



P48 Population Dynamics of *Vibrio vulnificus* in Postharvest Oysters

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Abstract

Quantitative and qualitative changes in the *Vibrio vulnificus* population in oysters (*Crassostrea virginica*) may affect the risk associated with consuming raw oysters. The objective of this study was to determine the effects of postharvest storage at 26°C on growth and qualitative characteristics of naturally occurring *V. vulnificus* in shellstock oysters. Oysters were collected monthly from May 1998 to April 1999, in Mobile Bay, AL; samples of 12 oysters were analyzed for *V. vulnificus* densities at 0, 5, 10, and 24 h postharvest. After 24 h storage at 26°C, 12 oysters were transferred to 3°C and subsequently analyzed after 14-17 d. Bacterial densities were determined by two direct plating techniques using alkaline-phosphatase- and digoxigenin-labeled-DNA probes, which targeted the species-specific cytolysin-hemolysin of *V. vulnificus*.

Isolates were examined for colony types (opaque or translucent) and LPS type. The two DNA probe methods were equivalent (r=0.89). From April to November, densities at harvest ranged from 220 to 4,000 CFU/g and <10 to 60 CFU/g from December to March. A 1.2 log₁₀ increase in *V. vulnificus* counts occurred between harvest and 10 h of storage at 26°C during April to November. Opaque *V. vulnificus* colonies predominated throughout the year. There appeared to be a greater prevalence of LPS untypable *V. vulnificus* strains during the winter and after refrigerated storage. The 1/5 LPS type (most common in clinical strains) was not detected during winter months. These results show that qualitative as well as quantitative changes may occur in the *V. vulnificus* population of postharvest oysters.

Introduction

The public health risk from consuming raw oysters may be influenced by qualitative as well as quantitative changes in *Vibrio vulnificus* populations within oysters postharvest. Qualitative characteristics (opaque/translucent colony type and lipopolysaccharide (LPS) types) in *V. vulnificus* may be used to indicate the presence or absence of important virulence determinants.

Implementation of harvesting or processing controls to eliminate the causative organisms is hindered by the lack of a reliable, well-defined virulence marker and an unknown infectious dose. Data are also lacking on the frequency of opaque vs. translucent *V. vulnificus* strains present in the environment as well as in postharvest oysters.

The objective of this study was to determine qualitative (capsule expression and LPS type) changes in *V. vulnificus* populations during postharvest storage of oysters at 26°C and 3°C.

Materials and Methods

Oyster Collection and Handling:

- Collected oysters (*Crassostrea virginica*) monthly (May 1998 to April 1999) from Mobile Bay, AL.
- Chilled 12 oysters on ice; held 60-80 oysters at ambient temperature on the boat.
- Transported oysters to FDA's Gulf Coast Seafood Lab, Dauphin Island, AL within 1 h of collection, and incubated at 26°C.
- Analyzed chilled oysters within 2 h to obtain harvest levels (0 h).
- 12 oysters (maintained at 26°C) were analyzed at 5, 10, and 24 h after harvest.
- After 24 h storage at 26°C, 12 oysters were transferred to 3°C and analyzed 14 to 17 d later.
- Oysters were scrubbed, shucked, mixed with an equal weight of sterile PBS, and blended 90 seconds in a sterile Waring blender jar.

V. vulnificus Enumeration by Direct Plating and VVAP Probe Detection (Direct-VVAP):

- Aliquots of oyster homogenate (0.20 g of a 1:1, w/w, taken directly from a blended sample, or 0.1 ml portions from ten-fold dilutions in PBS) were spread-plated onto *Vibrio vulnificus* agar plates and incubated at 35°C for 18-24 h (DePaola et al., 1997, J. Microbiol. Meth. 29:115-120).
- Filter preparation, hybridization, and colorimetric visualization were performed according to Wright et al. (1993, Appl. Environ. Microbiol. 59:541-546) and DePaola et al. (1997).
- The oligonucleotide probe (VVAP) conjugated with alkaline phosphatase, which targeted the cytolysin-hemolysin gene in *V. vulnificus*, was purchased from DNA Technology, Denmark.

- Alkaline phosphatase-positive colonies were visualized by incubation in NBT/BCIP substrate solution according to the manufacturer's instructions (Boehringer Mannheim Corp.).

V. vulnificus Enumeration by Direct Plating and VV-Dig Probe Detection (Direct VV-Dig):

- Nylon membranes, placed on TSAMS agar plates (TSA + 0.15% MgSO₄ + 2% extra salt), were spread-plated with the same dilutions of oyster homogenate used for the Direct-VVAP probe. After 3 h, transferred membranes to cellobiose-colistin (CC) agar plates and incubated at 40°C for 18-24 h (Høi et al., 1998, Appl. Environ. Microbiol. 64:1721-1724).

- Probe, filter preparation, and colorimetric detection were performed according to The Genius System User's Guide for Filter Hybridization (Version 2, 1992, Boehringer Mannheim Corp.). Color development in NBT/BCIP.

Lipopolysaccharide (LPS) Typing of Confirmed *V. vulnificus* Isolates:

- Monoclonal antibodies (MAb) were produced to recognize five unique types of LPS-associated O-antigens on the outer membrane surface of *V. vulnificus* (A. Zuppardo, LA DHH, New Orleans, LA provided the *V. vulnificus* LPS MAb used in this research).

- Enzyme immunoassay (EIA) technology was used to determine the LPS type of each confirmed *V. vulnificus* isolate.

Vibrio vulnificus Harvest Densities

Month	Direct-VVAP	Direct-VV Dig	Water Temp. (°C)	Salinity (ppt)
May	2800	1700	28.0	12.0
Jun	1000	ND	32.5	20.0
Jul	700	300	32.5	25.0
Aug	4000	2900	32.5	15.0
Sep	3400	1600	30.5	22.0
Oct	1380	580	27.0	18.0
Nov	900	220	22.0	20.0
Dec	10	30	20.0	18.0
Jan	10	<10	10.0	12.0
Feb	20	<10	18.0	4.0
Mar	60	<10	18.0	8.5
Apr	1400	250	24.0	8.7

ND = Not Determined

Number of Confirmed *V. vulnificus* Opaque (Op) and Translucent (Tr) Colonies by Month and Hours of Storage

Time	Jun Op/Tr	Jul Op/Tr	Aug Op/Tr	Sep Op/Tr	Oct Op/Tr	Nov Op/Tr	Dec Op/Tr	Jan Op/Tr	Feb Op/Tr	Mar Op/Tr	Apr Op/Tr	Total
0 h	8/0	5/0	30/1	6/3	27/9	13/13	6/0	0/0	14/0	0/0	25/20	134/46
5 h	9/0	5/0	29/1	9/1	27/8	6/5	13/2	0/0	23/0	1/0	29/12	151/29
10 h	11/0	1/0	9/2	0/0	10/1	5/6	0/0	0/0	0/0	0/0	34/14	70/23
24 h	0/0	2/1	7/1	1/0	13/2	12/23	0/1	2/3	1/3	2/0	26/8	66/42
14-17 d	0/0	0/0	11/1	5/7	21/5	5/2	13/3	0/0	5/1	3/1	30/4	93/24
Total	28/0	13/1	86/6	21/11	98/25	41/49	32/6	2/3	43/4	6/1	144/58	514/164

LPS Types of Confirmed *V. vulnificus* Isolates Grouped by Hours of Storage at 26°C

Hour	n	Type 1 and Type 5	Type 2	Type 3	Type 4	Total % Typable
0	192	16	46 (24%)	7	14	43%
5	193	20	25 (13%)	8	26 (13%)	41%
10	151	18	27 (18%)	4	22	47%
24	113	13	19	5	41 (36%)	69%
14-17 d	123	3	12 (10%)	4	8	22%
Total	772	70	129	28	111	

Results

0 h LPS Types and Percentages for Each Month

Month	Type 1 and Type 5	Type 2	Type 3	Type 4	Total % Typable
May (12)*	1	1	1	1	33%
Jun (8)	5 (63%)	2	1	0	100%
Jul (5)	1	1	0	1	60%
Aug (31)	5	10 (32%)	0	0	48%
Sep (9)	0	1	0	1	22%
Oct (36)	2	7 (19%)	2	7 (19%)	50%
Nov (26)	0	5 (19%)	1	4	38%
Dec (6)	0	0	0	1	17%
Jan (0)	0	0	0	0	0%
Feb (14)	0	0	0	0	0%
Mar (1)	0	0	0	0	0%
Apr (44)	2	19 (43%)	1	0	50%

* Number of confirmed *Vibrio vulnificus* isolates for the 0 hr time point.

LPS Types by Month

(All Time Points were Pooled within Each Month)

Month	n*	Type 1 and Type 5	Type 2	Type 3	Type 4	Total % Typable
May	44	8 (18%)	5	1	5	43%
Jun	28	9 (32%)	3	3	1	57%
Jul	14	5 (36%)	1	0	1	50%
Aug	92	11	21 (23%)	6	6	48%
Sep	32	4	5 (16%)	1	5 (16%)	47%
Oct	139	15	25 (18%)	4	16	43%
Nov	106	1	9	2	60 (57%)	68%
Dec	53*	5	5	1	7 (13%)	34%
Jan	5	0	1	1	1	60%
Feb	47	0	0	0	0	0%
Mar	12	0	3 (25%)	1	0	33%
Apr	200*	12	51 (26%)	8	9	40%

* No 14 d LPS data for these time points.

* Total number of *V. vulnificus* isolates for 0, 5, 10, 24 h and 14 d time points.

Conclusions

- From April to November, *V. vulnificus* harvest densities ranged from 220 to 4,000 CFU/g and <10 to 60 CFU/g from December to March.
- The two DNA probe methods (Direct VVAP and Direct VV-Dig) were equivalent to each other (r = 0.89).
- Opaque *V. vulnificus* predominated throughout the year.
- There was a greater prevalence of LPS untypable *V. vulnificus* strains during the winter and after refrigerated storage. Of the 772 total confirmed *V. vulnificus* isolates, 56 % were LPS untypable and 44 % were typable.
- LPS type 1/5 (most common in clinical strains) was not detected during the winter months.