

The effects of salinity, pH, temperature, and dissolved oxygen on sensitivity of PCR identification of T4 bacteriophage

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Bacteriophages are used as indicators of pathogenic bacteria in drinking, and wastewaters. They also show potential in limiting aquatic bacterial populations through their lytic properties. The effect of different water characteristics (salinity, pH, dissolved oxygen, and temperature) on the sensitivity of the PCR identification of virus particles were analyzed to determine at what levels bacteriophage can be detected in environmental samples. Results from this preliminary study indicate that a PCR bacteriophage detection technique has potential as a relatively efficient and economical indicator of coliform contamination in multiple aquatic environments. While further evaluation is needed, the protocol appears to function in both fresh water and saline environments, and a range of abiotic and flux conditions.

Introduction

Bacteriophages, also referred to as phages, are a class of viruses that only infect bacteria. It is estimated that they are the most abundant living entity on this planet, with estimates of their number between 10^{30} to 10^{32} viruses [1]. Every location on the planet that contains bacteria contains bacteriophages. Bacteriophages are critical to the control of bacterial populations and maintaining colony diversity. To understand the dynamics of a bacterial system, detailed understanding of phages must also be considered. Studies have shown that bacteria and phages have a co-evolutionary relationship, an arms race, as one evolves so does the other to counter the challenges each presents [2].

Bacteriophages are a very simplistic entity because they are not a living organism, and they can only replicate when they have infected a host, and as such are a parasite. They take over the cellular operations and use its genetic information to force the cell to produce more phage. After awhile the cell begins to swell with the number of phage particles multiplying in the host, and eventually the cell busts open and the phages begin diffusing for a new bacterial host to infect. In terms of organization, they are the most streamlined. Phages have a protein shell wrapped around their genetic material. Some phages are very simple in shape, but others take on a more science fiction-like appearance. One of these is T4 bacteriophage, a coliphage; a phage that infects *Escherichia coli* (*E. coli*).

E. coli is a common bacterium that is found in water that has been contaminated with fecal matter. Although all water samples have large number of bacteria, it is important to note that many of them are not pathogenic and are benign. This is true for fresh or salt water systems. It is reported that 10^7 phage particles are present per millilitre in coastal estuary systems [3]. These phages are important in limiting bacterial populations and maintaining water quality in these estuary systems.

Water quality issues are important concerns for coastal South Carolina. More than 60% of the nation's estuaries experience water quality problems [4]. This poor water quality may favour the phytoplankton blooms (including harmful algae) and occasional hypoxic/anoxic events (fish kills) that affect our estuaries [5]. Bacteriophages have been used as indicators to predict the presence of pathogenic bacteria in drinking, waste and recreational waters. Some groups have determined that coliphages are as adequate an indicator of faecal pollution as are

actual coliform bacteria counts [6]. Bacteriophages have also shown potential in limiting aquatic bacteria population through their lytic properties. Crothers-Stomps et al. concluded that the treatment of bacterial infection by bacteriophage, commonly referred to as phage therapy, has potential for use to control disease in aquaculture systems [7]. Advantageous properties of phage as therapeutic agents include self-replication, which results in increased concentrations as infection persists, and the narrow host of phage, which prevents harm to beneficial, naturally occurring microflora [8].

While an excellent bacterial pollution indicator in drinking and wastewater settings, little is known about the survival and persistence of bacteriophages in the harsher saline environments of seawater. While the US EPA first approved bacteriophages as surrogates for groundwater aquifer contamination in 1996 the ecology of coliphages in the natural environment remains poorly understood. Research on the survival of coliphages in aquatic ecosystems shows that while coliphages are more resistant to environmental stresses than *E. coli*; the phages remain sensitive to temperature, chemical treatments and salinity [9,10]. Reyes and Jiang found that coliphage occurrence was significantly different between freshwater, estuarine and coastal locations and correlated with water temperature, salinity and rainfall in the watershed [5].

The lower number of bacteriophage present in the saline environment requires a sensitive detection method to be an effective indicator. A polymerase chain reaction (PCR) protocol has been developed by the Richardson laboratory for identifying bacteriophage under ideal conditions in a laboratory. This technique targets two genes in the T4 bacteriophage genome, open reading frame 23 (ORF 23) and open reading 43 (ORF 43). Both of these genes are highly conserved in T-even bacteriophages. Currently the tests have able to detect five virus particles per testing sample on a regular basis, equating to about 230 viruses per millilitre of water sample collected (See Figure 1). The method has also been able to detect as low as two viral particles in testing sample, but the results were not consistent to be confident at that level of viral material.

This study evaluated whether some properties of seawater (salinity, pH and dissolved oxygen content) affected the sensitivity of this PCR method to detect bacteriophages in water collected from environmental samples. The effect of the different water characteristics on the sensitivity of the PCR identification

of bacteriophage particles was analyzed to determine what relationships could be detected in these environmental samples. The water flux from each collection site was considered when looking at the results. Also, this study looked at the effect seawater (salinity, pH, and dissolved oxygen content) has on bacteriophage presence in an estuary.

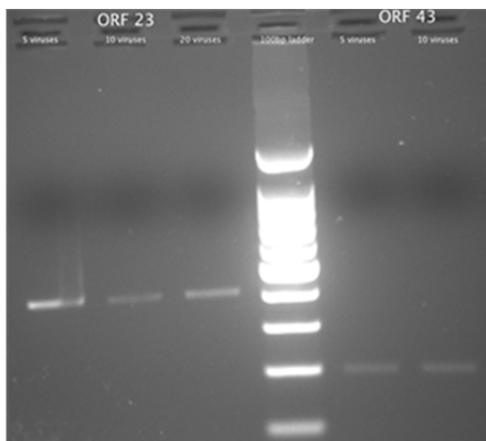


Figure 1. 2% agarose gel, 60volts for 1.5 hours

Method and Materials

Sample Collection

One water and one soil sample were collected from seven South Carolina aquatic sites each week. Two of these sites were located in an Atlantic salt marsh estuary (Hobcaw), and five were located in a moderate flow coastal river, the Waccamaw. The Waccamaw River sites included two low flux backwaters (Sterritt Swamp and Conway River Swamp) and five high flow sites (Reeve's Ferry, Conway Waterfront, Wachesaw Landing, and Hagley Landing). During collection, pH, water temperature, salinity, and dissolved oxygen of the water samples were recorded. Also recorded were weather conditions, air temperature, and other notable visual information at the time of collection. Samples were refrigerated until processing.

DNA Extraction

70µL of sample and 7µL of proteinase K were added to a micro centrifuge tube and incubated at room temperature for 45 minutes on an orbital shaker to expose the T4 bacteriophage DNA. Samples were exposed to a constant temperature of 96.1°C for 10 minutes in a hot block to inactivate the proteinase K and any remaining phage samples.

PCR Amplification and Testing

In a large PCR tube, 25µL of GoTaq Hot start master mix, 2µL of each forward primer (23L and 43L); 2µL of each reverse primer (23R and 43R) and 21µL of sample DNA were combined. The genetic material within the target open reading frames was amplified using a Biorad personal thermocycler. After amplification, the samples were run out on a 1% agarose gel

stained with ethidium bromide at 100 volts for one hour. The gel was imaged using a 100 base pair ladder as reference. A positive result for OFR 23 was shown by a band roughly 400 base pairs in length; a positive result for OFR 43 was shown by band at about 200 base pairs in length.

Results

In the estuarine samples (both soil and water) there was no significant difference in pH and salinity between positive and negative samples. However, temperature showed a significant inverse relationship to the proportion of ORF 43 positive results. The lower the temperature, the higher occurrence of bacteriophage detection was observed (Table 1).

Table 1. Statistical analysis of water and soil samples with the environmental variables recorded.

Variable	Positive Average	Negative Average	F-Test	Degrees Freedom	P-Value
pH	7.78	7.80	0.070	(1,76)	0.79
Temperature (°C)	10.36	17.11	8.24	(1,76)	0.01
Dissolved Oxygen (mg/L)	7.96	7.27	2.39	(1,76)	0.13
Salinity (PSU)	33.91	34.51	1.58	(1,76)	0.21

While not significantly different in terms of the rate of bacteriophage detection for water and soil samples, there was a significant inverse relationship between water temperature and dissolved oxygen concentration ($R^2 = 0.614$, $F_{(1,59)} = 93.9$, $P < 0.001$) at the Hobcaw sites. Rainfall data collected at Hobcaw showed that bacteriophage detection increased as 24 hour precipitation increased ($P = 0.017$) (Table 2).

Table 2. Twenty-four hour rainfall totals before the days of collections.

Variable	Positive Average	Negative Average	F-Test	Degrees Freedom	P-Value
24hr rainfall (mm/day)	11.76	2.63	6.02	(1,56)	0.02

In the coastal riverine environment, little variability in salinity was observed and had no apparent impact of the rate of T4 bacteriophage detection. While differences were observed in pH, water temperature and dissolved oxygen across the river sites, no significant patterns of T4 phage detection were associated with these abiotic variations (Data not shown).

In the coastal riverine environment, the confirmation of T4 bacteriophage presence occurred at a significantly higher rate (20.68%) in the low flux environment of the backwater swamp and primary embanked streamlet (Conway River Swamp and Sterritt Swamp) than in the high flux tertiary stream sites of the Waccamaw River (11.11%) (Reeve's Ferry, Conway Waterfront, Wachesaw Landing, and Hagley Landing) ($F_{44,57} = 0.601$, $P > 0.05$). This percent occurrence is similar to the 12.8% detection rate found in the estuarine marsh at Hobcaw Barony, where semi-diurnal tidal flux is high (Table 3).

Table 3. Results from the PCR testing (ORF 23 and ORF 43) for each site collected and sample collected.

Site	# samples	ORF 23 detected	ORF 43 detected	% ORF 23 detected	% ORF 43 detected	Flux Rate
Hobcaw A	48	4	6	8.3%	12.5%	Tidal
Hobcaw B	32	4	5	12.5%	15.6%	Tidal
Hobcaw C	6	0	0	0.0%	0.0%	Tidal
Lower River	6	0	0	0.0%	0.0%	High
Reeve's landing	24	1	3	4.2%	12.5%	High
Conway Waterfront	16	1	3	6.3%	18.8%	High
Conway River Swamp	33	7	10	21.1%	30.3%	Low
Sterritt Swamp	26	3	4	11.5%	15.4%	Low

T4 bacteriophage detection by PCR amplification was found to be more sensitive in water samples than in soil samples. Water samples showed a 21.3% detection rate compared to a 4.6% detection rate in soil samples (Table 4). PCR using ORF 43 demonstrated an enhanced sensitivity to T4 bacteriophage detection compared to ORF 23 expression (Table 4).

Table 4. Comparing sample type (water or soil) to which ORF was positive.

Sample type	# samples	ORF 23 detected	ORF 43 detected	% ORF 23 detected	% ORF 43 detected
Soil	65	1	3	1.5%	4.6%
Water	127	19	27	15.0%	21.3%
Total	192	20	30	10.4%	15.6%

In all samples that were positive, there was not a situation where ORF 23 was positive while ORF 43 was not. In the 10 cases where ORF 23 detection was negative while ORF 43 detection was positive, two were soil samples and eight were water samples. These 10 samples came from various flux conditions and river/estuary locations.

Discussion

The PCR bacteriophage detection technique demonstrated potential as a relatively efficient and economical indicator of coliform contamination in multiple aquatic environments. It was capable of identifying the presence of the T4 bacteriophage across a wide variety of salinities, pHs, water temperatures and dissolved oxygen concentrations.

The finding that lower water temperature was correlated with higher oxygen concentration in the estuary samples is in keeping with the higher gas solubility of lower temperature waters. This indicates that the positive bacteriophage identification rates found in colder waters may be associated in part with higher oxygen concentrations and not exclusively with lower water temperatures. With the higher concentration of oxygen in water, the physiologic conditions of the host *E. coli* may have been at a more favorable metabolic state for occurrence of a more productive burst size or faster lytic cycle completion.

The higher rates of identification of T4 bacteriophage following rainfall events in the estuarine samples are consistent with increased nutrient and fecal contamination runoff from the land during the initial phases of a rain event. These rain events may have produced a more productive environment for bacterial

growth in the estuary that was flushed only tidal flow. The lower incidence of positive bacteriophage identification following rainfall events in the river environment may have been indicative of increased dilution and flux in the river caused by the addition of rainfall and runoff which continued downstream. This is consistent with the finding of decreased T4 bacteriophage identification in high flux sites. Natural flushing may inhibit the capacity of the bacteriophage to infect any bacteria present. The completion of phage lytic infection is limited by adsorption. Phage adsorption rate is dependent on bacterial host and phage density. As flushing increases, the concentrations of both factors are decreased, which likely leads to decreased rate of phage adsorption and limitation of phage density. Taking into account the optimal growth conditions of the phage host, nutrient limitations through dilution of can reduce production of phage progeny by means of inhibition of lysis or reducing burst size, further decreasing viral density. The results indicated that T4 bacteriophage detection by PCR amplification is much more sensitive in water samples than in soil samples. The completion of the bacteriophage lytic cycle may have been limited by the rate at which it can adsorb to the host bacterium. Soil may have served as a physical barrier between the bacteriophage and its host, particularly in the higher flux environments. With limited ability of diffusion by the bacteriophage and its host in soil versus water, adsorption to the host may have been decreased, contributing to potential lower concentration of bacteriophage in soil. With no purification steps from crude soil samples, it is possible that metal ions and other chemical species could have remained in the sample to decrease Proteinase K and TAQ polymerase activity or disrupt the integrity of the target primer binding sites.

There was evidence of enhanced sensitivity to PCR detection expressed by ORF 43 compared to ORF 23. ORF 43 is more conserved than open reading frame 23. Higher conservation of open reading frame 43 among phage species can lead to a higher probability that the complementary sequence of ORF 43 primers will exist and a successful amplification will occur.

A major limitation of the study was the lack of associated bacterial identification. Without this correlation with bacterial presence and concentration, the actual selectivity and sensitivity of the PCR bacteriophage protocol as an indicator of coliform presence could not be assessed. The sampling period was also seasonally limited with estuarine samples taken between late August and March and riverine samples limited to the September–March period. This limited the variability in temperature,

rainfall and seasonal human impact that could affect coliform concentrations at the sample locations. These limitations are being addressed in a subsequent study where *E. coli* and coliform counts are collected for each water sample and the sampling period has been extended to cover all seasons.

Conclusions

Results from this preliminary study indicate that a PCR bacteriophage detection technique has potential as a relatively efficient and economical indicator of coliform contamination in multiple aquatic environments. While further evaluation is needed, the protocol appears to function in both fresh water and saline environments, and a range of abiotic and flux conditions.

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