cannon, et al., [36], while coliphage detection using CPA and CPB has been confirmed in Chapel Hill, North Carolina, an area relatively close in distance and climate to Coastal Carolina University [33]. Such identification of phages in diverse locations suggests that sampled bacteriophages appear widely distributed, as members of identified taxonomic groups using specific primer sets CPA, CPB, and CPO have not only been found in water sources, as suggested by Cannon, et al. [36], and Lee [33], but also amongst the human population, as confirmed and delineated by this study. Transportation schemes of coliphages between water sources, fecal matter, and the human population remain unknown and unquantified, albeit the presence of coliphages remains to be viewed as an indicator of fecal contamination.

The immense profusion of coliphages amongst the Coastal Carolina University population may be the result of relatively high population density and stress-induced immunosuppression, of which may increase susceptibility to bacterial infection and thus elevate localized phage production. Most notably, coliphages can serve as indicator species of fecal contamination, as aforementioned, and therefore may represent individual hygiene level at a given moment of time, and possibly susceptibility to bacterial colonization and thus phage proliferation.

The long-term study circumscribing bacteriophage population fluctuations strove to quantify primordial trends observed in local *S. aureus* and *E. coli* phage population dynamics. *Staphylococcus aureus* bacteriophage numbers were observed to be greatest in number in October of 2017, after which positive *S. aureus* sample numbers for both molecular (Figure 1) and microbial (Figure 2) tests declined. No positive PCR products were observed for coliphage samples from October 2017 to December 2017, albeit 4 positive coliphage PCR products were

observed starting in February of 2018. In March 2018, the last month of observation, 6 positive coliphage molecular samples were detected. A few positive coliphage plaque assays were observed from October 2017 through December 2017, albeit a tremendous escalation in positive samples was seen around February of 2018 (Figure 2). This small pilot study illustrated the prospective notion that coliphage occupancy is capable of representing temporal competition to *Staphylococcus aureus* phages (and vice versa), as *S. aureus* phage populations appear to decline while coliphage populations tend to escalate, as can be visualized by both Figure 1 and Figure 2. Furthermore, this pilot study provided insight into local phage population fluctuations, of which could also potentially be attributed to external factors such as UV exposure, physiological and biological stress, mechanical damage, along with the utilization of various skin products such as acne treatments and sun block.

Conclusion

Plaque assay results for both coliphage and *S. aureus* phage sporadic studies signify that broad detection of bacteriophages appears best with microbial techniques, albeit molecular methods remain valuable for sensitive detection of both lytic and lysogenic phages. Results indicated that an extensive lytic bacteriophage reservoir against *Staphylococcus aureus* can be found in the general populace of Coastal Carolina University, albeit the local *S. aureus* phage population remains dynamic in nature and appears to fluctuate immensely for unknown but speculative reasons. With the growing antibiotic resistant community-associated MRSA threat, harvesting and exploring the utility of bacteriophages, the natural bactericidal products crafted by billions of years of evolution, may prove to be immensely useful to humanity as a whole in its fight against antibiotic resistant infections.

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Notes and References

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