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Apparent Loss of *Vibrio vulnificus* from North Carolina Oysters Coincides with a Drought-Induced Increase in Salinity

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Despite years of successful isolation of *Vibrio vulnificus* from estuarine waters, beginning in 2007, it was extremely difficult to culture *V. vulnificus* from either North Carolina estuarine water or oyster samples. After employing culture-based methods as well as PCR and quantitative PCR for the detection of *V. vulnificus*, always with negative results, we concluded that this pathogen had become nearly undetectable in the North Carolina estuarine ecosystem. We ensured that the techniques were sound by seeding North Carolina oysters with *V. vulnificus* and performing the same tests as those previously conducted on unadulterated oysters. *V. vulnificus* was readily detected in the seeded oysters using both classes of methods. Furthermore, oysters were obtained from the Gulf of Mexico, and *V. vulnificus* was easily isolated, confirming that the methodology was sound but that the oysters and waters of North Carolina were lacking the *V. vulnificus* population studied for decades. Strikingly, the apparent loss of detectable *V. vulnificus* coincided with the most severe drought in the history of North Carolina. The drought continued until the end of 2009, with an elevated water column salinity being observed throughout this period and with *V. vulnificus* being nearly nonexistent. When salinities returned to normal after the drought abated in 2010, we were again able to routinely isolate *V. vulnificus* from the water column, although we were still unable to culture it from oysters. We suggest that the oysters were colonized with a more salt-tolerant bacterium during the drought, which displaced *V. vulnificus* and may be preventing recolonization.

Vibrio vulnificus is a halophilic bacterium capable of causing wound infections and fatal septicemia in humans (10, 24, 26). This organism is part of the normal flora in estuarine waters as well as in shellfish inhabiting those estuaries (26). Infections caused by *V. vulnificus* are the leading cause of seafood-borne deaths in the United States, typically resulting from the ingestion of oysters harboring the organism, and commonly result in primary septicemia with a fatality rate of >50%. Thus, *V. vulnificus* has the highest case fatality rate of any food-borne pathogen (2, 18, 25, 26).

The most important factors determining the *V. vulnificus* load in oysters are temperature and salinity. The temperature effect is easily seen in seasonal and experimental data (with temperatures of 13°C to 22°C being the most permissive to *V. vulnificus* survival). Salinity is also an important although less obvious factor affecting *V. vulnificus* levels (11, 13, 19, 20, 28, 30, 32).

Historically, *V. vulnificus* has been easily isolated from North Carolina and Gulf Coast estuarine waters and oysters (20, 27, 29, 30, 38–40), with plating onto selective media, such as cellobiose-polymyxin B-colistin (CPC) medium, with or without an enrichment step, being a commonly used procedure for the isolation of this organism from shellfish. Such *V. vulnificus*-specific media are used for primary isolation, but a confirmatory step employing molecular methods is typically used to verify the identification (8). However, beginning in the spring of 2007, colonies presumptively identified as *V. vulnificus* colonies on selective media could not be confirmed as this species. Coincidently, in 2007, North Carolina entered into the worst drought since recordkeeping began in 1895, significantly elevating estuarine salinity (22, 23). In the 6-year study that we report here, we describe the coincidence between extended extreme environmental changes and *V. vulnificus* oyster colonization and present a possible explanation for the continued lack of *V. vulnificus* isolation from North Carolina oysters despite a return of this species to North Carolina estuarine waters.

MATERIALS AND METHODS

Media. CPC$^+$ agar (a derivative of CPC agar) is both selective and differential for *V. vulnificus* (17, 39). Presumptive *V. vulnificus* colonies grown on CPC$^+$ medium were confirmed by subsequent PCR analysis, as described below (40). CHROMagar Vibrio (CHROMagar, Paris, France) is a chromogenic medium that distinguishes four *Vibrio* spp., including *V. parahaemolyticus*, *V. vulnificus*, *V. cholerae*, and *V. alginolyticus* (7, 21, 43). As with CPC$^+$ medium, a confirmation step must be conducted to confirm the species of the isolates.

Oyster collection. More than 650 oysters (*Crassostrea virginica*) from several North Carolina sites were collected by hand from the intertidal zone between 2005 and 2010, with spring, summer, fall, and winter harvest dates. Oysters were either sampled at a laboratory near the collection site within 2 h of harvest or placed into coolers with ice packs and sampled within 6 h of collection. Oysters from a Gulf Coast site at Dauphin Island, AL, were shipped overnight with ice packs and sampled within 2 h of arrival.

Oyster sampling. Oyster tissue was aseptically removed and homogenized in 20‰ artificial seawater (ASW) at a 1:1 weight-to-volume ratio using sterile blender cups (Warring, Torrington, CT). After homogenization, samples were diluted in sterile phosphate-buffered saline (PBS) and spread onto both CPC$^+$ agar and CHROMagar Vibrio and incubated at 40°C and 37°C, respectively.

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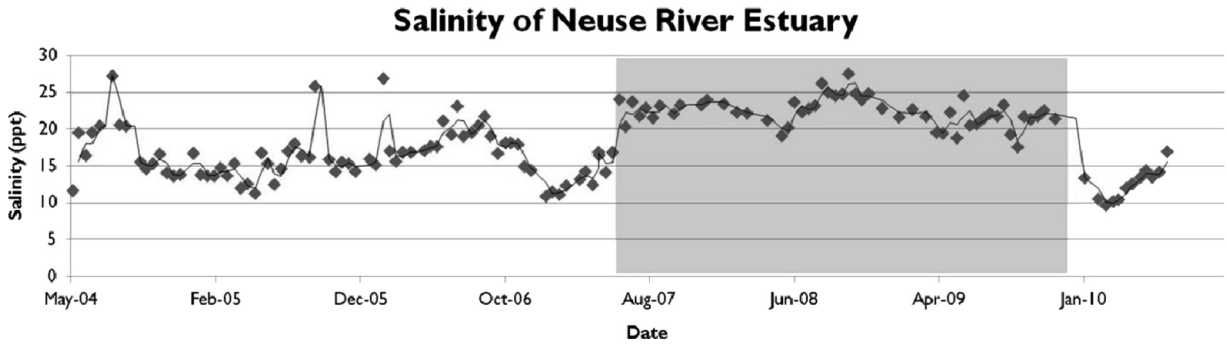

FIG 1 Biweekly salinity data from the Neuse River Estuary in North Carolina. The black line represents the monthly moving salinity average; the shaded area indicates the drought period.

Water sampling. Water samples (10 ml) were vacuum filtered onto 0.22-μm filters, which were aseptically placed onto the same media.

Species confirmation. Presumptive colonies were transferred onto heart infusion (HI) agar and grown overnight at 30°C. Each strain was subjected to PCR with primers amplifying the hemolysin gene *vvhA*, confirming the identification of the isolate as *V. vulnificus* (34, 40). Reactions were performed by using GoTaq polymerase (Promega, San Luis Obispo, CA) in a Techne Genius thermal cycler according to parameters suggested previously by Warner and Oliver (40). PCR products were visualized by gel electrophoresis on 1% agarose gels stained with ethidium bromide.

Seeding of oysters with *V. vulnificus*. Oysters from the North Carolina coast were fed 24 h prior to being removed from maintenance tanks and placed into two separate tanks with artificial seawater with 20‰ salinity at 23°C. *V. vulnificus* cells (of the E genotype) were added to one tank at a final concentration of $\sim 10^4$ CFU per ml. Another tank served as a control (no *V. vulnificus* inoculum). Oysters were allowed to take up *V. vulnificus* cells for 24 h. Oysters from both tanks were then removed, shucked, and homogenized as described above.

PCR and quantitative PCR analyses of oyster homogenates. Diluted oyster homogenates (1 ml) from four control oysters and one artificially infected oyster were treated with the Wizard genomic DNA purification system (Promega), and isolated DNA was subjected to both PCR (described above) and quantitative PCR (Q-PCR). Q-PCR was performed by using the QuantiTect SYBR green PCR kit (Qiagen, Valencia, CA) with a Lightcycler 2.0 instrument (Roche, Basel, Switzerland), employing E- and C-genotype-specific primers (40) in separate reaction mixtures. Reaction mixtures were heated to 95°C for 15 min to activate the polymerase. Reaction mixtures were then treated to 55 cycles consisting of denaturation at 94°C for 15 s, a 53°C annealing step for 30 s, and a 15-s extensions step at 72°C, followed by a single quantification read. Quantifications were calculated by using software bundled with the Lightcycler 2.0 instrument, using a standard curve generated by making seven 10-fold serial dilutions of target DNA.

Microbial identification. Colonies that presumptively appeared to be *V. vulnificus* positive on CPC$^+$ agar and/or CHROMagar Vibrio but which subsequently were confirmed not to be this species were subjected to genetic identification by PCR and by sequencing of the first 500 bp of the 16S rRNA gene (Accugenix, Newark, DE).

RESULTS

Sampling of North Carolina water and oysters. Estuarine salinity levels during the 2007-2009 drought (22.4‰ ± 1.9‰) were significantly higher ($P < 0.001$, determined by Student's *t* test) than those during nondrought periods (15.9‰ ± 3.5‰) (Fig. 1). Natural oyster samples plated onto CPC$^+$ medium resulted in 3,990 presumptive *V. vulnificus* isolates, which were subjected to PCR confirmation. In both 2005 and 2006, 40.7% of the presumptive isolates were positively confirmed to be isolates of this species, whereas in the drought period of 2007 to 2010, the rate of confirmation dropped to 0.7% or lower (Table 1). To ensure that the lack of *V. vulnificus* recovery was not due to deficiencies in the CPC$^+$ medium, we began utilizing an additional medium, CHROMagar Vibrio, in 2010 to collect bacteria from oyster samples. Only 4% of 456 presumptive colonies isolated on this medium were confirmed to be *V. vulnificus* isolates (Table 1). There was a highly significant difference between the number of presumptive isolates confirmed in drought years and the number confirmed in predrought years ($P < 0.001$ using chi-square analysis with a Yates correction for continuity). In addition, between 2006 and 2010, water samples were taken from the same North Carolina estuaries from which the oysters were harvested and plated onto CPC$^+$ agar. This generated a total of 2,404 presumptive *V. vulnificus* isolates. In 2006, before the drought began, 45.7% of isolates from these water samples were determined to be *V. vulnificus* isolates. In 2007, none of the isolates were confirmed to be *V. vulnificus* (although the sample size was small), and in 2008, only 2.4% were determined to be *V. vulnificus*. In the last part of 2009 and in 2010, after the drought period ended, the percentages of confirmed *V. vulnificus* isolates in water samples were 38.1% and 42.4%, respectively (Table 2), returning to predrought values.

Of the samples taken from oysters seeded with *V. vulnificus*, homogenized, and plated onto CPC$^+$ agar, 79 of 80 (99%) presumptive isolates were confirmed to be *V. vulnificus* isolates. Homogenates from oysters harvested from Gulf Coast waters between 2008 and 2010 and plated onto CPC$^+$ agar generated 131

TABLE 1 Presumptive *V. vulnificus* isolates, obtained from North Carolina oysters using either CPC$^+$ agar or CHROMagar Vibrio, confirmed as *V. vulnificus* following PCR analysis[a]

Isolation medium	Yr	No. of isolates tested	% confirmed *V. vulnificus* isolates
CPC$^+$ agar	2005	166	40.7
	2006	201	40.7
	2007	1,041	0.6
	2008	1,428	0.6
	2009	404	0.7
	2010	750	0.7
CHROMagar Vibrio	2010	456	4.0

[a] The shaded area indicates data from the drought period.

TABLE 2 Presumptive *V. vulnificus* isolates, obtained from North Carolina estuarine waters using CPC$^+$ agar, confirmed as *V. vulnificus* following PCR analysis[a]

Yr of isolation	No. of isolates tested	% confirmed *V. vulnificus* isolates
2006	138	45.7
2007	45	0
2008	245	2.4
2009 (September–December)	425	38.1
2010	1,551	42.4

[a] The shaded area indicates data from the period of drought.

TABLE 4 Molecular identification of false-positive isolates from CPC$^+$ agar and CHROMagar Vibrio using 16S rRNA gene sequencing[a]

Colony type	Sequence match (% alignment difference)
Presumptively positive on CPC$^+$ agar	*V. coralliilyticus* (1.3)
	V. nereis (1.4)
	V. tubiashii (1.7)
	V. sinaloensis (1.8)
Presumptively positive on CPC$^+$ agar and CHROMagar Vibrio	*V. mediterranei* (1.0)

[a] The species listed are hits from the proprietary Accugenix sequence library that aligned to our unknown sequences with less than a 2% difference.

presumptive *V. vulnificus* isolates, with an additional 10 isolates being obtained with CHROMagar Vibrio. While North Carolina oysters during this period yielded <1% confirmed *V. vulnificus* isolates, Gulf Coast isolates yielded >96% positive confirmation of *V. vulnificus* isolates (Table 3).

PCR and Q-PCR detection of *V. vulnificus* in oyster homogenates. Oysters (both natural and those seeded with *V. vulnificus*) were homogenized, and total DNA was extracted. PCR analysis of control oysters detected no *V. vulnificus* cells, while the spiked oyster homogenates produced PCR amplicons for the *V. vulnificus*-specific *vvhA* (hemolysin) and *vcgE* (virulence-correlated) genes (data not shown).

These same DNA extracts were also subjected to quantitative PCR analysis with primers specific for the E and C genotypes of *V. vulnificus* (40). The numbers of copies of the *V. vulnificus* C-genotype-specific gene (*vcgC*) were undetectable in all tested oysters (control and seeded). In contrast, the oysters seeded with E-genotype *V. vulnificus* cells contained enough *V. vulnificus* DNA to be detected by E-type-specific probes, yielding 1.3×10^4 copies per μl of concentrated sample (data not shown).

Sequence-based identification. Two false-positive (PCR-negative) isolates on CPC$^+$ agar were identified to the genus level based on 16S rRNA gene sequencing. Neither isolate was identified as *V. vulnificus* (>7% sequence mismatches). Differing by <2% sequence alignment (the top matches) were *V. coralliilyticus*, *V. mediterranei*, *V. nereis*, *V. tubiashii*, and *V. sinaloensis* (Table 4).

DISCUSSION

The isolation of *V. vulnificus* from the oysters and water of North Carolina estuaries has been routinely accomplished by our laboratory and others (1, 4, 20, 27, 29, 32, 41, 42). Historically, by use of CPC$^+$ agar, designed for the isolation of *V. vulnificus* from environmental samples (39), the organism has been easy to collect and identify from oysters in North Carolina. In 2007, the isolation of confirmed *V. vulnificus* colonies was extremely difficult, even though a large number of samples was tested. Concerned about a possible deficiency in the isolation medium, we further employed CHROMagar Vibrio, but this medium performed only slightly better. Both media yielded colonies that appeared to be *V. vulnificus* colonies, but very few were confirmed by molecular testing. The phenomenon of *V. vulnificus*-specific media losing specificity when samples contain a large number of competing *Vibrio* spp. was reported previously by Macian et al. (16), offering a possible explanation for the presence of false-positive *V. vulnificus* colonies on these typically reliable media. Due to the inability to isolate *V. vulnificus* using typically applied culture-based methods, we tested molecular methods of detection, including PCR and Q-PCR, on DNA extracted from oyster tissue. No *V. vulnificus* could be detected by either method.

To confirm that the isolation and confirmation techniques were sound, oysters were seeded with *V. vulnificus*. These oysters yielded confirmed *V. vulnificus* cells by culture and Q-PCR detection methods, providing evidence that the media and techniques were working correctly. As further verification of the methodology, Gulf Coast oysters were obtained and processed in the same fashion as North Carolina oysters, with confirmed *V. vulnificus* cells being easily recovered.

Having established that North Carolina oysters harbored extremely reduced numbers of *V. vulnificus* cells, we investigated potential events that might cause such a sudden and significant loss. The drought that occurred during our study period was the most severe since recordkeeping began in 1895 (23). These conditions, which began in the middle of 2007 and persisted until the end of 2009 (22), resulted in a long-term (>2 1/2 years) increase in the average salinity of the estuary. While previous increases of salinity to this level had occurred, these were short-term increases and unlike the extended drought that occurred during this study (R. T. Noble and H. Paerl, unpublished data).

Kaspar and Tamplin (11) determined previously that the survival of *V. vulnificus* decreased in seawater with salinity greater than 25‰. These findings were supported by *in situ* data reported by Motes et al. (20), showing that increases in salinity in Apalachicola Bay were linked with declines in rates of *V. vulnificus* recovery from oysters, and by Wetz et al. (42), who found that salinity-lowering storm events resulted in an increased rate of recovery of *V. vulnificus*. Consistent with such observations, Jones (S. H. Jones, presented at the Proceedings of the 1994 *Vibrio vulnificus* Workshop, Washington, DC) found that oysters moved to water

TABLE 3 Presumptive *V. vulnificus* isolates, obtained from Gulf Coast oysters using either CPC$^+$ agar or CHROMagar Vibrio, confirmed as *V. vulnificus* isolates following PCR analysis

Isolation medium	Yr	No. of isolates tested	% confirmed *V. vulnificus* isolates
CPC$^+$ agar	2008	80	96
	2009	31	98
	2010	20	100
CHROMagar Vibrio	2010	10	100

with elevated (25‰) salinity were cleared of *V. vulnificus*, and Motes and DePaola (19) reported similar results for oysters relayed from estuarine to offshore (32‰) sites. Such studies, however, have not examined the presence of *V. vulnificus* in high-salinity waters for periods lasting longer than 21 days (1, 9, 11, 13, 15, 19, 20, 32, 33, 36, 37, 44; S. H. Jones, presented at the Proceedings of the 1994 *Vibrio vulnificus* Workshop, Washington, DC), while our study suggests that long-term-elevated salinity (even less than 25‰, considered to be the upper limit of the *V. vulnificus* salinity preference) could negatively impact oyster colonization by *V. vulnificus*.

It is possible that other environmental changes, unaccounted for in this study, could have contributed to the loss of *V. vulnificus*. That considered, it is conceivable that the lengthy drought and shift in estuarine salinity either induced *V. vulnificus* to abandon the oyster habitat or had outright bactericidal effects. Either possibility would lead to a loss of *V. vulnificus* in oysters, leaving an empty niche for an organism with similar physiological characteristics but able to endure elevated salinities.

After the drought eased at the end of 2009, the salinity of the North Carolina estuary returned to normal. The number of presumptive *V. vulnificus* isolates from water samples that were confirmed to be isolates of this species quickly increased to pre-drought levels. Nevertheless, oysters harvested from these waters in 2009 and 2010 still contained extremely low numbers of *V. vulnificus* cells.

It is possible that by using enrichment, we would have been able to detect low numbers of *V. vulnificus* cells in the environment, but with the molecular and culture-based methods that we employed, we found levels in oysters to be nearly nondetectable. It is not known why *V. vulnificus* again became detectable in estuarine waters by our methods yet concentrations of *V. vulnificus* in the oysters that inhabit these waters remain mostly nondetectable. We speculate that the answer may lie in results obtained by our laboratory and many others over the last 30 years. Studies examining the uptake and depuration of *V. vulnificus* in seeded oysters have all reported that *V. vulnificus* cells are rapidly taken up but are not retained and are quickly depurated (3, 5, 6, 12, 14, 31, 35). If the oyster microflora is firmly established during the early stages of oyster development, transient bacterial cells acquired through gill filtration could be unable to establish residency in the oyster gut. The "original" population would likely be displaced only by extreme events, such as large and acute shifts in salinity, which occur when oysters are relayed to waters with much higher salinity (19, 20), or as in the moderate yet chronic salinity increases described in our study. If this is correct, the reemergence of a significant *V. vulnificus* population in adult North Carolina oysters may be observed only when oyster larvae produced after the drought conditions eased (i.e., after 2009) develop into adults, a period of ca. 2 years. This is a testable hypothesis which we intend to pursue over the next several years.

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