Food and Environmental Virology (2019) 11:101–112
https://doi.org/10.1007/s12560-019-09365-5

ORIGINAL PAPER

Bacteriophages Against Pathogenic Vibrios in Delaware Bay Oysters (*Crassostrea virginica*) During a Period of High Levels of Pathogenic *Vibrio parahaemolyticus*

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Received: 6 September 2018 / Accepted: 7 January 2019 / Published online: 31 January 2019

Abstract

Eastern oysters (*Crassostrea virginica*) from three locations along the Delaware Bay were surveyed monthly from May to October 2017 for levels of total *Vibrio parahaemolyticus*, pathogenic strains of *V. parahaemolyticus* and *Vibrio vulnificus*, and for strain-specific bacteriophages against vibrios (vibriophages). The objectives were to determine (a) whether vibriophages against known strains or serotypes of clinical and environmental vibrios were detectable in oysters from the Delaware Bay and (b) whether vibriophage presence or absence corresponded with *Vibrio* abundances in oysters. Host cells for phage assays included pathogenic *V. parahaemolyticus* serotypes O3:K6, O1:KUT (untypable) and O1:K1, as well as clinical and environmental strains of *V. vulnificus*. Vibriophages against some, but not all, pathogenic *V. parahaemolyticus* serotypes were readily detected in Delaware Bay oysters. In July, abundances of total and pathogenic *V. parahaemolyticus* at one site spiked to levels exceeding regulatory guidelines. Phages against three *V. parahaemolyticus* host serotypes were detected in these same oysters, but also in oysters with low *V. parahaemolyticus* levels. Serotype-specific vibriophage presence or absence did not correspond with abundances of total or pathogenic *V. parahaemolyticus*. Vibriophages were not detected against three *V. vulnificus* host strains, even though *V. vulnificus* were readily detectable in oyster tissues. Selected phage isolates against *V. parahaemolyticus* showed high host specificity. Transmission electron micrographs revealed that most isolates were ~60-nm diameter, non-tailed phages. In conclusion, vibriophages were detected against pandemic *V. parahaemolyticus* O3:K6 and O1:KUT, suggesting that phage monitoring in specific host cells may be a useful technique to assess public health risks from oyster consumption.

Keywords Bacteriophage · Oysters · *Crassostrea virginica* · *Vibrio parahaemolyticus* · *Vibrio vulnificus*

Introduction

The Eastern oyster (*Crassostrea virginica*) is a mainstay of the commercial shellfish industry on the U.S. East and Gulf Coasts. Despite their popularity, oysters occasionally cause

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illness from bacteria, viruses, and parasites (DePaola et al. 2010; Marquis et al. 2015) that become concentrated within their edible tissues. Over the years, the availability of oysters has been negatively impacted by outbreaks of *Vibrio parahaemolyticus*, which typically lead to closure of shellfish harvesting areas and product recalls. Events which trigger the proliferation of *V. parahaemolyticus* are not fully understood but include elevated seawater temperatures and low salinities (Kaneko and Colwell 1973; DePaola et al. 1990, 2003a; Johnson et al. 2010; Elmahdi et al. 2018; Whitaker et al. 2010).

Members of the genus *Vibrio* are Gram-negative, gammaproteobacteria that are indigenous to the marine environment. Depending on species, some vibrios are pathogenic to humans as well as fish, shellfish and other marine life. *V. parahaemolyticus* is considered the primary cause of shellfish-related bacterial illness in the U.S., while *Vibrio vulnificus* causes over 95% of seafood-related deaths in the U.S. (Centers for Disease Control and Prevention 2010, 2016). *V. parahaemolyticus* is most often transmitted by the consumption of raw or lightly cooked fish and shellfish, while *V. vulnificus* is transmitted by two routes: through the consumption of contaminated fish and shellfish, principally oysters, and through wound infections acquired in the marine environment. Other vibrios that cause disease in humans include *V. cholerae, V. alginolyticus, V. hollisae, V. metschnikovii, V. mimicus*, and the flesh-eating *Vibrionaceae Photobacterium damselae*. All of these vibrios can inhabit shellfish-growing areas, particularly during the warmer months. Virulence factors for the pathogenic serotypes of *V. parahaemolyticus* include the thermostable direct hemolysin (TDH) and the TDH-related hemolysin (TRH) coded for by the *tdh* and *trh* genes, respectively (Miyamoto et al. 1969; Shirai et al. 1990; Honda et al. 1988, 1991; Tada et al. 1992; Honda and Iida 1993). A species-specific marker for *V. parahaemolyticus* is the thermolabile hemolysin (TLH) and associated gene (*tlh*). Levels of tlh^+ *V. parahaemolyticus* exceeding a most probable number (MPN) count of 10,000/g can trigger enforcement actions by regulatory authorities (U.S. Food and Drug Administration 2017 revision). Pathogenic strains of *V. vulnificus* can be determined by the presence of the virulence-correlated gene type C (*vcgC*), since this clinical genotype is highly correlated with disease, but the environmental genotype (*vcgE*) is not (Rosche et al. 2005; Warner and Oliver 2008a, b).

Factors that appear to modulate *Vibrio* levels in oysters include several types of naturally occurring predatory bacteria (Richards et al. 2016, 2017) and bacteriophages (phages) (reviewed in Kalatzis et al. 2018; Zhang et al. 2018). Phages are bacterial viruses that are in exceedingly high levels in most environments. Phage isolates against vibrios (vibriophages) and other bacterial pathogens are increasingly being evaluated to reduce mortalities in aquaculture systems (reviewed in Oliveira et al. 2012; Richards 2014). Baross et al. (1978) identified and quantified phages against *V. parahaemolyticus* in Pacific oysters (*Crassostrea gigas*) from Oregon and Washington State and isolated phages from 35% of environmentally harvested Pacific oysters and most shucked market oysters (*Ostrea lurida*). Many phages have narrow host specificities often limited to members of a genus or species, or frequently just a few strains within a species. Vibriophages against *V. parahaemolyticus* strains were also isolated in Pacific oysters from British Columbia, Canada, where they were detected throughout the year (Comeau et al. 2005). Phages against *V. vulnificus* have also been isolated at high densities in Eastern oysters from the Gulf Coast (DePaola et al. 1997, 1998; Pellon et al. 1995). No studies were identified on the prevalence of vibriophages in Eastern oysters along the Mid-Atlantic Coast or the Delaware Bay. In the marine environment, one would expect phages against bacteria that are commonplace to be detected more frequently than phages against uncommonly found bacteria. Unfortunately, little is known about the influence of phages on the modulation of human pathogenic vibrios in seawater and shellfish.

The objectives of this study were to: (a) determine whether vibriophages against known strains or serotypes of clinical and environmental vibrios were detectable in oysters collected from three sites along the Delaware Bay from May to October 2017, and (b) whether vibriophage presence or absence in oysters corresponded with the abundance of total or clinical *V. parahaemolyticus* and clinical or environmental strains of *V. vulnificus*. Selected phage isolates were also characterized for host specificity against a variety of *Vibrionaceae* and by transmission electron microscopy.

Materials and Methods

Vibrio Host Cells

The vibrios used for initial screening for phages in oyster homogenates are listed in Table 1 along with *Vibrionaceae* used in follow-up host specificity testing of selected phage isolates. Together, these bacteria include a variety of important human and fish pathogens. *Vibrio* stock cultures were typically grown on Luria–Bertani agar or broth (Fisher Scientific, Park Lawn, NJ, USA) made with 30 ppt natural seawater that had been autoclaved and 0.22-µm filtered before use. Incubation was at 26 °C and broth tubes were swirled at 175 rpm.

Oyster Collection

Subtidal oysters were sampled monthly from May through October, 2017, inclusive, from three sites along the coast of

Table 1 Vibrios used in this study

Bacterial host cell	Designation[a]	Serotype/genotype	Origin	Source
For initial screening from oyster homogenate				
V. parahaemolyticus	RIMD2210633	O3:K6, *tdh*$^+$/*trh*$^-$	Japan	University of Delaware
	DIE12 052499	O1:KUT, *tdh*$^+$/*trh*$^+$	Alabama	FDA
	DAL 1094	Non-O3:K6, *tdh*$^-$/*trh*$^-$	Unknown	FDA
	ATCC 17802	O1:K1, *tdh*$^-$/*trh*$^+$	Japan	ATCC
V. vulnificus	364 Environmental		Hawaii	University of Florida
	367 Environmental		Hawaii	University of Florida
	1003 Clinical		Louisiana	University of Florida
For use in host specificity studies of isolated phages				
V. parahaemolyticus	ATCC 49529	O4:K12	California	ATCC
V. vulnificus	362 Environmental		Hawaii	University of Florida
Vibrio cholerae		O1	Unknown	USDA
		O139	Unknown	ICDDR-B
Vibrio alginolyticus	FDA 22		Unknown	FDA
	ATCC 17749		Japan	ATCC
V. hollisae	89A 1960		Unknown	FDA
V. metschnikovii	ATCC 7708		Unknown	ATCC
V. mimicus	ATCC 33654		Louisiana	ATCC
Photobacterium damselae ssp. *damselae*			Delaware	USDA

ATCC American type culture collection, Manassas, VA, *FDA* U.S. Food and Drug Administration, Dauphin Island, AL, *tdh* thermostable direct hemolysin (TDH) gene, *trh* TDH-related hemolysin gene, *USDA* U.S. Department of Agriculture, Dover, DE, *ICDDR-B*, International Center for Diarrhoeal Disease Research—Bangladesh, Dhaka, Bangladesh

the Delaware Bay: a dock on the Broadkill River in Lewes, DE (decimal degrees: 38.7907423°, −75.1642248°), a dock on the Murderkill River in Bowers, DE (39.057232; −75.3991030) and a dock in the town of Slaughter Beach, DE, USA (38.947050; −75.314571) (Fig. 1). Oysters were collected monthly from each study site and were immediately placed in Ziploc bags (SC Johnson & Sons, Racine, WI, USA) and chilled in an insulated cooler with ice packs. A sheet of bubble wrap was placed between the oysters and the ice to prevent direct contact of samples with the ice packs. Shipping temperature of the cooler was monitored using a SmartButton data logger (ACR Systems, Inc., Surrey, British Columbia, Canada) and the temperature was always maintained at < 10 °C, where 10 °C is considered the minimum temperature for *Vibrio* growth during transport. Oysters were then transported to the laboratory within 3 h prior to analysis.

Most Probable Number–PCR Assays for Vibrios

Vibrio parahaemolyticus and *V. vulnificus* were detected in oysters by the most probable number (MPN) technique (Blodgett 2010) according to the National Shellfish Sanitation Program (NSSP) Guide for the Control of Molluscan Shellfish (2017 revision) followed by confirmation of growth-positive enrichment tubes using qPCR. In essence, oysters and their liquor were diluted tenfold in phosphate-buffered saline, homogenized at high speed for 1 min and serially diluted tenfold to 10^{-6} in PBS. One gram of homogenate and 1 ml from all dilution tubes were inoculated into triplicate MPN tubes containing sterile alkaline peptone water (APW) consisting of 1% peptone, 1% NaCl, pH 8.5, according to the U.S. Food and Drug Administration's (FDA) Bacteriological Analytical Manual (Blodgett 2010). Incubation was at 35 °C for up to 48 h. One millilitre of culture media from each growth-positive tube was boiled for 10 min and 2 µl was subjected to qPCR in reaction volumes totaling 25 µl (Jones et al. 2009; Audemard et al. 2018). qPCR was performed for *tlh*$^+$, *tdh*$^+$ and *trh*$^+$ *V. parahaemolyticus* using PCR primers and probe sequences and PCR amplification conditions as described in previous studies (Nordstrom et al. 2007; Kinsey et al. 2015). An internal amplification control (IAC) was used to ensure PCR integrity to detect any false-positive samples. The same MPN tubes were screened by PCR for the *vcgC* gene of *V. vulnificus*. Probes, primers and cycling conditions for the *vcgC* gene were as described by Baker-Austin et al. (2010), except that the initial activation time was changed from 10 to 3 min. All primers were synthesized by Integrated DNA Technologies (Skokie, IL, USA) and probes were manufactured by Life Technologies (Carlsbad, CA, USA). qPCR was performed using iTaq Universal Probes Supermix (Bio-Rad

Fig. 1 Map of the Delaware Bay showing oyster collection sites in Delaware at Bowers, Slaughter Beach and Lewes

Laboratories, Hercules, CA, USA) containing iTaq DNA polymerase, dNTPs, and $MgCl_2$ according to the manufacturer's instructions in 25 µl reaction volumes. PCR was performed on an Applied Biosystems (ABI) cycler (7500 Real-time PCR System, Thermo Fisher Scientific, Carlsbad, CA, USA). Positive and negative controls were used for each PCR cycle. After completion of PCR, the number of MPN-positive tubes that showed amplification of virulence genes was recorded and an MPN index was generated using standard FDA procedures and tables (Blodgett 2010).

Phage Enrichments and Plaque Assays

Oyster homogenates from which *V. parahaemolyticus* and *V. vulnificus* quantifications were derived were screened for vibriophages after centrifugation and filtration to remove debris and non-vibrio bacteria, prior to enrichment in potential host vibrios. Enrichment was considered necessary since direct enumeration by plaque assay often leads to negative results if phage titers are at or below the detection limit. Bacterial overgrowth is usually encountered when testing oyster homogenates directly; therefore, vibriophage detection was achieved through an enrichment-plaque assay process. For enrichment, the same oyster homogenates used for the previously described *Vibrio* MPN-PCR assays were centrifuged at 15,000×*g*, 15 min at 4 °C and 15 ml of each supernatant was passed through low-protein-binding Durapore 0.22-µm polyvinylidene difluoride (PVDF) Millex GV syringe filters (Merck Millipore, Tullagreen, Carrigtwohill, Co. Cork, Ireland). 15 ml of the filtered homogenate was inoculated into an equal volume of double strength Luria–Bertani broth made with diluted seawater (one part seawater and two parts distilled water) to give a final salt concentration of 30 ppt. A 500 µl aliquot of each host *Vibrio* culture that had been grown to an $OD_{600} = 0.6–0.8$ was also inoculated into each mix and phage enrichment proceeded overnight at 26 °C, 175 rpm. After incubation, the enrichment was centrifuged and 0.22-µm filtered, as previously described, and the filtrate was tested against each host using a modified double agar plaque assay (Adams 1959). Media for the plaque assay was as follows: the bottom agar consisted of (per 100 ml): 1.0 g tryptone (Fisher Scientific), 100 mg dextrose (Fisher Scientific), 2 g of NaCl, 1.5 g of Sigma Type II Agarose (Sigma-Aldrich, St. Louis, MO, USA), and 100 ml dH_2O. 15 ml of autoclaved bottom agar was added per 100-mm diameter petri dish. Top agar consisted of (per 100 ml): 1 g tryptone, 100 mg dextrose, 2 g NaCl, 600 mg Sigma Type II Agarose (Sigma-Aldrich), 50 mg $MgCl_2 \cdot 6H_2O$, 500 mg yeast extract (Fisher Scientific), and 37 mg $CaCl_2 \cdot 2H_2O$ (Sigma-Aldrich). For each plaque assay, 500 µl of the filtered enrichment and 250 µl of a host bacterial broth culture at $OD_{600} = 0.6–0.8$ were added to a tube containing 15 ml of liquified top agar maintained at approx. 48 °C, the tube was capped and inverted 3–4 times to mix, and the agar was poured onto the bottom agar. After agar solidification, plates were inverted and incubated at 26 °C for 48 h after which plates containing plaques were recorded as positive. Individual plaques were occasionally picked and purified for subsequent host specificity testing and for electron microscopy.

Morphological Characterization of Phages

Selected phages were characterized by transmission electron microscopy (TEM). Nine plaque-purified phage isolates were centrifuged at 2800×*g* for 10 min and the supernatants were filtered through low-protein-binding Durapore 0.45-µm PVDF Millex-HV syringe filters (Merck Millipore) to remove debris. Test samples (5–10 µl) were applied to the surface of Parafilm (Pechiney Plastic Packaging, Menasha, WI, USA) and Formvar (Electron Microscopy Sciences, Fort Washington, PA, USA)-coated copper grids (400 mesh, Ted Pella Inc., Redding, CA, USA), placed on the test sample for ≤ 15 s, blotted on Whatman filter paper, and negatively stained for 3–5 s with 1% phosphotungstic acid (Polysciences Inc., Warrington, PA) at pH 7.0. They were blotted again, allowed to air dry, and examined under

a Philips CM12 transmission electron microscope (Philips, Eindhoven, The Netherlands) at an accelerating voltage of 80 kV. Images were collected with a 4000M-T1-GE-AMT detector (DVC Co., Austin, TX) and processed with AMT V600 software (AMT, Danvers, MA, USA).

Host Specificity Testing

Selected phage isolates were further characterized by spot testing to determine their host specificities against seven different *Vibrio* spp. and *Photobacterium damselae* subsp. *damselae* (Table 1). The spot testing procedure was a modified double agar assay, similar to the plaque assay procedure described above. In essence, the bottom agar was allowed to cool and the top agar plus host bacteria ($OD_{600} = 0.6–0.8$) was poured to create lawns of potential host bacteria. After the top agar solidified, duplicate 2 µl drops of each phage isolate were added to the surface of the plate and the plates were incubated at 26 °C for up to 48 h and observed for areas of clearing (plaques).

Results

Abundances of Total and Pathogenic *V. parahaemolyticus* in Oysters

Subtidal oysters collected from three sites in the Delaware Bay throughout the course of this study (May–October, 2017) all contained *V. parahaemolyticus* with the species-specific *tlh* gene and the *V. parahaemolyticus* pathogen genes *tdh* and *trh* (Table 2). They also contained the *V. vulnificus* pathogenicity marker gene *vcgC* (Table 2). Abundances of tlh^+ *V. parahaemolyticus*, were within the normal regulatory limits of < 10,000 MPN/g, except for the Lewes harvesting site in July when an extraordinarily high abundance of tlh^+ *V. parahaemolyticus* (92,400 MPN/g), was detected (Table 2). Individual oysters were previously found to have high levels of *V. parahaemolyticus* (Klein and Lovell 2017); however, we are unaware of any studies that have documented a level as high as 92,400 MPN/g. The abundances of *V. parahaemolyticus* containing *tdh* or *trh* genes were exceedingly high in July at the Lewes site (214 and 211 MPN/g, respectively). Levels over 10 MPN/g are considered extraordinarily high (DePaola et al. 2000). Consequently, the July levels of over 200 MPN/g in this present study should raise health concerns. Other instances when tdh^+ *V. parahaemolyticus* levels were > 10 MPN/g were in June and July at the Lewes and Bowers sites where tdh^+ were ≥ 23.9 MPN/g and in May at Slaughter Beach with $tdh^+ = 21.1$ MPN/g. The trh^+ levels in June–August at Lewes ranged from 14.7 to 211 MPN/g and at Bowers from 14.7 to 42.7 MPN/g; however, counts at Slaughter Beach never exceeded 9.3 MPN/g throughout the study (Table 2). The frequency of tdh^+ and trh^+ detection in oysters was 100% at all sampling sites, far higher than found in the Northeast, West Coast, and Gulf Coasts of the U.S. (DePaola et al. 2000) or the Chesapeake Bay (Elmahdi et al. 2018).

The high abundances of *tlh* (92,400 MPN/g) as well as of *tdh* (214 MPN/g) and *trh* (211 MPN/g)-positive *V. parahaemolyticus* for the oysters from Lewes corresponded with the highest water temperature (25.6 °C) and the lowest DO (4.24 mg/L) for that site (Table 2). The second highest *tdh* and *trh* readings (42.7 and 93.3 MPN/g from June) also corresponded to the second highest temperature (24.7 °C) and the second lowest DO (5.41 mg/L) at the Lewes site. Results at the Bowers site were similar where the 2 months with the highest *tdh* and *trh* levels (June and July) corresponded to the highest temperatures and lowest DO levels recorded for that site (Table 2), suggesting some relationship between elevated temperature, low DO and high levels of potentially pathogenic *V. parahaemolyticus*. In contrast, the only high count at the Slaughter Beach site was for *tdh* (21.1 MPN/g) in May and that corresponded to the lowest temperature of the entire study (14.4 °C) and the highest DO recorded (5.88 mg/L). Turbidity results were also collected (Table 2) but did not appear to affect the abundance of *tlh, tdh* or *trh* strains. The amount of environmental data collected is too limited for accurate statistical correlations between *tlh, tdh* and *trh* abundances and environmental parameters.

Abundance of Pathogenic *V. vulnificus* in Oysters

Vibrio vulnificus containing the *vcgC* pathogenicity marker gave the highest count (1470 MPN/g) in August at Bowers, while July and September abundances were 276 and 427 MPN/g, respectively (Table 2). High counts of ~ 1000 *vcgC*-positive *V. vulnificus*/g were previously reported in oysters from North Carolina (Warner and Oliver 2008b). The August sampling at Lewes gave a count of 204 MPN/g, while the remaining oyster samples at all three sites contained ≤ 42.7 MPN/g. The frequency of oyster samples containing $vcgC^+$ *V. vulnificus* was 100% for Lewes and Bowers oysters over the course of the study and 83.3% for Slaughter Beach oysters (Table 2). It is interesting that the high abundances of *tlh, tdh* and *trh* in Lewes oysters in July, corresponded with low levels of $vcgC^+$ *V. vulnificus* (4.3 MPN/g) during that same period; however, the abundance of $vcgC^+$ *V. vulnificus* increased to 204 MPN/g in August when *tlh, tdh* and *trh* levels had dropped to 1120, 9.3 and 14.7 MPN/g, respectively (Table 2).

Detection of Vibriophages in Oysters

Results of phage isolations against three serotypes of *V. parahaemolyticus* are shown in Table 2. The frequency of phage

Table 2 MPN–PCR counts of *tlh*$^+$ and virulence-associated (*tdh*$^+$ and *trh*$^+$) *V. parahaemolyticus* in oysters from three collection sites, corresponding phages isolated against three *V. parahaemolyticus* serotypes, levels of pathogenic *vcgC*$^+$ *V. vulnificus*, and seawater parameters at time of oyster collection

Location/oyster collection dates	MPN–PCR of *V. parahaemolyticus*			Phages isolated against the following *V. parahaemolyticus* host serotypes			MPN–PCR of *V. vulnificus*	Seawater parameter at time of harvest			
	tlh$^+$ (MPN/g)	*tdh*$^+$ (MPN/g)	*trh*$^+$ (MPN/g)	O3:K6	O1:KUT	O1:K1	*vcgC* (MPN/g)	Temp. (°C)	Salinity (ppt)	Dissolved oxygen (mg/l)[a]	Turbidity (NTU)
Lewes											
5/23/17	24	4.3	0.9	+	+	ND[a]	0.9	17.0	17.0	7.86	29.2
6/13/17	933	42.7	93.3	+	+	−	34.7	24.4	23.2	5.41	25.2
7/11/17	92,400	214.0	211.0	+	+	+	4.3	25.6	24.3	4.24	26.0
8/15/17	1120	9.3	14.7	−	+	−	204.0	23.3	25.0	6.16	26.3
9/12/17	4270	4.3	7.4	+	+	+	4.3	16.1	21.3	6.72	26.2
10/10/17	204	0.4	2.3	−	+	+	0.4	22.8	23.1	ND	28.2
Bowers											
5/30/17	93	4.3	9.3	+	+	ND	3.8	15.5	18.7	6.01	23.1
6/20/17	2390	42.7	42.7	+	+	−	9.3	23.3	26.0	5.04	27.3
7/18/17	427	23.9	24.0	+	+	+	276.0	23.9	27.4	4.62	27.4
8/15/17	286	1.5	14.7	+	+	+	1470.0	23.3	25.6	6.28	19.8
9/12/17	741	1.1	0.4	+	+	+	427.0	17.8	20.5	6.24	16.6
10/10/17	4270	1.5	0.7	−	+	+	9.3	20.6	22.9	5.21	28.2
Slaughter Beach											
5/30/17	147	21.1	9.3	+	+	ND	0.0	14.4	18.7	5.88	26.2
6/27/17	240	4.3	2.3	+	+	+	4.3	20.0	24.7	3.76	24.7
7/25/17	240	2.3	7.4	+	+	+	7.4	21.7	27.4	2.46	24.3
8/15/17	933	2.3	2.3	+	+	+	38.4	23.3	25.5	5.77	20.7
9/12/17	43	0.9	2.9	+	+	+	42.7	17.2	20.7	5.69	19.1
10/10/17	1470	2.9	2.9	+	+	+	2.9	22.8	23.2	ND	18.1

[a]ND signifies that the specific test was not done

isolation against *V. parahaemolyticus* O1:KUT was 100% at all three sampling sites. Phages against *V. parahaemolyticus* O3:K6 were identified in all monthly oyster samples from Slaughter Beach, 5 of 6 (83.3%) monthly samples from Bowers and 4 of 6 (66.7%) samples from Lewes (Table 2). There was no testing for phages against strain O1:K1 during the 1st month of sampling. The remaining 5 months of sampling revealed that all (100%) of the samples contained phages against O1:K1 at Slaughter Beach, 4 of 5 (80%) of the samples were positive against O1:K1 at Bowers, and 3 of 5 (60%) of the samples were positive at Lewes (Table 2). A fourth strain of *V. parahaemolyticus* known as DAL 1094 was also used as a host, but no phages against it were detected (not included in Table 2). The frequencies of phage isolation between the three sites were clearly different.

On a monthly basis, phages were isolated against the O3:K6 serotype at Slaughter Beach in October but were not detected at the Bowers or Lewes sites in October, even though seawater temperatures and salinities at the time of oyster collection were relatively consistent among the sites ranging from 20.6–22.8 °C and 22.9–23.2 ppt salinity (Table 2). Seawater turbidity at Slaughter Beach was only 18.1 NTU compared to the other sites which were both 28.2 NTU (Table 2). Unfortunately, the inability to quantify phages against the individual *V. parahaemolyticus* serotypes, necessitated by the need for phage enrichment for detection, prevents correlations between phage numbers and water quality parameters. The absence of phages specific for *V. parahaemolyticus* O1:K1 from Lewes in June and August, and from Bowers in June contrasts with the presence of phages against O1:K1 during these same months at Slaughter, again demonstrating spatial differences (Table 2). The causes of this variability are not expected to be associated with the methods used, since enrichment procedures should greatly increase the chances of detecting vibriophages even when the vibriophages are in low numbers within the oysters.

Host Specificity of Phages

Nine selected phage isolates were tested for host specificity toward a variety of human pathogenic vibrios (listed in Table 1) as well as against the human pathogen *P. damselae* subsp. *damselae*, a flesh-eating marine bacterium in the *Vibrionaceae* family that is capable of causing necrotizing fasciitis (Hundenborn et al. 2013) (Table 3). Phages were isolated from all three oyster-harvesting sites with phages VP1, VP2, VP6–VP9 obtained from Lewes; VP3 and VP4

Table 3 Host specificity of nine isolated *V. parahaemolyticus* phages

Test vibrios	Phage strain[a]								
	VP1	VP2	VP3	VP4	VP5	VP6	VP7	VP8	VP9
V. parahaemolyticus									
O3:K6, RIMD2210633	+	+	+	+	+	−	−	−	−
O1:KUT, DIE12 052499	−	−	−	−	−	+	+	+	−
O1:K1, ATCC 17802	−	−	−	−	−	−	−	−	+
DAL 1094	−	−	−	−	−	−	−	−	−
O4:K12, ATCC 49529	−	−	−	−	−	−	−	−	−
V. vulnificus									
MLT 362	−	−	−	−	−	−	−	−	−
MLT 364	−	−	−	−	−	−	−	−	−
MLT 367	−	−	−	−	−	−	−	−	−
MLT 1003	−	−	−	−	−	−	−	−	−
Vibrio cholerae									
V. cholera O1	−	−	−	−	−	−	−	−	−
V. cholera O139	−	−	−	−	−	−	−	−	−
Vibrio alginolyticus									
FDA 22	−	−	−	−	−	−	−	−	−
ATCC 17749	−	−	−	−	−	+	−	−	+
Other bacterial species									
V. hollisae CFSAN 89A 1960	−	−	−	−	−	−	−	−	−
V. metschnikovii ATCC 7708	−	−	−	−	−	−	−	−	−
V. mimicus ATCC 33654	−	−	−	−	−	−	−	−	−
Photobacterium damselae ssp. *damselae*	−	−	−	−	−	−	−	−	−

[a]Phages VP1, VP2, VP6–VP9 were from the Lewes site; VP3 and VP4 were from the Slaughter Beach site; and VP5 was from the Bowers site

from Slaughter Beach; and VP5 from Bowers. The phages were highly species and serotype/strain specific (Table 3). Phages VP1–VP5 were monospecific for *V. parahaemolyticus* O3:K6; they were unable to produce lytic plaques in any of the other four strains of *V. parahaemolyticus* (Table 3). Phages VP7 and VP8 were monospecific for *V. parahaemolyticus* O1:KUT. Only two phages demonstrated the ability to infect two *Vibrio* species. The first was VP6 which infected *V. parahaemolyticus* O1:KUT and *V. alginolyticus* ATCC 17749. The second was VP9 which infected *V. parahaemolyticus* O1:K1 (ATCC 17802) and *V. alginolyticus* ATCC 17749. Although isolates VP6 and VP9 were able to infect *V. alginolyticus* ATCC 17749, neither was able to infect another *V. alginolyticus* strain (FDA 22). *Vibrio alginolyticus* is a well-known fish and shellfish pathogen but it also causes human skin and ear infections from water activities (Jacobs Slifka et al. 2017). Cross-specificity between *V. parahaemolyticus* phages obtained from oysters and *V. alginolyticus* have been reported previously, presumably because of the close genetic relationship between the two species (Comeau et al. 2005). None of the phages infected *V. parahaemolyticus* DAL 1094 or the O4:K12 serotype, or *V. vulnificus, V. cholerae* O1 or O139, *V. hollisae, V. metschnikovii, V. mimicus*, or *P. damselae* subsp. *damselae* (Table 3). Clearly, there was very narrow host specificity of the *V. parahaemolyticus* phages toward other *V. parahaemolyticus* strains. It should also be noted than none of the isolated phages were infectious toward *V. parahaemolyticus* O4:K12, a serotype which is becoming more commonly found in U.S. coastal waters and which has been attributed to outbreaks of vibriosis (DePaola et al. 2003b). Given the high host specificity of the phage isolates, the inability to detect phages against the O4:K12 serotype was expected, since this strain was not used in initial enrichments of phages from the oyster tissues.

Phage Morphology

Phages were imaged by transmission electron microscopy and representative images for VP2, VP6, VP7 and VP9 are shown in Fig. 2. Most phages were non-tailed with rounded or icosahedral capsids approx. 60 nm in diameter as shown for VP2, VP7 and VP9. Phage VP2 capsid was clearly icosahedral with peplomers extending from its apices. Non-tailed phages have been described in marine systems previously (Holmfeldt et al. 2013; Kauffman et al. 2018). Only one phage (VP6) contained a typical head and tail structure (Fig. 2). The head of VP6 was generally 60 nm in diameter while tail lengths were highly variable with a mean length of 44 nm. There was no contraction of the tail and the tails were relatively long; therefore, it can be concluded that this phage is a member of the *Siphoviridae* family.

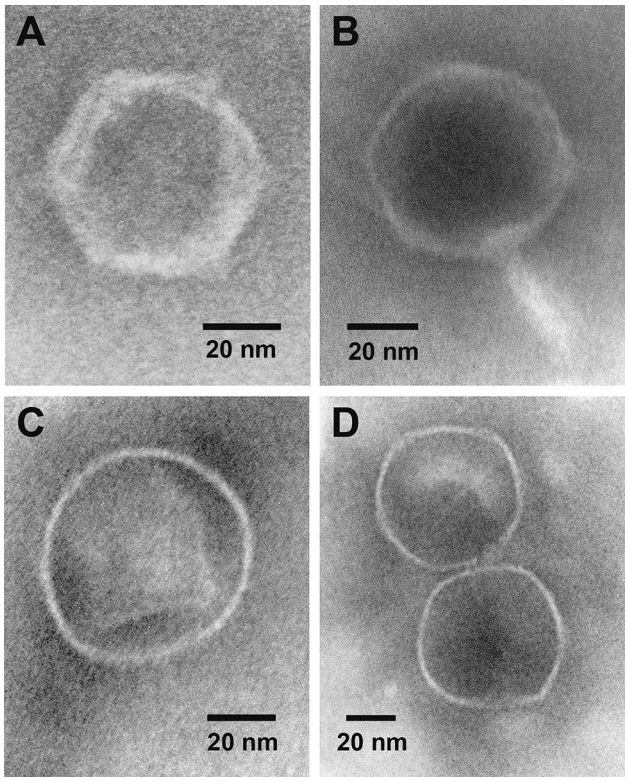

Fig. 2 Morphology of selected phages by transmission electron microscopy. Phages are designated **a** VP2, **b** VP6, **c** VP7 and **d** VP9. VP6 (**b**) is the only tailed phage observed and is likely a member of the *Siphoviridae* family, since there was no evidence of tail contraction. The individual scale bars indicate 20 nm

Discussion

The first objective of this study was to determine whether phages against known strains or serotypes of clinical and environmental vibrios were detectable in oysters collected from three sites along the Delaware Bay from May to October 2017. Phages against three of the four *V. parahaemolyticus* strains were readily detectable. Phages against *V. parahaemolyticus* O1:KUT were detected during all monthly samplings at all three sites. In a study of seawater, sediment and oysters from the nearby Chesapeake Bay, Maryland (USA), researchers found that 37.5% of the *V. parahaemolyticus* isolates were the O1:KUT serotype (Chen et al. 2017). In our study, phages against *V. parahaemolyticus* O3:K6 were detected 83.3% of the time, while phages against the O1:K1 serotype were frequently isolated (in 12 of 15 samples, 80%); although, their absence from oysters was noted from Lewes in June and August and from Bowers in June (Table 2). O1:K1 was detected in Slaughter Beach oysters for all samplings (Table 2). The presence of phages against particular *V. parahaemolyticus* serotypes during some months, coupled with the high host specificity of the vibriophages tested,

suggest that these serotypes were likely present in the oysters.

Oyster enrichments contained phages against all three *V. parahaemolyticus* serotypes (O3:K6, O1:KUT, and O1:K1) 66.6% of the time, indicating the widespread presence of these serotypes in Delaware Bay oysters. The O3:K6 serotype is noteworthy because it is a pandemic strain originally isolated in India in 1996 (Okuda et al. 1997). It rapidly spread worldwide (Nair et al. 2007), including the United States, where it has caused illness from oyster consumption (Centers for Disease Control and Prevention 1999; Daniels et al. 2000). Similarly, some of the *V. parahaemolyticus* O1:KUT isolates are known pandemic strains (Chowdhury et al. 2004; Preeprem et al. 2018). If the detection of phages against a particular serotype of *V. parahaemolyticus* is dependent on the presence of that serotype in the oysters, then the presence of phages against invasive strains of *V. parahaemolyticus*, like the O3:K6 and O1:KUT strains, in oysters during most of the summer months should trigger public health concern and call for additional studies. Likewise, the inability to detect phages against the O3:K6 serotype in oysters from Lewes during August and October and from Bowers in October (Table 2) suggests the absence of host *V. parahaemolyticus* O3:K6 from the oysters during those periods. Further study is needed to determine if phage detection against specific *V. parahaemolyticus* serotypes can be used to assess potential public health risk from oyster consumption. No phages were detected in oysters against *V. parahaemolyticus* DAL 1094.

Although phages against *V. vulnificus* have been previously isolated in the Gulf of Mexico from a variety of matrices including oysters and seawater (DePaola et al. 1997, 1998; Pellon et al. 1995), we did not detect vibriophages against three strains of *V. vulnificus* host cells (strains 364, 367 and 1003) (Table 1). *V. vulnificus* 1003 is a clinical strain that was isolated from Louisiana, along the U.S. Gulf Coast, while the other two strains used in the present study were well-characterized environmental isolates from Hawaii (Tamplin et al. 1996) (Table 1). In retrospect, *V. vulnificus* isolates from local sources may have made better hosts; however, clinical strains of *V. vulnificus* are seldom acquired from the Delaware Bay or adjacent areas due to lower seawater temperatures than those found along the Gulf Coast. Consequently, clinical *V. vulnificus* isolates from this region are not generally available for use as phage host cells. The use of arbitrarily isolated vibrios from oysters as potential host cells was not considered, since our goal was to determine if vibriophages against pathogenic *Vibrio* species could be detected in Delaware oysters. There is little benefit to detecting phages against arbitrarily isolated vibrios that have not been characterized and whose virulence is unknown.

If we make a logical assumption that vibriophages are present in oysters when there are host vibrios present (since vibriophages would require host bacteria to replicate), then we could also assume that phages are absent when host vibrios are absent. This would suggest that the inability to isolate any phages specific for *V. parahaemolyticus* DAL 1094 or the three strains of *V. vulnificus* used for screening oysters was because these strains were not present in the oysters from any of the sampling sites during this study. It can be concluded that oysters along the Delaware Bay consistently carry phages against highly pathogenic *V. parahaemolyticus* over the summer months and that these phages are quite species and strain specific. Phage presence likely reduces the abundance of *V. parahaemolyticus* to some degree within oysters; however, it is clear from this study that phage presence does not eliminate total or pathogenic strains of *V. parahaemolyticus*. This is likely because the oysters contain a broad spectrum of different *V. parahaemolyticus* serotypes, not just those tested in this study.

The second objective of this study was to determine whether vibriophage presence or absence in oysters corresponded with the abundance of total or pathogenic *V. parahaemolyticus* and clinical or environmental strains of *V. vulnificus*. This determination was facilitated by unexpectedly high abundances of *tdh*$^+$, *trh*$^+$, and *tlh*$^+$ *V. parahaemolyticus* at Lewes in July, where counts were 214, 211 MPN/g and 92,400 MPN/g, respectively. The presence or absence of phages against *V. parahaemolyticus* O3:K6, O1:KUT and O1:K1 did not correspond with the levels of potentially pathogenic *tdh*$^+$ and *trh*$^+$ *V. parahaemolyticus* detected in the oysters. For instance, phages against *V. parahaemolyticus* O3:K6, O1:KUT and O1:K1 were detected when the *tdh*$^+$ and *trh*$^+$ strains of *V. parahaemolyticus* were very high (>200 MPN/g) as well as very low (≤ 4.3 MPN/g) in Lewes (Table 2). Slaughter Beach had the lowest abundances of *tdh* and *trh* positive *V. parahaemolyticus*; however, phages against three of the four host cells tested were detected each month throughout the sampling period. Similarly, phages against these three *Vibrio* serotypes were regularly detected regardless of whether the *tlh* abundances were high (92,400 MPN/g) or low (43 MPN/g). There can be no direct correlation calculated between the vibrios and the phages because *Vibrio* counts were determined quantitatively, while phage counts were obtained from enrichment samples, which do not give any indication of the initial levels of phages present in the sample. Our initial thinking was that phage detection would be infrequent when total *Vibrio* levels were low, but to the contrary, phage detection was frequent regardless of *Vibrio* levels.

The virulence of *V. parahaemolyticus* (as suggested by the presence or absence of the *tdh* and *trh* hemolysin genes) did not appear to affect phage infectivity, since phages were able to infect the O3:K6 (*tdh*$^+$/*trh*$^-$), O1:KUT (*tdh*$^+$/*trh*$^+$) and O1:K1 (*tdh*$^-$/*trh*$^-$) strains, but they could not infect the

DAL 1094 strain, which is also *tdh*$^-$/*trh*$^-$, like the O1:K1 strain.

The extremely high levels of *tlh, tdh* and *trh*-positive *V. parahaemolyticus* in oysters from Lewes in July was a cause for concern, so we contacted the New Jersey Department of Environmental Protection (NJ DEP) in Leeds Point, NJ, to determine if they too were finding such high levels in Delaware Bay oysters in July. They regulate shellfish harvesting along the New Jersey portion of the Bay. They provided information on their routine analyses of oysters from commercial harvesting sites in the Bay. Their assays were conducted following essentially the same MPN–PCR procedures used in the present study. The NJ DEP results for July 2017 revealed high *tdh* and *trh* levels of 214 and 74.9 MPN/g, respectively, but *tlh* levels were only 462.2 MPN/g, compared to our finding of 92,400 MPN/g. Confirmation of such high abundance of both *tdh*$^+$ and *trh*$^+$ *V. parahaemolyticus* on the New Jersey side of the Bay supports our findings of high levels (214 *tdh*$^+$ and 211 *trh*$^+$ MPN/g) on the Delaware side of the Bay for July 2017. For unknown reasons, there were substantial differences in abundances of total *V. parahaemolyticus* (*tlh*$^+$) in New Jersey versus Delaware in July. Oyster harvesting in the U.S. is regulated based on several parameters, including having *tlh*$^+$ *V. parahaemolyticus* below 10,000 MPN/g (U.S. FDA 2017 revision); however, there are no MPN limits for potentially pathogenic *V. parahaemolyticus* carrying the *tdh*$^+$ and *trh*$^+$ hemolysin genes, thus bringing the use of *tlh* alone into question.

In conclusion, this study demonstrated the ability to detect phages against highly pathogenic *V. parahaemolyticus* serotypes in Delaware Bay oysters. To our knowledge, this is the first time that phages against epidemic strains of *V. parahaemolyticus* have been isolated in the Delaware Bay. Results suggest the possible presence of the pathogens themselves in Delaware Bay oysters, which has major safety implications. More quantitative assessments of phage levels against pathogenic vibrios in oysters is needed. Further research is also needed to determine if oysters and other shellfish may be monitored for a broad range of pathogens by detecting phages in appropriate host cells. With further research, it may be possible to regulate shellfish and their growing waters by identifying and quantifying phages against specific *V. parahaemolyticus* serotypes and other pathogens of interest. Currently, presence or absence testing of enriched cultures for vibriophages cannot be directly correlated with total and pathogenic *Vibrio* levels as determined by MPN–PCR, thus highlighting the need to develop more quantitative phage assay methods for oyster tissues.

Acknowledgements We thank Esam Almuhaideb, Department of Agriculture, Food and Resource Sciences, University of Maryland Eastern Shore, Princess Anne, MD, for confirmatory analysis of selected data. We also thank Devin Mendez, College of Agriculture, Science and Technology, Delaware State University, Dover, DE, for graphing assistance. Support for this project was provided by a USDA Capacity Building Grant Award no. 2014-38821-22430 (SP) and by USDA, ARS intramural funds under CRIS 8072-42000-081-00D (GPR).

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