Apparent loss of *Vibrio vulnificus* in North Carolina oysters coincides with drought-induced increase in salinity

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Abstract

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 and PCR and quantitative PCR for 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 the techniques were sound by seeding North Carolina oysters with *V. vulnificus* and performing the same tests as 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 elevated water column salinity observed throughout this period, and with *V. vulnificus* being nearly non-existent. When salinities returned to normal after the drought abated in 2010, we were again able to routinely isolate *V. vulnificus* from the water column, though still unable to culture it from oysters. We suggest the oysters were colonized with a more salt-tolerant bacterium during the drought, which displaced *V. vulnificus* and may be preventing recolonization.
**Introduction**

*Vibrio vulnificus* is a halophilic bacterium capable of causing wound infections and fatal septicemia in humans (10, 26, 28). This organism is normal flora in estuarine waters as well as in shellfish inhabiting those estuaries (28). Infections caused by *V. vulnificus* are the leading cause of seafood-borne deaths in the United States, typically resulting from ingestion of oysters harboring the organism, and commonly result in primary septicemia carrying a fatality rate > 50%. Thus, *V. vulnificus* has the highest case fatality rate of any foodborne pathogen (2, 19, 27, 28).

The most important factors determining the *V. vulnificus* load in oysters are temperature and salinity. The temperature effect is easily seen through seasonal and experimental data (with temperatures of 13-22°C being the most permissive to *V. vulnificus* survival). Salinity is also an important, though less obvious factor affecting *V. vulnificus* levels (12, 14, 20, 21, 30, 32, 34).

Historically, *V. vulnificus* has been easily isolated from North Carolina and Gulf Coast estuarine waters and oysters (21, 29, 31, 32, 40, 41, 42), with plating onto selective media, such as CPC, 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 employed to verify identification (8). However, beginning in the spring of 2007, colonies presumptively identified as *V. vulnificus* 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 (23, 25). In the six-year study we report here, we describe the coincidence between extended extreme environmental changes and *V. vulnificus* oyster colonization, and present a possible explanation for 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+ (a derivative of CPC agar) is both selective and differential for *V. vulnificus* (18, 41).

Presumptive *V. vulnificus* colonies grown on CPC+ are confirmed by subsequent PCR analysis described below (42). 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, 22, 45).

As with CPC+, 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 two hours of harvest or were placed in coolers with ice packs and sampled within 6 hours of collection. Oysters from a Gulf Coast site at Dauphin Island, Alabama, were shipped overnight with ice packs and sampled within two hours of arrival.

Oyster sampling

Oyster tissue was aseptically removed and homogenized in 20‰ ASW at 1:1 weight: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+ and CHROMagar™ Vibrio and incubated at 40°C and 37°C, respectively.

Water sampling

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

Species Confirmation
Presumptive colonies were transferred to heart infusion (HI) agar and grown overnight at 30°C.

Each strain was subjected to a PCR reaction with primers amplifying the hemolysin gene, vvhA, confirming the isolate as *V. vulnificus* (36, 42). Reactions were performed using GoTaq polymerase (Promega, San Luis Obispo, CA) in a Techne Genius thermal cycler using the parameters suggested by Warner and Oliver (42). 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 hours prior to being removed from maintenance tanks and placed into two separate tanks with 20‰ salinity artificial sea water at 23°C. *V. vulnificus* cells (of the E-genotype) were added to one tank at a final concentration of ~10⁴ colony forming units (CFU) per ml. Another tank served as a control (no *V. vulnificus* inoculum). Oysters were allowed to take up *V. vulnificus* cells for 24 hours. Oysters from both tanks were then removed, shucked and homogenized as described above.

**PCR and quantitative-PCR analysis of oyster homogenate**

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 subjected to both PCR (described previously) and quantitative PCR (Q-PCR). Q-PCR was performed using the QuantiTect SYBR Green PCR kit (Qiagen, Valencia, CA) using a Lightcycler 2.0 (Roche, Basel, Switzerland), employing E and C genotype-specific primers (42) in separate reactions. Reactions were heated to 95°C for 15 minutes to activate the polymerase. Reactions were then treated to 55 cycles consisting of 94°C denaturation for 15 seconds, a 53°C annealing step for 30 second, and a 15 second extensions step at 72°C followed by a single quantification read. Quantifications were calculated using software bundled with the Lightcycler 2.0 using a standard curve generated by making seven 10-fold serial dilutions of target DNA.
Microbial identification

Colonies that presumptively appeared positive as *V. vulnificus* on CPC+ and/or CHROMagar™ Vibrio, but which subsequently were not confirmed to be this species, were subjected to genetic identification by PCR and by sequencing the first 500 base pairs 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 greater (p<.001, student’s t-test) than during non-drought periods (15.9‰± 3.5‰, Figure 1). Natural oyster samples plated onto CPC+ resulted in 3990 presumptive *V. vulnificus* isolates which were subjected to PCR confirmation. In both 2005 and 2006, 40.7% of the presumptive isolates were positively confirmed as this species, whereas in the drought period 2007-2010, confirmation dropped to 0.7% or less (Table 1).

To ensure the lack of *V. vulnificus* recovery was not due to deficiencies in the CPC+ medium, in 2010 we began utilizing an additional medium, CHROMagar™ Vibrio, to collect bacteria from oyster samples. Only 4% of 456 presumptive colonies isolated on this medium were confirmed to be *V. vulnificus* (Table 1).

There was a highly significant difference between the number of presumptive isolates confirmed in drought years versus the number confirmed from pre-drought years (p<0.001 using Chi-square analysis with 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+.

This generated 2404 total presumptive *V. vulnificus* isolates. In 2006, before the drought began, 45.7% of these water samples were determined to be *V. vulnificus*. In 2007, none of the isolates were confirmed (although the sample size was small) and in 2008, only 2.4% were determined to be *V. vulnificus*.
V. vulnificus. In the last part of 2009 and in 2010, after the drought period ended, the confirmed
percentages of water samples were 38.1% and 42.4%, respectively (Table 2), returning to pre-drought
values.

Of samples taken from oysters seeded with V. vulnificus, homogenized, and plated onto CPC+, 79
of 80 (99%) presumptive isolates were confirmed as V. vulnificus. Homogenates from oysters harvested
from Gulf Coast waters between 2008 and 2010 and plated onto CPC+ agar generated 131 presumptive
V. vulnificus isolates with an additional 10 isolates obtained from CHROMagar™ Vibrio. While North
Carolina oysters during this period yielded <1% confirmed V. vulnificus, Gulf Coast isolates yielded >96%
positive confirmation as V. vulnificus (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 the 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 (42). The number 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 1.3x10^4 copies per μl of concentrated sample (data not shown).

Sequence-based identification

Two false positive (PCR-negative) isolates on CPC+ were identified to the genus level based on
16S rDNA sequencing. Neither was identified as V. vulnificus (>7% sequence mismatch). Differing by <2%
sequence alignment (the top matches) were V. coralliiyticus, V. mediterranei, V. neresis, V. tubiashii, and
V. sinaloensis (Table 4).
Discussion

Isolation of *V. vulnificus* from the oysters and water of North Carolina estuaries has been routinely accomplished by our lab and others (1, 4, 21, 29, 31, 34, 43, 44). Historically, using CPC+ agar designed for the isolation of *V. vulnificus* from environmental samples (41), 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 as *V. vulnificus*, 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. has been reported previously by Macian *et al.* (17), 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 QPCR, 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* by culture and QPCR detection methods, providing evidence that the media and techniques were working correctly. Further verifying the methodology, Gulf Coast oysters were obtained and processed in the same fashion as North Carolina oysters, with confirmed *V. vulnificus* cells 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 record keeping began in 1895 (25). These conditions, which began in the middle of 2007 and persisted until the end of 2009 (23),...
resulted in a long-term (>2 ½ y) increase in average salinity of the estuary. While previous increases of salinity to this level had occurred, these were short-term and unlike the extended drought that occurred during this study (24).

Kaspar and Tamplin (12) determined that *V. vulnificus* survival decreased in seawater with salinity greater than 25‰. These findings were supported by *in situ* data by Motes *et al.* (21) showing that increases in salinity in Apalachicola Bay were linked with declines in *V. vulnificus* recovered from oysters, and by Noble *et al.* (44) who found that salinity-lowering storm events resulted in increased recovery of *V. vulnificus*. Consistent with such observations, Jones (11) found that oysters moved to elevated (25‰) salinity waters were cleared of *V. vulnificus*, and Motes and DePaola (20) reporting similar results in 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, 12, 14, 16, 20, 21, 34, 35, 38, 39, 46), while our study suggests that long-term elevated salinity (even less than 25‰, considered to be the upper limit of *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 else 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 NC estuary returned to normal. The number of *V. vulnificus* presumptive isolates from water samples which were confirmed as 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 using enrichment, we would have been able to detect low numbers of *V. vulnificus* in the environment, but using the molecular and culture based methods we employed we found levels in oysters to be nearly non-detectable. It is not known why *V. vulnificus* again became detectable in estuarine water by our methods yet concentrations of *V. vulnificus* in the oysters that inhabit these waters remain mostly non-detectable. We speculate that the answer may lie in results obtained by our lab and many others over the last 30 years. Studies examining 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, 13, 15, 33, 37). 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 only be displaced by extreme events, such as large and acute shifts in salinity which occur when oysters are relayed to much higher salinity waters (20, 21), or as in the moderate yet chronic salinity increases described in our study. If this is correct, then the re-emergence of a significant *V. vulnificus* population in adult North Carolina oysters may only be observed when oyster larvae produced after the drought conditions (*i.e.* >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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Converse, Kathy Conn, Curt Stumpf, Sarah Rhodes, and Sarah Hatcher. We also thank Andy DePaola for providing Gulf Coast oysters and J. Vaun Mcarthur of the Savannah River Ecology Lab for critical review of our manuscript. This material is based on work supported by the Cooperative State Research, Education, and Extension Service, U.S. Department of Agriculture, under Award Nos. 2007-35201-1838 and 2009-03571, and by the National Science Foundation/National Institutes of Health Joint Program in “Ecology of Infectious Disease” under Grant No. OCE-0813147. Any opinions, findings, conclusions, or recommendations expressed in this publication are those of the authors and do not necessarily reflect the views of the U.S. Department of Agriculture or the National Science Foundation.

References


Figure 1: Biweekly salinity data from the Neuse River Estuary in NC. Black line represents the monthly moving salinity average; shaded area indicates the drought period.
Table 1: Presumptive *V. vulnificus* isolates, obtained from North Carolina oysters using either CPC+ or CHROMagar™ Vibrio, confirmed as *V. vulnificus* following PCR analysis. Shaded area indicates period of drought.

<table>
<thead>
<tr>
<th>Year</th>
<th>2005</th>
<th>2006</th>
<th>2007</th>
<th>2008</th>
<th>2009</th>
<th>2010</th>
<th>2010</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. Tested</td>
<td>166</td>
<td>201</td>
<td>1041</td>
<td>1428</td>
<td>404</td>
<td>750</td>
<td>456</td>
</tr>
<tr>
<td>% Confirmed</td>
<td>40.7%</td>
<td>40.7%</td>
<td>0.6%</td>
<td>0.6%</td>
<td>0.7%</td>
<td>0.7%</td>
<td>4%</td>
</tr>
</tbody>
</table>
Table 2: Presumptive *V. vulnificus* isolates obtained from North Carolina estuarine waters using CPC+ and confirmed as *V. vulnificus* following PCR analysis. Shaded area indicated period of drought.

<table>
<thead>
<tr>
<th>Year</th>
<th>2006</th>
<th>2007</th>
<th>2008</th>
<th>2009 (Sept. – Dec.)</th>
<th>2010</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. Tested</td>
<td>138</td>
<td>45</td>
<td>245</td>
<td>425</td>
<td>1551</td>
</tr>
<tr>
<td>% Confirmed</td>
<td>45.7%</td>
<td>0%</td>
<td>2.4%</td>
<td>38.1%</td>
<td>42.4%</td>
</tr>
</tbody>
</table>
Table 3: Presumptive *V. vulnificus* isolates, obtained from Gulf Coast oysters using either CPC+ or CHROMagar™ Vibrio, and confirmed as *V. vulnificus* following PCR analysis.

<table>
<thead>
<tr>
<th>Year</th>
<th>No. Tested</th>
<th>% Confirmed</th>
<th>Isolated using CPC+</th>
<th>Isolated using CHROMagar™ Vibrio</th>
</tr>
</thead>
<tbody>
<tr>
<td>2008</td>
<td>80</td>
<td>96%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2009</td>
<td>31</td>
<td>98%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2010</td>
<td>20</td>
<td>100%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2010</td>
<td>10</td>
<td>100%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 4: Molecular identification of false positive isolates from CPC+ and CHROMagar™ Vibrio using 16S rDNA gene sequencing. Species listed are hits from the proprietary Accugenix sequence library that aligned to our unknown sequences with less than 2% difference.

<table>
<thead>
<tr>
<th>Colony type</th>
<th>Presumptively positive on CPC+</th>
<th>Presumptively Positive on CPC+ and CHROMagar™ Vibrio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sequence match</td>
<td>V. coralliilyticus (1.3%)</td>
<td>V. mediterranei (1.0%)</td>
</tr>
<tr>
<td>(alignment difference)</td>
<td>V. nereis (1.4%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>V. tubiashii (1.7%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>V. sinaloensis (1.8%)</td>
<td></td>
</tr>
</tbody>
</table>