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An Overview of *Vibrio vulnificus* and *Vibrio parahaemolyticus*

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ABSTRACT: The Vibrionaceae are environmentally ubiquitous to estuarine waters. Two species in particular, *V. vulnificus* and *V. parahaemolyticus*, are important human pathogens that are transmitted by the consumption of contaminated molluscan shellfish. This document provides a comprehensive review of the current state of knowledge about these important foodborne disease agents. Topics include the epidemiology of human disease; biotypes and virulence factors; cultural and molecular-based detection methods; phenotyping and genotyping approaches; microbial ecology; and candidate control strategies. Recent international risk assessment efforts are also described. The reader will gain an understanding of why these organisms pose a public health risk and how improving our understanding of their behavior in the environment and the host can aid in reducing that risk in the future.

Introduction

In the United States, contaminated seafood is responsible for 26.5% of all foodborne disease outbreaks (Mead and others 1999) with the majority of these illnesses associated with the consumption of raw bivalve molluscan shellfish (Cook 1991). Bivalves, including oysters, clams, mussels, and cockles, are filter-feeding organisms that pump seawater through their digestive systems to obtain oxygen and food and, in this process, accumulate and concentrate microorganisms. These organisms can be harmless commensals as well as pathogens, the most significant of which are the human enteric viruses and the pathogenic *Vibrio* species. Since shellfish are frequently consumed whole and raw, they can serve as passive carriers of foodborne disease agents.

Vibrio species designations

The genus *Vibrio* is in the family Vibrionaceae, which also includes the genera *Aeromonas, Plesiomonas*, and *Photobacterium* (Atlas 1997). All vibrios are ubiquitous in the marine environment and all species except *Vibrio cholerae* and *Vibrio mimicus* require sodium chloride supplementation of media for growth. There are 30 species in the genus *Vibrio*; 13 of these are pathogenic to humans, including *V. cholerae, V. mimicus, V. fluvialis, V. parahaemolyticus, V. alginolyticus, V. cincinnatiensis, V. hollisae, V. vulnificus, V. furnissii, V. damsela, V. metshnikovii*, and *V. carchariae*. All of the pathogenic vibrios have been reported to cause foodborne disease, although *V. cholerae* O1, *V. parahaemolyticus*, and *V. vulnificus* are considered the most significant agents. Members of the *Vibrio* genus are straight or curved Gram-negative, nonspore-forming rods, 0.5 to 0.8 μm in width and 1.4 to 2.6 μm in length (McLaughlin 1995). However, when they are grown in the laboratory, they frequently revert to straight rod morphology (Atlas 1997). Vibrios are motile by a single polar flagellum and are aerobic or facultatively anaerobic. Most species produce oxidase and catalase and ferment glucose without producing gas (McLaughlin 1995). *V. vulnificus* is similar phenotypically to *V. parahaemolyticus* (Oliver 1989). The 2 most distinctive characteristics of *V. vulnificus* are fermentation of lactose and production of β-D-galactosidase and these biochemical tests for them can be used to distinguish it from the related *V. parahaemolyticus* (Hollis and others 1976).

Classification of *V. vulnificus* strains

Historically, *V. vulnificus* strains have been classified by biotyping, a technique based on a combination of different phenotypic, serologic, and host range characteristics. Biotype 1 can be found in warm marine waters and was initially thought to be the only biotype associated with human infection (Blake and others 1980). Biotype 1 strains are pathogenic to humans, have different immunologically distinct lipopolysaccharide (LPS) types, and are indole positive (Biosca and others 1996). Biotype 2 was first thought to be pathogenic only to eels (Tison and others 1982), but this was later disputed based on human clinical evidence (Veenstra and others 1992; Amaro and Biosca, 1996). In addition, Amaro and others (1992a, 1992b) compared the 2 biotypes, finding that biotype 2 strains were able to adhere to human and fish cell lines and were highly cytotoxic. In addition, biotype 2 strains were more virulent for mice ($LD_{50} = 10^5$ to 10^6 CFU) when compared to biotype 1 strains ($LD_{50} = 10^8$ CFU). In general, biotype 2 strains have the following characteristics: pathogenic to both humans and eels; expression of a common LPS type; and negative indole reaction (Biosca and others 1996). In 1996, *V. vulnificus*

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biotype 3 was first described when it was associated with an outbreak involving 62 Israeli patients with either wound infection or septicemia (Bisharat and others 1999, 2005). To date, human disease caused by biotype 3 has not been associated with food consumption.

There appears to be a relationship between different 16S rRNA sequences and the virulence of *V. vulnificus* and this has been used as a means of strain typing as well. The sequence of 16S rRNA is highly conserved among all organisms and is commonly used to discern the evolutionary relationships among prokaryotes. Various regions within the rRNA genes evolve at slightly different rates, resulting in alternating regions of nucleotide conservation and variability (De Rijk and others 1992; Van de Peer and others 1996). Recently, Nilsson and others (2003) reported differences in rRNA sequences between clinical and environmental *V. vulnificus* strains. These data showed that two 16S rRNA types (designated A and B) contain a 492 bp-amplified region which has an *Alu*I cleavage site after nucleotides 202 and 244, and a *Hae*III cleavage site after nucleotides 168 and 372. The difference between types A and B is that the type A sequence has an additional *Alu*I cleavage site after nucleotide 140, whereas type B has an additional *Hae*III site after nucleotide 147. In general, the B sequence is more highly associated with clinical strains and the A sequence is associated with environmental isolates. Additionally, Lin and Schwarz (2003) found more type A strains to be isolated in June and July, while more type B strains were isolated in September.

Pathogenicity of *V. vulnificus*

V. vulnificus virulence is multifaceted and not well understood. Indeed, many virulence factors have been reported for this organism, including (1) a polysaccharide capsule; (2) various extracellular enzymes; (3) exotoxins; and (4) the ability to obtain iron from transferrin (Linkous and Oliver 1999; Gulig and others 2005). The absence of estrogen has also been cited as a host factor linked to increased risk of infection (Linkous and Oliver 1999).

The presence of a capsule, which is also related to colony opacity, is probably the best-known virulence factor for *V. vulnificus*. *V. vulnificus* is an extracellular pathogen that relies on its polysaccharide capsule to avoid phagocytosis by host defense cells (Linkous and Oliver 1999; Strom and Paranjpye 2000; Gulig and others 2005). The transformation of encapsulated isolates to the nonencapsulated form is dependent on growth phase and temperature, which in turn affect bacterial cell morphology. For instance, Wright and others (1990) found an increase in the expression of capsular polysaccararide (CPS) during the logarithmic growth phase and a decrease during the stationary phase of growth for a clinical isolate of *V. vulnificus*. Also, there was significant expression of CPS observed for cells grown at 30 °C as compared to those grown at 37 °C. Encapsulated isolates have opaque colony morphology but can undergo a reversible phase variation to the translucent colony phenotype, which is correlated with reduced CPS production (Wright and others 1990; Strom and Paranjpye 2000). Wright and others (1990) reported that nonencapsulated strains (clinical) produced by transposon mutagenesis had a lethal dose over 4 times higher than that of the encapsulated strains. Research has shown that infection with *V. vulnificus* elicits an antibody response specific to the capsule (Foire and others 1992) and *V. vulnificus,* like other bacteria, relies on the capsule to resist host defenses during systemic disease.

There is evidence that several extracellular enzymes play a role in *V. vulnificus* pathogenicity. Moreno and Landgraf (1998) reported that the enzymes lecithinase, lipase, caseinolytic protease, and DNase were present in > 90% of the *V. vulnificus* strains screened, all of which were isolated from seafood samples. The protease may be particularly important, as Oliver and others (1986) found that 91% of the clinical and environmental strains of *V. vulnificus* screened produced a protease that was capable of breaking down native albumin, hypothesizing that this protease might be involved in promoting systemic infection. A separate metalloprotease containing a zinc atom is able to degrade a number of biologically important host-associated proteins, including elastin, fibrinogen, and plasma protease inhibitors (Miyoshi and others 1995). The most dramatic pathological action of the metalloprotease is its vascular permeability-enhancing action (Shinoda and Miyoshi 2000).

The exotoxin hemolysin/cytolysin produced by *V. vulnificus* has been the most studied virulence marker. Hemolysin/cytolysin, encoded by a gene designated *vvh*A (other abbreviations are *cth* and *hha*), is a heat-labile enzyme that lyses mammalian erythrocytes and is cytotoxic to a variety of mammalian tissue culture cell lines (Gray and Kreger 1985; Strom and Paranjpye 2000). The *vvhA* protein displays 65% and 60% amino acid sequence similarity to the *V. cholerae* El Tor hemolysin and *V. cholerae* non-O1 cytolysin, respectively (Yamamoto and others 1990; Wright and Morris 1991; Strom and Paranjpye 2000). Gray and Kreger (1986) reported antibodies specific to the *V. vulnificus* hemolysin in the blood of infected mice, suggesting that the enzyme plays a role in pathogenicity. Later, Gray and Kreger (1987) demonstrated that mice injected with hemolysin developed skin damage similar to that of infected humans. Lee and others (2004) found that 20% normal pooled human serum significantly inhibited hemolytic and cytotoxic activities of the *vvhA* protein, suggesting that it could be inactivated *in vivo* and that its activity might be compromised by serum constituents such as cholesterol. When these same investigators inoculated mice intraperitoneally with 10^7 CFU of a clinical *V. vulnificus* isolate, they observed the expression of the *vvh*A gene product in bacterial cells isolated from host livers, suggesting that the protein itself is produced *in vivo* and in association with particular tissues.

The amount of iron available in the host is an important factor influencing the lethality of *V. vulnificus*. Wright and others (1981) showed that the intraperitoneal LD_{50} was reduced from 10^6 CFU to 1 CFU in iron-treated mice. Later, Reyes and others (1987) classified both clinical and environmental strains of *V. vulnificus* into categories of virulent and avirulent, with the former demonstrating a lethal infectious dose of $< 10^5$ CFU/mL, while the latter failed to kill suckling mice at doses $> 10^9$ CFU/mL, although route of administration was an important mitigating factor. Morris and others (1987) found that none of the *V. vulnificus* strains (clinical and environmental) tested was capable of growth in iron-limited media in the presence of 30% saturated transferrin; however, some strains were able to grow in the presence of 100% saturated transferrin. These investigators hypothesized that the increased saturation of transferrin, either through an excess of iron or through a relative decrease in the amount of transferrin, may be associated with the pathogenesis of *V. vulnificus* (Morris and others 1987; Brennt and others 1991). Transferrin is an iron transport protein and, because free iron is virtually absent in the body, pathogenic bacteria like *V. vulnificus* may have evolved mechanisms to scavenge iron from the iron transport proteins (Strom and Paranjpye 2000). Alternatively, they may use iron-scavenging siderophores and proteins that can serve as iron donors (such as phenolate and hydroxamate [Simpson and Oliver 1983] and hemoglobin, methemoglobin, and hematin [Helms and others 1984]) (Gulig and others 2005). Stelma and others (1992) used the iron-overloaded mouse model to characterize the virulence of various *V. vulnificus* strains of clinical and environmental origin, finding that iron-overloaded mice died after challenge with lower doses ($< 10^2$ CFU) of a virulent strain as compared to higher doses ($> 4.0 \times 10^3$ CFU) of an avirulent strain. Starks and others (2000) found that 3 clinical strains and

3 attenuated isolates of *V. vulnificus* from oysters or seawater caused identical skin lesions in subcutaneously inoculated iron dextran-treated mice; however, the inocula required for identical frequency and magnitude of infection were at least 350-fold higher for the environmental strains. The investigators' data suggested that the difference between these clinical and environmental strains might be related to their ability to grow in the host and/or susceptibility to host defenses. In addition, Starks and others (2000) reported that clinical and environmental strains of *V. vulnificus* required 10^5-fold higher inocula to cause an identical disease process in normal mice as compared to those treated with iron dextran. However, DePaola and others (2003), who evaluated strains of *V. vulnificus* obtained from market oysters and from oyster-associated primary septicemia cases, found that 88% of all the strains characterized were virulent when subcutaneously inoculated into iron dextran-treated mice, suggesting little strain-to-strain variability in the infection process when animals cannot appropriately metabolize iron. Recently, Choi and others (2006) conducted a study on the cyclic AMP-cAMP receptor protein (CRP) complex by creating a *crp* deletion mutant to study the role this complex plays in *V. vulnificus* virulence. They found that *V. vulnificus* growth decreased under iron-limited conditions. The vulnibactin-mediated iron-uptake system was suppressed along with the transcription of the *vis* and *vuuA* genes, and growth was suppressed on transferrin-bound iron and in cirrhotic ascites. Furthermore, all the defects of the *crp* mutant were restored by in-trans complementation of the wild-type *crp* gene. These data suggest that the CRP complex plays an important role in iron utilization (Choi and others 2006).

Epidemiological evidence suggests that men are more susceptible to *V. vulnificus* infection than women. For instance, Shapiro and others (1998) reported that 86% of the reported cases of *V. vulnificus* infection occurred in men. Eighty-five percent of individuals who develop endotoxic shock from *V. vulnificus* are males (Oliver 1989; Merkel and others 2001). Although this may be due to the fact that men are more likely to consume raw oysters, or that men are more likely to have underlying liver disease, a recent study by Merkel and others (2001) offers an alternative explanation related to the protective effect of estrogen. In this study, the investigators showed that male rats injected with *V. vulnificus* LPS had an 82% fatality rate whereas normal female rats treated identically had a fatality rate of only 21%. When these female rats were ovariectomized, thereby lowering their estrogen levels, fatality rates increased to 75% (Merkel and others 2001). When ovariectomized female mice were treated with subsequent estrogen replacement therapy, a decrease in mortality rates was observed, making the mortality rates of hormonally treated ovariectomized females similar to those of the nonovariectomized female mice (38% and 21%, respectively). Furthermore, gonadectomized male mice died at the same rate as nongonadectomized males. However, when gonadectomized male mice were treated with estrogen, a decrease in the mortality rate occurred (from 80% down to 50% mortality, respectively). Protection in these male mice increased with increasing estrogen dose. Taken together, the data of Merkel and others (2001) suggest that estrogen provides protection against *V. vulnificus* endotoxic shock.

Classification of *V. parahaemolyticus* strains

Historically, the species *V. parahaemolyticus* has been further classified based on serotype, which is discussed subsequently. More recently, classifications have been made based on the presence of particular genes, some of which correlate with pathogenicity. For general species delineation, the thermolabile hemolysin (*tlh*) gene is used. *V. parahaemolyticus* strains are considered "pathogenic" if the thermostable direct hemolysin (*tdh*) and/or TDH-related hemolysin (*trh*) genes are present. These genes, and their relationship to pathogenicity, are discussed in greater detail below.

Pathogenicity of *V. parahaemolyticus*

Many virulence factors are thought to play a role in the pathogenicity of *V. parahaemolyticus*, including those associated with beta-hemolysis, adherence factors, various enzymes, and the products of the *tdh, trh,* and *ure* genes. Historically, *V. parahaemolyticus* pathogenicity has been associated with the Kanagawa phenomenon (KP), which is observed as beta-hemolysis on Wagatsuma agar. Virtually all clinical isolates of *V. parahaemolyticus* are KP-positive, whereas only 1% to 2% of environmental strains are KP-positive (Sakazaki and others 1968; Miyamoto and others 1969; Nishibuchi and Kaper 1995). It is now known that the Kanagawa reaction is caused by the thermostable direct hemolysin (TDH) protein (Nishibuchi and Kaper 1995), so named because it is not inactivated by heat (100 °C for 10 min) and because its hemolytic activity is not enhanced by the addition of lecithin, suggesting direct activity on erythrocytes (Sakurai and others 1973; Nishibuchi and Kaper 1995). Kaper and others (1984) were the first to clone the gene encoding the TDH protein (designated *tdh* 1) from *V. parahaemolyticus* strain WP1, which was clinical in origin. This group subsequently used probes derived from this gene to identify *tdh* genes in other *V. parahaemolyticus* strains. Later, Hida and Yamamoto (1990) found that *V. parahaemolyticus* strain WP1 actually contained a 2nd and distinct *tdh* gene, designated *tdh* 2. A survey conducted by Nishibuchi and Kaper (1990) showed that all KP-positive (clinical) *V. parahaemolyticus* strains do indeed contain 2 *tdh* genes, whereas *V. parahaemolyticus* strains (clinical and environmental) that show weak hemolysis on Wagatsuma agar and are considered to be only KP-intermediate have only 1 *tdh* gene. When looking at KP-negative strains, most of which are of environmental origin, 16% of these strains contained 1 copy of the *tdh* gene, while the rest of the KP-negative strains did not have the *tdh* gene, suggesting that most KP-negative strains cannot produce the TDH protein (Nishibuchi and others 1985; Nishibuchi and Kaper 1995). Occasionally, isolates of other *Vibrio* spp., including *V. hollisae, V. cholerae* non-O1, and *V. mimicus,* have been found to carry the *tdh* gene (Nishibuchi and Kaper 1995).

Regardless of the importance of the Kanagawa factor and the TDH protein, KP-negative strains of *V. parahaemolyticus* have occasionally been associated with outbreaks of gastroenteritis. Honda and others (1987, 1988) reported that some KP-negative strains of *V. parahaemolyticus* associated with illness in humans produced a TDH-related hemolysin (designated TRH) which was similar but not identical to the TDH protein. The TRH protein was first found in O3:K6 strains. Furthermore, this new hemolysin, which was mostly associated with environmental *V. parahaemolyticus* isolates, was responsible for significant lethality in the mouse model when the animals were challenged by intraperitoneal injection (Sarkar and others 1987). The gene corresponding to this protein was designated *trh.* There is about a 69% similarity in nucleotide sequence when comparing the *trh* and *tdh* genes, suggesting that they evolved from a common ancestor (Honda and others 1988; Nishibuchi and others 1989). In addition, evidence exists that there are multiple forms of the *trh* gene among some *Vibrio spp.* which differ in nucleotide sequence and whose corresponding proteins differ in hemolytic activity, but which appear to be derived from a common ancestor (Kishishita and others 1992). Some clinical isolates were shown to contain both the *tdh* and *trh* genes, whereas most environmental isolates do not contain *tdh* or *trh* genes (Xu and others 1994). In a series of deletion mutation experiments, investigators deleting all or part of the *trh* gene observed that the hemolytic activity of the

protein was lost; however, the mutants were still somewhat active in cytotoxity assays and caused partial fluid accumulation in ligated rabbit small intestines (Ming and others 1994). These data suggest that virulence factors in addition to TRH and TDH are involved in the pathogenicity of *V. parahaemolyticus*. However, the CDC recently noted that *V. parahaemolyticus* strains lacking both the *tdh* and *trh* genes were associated with more severe cases of *V. parahaemolyticus* infection, many of which required hospitalization (Yu and others 2006).

Early work suggested that "adhesiveness" appears to play an important role in *V. parahaemolyticus* pathogenicity. Hackney and others (1980) found that all clinical and environmental strains of *V. parahaemolyticus* that they tested were capable of adhering to human fetal intestinal (HFI) cells, although the degree of adherence was variable. Strains isolated from patients were observed to have high adherence capability regardless of their Kanagawa reaction, whereas Kanagawa-negative strains isolated from seafood exhibited the weakest adherence. Yamamoto and Yokota (1989) reported that the ability of *V. parahaemolyticus* clinical isolates to adhere to human small intestinal mucosa correlated roughly with hemagglutinin levels in human or guinea pig erythrocytes.

Many enzymes are thought to play a role in the pathogenicity of *V. parahaemolyticus*. Baffone and others (2001) examined several enzymatic (lipase, gelatinase, and hemolysin), biological (adhesiveness, cytotoxicity, and enterotoxicity), and enteropathogenic activities of *V. parahaemolyticus* strains isolated from seawater, finding that virtually all strains tested had lipase and gelatinase activity, whereas only 10% were positive for hemolysin activity. As many as 80% and 90% of the *V. parahaemolyticus* isolates screened had adhesive and cytotoxicity capabilities, respectively. Furthermore, 30% of the *V. parahaemolyticus* strains were pathogenic to white mice using the ileal loop assay, while 60% of strains were lethal to adult mice using the whole animal bioassay.

It has been suggested that urea hydrolysis may be used as a marker to predict potentially virulent strains of *V. parahaemolyticus*. Abbot and others (1989) first reported this phenomenon, finding that the urease-positive phenotype was associated with the O4:K12 serotype. Kaysner and others (1994a) reported that *tdh*-positive isolates of clinical and environmental origin were also urease-positive, while Osawa and others (1996) reported that all clinical and environmental strains carrying the *trh* gene tested positive for urease. Iida and others (1997) found that the *ure* gene was responsible for urease production in *V. parahaemolyticus* and that the *ure* and *trh* genes were genetically linked, as demonstrated by restriction endonuclease digestion. A later study revealed close proximity of the *tdh*, *trh*, and *ure* genes on the chromosome of pathogenic (clinical) *V. parahaemolyticus* strains (Iida and others 1998). These data suggest the presence of a pathogenicity island, which may have occurred as a consequence of gene transfer, because the GC content of the *tdh* and *trh* genes is considerably lower than the mean GC content of the genomic DNA of *V. parahaemolyticus*.

The means of transfer of this putative pathogenicity island has motivated recent research endeavors. One hypothesis is the role of filamentous phage in gene transfer. For instance, Southern blot hybridization has demonstrated the integration of a filamentous phage genome into chromosomal DNA of *V. parahaemolyticus* (Chang and others 1998) and others have shown filamentous phage specifically associated with the pandemic *V. parahaemolyticus* strains (O3:K6, O4:K68, and O1:K untypeable) (Iida and others 2001; Chang and others 2002). Gene transfer by plasmid is another means by which *V. parahaemolyticus* could have obtained genes associated with pathogenicity. For instance, it is well documented that the *tdh* gene is found in many *Vibrio* species (Nishibuchi and others 1985, 1990, 1996; Honda and others 1986). Some investigators favor plasmid-mediated gene transfer between *V. parahaemolyticus* and *V. cholera* non-O1 *tdh* genes (Honda and others 1986; Baba and others 1991), while others do not (Nishibuchi and others 1985; Nishibuchi and Kaper 1990). Nonetheless, there is evidence that the *tdh* genes of many *Vibrio* species are flanked by insertion sequence-like elements (Baba and others 1991; Terai and others 1991), suggesting that the *tdh* genes may be derived from a common ancestral source and may be readily transposed within chromosomes. Lin and others (1993) reported that *V. parahaemolyticus* AQ3815 contains a *toxRS* operon, a regulatory gene that controls the expression of the *tdh* gene, similar to *V. cholerae*.

Recent sequencing efforts have aided in elucidation of the relationships between *Vibrio* species, which contain 2 circular chromosomes (Yamaichi and others 1999). Tagomori and others (2002) compared the genetic maps of KP-positive *V. parahaemolyticus* strain KX-V237, *V. parahaemolyticus* AQ4673 (clinical), and *V. cholerae* N16961, finding that the genomes of KX-V237 and AQ4673 were very similar. The large chromosomes of KX-V237 and *V. cholerae* N16961 were similar, although the small chromosomes were less so. Similarly, Makino and others (2003) found that, when comparing sequences associated with the *V. parahaemolyticus* genome to those of *V. cholerae*, there were apparently many rearrangements within and between the 2 chromosomes. The genes for the type III secretion system (TTSS) were identified in the genome of *V. parahaemolyticus*, but not in *V. cholerae*. The TTSS is a central virulence factor for diarrhea-causing bacteria such as *Shigella*, *Salmonella*, and enteropathogenic *Escherichia coli*. These data suggest that TTSS might be a mechanism associated with *V. parahaemolyticus* infection, one considerably different from the mechanism of disease caused by *V. cholerae*. In a recent study, Ono and others (2006) showed that *V. parahaemolyticus* RIMD2210633 (clinical) contains 2 sets of the gene clusters (TTSS1 and TTSS2) that encode for the TTSS.

Serovars of *V. parahaemolyticus*

Serotyping of *V. parahaeomolyticus* is done using antibodies specific to O (somatic) and K (capsular) antigens; all *V. parahaemolytiucs* strains share a common H (flagellar) antigen. To date, 12 O antigen types and over 70 K antigen types have been described, though many strains remain untypable (Kaysner and DePaola 2001). Furthermore, five of the K antigens have been found to occur with either of 2 O group antigens, yielding 76 recognized serotypes (Table 1).

In 1996, a unique serovar (O3:K6) of *V. parahaemolyticus* abruptly appeared in Calcutta, India (Okuda and others 1997).

Table 1 — Reported serotypes of *V. parahaemolyticus* (FDA BAM 2001)

O antigen	K antigen
1	1, 25, 26, 32, 38, 41, 56, 58, 64, 69
2	3, 28
3	4, 5, 6, 7, 27, 30, 31, 33, 37, 43, 45, 48, 54, 57, 58, 59, 65
4	4, 8, 9, 10, 11, 12, 13, 34, 42, 49, 53, 55, 63, 67
5	5, 15, 17, 30, 47, 60, 61, 68
6	6, 18, 46
7	7, 19
8	8, 20, 21, 22, 39, 70
9	9, 23, 44
10	19, 24, 52, 66, 71
11	36, 40, 50, 51, 61
12	52

A total of 134 strains of *V. parahaemolyticus* collected between 1994 and 1996 during active surveillance among hospitalized patients in Calcutta were classified as serovar O3:K6. The so-called Calcutta O3:K6 strain was very different from other O3:K6 strains isolated from Asian travelers between 1982 and 1993; however, the Calcutta O3:K6 strain was indistinguishable from other O3:K6 isolates obtained between 1995 and 1996 from Southeast Asian countries. This suggested that a unique O3:K6 clone may have become prevalent worldwide in the late 1990s (Okuda and others 1997; Bag and others 1999). In addition to the appearance of this new O3:K6 serovar, strains of serovars O4:K68 and O1:K untypeable (KUT) have been associated with an increased incidence of *V. parahaemolyticus* infections worldwide. Furthermore, these strains (serovars newly emerged O3:K6, O4:K68, and O1:K untypeable) appear to be highly similar by restriction fragment length polymorphism-pulsed field gel electrophoresis (RFLP-PFGE) and arbitrarily primed polymerase chain reaction (AP-PCR) (Okura and others 2003). After the appearance of these pandemic strains in India, they spread to many Asian countries. In Vietnam, from 1997 to 1999, 49% of 523 *V. parahaemolyticus* strains isolated from hospitalized patients were pandemic strains. During this survey, there was an obvious transition of prevalence between the pandemic strains, with O3:K6, O4:K68, and O1:K25 serotypes being more prevalent during 1997, 1998, and 1999, respectively (Chowdhury and others 2004). From 1998 to 200 in Bangladesh and Thailand, 66 strains of *V. parahaemolyticus* were isolated from patients and 14 different serotypes were identified (Bhuiyan and others 2002). Taiwan observed an increase in food-borne outbreaks during 1996 to 1999 with the new *V. parahaemolyticus* serovar O3:K6 accounting for 50.1% to 83.8% of annual *V. parahaemolyticus* infections (Chiou and others 2000). Wong and others (2000) compared O3:K6 strains from India, Japan, Taiwan, and Korea by RFLP-PFGE and found 13 different patterns. Cluster analysis revealed 2 distinct cluster groups; 1 group contained all strains isolated before 1996 and a 2nd group consisted of strains isolated after 1996. This was the 1st report that demonstrated that the new O3:K6 strains from Korea, Taiwan, Japan, and India were genetically related. Chowdhury and others (2000) showed that some strains with serotypes O4:K68 and O1:KUT have RFLP-PFGE patterns similar to the pandemic O3:K6 strains, suggesting that they may have originated from the Calcutta O3:K6 pandemic strain. In a recent review, Nair and others (2007) postulated that other serotypes with identical genotypes and molecular profiles to those of O3:K6 emerged from a single O3:K6 serotype. These were collectively referred to as "serovariants" of O3:K6. These serovariants appeared to have diverged from the O3:K6 isolates by alteration of the O and K antigens, and they constitute what are now considered as pandemic O3:K6 strains.

During 1998 there were *V. parahaemolyticus* outbreaks in the United States associated with pandemic O3:K6 strains (DePaola and others 1998). Matsumoto and others (2000) compared pandemic O3:K6 strains from North America and Asia using molecular methods and demonstrated that the North American strains were indistinguishable from the Asian strains. This was a significant finding as never before had *V. parahaemolyticus* been considered pandemic (Matsumoto and others 2000). The pandemic O3:K6 serotype was implicated in 2 outbreaks in Chile in 1998 and 2004 (Gonzalez-Escalona and others 2005). Quilici and others (2005) reported the presence of the pandemic *V. parahaemolyticus* O3:K6 serovar in France and, during this same time frame, Martinez-Urtaza and others (2005) described a pandemic O3:K6 outbreak in Spain, suggesting that this serovar had spread to Europe. Ansaruzzaman and others (2005) reported the 1st appearance of the pandemic serovars of *V. parahaemolyticus* in sub-Saharan Africa, with 42 cases of *V. parahaemolyticus* in Beira, Mozambique, from February to May 2004. Of the 42 isolates, 32 belonged to the O3:K6 serotype, 2 belonged to the O4:K68 serotype, and the remaining 8 isolates did not belong to any of the known pandemic serovars. In 2005, Fuenzalida and others (2006) described the largest *V. parahaemolyticus* outbreak ever reported (about 11000 cases), caused by the pandemic O3:K6 strains and associated with Chilean shellfish consumption. However, analysis of shellfish isolates showed only 3/50 samples positive for *V. parahaemolyticus* contained detectable levels of pandemic O3:K6 strains. Nonpathogenic *V. parahaemolyticus* was isolated from the majority of samples and was separated into 14 distinct groups by direct genome restriction enzyme analysis (DGREA); these were clearly distinguishable from the pandemic clone.

Epidemiology

There are 3 major clinical manifestations of *Vibrio* infection: wound infection, primary septicemia, and gastroenteritis. Although both *V. vulnificus* and *V. parahaemolyticus* cases occur sporadically, the former are almost always sporadic while the latter can also occur in outbreak settings. Desenclos and others (1991) used the case control study design to estimate the annual incidence of all *Vibrio* infection at 95.4 per million for raw oyster consumers with liver disease, 9.2 per million for raw oyster consumers without liver disease, and 2.2 per million for those who do not consume raw oysters. Another case control study conducted by Hlady and Klontz (1996) reported disease manifestation proportions of 51%, 24%, and 17% for gastroenteritis, wound infection, and septicemia, respectively. Fatality rates were only 1% for gastroenteritis, but were 5% for wound infection and 44% for septic disease. Sixty-eight percent of gastroenteritis and 83% of primary septicemia cases were associated with raw oyster consumption. Ninety-one percent of the primary septicemia cases and 86% of the wound infections occurred in April through October, with 48% of those with primary septicemia reporting pre-existing liver disease (Hlady and Klontz 1996). Possibly, as a consequence of recent climate events such as El Niño, which caused the water temperatures to be warmer than normal, about 20% of all *V. vulnificus* primary septicemia cases since 2000 have occurred in November (M. Glatzer, personal communication, 2006).

Gastroenteritis. When *V. vulnificus* and *V. parahaemolyticus* are isolated from stool alone, they are characterized as causing gastroenteritis (Strom and Paranjpye 2000). Gastroenteritis caused by *V. vulnificus* and *V. parahaemolyticus* may go unreported since the disease is not usually life-threatening and symptoms are typically not severe enough to warrant medical attention. In a study conducted by Hlady and Klontz (1996), *V. parahaemolyticus*, *V. cholerae*, *V. hollisae*, *V. mimicus*, and *V. fluvialis*, as well as *V. vulnificus*, were all associated with the gastrointestinal disease syndrome. *V. parahaemolyticus* is the vibrio most often associated with gastroenteritis. In fact, *V. parahaemolyticus* seafood-borne gastroenteritis is the leading cause of foodborne disease outbreaks in Taiwan and Japan (Pan and others 1997). Chiou and others (2000) reported that 542 out of 850 outbreaks in Taiwan between 1995 and 1999 were caused by *V. parahaemolyticus*; with 40 serovars (primarily O3:K6) represented. Su and others (2005) reported that, during 1995 to 2001, there were 2057 cases of *V. parahaemolyticus* in northern Taiwan; the majority (99.4%) of *V. parahaemolyticus* strains could be identified by K serotyping, with 55.2% representing the K6 serovar.

Gastroenteritis outbreaks caused by *V. parahamolyticus*. Historically, *V. parahaemolyticus* has been associated primarily with sporadic disease in the United States; however, large gastroenteritis outbreaks have occurred. Early on, postcooking contamination

of crustaceans was associated with outbreaks. In the late 1990s, there was a shift toward links to the consumption of raw oysters. In a 1981 outbreak in Washington and Oregon, raw oysters from Willipa Bay, Wash., were implicated. All 5 isolates obtained from the feces of individuals showing gastrointestinal symptoms hydrolyzed urea, were KP-positive, and belonged to serotype O4:K12 (Nolan and others 1984). In 1988, *Vibrio* surveillance began in 4 Gulf Coast states (Alabama, Florida, Louisiana, and Texas) and by the end of that year, 34 *V. parahaemolyticus* cases had been reported with 1 case of septicemia, 26 cases of gastroenteritis, and 6 wound infections (Levine and others 1993). Between 1988 and 1997, a total of 345 cases of *V. parahaemolyticus* infection were reported to the CDC by the Gulf Coast *Vibrio* Surveillance System. Of these cases, 59% were gastroenteritis, 34% were wound infections, and 5% were septicemia (Daniels and others 2000).

In 1997, a culture-confirmed outbreak of *V. parahaemolyticus* occurred in North America and resulted in 209 cases, all attributable to the consumption of oysters harvested from coastal waters of California, Oregon, Washington, and British Columbia. Many different serotypes were isolated from patients, some of which matched those identified from oyster samples (CDC 1998). The following year, another multistate outbreak associated with the consumption of raw oysters harvested from the Galveston Bay, Tex., occurred. In this case, *V. parahaemolyticus* infections were reported in 296 Texas residents and 120 individuals from 12 other states. Subsets of the clinical isolates collected were all identified as the *V. parahaemolyticus* pandemic serotype O3:K6, which contained the *tdh* gene. Although none of the oyster isolates had RFLP-PFGE patterns matching the clinical strains, the RFLP-PFGE patterns of the Galveston Bay and the Asian *V. parahaemolyticus* pandemic O3:K6 strains were shown to be distinct but closely related (Matsumoto and others 2000). Consumption of shellfish or crustaceans harvested from Long Island, N.Y., waters were implicated in another *V. parahaemolyticus* outbreak in 1998. In this case, 12 *V. parahaemolyticus* clinical isolates were identified as the pandemic O3:K6 serotype (CDC 1999). In 2006, another *V. parahaemolyticus* outbreak occurred in New York, Oregon, and Washington, with a total of 177 cases of which 72 were confirmed. In this outbreak, the strains implicated were not of the pandemic serotype. Contaminated oysters and clams harvested from Washington and British Columbia sites were linked in the traceback investigation (CDC 2006).

Following the outbreaks in Washington, Texas, and New York in 1997 and 1998, DePaola and others (2000) tested shellfish from the same location as the outbreaks for total *V. parahaemolyticus* and pathogenic (*tdh* and/or *trh*) strains. These investigators recovered *V. parahaemolyticus* in 77% of the Pacific Northwest oyster samples tested, with pathogenic strains detected at densities of < 10 MPN/g in only 15% of the 1997 samples, and no pathogenic strains detected in the 1998 samples. However, all Texas oyster samples tested positive for *V. parahaemolyticus*, most with densities ranging between 100 and 1000 MPN/g; 1 sample had a density of 23000 MPN/g. Only 2 samples tested positive for pathogenic strains. New York samples had total *V. parahaemolyticus* densities ranging from <10 to 120 MPN/g but no samples tested positive for pathogenic strains. These data show that the levels of *V. parahaemolyticus* vary widely in different harvesting locations, and that the proportion of pathogenic strains is generally quite small and they are frequently nondetectable.

In 2004 July, an outbreak of *V. parahaemolyticus* occurred in Alaska; 62 people were reported as having gastroenteritis associated with consumption of raw oysters harvested from Alaskan waters and served during a cruise. Nine stool samples were confirmed as positive for *V. parahaemolyticus* and 8 isolates were sent to the CDC for typing; *V. parahaemolyticus* O6:K18 was identified as 7 of the 8 clinical isolates. All oyster samples taken from an implicated cruise ship were positive for *tdh*, and 4 different serotypes (O6:K18, O1:K9, O5:K17, and O10:K68) were represented. Ninety-six oyster samples were taken from Alaskan farms and 31 samples were positive for *V. parahaemolyticus*. All samples positive for *V. parahaemolyticus* came from farms in the Prince William Sound and southeastern Alaska. In this case, 11 serotypes were identified, but all O6:K18 isolates came from a single farm. The RFLP-PFGE patterns obtained for the clinical and oyster isolates were highly related. The RFLP-PFGE pattern of O6:K18 isolates observed in this outbreak was similar to that of the O6:K18 isolates found in Pudget Sound, suggesting possible spread of these strains by such routes as discharge of ballast water, migration of marine animals, or sea birds. Interestingly, all oysters were harvested when the mean daily water temperature was 15 °C or greater; previously, it was thought that Alaskan waters were too cold to harbor *V. parahaemolyticus* (McLaughlin and others 2005).

Wound infections. *V. vulnificus* and *V. parahaemolyticus* are most often associated with wound infections, although Hlady and Klontz (1996) reported that other *Vibrio* species can occasionally be responsible for this disease syndrome. Wound infections are defined as those cases where a patient incurred a wound before or during exposure to seawater, seafood drippings, or punctures from spines or bones, and from which *V. vulnificus* or *V. parahaemolyticus* was subsequently cultured from that wound, blood, or an otherwise normally sterile site (Strom and Paranjpye 2000). The majority of wound infections, whether caused by *V. vulnificus* or *V. parahaemolyticus*, occur in fishermen and seafood processors. In a study conducted by Strom and Paranjpye (2000), 69% of wound infections appeared to be related to occupational exposures among oyster shuckers and commercial fishermen.

Atypical infections. There have been some atypical infections caused by *Vibrio* spp. reported in the literature. Vartain and Septimus (1990) were the first to describe osteomyelitis caused by *V. vulnificus* in a person who scraped his leg on a rock in brackish water. The patient initially developed a wound infection; 13 wk later the bone was infected. An ocular infection caused by *V. vulnificus, Plesiomonas shigelloides,* and *Shewanella putrefaciens* occurred when a fisherman was struck in the eye with a fishhook (Butt and others 1997). Johnson and Arnett (2001) reported a case of septic arthritis in a patient who consumed oysters the day before onset and *V. vulnificus* was isolated from blood and synovial fluid around an arthritic wrist. A fatal case of *V. vulnificus*-associated meningoencephalitis occurred in a patient who consumed raw fish and had a history of chronic liver disease (Kim and others 2003). Penland and others (2000) reported 17 cases of trauma-associated ocular infections, including 7 caused by *V. vulnificus,* 5 by *V. alginolyticus*, 3 by *V. parahaemolyticus,* and 1 each by *V. albensis* and *V. fluvialis*. The 1st case of a *V. vulnificus* ocular infection not associated with trauma was recently reported in Korea and linked to raw fish consumption (Jung and others 2005).

Primary septicemia caused by *V. vulnificus*. Primary septicemia caused by *V. vulnificus* is usually associated with the consumption of raw shellfish and is defined as a systemic illness characterized by fever and shock and in which *V. vulnificus* is isolated from blood or an otherwise sterile site (Strom and Paranjpye 2000). Although most often caused by *V. vulnificus*, Hlady and Klontz (1996) showed that *V. cholerae* non-O1 and *V. parahaemolyticus* can cause septic disease. Fortunately, these infections are relatively rare and, on average, there are 32 *V. vulnificus* culture-confirmed primary septicemia cases reported to CDC annually, with nearly all of these associated with the consumption of oysters

harvested from the Gulf of Mexico. Because this does not include cases for which there is no food history, the CDC estimates approximately 100 primary septicemia cases per year in the United States (Mead and others 1999). The Korean CDC estimates 40 to 70 confirmed cases annually in that country (Korea Center for Disease Control and Prevention 2004). This apparent higher incidence of *V. vulnificus* infections in Korea may be the result of greater exposure due to high consumption of raw seafood or a higher prevalence of predisposing factors. It is well recognized that there are specific risk factors for the development of *V. vulnificus* sepsis (Hlady and Klontz 1996). Not only is raw oyster consumption a risk factor, but underlying liver diseases, including cirrhosis, damage to the liver due to alcoholism, and chronic hepatitis, are strong predictors for fatal outcomes of *V. vulnificus* sepsis, with 80% of those who die from the infection falling into these risk groups (Shapiro and others 1998; Strom and Paranjpye 2000).

Methods of isolation and detection

Culture methods. Multiple methods are recommended for the detection and/or enumeration of *Vibrio* species. The FDA Bacteriological Analytical Manual (BAM) (Kayser and DePaola 2001) cites standard procedures for the recovery of *V. vulnificus* and *V. parahaemolyticus* from raw molluscan shellfish. For enumeration, most probable number (MPN) analysis (Figure 1) or direct plating on nonselective media followed by DNA colony hybridization are the 2 techniques most frequently used (Figure 2). Briefly, MPN analysis for the enumeration of either organism is done by 10-fold serial dilution of shellfish samples in phosphate-buffered saline (PBS), followed by inoculation of dilutions in alkaline peptone water (APW), typically in triplicate. APW is incubated at 35 to 37 °C for 18 to 24 h and tubes positive for growth are streaked onto modified cellobiose-polymyxin B-colistin (mCPC) agar (for isolation of *V. vulnificus*) and/or thiosulfate-citrate-bile salts-sucrose

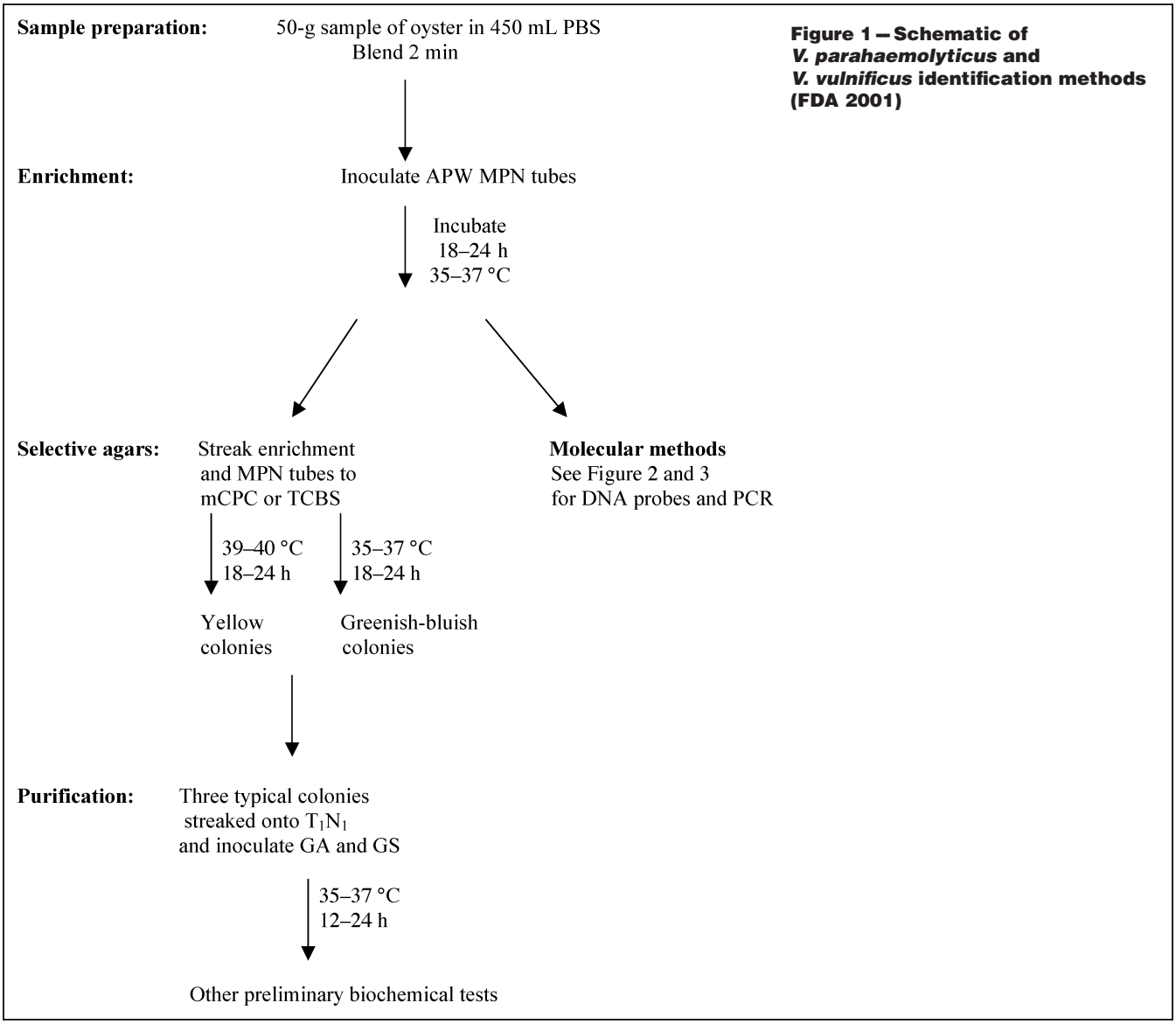

Figure 1 — Schematic of *V. parahaemolyticus* and *V. vulnificus* identification methods (FDA 2001)

(TCBS) agar (for isolation of *V. parahaemolyticus*). The mCPC and TCBS plates are incubated for 18 to 24 h at 39 to 40 °C and 35 to 37 °C, respectively, followed by examination for typical colonies. For biochemical identification, 3 or more typical colonies from each agar type are subjected to oxidase, arginine-glucose slant (AGS), ornithine decarboxylase, O/129 Vibriostat sensitivity, and the ONPG tests (Table 2). Alternatively, biochemical profiles can be obtained using API 20E (bioMerieux Inc., Hazelwood, Mo., U.S.A.) strips. As an alternative to biochemical identification, the FDA BAM suggests the use of species-specific alkaline phosphatase-labeled DNA probes (Figure 2) or PCR (Figure 3). Probes targeting the cytolysin gene (*vvhA*) are used for the identification of *V. vulnificus*, while those targeting sequences for the *tlh* can be used to identify *V. parahaemolyticus*.

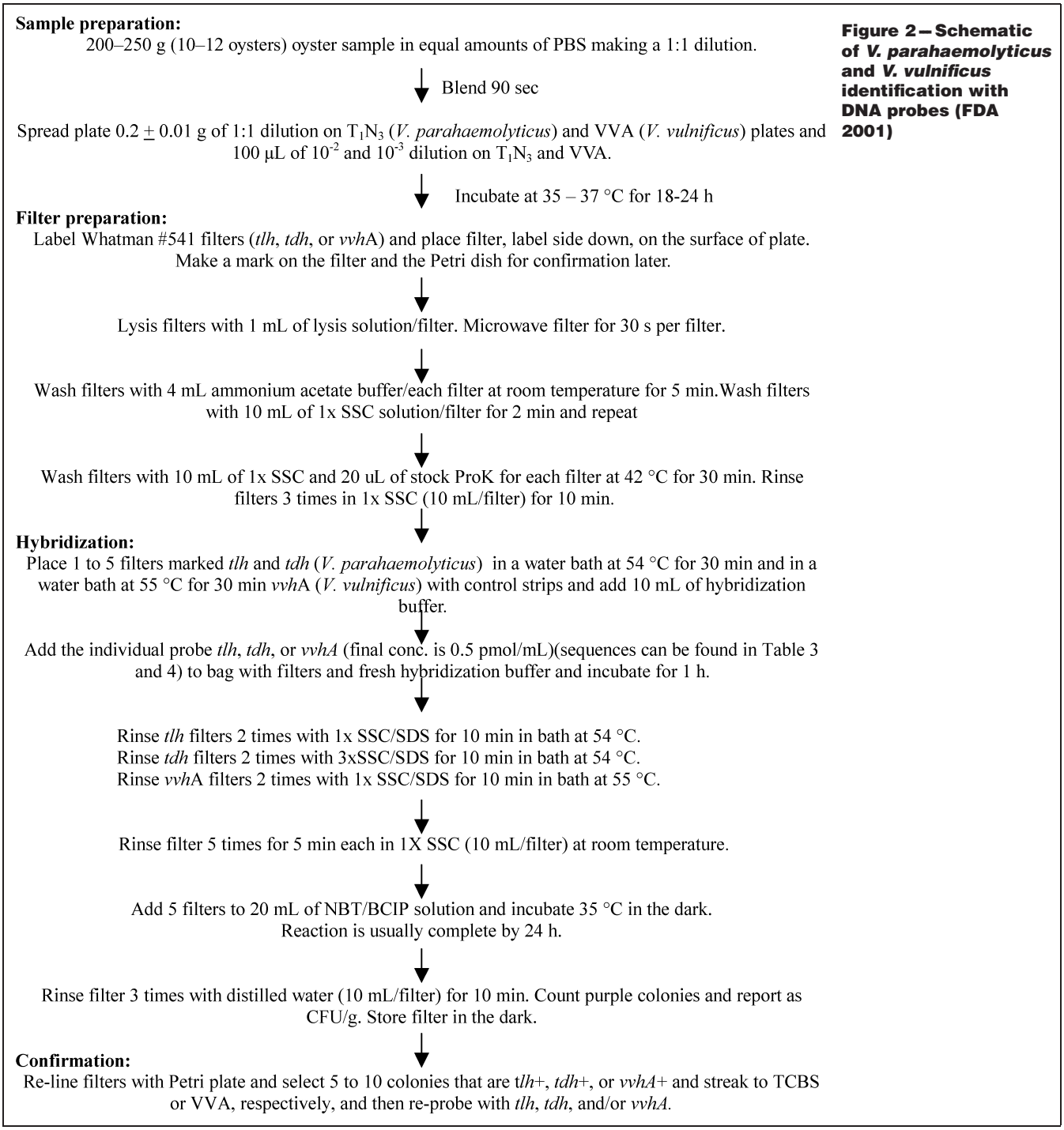

Figure 2—Schematic of *V. parahaemolyticus* and *V. vulnificus* identification with DNA probes (FDA 2001)

Identification of the "virulent" *V. parahaemolyticus* strains can be done by hybridization or PCR targeting the *tdh* (Kayser and DePaola 2001) and/or *trh* genes (Nordstrom and others 2006).

BAM methods are recommended for official analysis but may not reflect the latest technology or optimal methodology for detection of *V. vulnificus* and *V. parahaemolyticus* in naturally contaminated shellfish. Investigators have compared a variety of methodological alternatives (Alam and others 2001), including different dilution and enrichment buffers (Hagan and others 1994; Azanza and others 1996) and plating media (Oliver 1981; Oliver and others 1992; Hoi and others 1998a, 1998b; Cerda-Cuellar and others 2000). Direct plating remains difficult because of the large amount of natural microflora that may also grow on selective media. Micelli and others (1993) developed an alternative method for direct plaing of *V. vulnificus* from oyster homogenates. Using their so-called *V. vulnificus* enumeration (VVE) medium that contained Oxgall, sodium cholate, sodium taurocholate, and potassium tellurite, they reported reduction of 61% to 99% of marine-associated background microflora without adversely affecting the recovery of *V. vulnificus*. Detection limits were as few as 10 culturable *V. vulnificus* cells in 100 g of shellfish and compared favorably to MPN enrichment approaches with a shorter time to result. Recently, a chromogenic medium (Bio-Chrome Vibrio medium, BCVM, BioMedix, Pomona, Calif., U.S.A.) was developed to differentiate *V. parahaemolyticus* from other *Vibrio* species (Hara-Kudo and others 2001) and its efficacy has since been validated (Duan and Su 2005; Su and others 2005).

Molecular-based detection methods

DNA hybridization. Molecular-based methods, which rely on detection of specific gene targets by a variety of methods, have aided in the rapid identification and discrimination of *Vibrio* species from one another. See Tables 3 and 4 for details about gene targets and primers/probes for detection. Nishibuchi and others (1985) were the first to report a specific DNA probe for the detection of *V. parahaemolyticus*, which targeted the *tdh* gene but cross-reacted with some KP-negative strains. Soon thereafter, Nishibuchi and others (1986) evaluated 4 synthetic oligodeoxyribonucleotide probes corresponding to different regions of the *tdh* gene and demonstrated that under stringent hybridization conditions, two of the probes were capable of distinguishing KP-positive from negative or weakly positive strains. Lee and others (1992) developed a different oligonucleotide probe targeting the *tdh* gene and found that this probe identified 89 of 95 *V. parahaemolyticus* isolates. McCarthy and others (1999) reported that an alkaline phosphatase-labeled probe targeting the *tlh* gene correctly identified all 124 vibrio strains tested. Gooch and others (2001) used alkaline phosphatase (AP)-labeled *tlh* and digoxigenin-labeled *tlh* probes for DNA probe colony hybridization to enumerate *V. parahaemolyticus* after direct plating onto T_1N_3 (1% tryptone, 3% NaCl, 2% agar) medium, finding similar results to those obtained using the BAM MPN method. At low *V. parahaemolyticus* densities, the MPN method was more sensitive (3 MPN/g for a 0.1 g) than direct plating methods (10 CFU/g for a 0.1g sample). Nordstrom and DePaola (2003) reported that spread-plating on T_1N_3 after APW enrichment followed by colony hybridization using AP-labeled *tdh* probes was superior for the recovery of pathogenic *V. parahaemolyticus* when compared to a more conventional streak plate method. Ellison and others (2001) used the BAM-MPN and a direct plating procedure followed by DNA probe colony hybridization using an AP-labeled *tlh* probe (direct-VPAP) to determine *V. parahaemolyticus* levels in retail oysters from Florida. Although the correlation between methods was good, the direct-VPAP method was more rapid and precise.

Wright and others (1993) developed an AP-labeled DNA probe (VVAP) targeting the cytolysin (*vvh*A) gene of *V. vulnificus* which effectively differentiated the organism from other *Vibrio* species. DePaola and others (1997) applied VVAP for DNA colony hybridization following direct plating of Gulf of Mexico oysters onto *V. vulnificus* agar (VVA) and designated this method as direct-VVAP. The direct-VVAP and the BAM MPN methods were compared for enumeration of *V. vulnificus* levels in Gulf Coast oysters. The methods were in agreement > 90% of the time and the direct-VVAP approach was more rapid and precise than BAM MPN, although it did have a higher limit of detection (DePaola and others 1997). Cerda-Cuellar and others (2000) developed a probe specific to the 16S rDNA gene of *V. vulnificus* and successfully used it to distinguish this organism from other species of the *Vibrio* genus. For enumeration of *V. vulnificus* and *V. parahaemolyticus* in water samples, a hydrophobic grid membrane filtration (HGMF) technique has been applied in conjunction with cultural (DePaola and others 1988) and molecular (Kaysner and others 1994b) detection approaches. For example, Banerjee and others (2002) demonstrated that enumeration of *V. parahaemolyticus* and *V. vulnificus* from water samples could be achieved in 1 d by DNA probe colony hybridization of HGMF colony lifts using digoxigenin-labeled probes specific for *tlh* and *vvh*A genes of *V. parahaemolyticus* and *V. vulnificus*, respectively.

Polymerase chain reaction. Conventional PCR and real-time PCR have also been used to identify *V. parahaemolyticus* (Table 3) and *V. vulnificus* (Table 4). Brauns and others (1991) detected culturable and nonculturable *V. vulnificus* by PCR amplification using primers flanking the cytotoxin-hemolysin (*vvhA*) gene. In this case, as little as 72 pg and 31 ng of DNA from culturable

Table 2—Preliminary biochemical tests

Test	*V. parahaemolyticus*	*V. vulnificus*
TCBS agar	Green	Green
mCPC agar	No growth	Yellow
CC agar	No growth	Yellow
AGS	KA	KA
Oxidase	+	+
Arginine dihydrase	−	−
Ornithine decarboxylase	+	+
Lysine decarboxylase	+	+
0% NaCl	−	−
3% NaCl	+	+
6% NaCl	+	+
8% NaCl	+	−
10% NaCl	−	−
Growth at 42 °C	+	+
Sucrose	−	−
D-Cellobiose	V	+
Lactose	−	+
Arabinose	+	−
D-Mannose	+	+
D-Mannitol	+	V
ONPG	−	+
Voges Proskauer	−	−
10 μg O/129	R	S
150 μg O/129	S	S
Gelatinase	+	+
Urease	V	−

KA = slant alkaline/but slightly acidic; V = variable; R = resistant; and S = sensitive (FDA 2001).

cells and nonculturable cells, respectively, could be detected. Lee and others (1995) developed a species-specific PCR assay to differentiate *V. parahaemolyticus* from *V. alginolyticus* using a DNA region (pR72H) that is present in *V. parahaemolyticus* and absent in *V. alginolyticus*. The sensitivity of the PCR was approximately 1 CFU using purified chromosomal DNA in the amplification reactions, with a high degree of specificity. Karunasagar and others (1996) developed a PCR assay targeting the *tdh* gene, reporting detection limits > 10^4 CFU/g of *V. parahaemolyticus* when applied to lysates prepared directly from fish homogenates. Improved detection sensitivity (< 10 CFU/mL) was obtained by performing PCR after an 8-h enrichment in APW. Dileep and others (2003) compared conventional cultural methods and PCR targeting the *toxR* gene for the detection of *V. parahaemolyticus* in various seafood products; these investigators found that PCR performed better if it was preceded by a 6-h culture enrichment.

A number of multiplex PCR assays have been developed for detection of the pathogenic vibrios. Brasher and others (1998) designed a multiplex PCR assay to simultaneously detect *V. vulnificus*, *V. cholerae*, and *V. parahaemolyticus* (species specific) based on amplification of regions corresponding to gene targets *vvhA*, *ctx*, and *tlh*, respectively. When applied to artificially inoculated oyster homogenates, these investigators were able to detect < 10^1 to 10^2 CFU/g after a 6-h enrichment. Wang and others (1997) developed a PCR method able to detect 13 different foodborne pathogens, including *V. cholerae* (*ctx*), *V. parahaemolyticus* (pR72H fragment), and *V. vulnificus* (*vvhA*), with detection limits of 40, 4, and 100 cells per reaction, respectively. Bej and others (1999) designed a multiplex PCR assay to detect total and pathogenic strains of *V. parahaemolyticus* using *tlh*, *tdh*, and *trh* genes as targets. This assay gave the expected reactions on 111 isolates of *V. parahaemolyticus* and the investigators found that, in a few cases, the presence of the *tdh* gene was not associated with the Kanagawa phenomenon. The investigators reported that the detection limit for all 3 genes was between 10^1 and 10^2 CFU per 10 g when the assay was applied to seeded oysters that were pre-enriched for 6 h (Bej and others 1999).

The open reading frame (ORF8), derived from a filamentous phage (f237), has been exclusively associated with pandemic *V. parahaemolyticus* strains (Nasu and others 2000). The ORF8 sequence is distinct from other sequences in the database, but the phage itself is similar to the CTX phage that carries the genes that encode for cholera enterotoxin (*ctx*AB), an important virulence marker of *V. cholerae* (Waldor and others 1996). Interestingly, the ORF8 sequence was only detected by colony hybridization using a digoxigenin-labeled DNA probe in pandemic O3:K6 strains isolated after 1996 (Nasu and others 2000). Iida and others (2001) used the same method to evaluate 96 *V. parahaemolyticus* strains and found 53 isolates positive for the ORF8 sequence. These 53 isolates were represented by the O3:K6, O4:K68, and

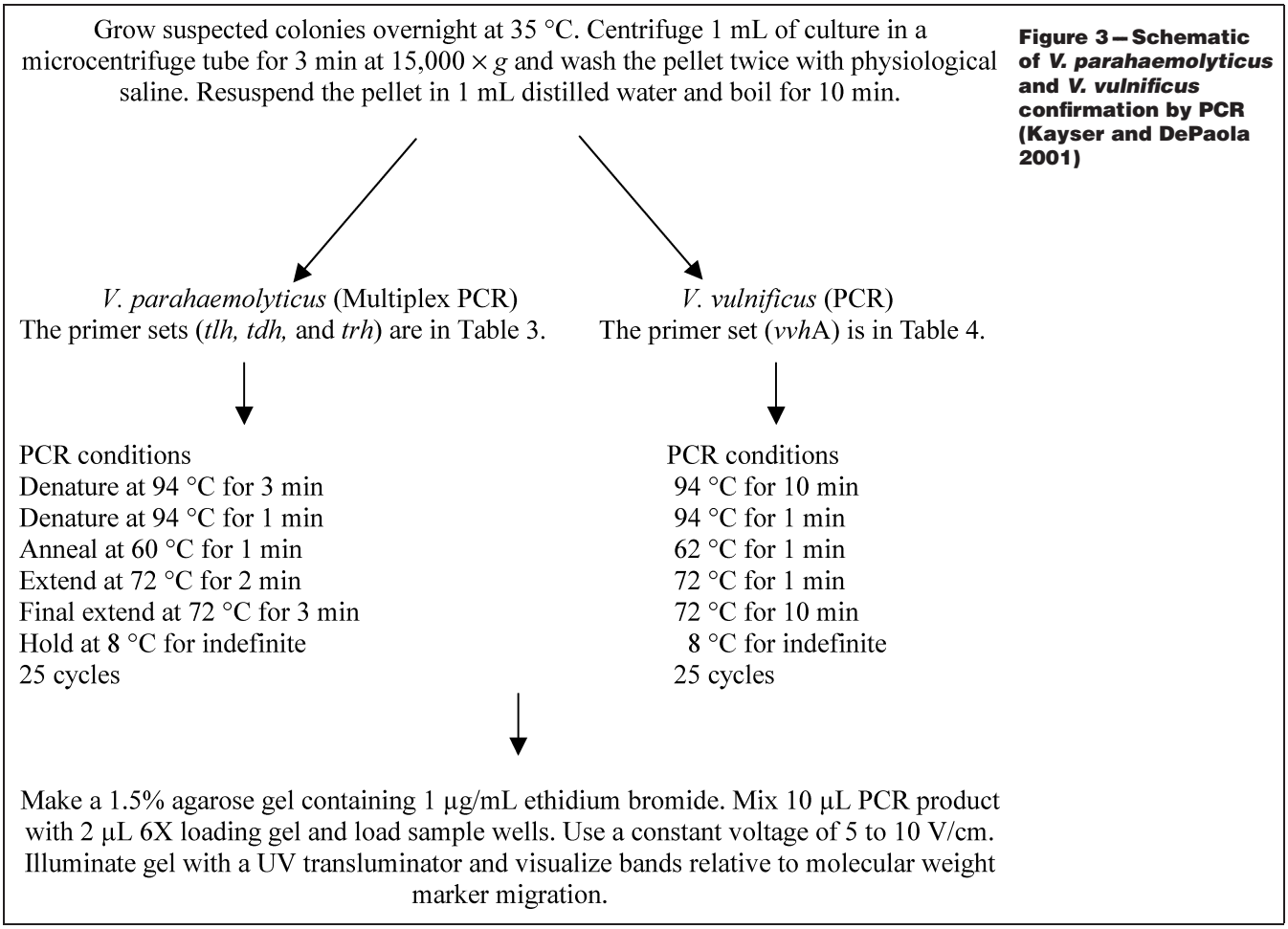

Figure 3—Schematic of *V. parahaemolyticus* and *V. vulnificus* confirmation by PCR (Kayser and DePaola 2001)

Table 3—Molecular methods and sequences used to identify *V. parahaemolyticus*

Gene	Location	Sequence	Application	Reference
tdh	330 to 350	5′-CCATCTGTCCCTTTTCCTGCC-3′	DNA hybridization	Nishibuchi and others (1986)
	504 to 524	5′-GGTACTAAATGGTTGACATCC-3′		
	685 to 702	5′-CCAAGTAAAATGTATTTGG-3′		Kaysner and others (1994)
	735 to 754	5′-GCATATGAGAGTGGTAGTGG-3′		
tdh	1275 bp	5′-GCTAAGTTTGTTGGTGAAGAT-3′	DNA hybridization	Lee and others (1992)
tlh	904 to 927	Forward	DNA hybridization	*McCarthy and others (1999),
		*5′-AAAGCGGATTATGCAGAAGCACTG-3′		*Gooch and others (2001),
		Reverse		*Ellison and others (2001),
		5′-GCTACTTTCTAGCATTTTCTCTGC-3′		*Nordstrom and DePaola (2003)
			PCR	Brasher and others (1998)
			Multiplex PCR	Bej and others (1999)
				Kayser and DePaola (2001)
pR72H	140 to 526	Forward	PCR	Lee and others (1995)
		5′-TGCGAATTCGATAGGGTGTTAACC-3′		
		Reverse		
		5′-CGAATCCTTGAACATACGCAGC-3′		
tdh2	85 to 719	Forward	PCR	Karunasagar and others (1996)
		5′-TTTCATGATTATTCAGTT-3′		
		Reverse		
		5′-TTTGTTGGATATACACAT-3′		
Genomic DNA	Not described	Forward	PCR	Wang and others (1997)
		5′-GAATTCGATAGGGTGTTAACC-3′		
		Reverse		
		5′-ATCCTTGAACATACGCAGC-3′		
tdh	Not described	5′-GGTTCTATTCCAAGTAAAATGTATTTG-3′	Hybridization	Kayser and DePaola (2001)
*tox*RS/old	Not described	Forward	PCR	Osawa and others (2002)
		5′-TAATGAGGTAGAAACG-3′		Okura and others (2003)
		Reverse		
		5′-ACGTAACGGGCCTACG-3′		
*tox*RS/new	Not described	Forward	GS-PCR	Matsumoto and others (2000)
		5′-TAATGAGGTAGAAACA-3′		Bhuiyan and others (2002)
		Reverse		Osawa and others (2002)
		5′-ACGTAACGGGCCTACA-3′		Okura and others (2003)
*tox*R	609 to 958	Forward	PCR	Dileep and others (2003)
		5′-GTCTTCTGACGCAATCGTTG-3′		
		Reverse		
		5′-ATACGAGTGGTTGCTTGCTGTCATG-3′		
ORF8	823 to 1192	Forward	PCR	Myers and others (2003)
		5′-AGGACGCAGTTACGCTTGATG-3′		
		Reverse		
		5′-CTAACGCATTGTCCCTTTGTAG-3′		
		Probe	Real-time	Ward and Bej (2006)
		5′-FAM-AAGCCATTAACAGTTGAAGGCGTTGA		
		CT-BHQ1		
ORF8	Not described	Forward	Colony hybridization	Nasu and others (2000);
		5′-GTTCGCATACAGTTGAGG-3′	PCR	Iida and others (2001)
		Reverse		Yeung and others (2003)
		5′-AAGTACAGCAGGAGTGAG-3′		Okura and others (2003)
tlh	Not described	Forward	Real-time PCR	Davis and others (2004)
		5′-CGAGAACGCAGACATTACGTTC-3′		Kaufman and others (2004)
		Reverse		
		5′-TGCTCCAGATCGTGTGGTTG-3′		
		Probe		
		5′-FAM-TCGCCGCTGACAATCGCTTCTCAT-BHQ1-3′		
tdh	Not described	Forward	Multiplex PCR	Bej and others (1999)
		5′-GTAAAGGTCTCTGACTTTTGGAC-3′		Kayser and DePaola (2001)
		Reverse		
		5′-TGGAATAGAACCTTCATCTTCACC-3′		

Continued

Table 3—Continued

Gene	Location	Sequence	Application	Reference
tdh	Not described	Forward 5′-AAACATCTGCTTTTGAGCTTCCA-3′ Reverse 5′-CTCGAACAACAAACAATATCTCATCAG-3′ Probe 5′-FAM-TGTCCCTTTCCTGCCCCCGG-TAMRA-3′	Real-time PCR	Blackstone and others (2003)
tdh	Not described	Forward 5′-CATCTTCGTACGGTTTTCTTTTTACA-3′ Reverse 5′-TCTGTCCCTTTTCCTGCCC-3′ Probe 5′-FAM-TCTCGAACAACAAACAATATCTCATCA GAACCG-BHQ1-3′	Real-time PCR	Davis and others (2004)
trh	Not described	Forward 5′-TTGGCTTCGATATTTTCAGTATCT-3′ Reverse 5′-CATAACAAACATATGCCCATTTCCG-3′	Multiplex PCR	Bej and others (1999) Kayser and DePaola (2001)
trh	Not described	Forward 5′-GCCAAGTGTAACGTATTTGGATGA-3′ Reverse 5′-TGCCCATTTCCGCTCTCA-3′ Probe 5′-FAM-ACGCCAGATATTTCGTCAATGTCGA AGC-BHQ1-3′	Real-time PCR	Davis and others (2004)
trh	Not described	5′-ACTTTGCTTTCAGTTTGCTATTGGCT-′3	DNA hybridization	Nordstrom and others (2006)
*gyr*B	Not described	Forward 5′-TGAAGGT-TTGACTGCCGTTGT-3′ Reverse 5′-TGGGTTTTCGACCAAGAACTCA-3′ Probe 5′-FAM-TTCTCACCCATCGCCGATTCAACCG C-TAMRA-3′	Real-time PCR	Cai and others (2006)
tlh	781 to 1230	Forward 5′-AAAGCGGATTATGCAGAACTG-3′ Reverse 5′-GCTACTTTCTAGCATTTTCTCTGC-3′ Probe 5′-TexR-AAGAACTTCATGTTGATGACA CT-BHQ2-3′	Hybridization PCR Real-time PCR	Kayser and DePaola (2001) Kayser and DePaola (2001) Ward and Bej (2006)
tdh	170 to 438	Forward 5′-CCATCCATACCTTTTCTTTCTCC-3′ Reverse 5′-ACTGTCATATAGGCGCTTAAC-3′ Probe 5′-TET-TATTTGTTGTTAGAAATACAACA AT-BHQ1-3′	Real-time PCR	Ward and Bej (2006)
trh	82 to 287	Forward 5′-GTATAGGTCTCTGACTTTTGGAC-3′ Reverse 5′-CTACAGAATTATAGGAATGTTGAAG-3′ Probe 5′-Cy5-ATTTTACGAACACAGCAGAAT-Iowa Black RQ-3′	Real-time PCR	Ward and Bej (2006)
*tox*R	Not described	Forward 5′-GACGCAATCGTTGAACCAGAA-3′ Reverse 5′-GCAAATCGGTAGTAATAGTGCCAA-3′ Probe 5′-VIC-AAAGCACCTGTGGCTTCTGCTG- TAMRA-3′	Real-time PCR	Takahashi and others (2005)

O1:KUT pandemic strains, but not in nonpandemic strains of any other serovar. Although these 2 studies used hybridization rather than PCR, Myers and others (2003) later developed a PCR assay targeting ORF8 specifically for the detection of the pandemic *V. parahaemolyticus* O3:K6 clone. The specificity of this PCR assay was confirmed only DNA from pathogenic *V. parahaemolyticus* O3:K6 pandemic isolates (after 1996) could be amplified, while the primers did not amplify the older (prior to 1996), non-O3:K6 *V. parahaemolyticus* strains, other *Vibrio* spp., or any other non-*Vibrio* spp. screened. Myers and others (2003) detected 10^3 CFU pandemic *V. parahaemolyticus* O3:K6/100 mL of seeded Gulf waters. At about the same time, Yeung and others (2003) used oligonucleotide primers for ORF8 with conventional PCR and correctly identified 39 *V. parahaemolyticus* pandemic isolates out of 78 total *V. parahaemolyticus* isolates, all of which contained the *tlh* gene.

Sequences corresponding to the *tox*RS operon have also been used as the target for PCR assays to identify pandemic *V. parahaemolyticus* (Table 3). The *tox*R is a regulatory gene of toxigenic *V. cholerae* (Miller and others 1987), but Lin and others (1993) found a *tox*R gene in *V. parahaemolyticus*. This gene had homology to the *tox*R of *V. cholerae*, which appears to promote the expression of the *tdh*2 gene and, to a lesser extent, the *tdh*1 gene. Matsumoto and others (2000) found that pandemic strains of *V. parahaemolyticus* have sequence substitutions at 7 base positions within the *tox*RS operon (*tox*RS/*new*). They developed a PCR method that targeted 2 of the base positions unique to the pandemic O3:K6 strain, resulting in an assay capable of differentiating the pandemic clone, including divergent serotypes from the old O3:K6 strains, from other nonpandemic strains. Okura and others (2004) developed a PCR assay to identify the pandemic group of *V. parahaemolyticus* using a marker derived from the group-specific sequence of an arbitrarily primed-PCR fragment that encodes for a "hypothetical protein." These PCR assays identified only the pandemic strains and further differentiated 82 *V. parahaemolyticus* strains (38 pandemic and 44 nonpandemic).

The performance of PCR assays based on ORF8 and *tox*RS/*new* sequences for differentiating pandemic *V. parahaemolyticus* strains has been examined by Osawa and others (2002). The investigators found that the ORF8 assay detected only the pandemic clone, while the *tox*RS/*new* assay detected all pandemic clone isolates and 4 strains isolated between 1982 and 1988, the latter of which were also untypeable by RFLP-PFGE. However, Bhuiyan and others (2002) disputed these findings when they reported that the ORF8 assay failed to identify 8 pandemic O3:K6 strains and one O4:K68 strain. Okura and others (2003) found *tox*RS/*new* sequences in 4 O3:K6 strains that did not contain *tdh* and ORF8 absent in 3 pandemic O3:K6 strains, while Chowdhury and others (2004) found ORF8 missing in 10% of the pandemic strains they tested. These studies indicate that neither *tox*RS nor ORF8 can be relied upon exclusively to differentiate pandemic *V. parahaemolyticus* from nonpandemic strains.

Table 4—Molecular methods and sequences used to identify *V. vulnificus*

Gene	Location	Sequence	Application	Reference
Cytolysin	1857 to 1880	5′-CTGTCACGGCAGTTGGAACCA-3′	DNA hybridization	Yamamoto and others (1990), Wright and others (1993)
vvhA	726 to 1113	Forward 5′-CGCTCACTGGGGCAGTGGCTG-3′ Reverse 5′-CCGTTAACCGAACGACCCGC-3′	PCR	Brauns and others (1991)
Cytolysin	3.2 kb	Entire plasmid pCVD702	DNA hybridization	Kaysner and others (1994)
*vvh*A	Not described	5′-GAGCTGTCACGGCAGTTGGAACCA-3′	DNA hybridization	Kayser and DePaola (2001)
vvhA	731 to 1113	Forward 5′-ACTGGGCAGTGGCT-3′ Reverse 5′-GCCGTTAACCGAACCA-3′ Probe 5′-ROXAACTATCGTGCACGCTTTGGTACCGT-BHQ2-3′	PCR Real-time PCR	Wang and others (1997) Panicker and Bej (2005)
vvhA	785 to 990	Forward 5′-CAACTTCAAACCGAACTATGAC-3′ Reverse 5′-CCAGTCGATGCGAATACGTTG-3′ Probe 5′-FAM-AACTATCGTGCA CGC TTTGGTACCGT-BHQ-3′	PCR Real-time PCR EMA real time	Brasher and others (1998) Panicker and others (2004) Panicker and Bej (2005)
*vvh*A	785 to 1303	Forward 5′-CCGCGGTACAGGTTGGCGCA-3′ Reverse 5′-CGCCACCCACTTTCGGGCC-3′	PCR	Kayser and DePaola (2001)
16S DNA	618 to 641	5′-GTCTGCCAGTTTCAAATGCAGTTC-3′	DNA hybridization	Cerda-Cuella and others (2000)
vvhA	786 to 990	Forward 5′-TTATGCTGAGAACGGTGACA-3′ Reverse 5′-TTTTATCTAGCCCCAAACTTG-3′ Probe 5′-CCGTTAACCGAACCA CCCGCAA-BHQ-3′	Real-time PCR	Campbell and Wright (2003) Panicker and Bej (2005)

Real-time PCR. Real time-PCR allows for the confirmation of amplicon identity while the amplification reaction is progressing, thereby bypassing time-consuming electrophoresis and hybridization methods. The method is considered quantitative by some, although when applied to detection of pathogens in food samples, this has yet to be realized. Real time-PCR has recently been applied to the detection and identification of *Vibrio parahaemolyticus* (Table 3). Blackstone and others (2003) were the first to report such an assay when they developed a method to detect pathogenic *V. parahaemolyticus* by targeting the *tdh* gene in a TaqMan format. When applied to enrichments of naturally contaminated oysters, the real-time PCR method was significantly more sensitive when compared to a streak plate/probe method. In addition, the real-time assay was faster and less resource intensive. Davis and others (2004) developed a TaqMan multiplex real time-PCR method targeting the *tlh*, *tdh*, and *trh* genes of *V. parahaemolyticus* using TaqMan probes with different labels. This assay was used to identify *V. parahaemolyticus* as the etiological agent in a foodborne disease outbreak associated with consumption of contaminated mussels. Kaufman and others (2004) found a strong correlation between cycle threshold and log concentration when using a real-time TaqMan PCR method targeting the *tlh* gene to detect *V. parahaemolyticus* in oyster mantle fluid. Recently, a TaqMan real-time PCR assay targeting the *toxR* gene was developed to quantify total *V. parahaemolyticus* in shellfish and seawater (Takahashi and others 2005). These investigators found the method to be specific for *V. parahaemolyticus* and reported a correlation between cycle threshold and $\log_{10}$ of *V. parahaemolyticus* cell number. This real-time PCR method was compared to the MPN cultural method for detection of *V. parahaemolyticus* in blue mussel and short-neck clams; 3 of the 10 samples which contained < 5 MPN/g by the cultural method were not detected by PCR, while 5 of the 10 samples gave similar results with both methods (Takahashi and others 2005). Cai and others (2006) developed a TaqMan real-time PCR method targeting the *gyr*B gene, which is well conserved in *V. parahaemolyticus* and has a single gene copy. The method had a detection limit of 1 CFU per PCR reaction when applied to pure culture and 6 to 8 CFU per PCR reaction in spiked raw oyster. The method was used to evaluate 300 seafood samples and 97 were PCR-positive for *V. parahaemolyticus*; only 78 samples were positive using a conventional culture method. Ward and Bej (2006) developed a TaqMan multiplex real-time PCR method targeting the *tlh,* ORF8, *tdh*, and *trh* genes of *V. parahaemolyticus* for identification of the organism in shellfish. This method identified total and pathogenic *V. parahaemolyticus* with detection limits of 1 CFU/g of oyster after overnight enrichment (16 h).

Real-time PCR has also been used for identification of *V. vulnificus* (Table 4). Campbell and Wright (2003) developed a TaqMan real-time PCR assay targeting the cytolysin gene (*vvhA*) of *V. vulnificus* and found this method to be specific after examination of 28 *V. vulnificus* strains and 22 non-*V. vulnificus* strains; the detection limit was 72 fg/μL of genomic DNA. When compared to the colony lift hybridization using the VVAP gene probe, the 2 methods correlated well and had similar sensitivity (Campbell and Wright 2003). Panicker and others (2004) developed a SYBR Green-based real-time PCR method targeting the hemolysin (*vvh*) gene of *V. vulnificus* and applied it to the detection of the organism in shellfish and Gulf waters. They reported no cross-reactivity with other *Vibrio* and non-*Vibrio* bacterial strains. The minimum detection limit of the assay was 10^2 CFU *V. vulnificus*/g of oyster tissue homogenate, or 10^2 CFU/10 mL water, as applied to samples without prior cultural enrichment. Improved detection limits (1 CFU/g) were obtained when samples were enriched for 5 h. The entire method took only 8 h, including sample processing, enrichment, and real-time PCR. Panicker and Bej (2005) compared 3 sets of oligonucleotide primers for detection of the *V. vulnificus vvhA* gene in the TaqMan real-time PCR format. Two of the 3 primer sets (set 1: F-vvh785/R-vvh990 and set 2: F-vvh731/R-vvh1113 primers with P-vvh874) were specific for *V. vulnificus*. Detection limits of 1 pg/μL of purified DNA, 10^3 CFU/mL of pure culture, and 1 CFU/g of oyster (after a 5-h enrichment) were achieved. Recently, Wang and Levin (2006) reported a TaqMan real-time PCR assay that discriminated between viable and nonviable *V. vulnificus* cells using the DNA intercalating agent ethidium monoazide (EMA).

Strain typing methods

Many different methods have been applied to *V. parahaemolyticus* and *V. vulnificus* strain typing. In an early study, Tamplin and others (1996) reported a high degree of variation in RFLP-PFGE profiles of 53 clinical and 78 environmental isolates of *V. vulnificus*. Ryang and others (1999) reported similar genetic diversity using RFLP-PFGE to type clinical *V. vulnificus* strains in Korea. Both studies reported slightly less diversity for other typing methods such as ribotyping (Tamplin and others 1996) and random amplified polymorphic DNA (RAPD) analysis (Ryang and others 1999). RFLP-PFGE has been used to identify the vehicle of *V. vulnificus* infection and to study the relationship between patient isolates. Overall, infection appears to result from the proliferation of a single strain, although clinical strains from different patients are frequently unique (Jackson and others 1997). Warner and Oliver (1999) used RAPD analysis to differentiate various *Vibrio* species, finding a great degree of heterogeneity in banding patterns, even within a specific species (*V. vulnificus* in particular). Arias and others (1998) recommended RAPD PCR for the differentiation of phenotypically atypical *V. vulnificus* strains as a simpler and slightly less discriminatory method, while recommending ribotyping for finer discrimination between isolates. Others confirmed the great degree of diversity seen with RAPD PCR, noting little correlation between strain source and RAPD pattern (Lin and others 2003). The same can be said for arbitrarily primed (AP)-PCR (Vickery and others 2000). It is also clear that biotype designations do not always correlate with phylogenies generated by molecular typing methods (Gutacker and others 2003).

Much of the typing work for *V. parahaemolyticus* has been done by Wong and colleagues. These investigators examined 130 *V. parahaemolyticus* isolates from Taiwan by RFLP-PFGE, finding 14 RFLP-PFGE types and 39 patterns; domestic clinical isolates were clustered into 4 types and showed little similarity to foreign clinical strains and domestic environmental strains (Wong and others 1996). When they used RFLP-PFGE to group 315 *V. parahaemolyticus* isolates from contaminated seafood, 96 patterns and 22 types were obtained. There was little relationship between RFLP-PFGE type and strain origin (Wong and others 1999). Wong and others (2000) also used RFLP-PFGE to characterize Taiwanese clinical isolates, reporting 57 patterns grouped into 19 types, with 5 of these types containing 76% of the isolates and a clear and distinct type for the pandemic O3:K6 strains. The ability of RFLP-PFGE to differentiate between pandemic O3:K6 and non-O3:K6 isolates was confirmed by Yeung and others (2002) and Wong and others (2000). Marshall and others (1999) compared RFLP-PFGE, enterobacterial repetitive intergenic consensus sequence (ERIC) PCR, ribotyping, and RFLP-PFGE on patient and environmental isolates associated with a 1997 *V. parahaemolyticus* outbreak in Canada and found no single method to be superior. In general, ERIC PCR and ribotyping were less discriminatory, whereas RFLP-PFGE was extremely discriminatory. Likewise, Wong and others (2001) evaluated 3 PCR-based *V. parahaemolyticus* typing methods, finding ribosomal gene spacer sequence (RS)-PCR a more practical method

than ERIC PCR because it generated fewer bands and patterns. More recently, Hara-Kudo and others (2003) concluded that, based on RFLP-PFGE, TDH-negative isolates were rather distant from TDH-positive isolates, and that TDH-positive strains were closely related to one another, regardless of serovar. DePaola and others (2003) used ribotying and serotyping to characterize *V. parahaemolyticus* isolates derived from clinical, environmental, and food sources and found no relationship between serogroup and ribogroup. Certain serogroups and ribogroups contained both clinical and environmental isolates, while others just contained environmental isolates, implying that certain serotypes or ribotypes may be more relevant to human disease. Isolates from the Pacific Coast of North America appeared to be a distinct population from those found near the Gulf and Atlantic Coasts.

In recent outbreaks, a new method based on direct genome restriction enzyme analysis (DGREA) has been used to group *V. parahaemolyticus* isolates. The method involves digestion of bacterial DNA with a 6-base restriction endonuclease that generates 30 to 40 fragments of sizes ranging from 500 to 2500 bp in length. These are separated using polyacrylamide gel electrophoresis and banding patterns visualized by silver nitrate staining. Fuenzalida and others (2006) found that DGREA was able to discriminate different clones of *V. parahaemolyticus*, with cluster analysis identifying 16 different groups; only 2 groups corresponded to the pandemic O3:K6 isolates. DGREA results had discriminatory power similar to that of RFLP-PFGE.

Ecology

Vibrio species are ubiquitous in estuarine waters and can frequently be isolated in high numbers from bivalves, crustaceans, finfish, sediment, and plankton (Kelly 1982: Oliver and others 1982; Tamplin and others 1982; O'Neil and others 1992; DePaola and others 1994). In general, higher densities of the organisms are found in oyster digestive tissue (Tamplin and Capers 1992; DePaola and others 1997) as compared to muscle tissue. Considerable oyster-to-oyster variability in vibrio levels has been noted. For example, Kaufman and others (2003) observed occasional "hot" oysters containing *V. parahaemolyticus* levels >10-fold higher than those of oysters harvested at the same time and within a 1-m^2 proximity (Kaufman et al. 2003).

Both organisms have been isolated from U.S. waters as far north as the Great Bay of Maine (*V. vulnificus*) (O'Neil and others 1992), Alaska (*V. parahaemolyticus*) (McLaughin and others 2005), and Long Island, New York (*V. parahaemolyticus*) (Tepedino 1982). Lower densities of *V. vulnificus* and *V. parahaemolyticus* were isolated from Pacific, Canadian, and North Atlantic waters where the water temperatures were generally cooler year round; higher densities were found in mid-Atlantic, Chesapeake Bay, and Gulf of Mexico waters where the water temperatures were warmer year round (Kaysner and others 1987; O'Neil and others 1992; Cook 1994; DePaola and others 1994; Wright and others 1996; Motes and others 1998).

Seasonal temperature and salinity. Both temperature and salinity play important and interrelated roles in the levels of *Vibrio* spp. Kelly and Stroh (1988) reported that *V. parahaemolyticus* was found in Pacific Northwest coastal waters only during the summer months, when water temperatures were above 17 °C and salinities were below 13 ppt. Further research by Kaspar and Tamplin (1993) demonstrated that at salinities between 5 and 25 ppt, *V. vulnificus* levels increased; however, when salinities were 30, 35, and 38 ppt, *V. vulnificus* levels decreased by 58%, 88%, and 83%, respectively. The same trend was reported by Motes and others (1998), who observed lower numbers of *V. vulnificus* at salinities above 28 ppt, which is typical of some Atlantic coastal sites in North and South Carolina. This high salinity may explain in part why *V. vulnificus* cannot be isolated routinely in oysters harvested from waters off these shores. High *V. vulnificus* levels, however, were found in oysters harvested from intermediate salinities between 5 and 25 ppt (Motes and others 1998).

Regardless of the role of salinity, temperature probably has the most important effect on the prevalence and levels of the pathogenic vibrios. The growth of *V. vulnificus* is favored by relatively high temperatures and the organism has an optimum growth temperature of 37 °C (Kelly 1982). Kaspar and Tamplin (1993) reported that *V. vulnificus* grew in the temperature range of 13 to 22 °C. Wright and others (1996) were able to culture *V. vulnificus* from estuarine waters of Chesapeake Bay collected at temperatures as low as 8 °C; however, *V. vulnificus* was not recovered at temperatures lower than 12.5 °C from Gulf of Mexico waters (Simonson and Siebeling 1986). In general, *V. vulnificus* is isolated infrequently from surface water samples from the Gulf of Mexico in January through March, when water temperatures are below 20 °C (Kelly, 1982). Peak recovery of *V. vulnificus* occurs in September, and there is substantial seasonal variation in prevalence and levels of the organism. Studies have demonstrated that during the summer months, *V. vulnificus* levels were similar (about 10^4 CFU/g) in oysters harvested from the Gulf of Mexico and Mid Atlantic states, but the levels were considerably higher in the Gulf of Mexico for other seasons (Cook and others 2002). Virulent strains of *V. vulnificus* have been found on the West Coast, although not as frequently or in as high numbers as from Gulf and Atlantic Coast waters (Kaysner and others 1987). Likewise, clams harvested from the northeastern U.S. coast and all U.S. West Coast waters had comparatively lower levels of *V. vulnificus* (Brenton and others 2001), perhaps due to the lower mean temperatures of these waters.

Seasonal and regional variation in the prevalence and levels of *V. parahaemolyticus* has also been noted. As is the case for *V. vulnificus*, the levels of *V. parahaemolyticus* in Gulf Coast oysters also peaks during the summer, followed by a gradual reduction in the colder months of the year (Motes and others 1998). DePaola and others (1990) compared seasonal levels of *V. parahaemolyticus* in Pacific, Gulf, and Atlantic Coast waters and oyster samples. The data showed strong correlations between water temperature and *V. parahaemolyticus* levels. The Gulf Coast had the warmest mean water temperature (22 °C) and highest mean *V. parahaemolyticus* levels of 11000 CFU/100 g (oysters) and 44 CFU/100 mL (water), while the Pacific coast water was the coldest (15 °C) and was associated with lower levels of *V. parahaemolyticus* (2100 CFU/100 g for oysters and 2 CFU/100 mL for water). Kaufman and others (2003) reported total *V. parahaemolyticus* levels immediately after harvest during June, July, and September to range from 200 to 2000 CFU/g in 90% of the oysters tested. Cook and others (2002) detected *V. parahaemolyticus* in 94.2% of shellfish taken from waters that were above 25 °C, but the organism was present in only 14.9% of shellfish samples harvested from waters that were below 10 °C. Gooch and others (2002) found that when water temperature at harvest was above 20 °C (April through December), the mean density of *V. parahaemolyticus* was 13000 CFU/100 g, whereas, when water temperatures were below 20 °C (January through March), the mean density was approximately 1-$\log_{10}$ lower, at 1500 CFU/100 g. DePaola and others (2003) reported similar seasonal trends in total *V. parahaemolyticus* for 2 sampling sites in Alabama. They also found that pathogenic *V. parahaemolyticus* (*tdh*+) strains constituted a higher percentage of the *V. parahaemolyticus* population when water temperatures and total *V. parahaemolyticus* levels were lower. The levels of pathogenic strains (*tdh*-positive) ranged from 10 to 20 CFU/g in 40% of the oysters harvested during June and July but pathogenic strains were nondetectable in oysters harvested in September. However, after storage at 26 °C for 24 h, pathogenic

V. parahaemolyticus was detected at levels of > 100 CFU/g in some oysters collected in June and July but remained nondetectable in oysters collected in September (Kaufman and others 2003).

The role of aquatic wildlife and zooplankton. Aquatic birds may be a vector for *Vibrio* spp., especially during the winter months. For example, *V. cholerae* has been isolated from aquatic birds at low levels and studies have reported the presence of non-O1 *V. cholerae* in ducks (Bisgaard and Kristen 1975) and gulls (Lee and others 1981) during the winter, when *Vibrio* spp. were not found in the water column. However, there is less information available regarding the role of aquatic birds in the persistence and/or spread of *V. parahaemolyticus* and *V. vulnificus*. In one study, non-O1 *V. cholerae*, *V. parahaemolyticus*, and other lactose-positive vibrios were isolated from bird feces (Roberts and others 1984). A later study by Buck (1990) reported *Vibrio* spp. in association with gulls and pelicans, while Miyasaka and others (2005) found a higher percentage of *V. parahaemolyticus* positive samples (55.4%) compared to *V. vulnificus* positive samples (14.1%) in wild aquatic birds in Japan during the winter months. In virtually all instances, the level of vibrios in bird populations was quite low.

Although *V. vulnificus* levels are higher in the estuarine environment during the warm summer months, the organism persists throughout the year. There are many ways in which *V. vulnificus* and *V. parahaemolyticus* can survive. Vanoy and others (1992) and Wright and others (1996) found *V. vulnificus* in plankton, suggesting that this bacterium may inhabit habitats similar to *V. cholerae* and *V. parahaemolyticus*. *V. vulnificus* also persists in marine sediment, suggesting winter survival in the floc zone at the sediment interface; when conditions are more conducive for growth (summer months), *V. vulnificus* will then colonize plankton (Vanoy and others 1992). DePaola and others (1994) isolated *V. vulnificus* throughout the winter months from the intestines of estuarine fish from the Gulf of Mexico, at densities higher than those found in oysters, sediment, or seawater.

Indeed, the relationship between zooplankton and *Vibrio* spp. may explain the year-round persistence of the vibrios. It is well documented that *Vibrio* spp. make up a significant portion of the natural microflora of zooplankton, especially zooplankton with chitinous exoskeleton such as copepods (Huq and others 1983; Sakar and others 1983; Chowdhury and others 1989; Carli and others 1993). Huq and others (1983) found higher numbers of vibrios associated with zooplankton than were found in the surrounding water column. A study conducted by Watkins and Cabelli (1985) demonstrated that only chitin and net zooplankters (live or dead) supported the growth of *V. parahaemolyticus* in estuarine water. Heidelberg and others (2002) found a diverse group of bacteria associated with zooplankton, with higher levels of bacteria associated with zooplankton during the cooler months of the year. However, the majority of these organisms were *V. cholerae*, *V. mimicus*, and *V. vulnificus*.

Extracellular proteins produced by *V. vulnificus* are also important in the organism's ability to survive in the estuarine environment and perhaps cause disease in infected hosts. For example, *V. vulnificus* exports a chitinase that may be used by the bacterium to colonize and adhere to the chitin exoskeletons of zooplankton. The metalloprotease and hemolysin may allow the organism to colonize and multiply in molluscan shellfish by breaking down tissue at the site of colonization, promoting release of necessary nutrients (Strom and Paranjpye 2000).

Bacteriophages. Bacteriophages are abundant in the marine environment, and those specific for the pathogenic *Vibrio* spp. are no exception (Suttle and others 1990; Boehme and others 1993; Jiang and Paul 1994). For example, Moebus and Nattkemper (1983) isolated 366 phages from the Altantic, 362 of which initiated infection in bacteria belonging to the *Vibrionaceae* family. Furthermore, 280 of these phages were specific for the *Vibrio* spp. Pelon and others (1995) isolated 9 phage strains specific for *V. vulnificus*, with patterns of susceptibility varying with specific *V. vulnificus* strain. Based on these results, the same investigators (Luftig and Pelon, 1996) attempted to use these 9 bacteriophage strains to reduce *V. vulnficus* populations in estuarine water, finding that *in vitro* exposure to 1.0 mL of the pooled phages reduced *V. vulnificus* levels by 5-$\log_{10}$. DePaola and others (1997) identified phages; infecting *V. vulnificus* in estuarine waters, sediments, plankton, crustacea, and the intestines of finfish and molluscan shellfish harvested from the Gulf Coast. The latter habitat had the highest abundance of phages; however, the lowest densities of phages were in the hemolymph and mantle fluid of oysters. Estimates of abundance ranged from 10^1 to 10^5 PFU/g of oyster tissue.

As is the case for *V. vulnificus* bacteriophages, those infecting *V. parahaemolyticus* are abundant and diverse, having been isolated from the coastal waters of Laos, Hawaii, Florida, and the Pacific and Atlantic coasts of North America (Sklarow and others 1973; Baross and others 1978; Kellogg and others 1995; Nakasone and others 1999; Hardies and others 2003; Comeau and others 2005). However, *V. parhaemolyticus* bacteriophages were not detected in the sediment and only found at low levels in waters off the coast of British Columbia (Comeau and others 2006). Koga and others (1982) isolated 18 bacteriophages infectious to *V. parahaeomlyticus* and reported 4 different morophological groups. Furthermore, there appeared to be no correlation between O and K serotype of *V. parahaeomlyticus* strains and host range of phages (Koga and others 1982). Like *V. vulnificus* phages, Comeau and others (2005) found 13 phages specific for *V. parhaemolyticus* to be consistently higher (0.5×10^4 to 11×10^4 virus/cm^3) year round in oysters compared to sediment and water. In a later study, the same investigators (Comeau and others 2006) demonstrated that *V. parahameolyticus* phages infect between 4 and 13 *V. parahaemolyticus* strains with a unique host range pattern. There also appears to be a relationship between host range and season (Comeau and others 2005). Using these same 13 phages to control *V. parahaemolyticus* in the environment, the investigators achieved reduction of the organism by 74%, 62%, and 30% in sediment, oysters, and the water column, respectively. However, bacteriophage treatment has yet to be realized as a practical method to control *Vibrio* contamination in oysters or their waters.

Starvation and the viable but nonculturable (VBNC) state. Marden and others (1985) were the first to characterize the behavior of marine bacteria to starvation. In fact, starvation is one of several stresses (in addition to cold temperature and suboptimal pH) (Gauthier 2000) that can induce the so-called viable but nonculturable (VBNC) state. Starvation combined with cold stress may be particularly effective in inducing VBNC (Linder and Oliver 1989). This term describes bacterial cells that do not form colonies on high-nutrient solid media, but are considered alive because metabolic activity can still be detected (Gauthier 2000; Oliver 2000). The VBNC state can be contrasted to cell injury in that injured cells lose their ability to grow on selective media, but can still be cultured on nutrient-rich media; VBNC cells cannot be cultured at all. Significant effort has gone into characterizing the VBNC state as related to the survival and virulence of *V. cholerae* and *V. vulnificus* (Oliver and Bockian 1995; Wong and others 2004; Asakura and others 2007). There is, however, evidence that *V. parahaemolyticus* also enters the VBNC state (Jiang and Chai 1996; Wong and Wang 2004).

There are physiological manifestations associated with entry into the VBNC state. Morphologically, *V. vulnificus* cells in the VBNC state are small cocci (0.3 μm), whereas after resuscitation

the cells become rod shaped (3 μm in length and 0.7 in width) (Linder and Oliver 1989; Nilsson and others 1991). VBNC cells also clump, suggesting the production of exopolysaccharides resulting in an outer membrane that is "blebbed" (Johnston and Brown 2002). Blebbing is a modification to the outer membrane that is frequently associated with bacterial resistance mechanisms (Jones and others 1989). Oliver and Colwell (1973) observed that, as temperature decreased, there was a proportional increase in the amount of unsaturated fatty acids in the cell membrane of *V. vulnificus*. Indeed, the palmitic (C_{16}) plus palmitoleic ($C_{16:1}$) fatty acid content was decreased by 57%, whereas short-chain fatty acid content increased from 5.4% to 29.0% as cells were entering the VBNC state (Linder and Oliver 1989). Wong and others (2004) found differences in the activities of 2 enzymes and in the fatty acid profiles of *V. parahaemolyticus* ST550 cells based on culturability status. During the 1st wk of exposure to starvation conditions, an increase in $C_{15:0}$ fatty acid content and a decrease in $C_{16:1}$ content was observed. Also, the enzyme superoxide dismutase became nondetectable in the VBNC state, while the cellular concentration of glucose-6-phosphate dehydrogenase did not change upon entry into the VBNC state.

Clearly, the VBNC state is a mechanism for bacteria to survive adverse conditions and there is evidence that stress conditioning impacts both induction of and speed at which cells enter the VBNC state. For example, Bryan and others (1999) observed that *V. vulnificus* entered the VBNC state when the temperature was shifted from 35 to 6 °C; however, when the culture was subjected to 15 °C prior to further temperature downshift, the cells remained culturable. Oliver and others (1991) reported that when *V. vulnificus* cells were prestarved for 24 h at room temperature and subsequently exposed to 5 °C, they failed to enter the VBNC state, whereas cells starved for the same period at 5 °C did enter the VBNC. When cells were starved for only 1, 2, and 4 h before exposure to 5 °C, the cells also entered the VBNC state, but at a slower rate.

Vibrio species that have entered the VBNC state can usually be revived within 3 d after a temperature shift to 21 °C. A leading theory to explain VBNC is that it is associated with increased sensitivity to hydrogen peroxide. This was first reported by Whiteside and Oliver (1997) who noted that VBNC cells of *V. vulnificus* could not be resuscitated after temperature upshift to 22 °C if suspended in nutrient-rich broth, but could be resuscitated in minimal media, such as artificial seawater (ASW), most likely due to the presence of peroxide byproducts occurring during media sterilization. It is well documented that injured cells frequently demonstrate an increased sensitivity to the toxic effects of hydrogen peroxide, a phenomenon that can be ameliorated by media supplementation with sodium pyruvate or catalase (Baird-Parker and Davenport 1965; Rayman and others 1978). Bogosian and others (2000) were the first to supplement media with catalase or pyruvate to promote the recovery of *V. vulnificus*, noting that higher culturable cell counts were observed after such supplementation. More recently, Kong and others (2004) constructed a deletion mutant of *V. vulnificus* which lacked catalase (*oxyR*) activity. When compared to the wild-type strain, the investigators showed that low temperature inhibited catalase activity, which likely contributed to loss of culturability. The loss of the superoxide dismutase activity in *V. parahaemolyticus* strains having entered the VBNC state provides further evidence for increased sensitivity to hydrogen peroxide (Wong and others 2004).

Some have speculated that the VBNC state does not really exist, but instead some viable cells remain and when the sample is subjected again to a more favorable environment, those residual viable cells replicate and become detectable on microbiological media. Bogosian and others (2000) conducted a series of experiments to address this issue. Specifically, they demonstrated that when warmed to room temperature, VBNC cells that could be cultured on pyruvate-supplemented media were able to use the nutrients provided by the dead cells to support the formation of more than 1 progeny cell. However, when the hydrogen peroxide-sensitive cell population declined to nondetectable levels on pyruvate-supplemented media, leaving only nonculturable cells present, warming did not lead to cell growth (Bogosian and others 2000).

The relationship between the VBNC state and virulence also is of great interest. Colwell and others (1996) fed VBNC cells of *V. cholerae* to human volunteers and observed an absence of disease but low levels of fecal shedding. Linder and Oliver (1989) reported that VBNC cells of *V. vulnificus* lost virulence in the mouse model; however, a low level of inoculum (5×10^4 cells) by the intraperitoneal route was used in these experiments. In a later study, Oliver and Bockian (1995) showed that intraperitoneal injection of mice with a total of 10^5 VBNC cells of *V. vulnificus* was lethal. In an effort to determine if VBNC cells remained pathogenic, 3 strains (*V. alginolyticus*, *V. parahaemolyticus* (environmental origin), and *V. parahaemolyticus* ATCC 43996) were induced into the VBNC state, followed by intragastric inoculation of 8 Balb/C mice for each bacterial strain. In this experiment, isolation and confirmation was obtained in 25% of the mice challenged with *V. alginolyticus*, 37.5% of mice challenged with *V. parahaemolyticus*, and 50% of mice challenged with *V. parahaemolyticus* ATCC 43996. In addition, when the strains were first inoculated in the mouse model, they caused fluid accumulation and expressed virulence characteristics (hemolysin production, adhesiveness, and cytotoxicity). However, when the strains were reisolated from the mice, grown in BHI broth, and then injected into the rat ileal loop, virulence factor expression (hemolysin production, adhesiveness, and cytotoxicity) was lost. Nonetheless, after 2 consecutive passages in the rat ileal loop model, virulence characteristics were reactivated. This is important because it suggests that VBNC cells retain their ability to express proteins associated with pathogencity, although such expression may be transient and/or unpredictable (Baffone and others 2003).

Stress response: pH and refrigerated storage. It is well documented that vibrios in the VBNC state are more resistant to sublethal stressors. For example, *V. parahaemolyticus* cells induced into the VBNC state by exposure to cold temperatures were observed to be more resistant to thermal inactivation (42 and 47 °C), low salinity, and acid inactivation (pH 4) (Wong and Wang 2004). Koga and Takumi (1995) reported that *V. parahaemolyticus* cells in the starved state were more resistant to other environmental stresses such as heat (47 °C) and osmotic pressure.

Such stress response may be of concern when using processing methods intended to reduce the levels of vibrios in raw or minimally processed molluscan shellfish. With regards to the effect of pH, Karem and others (1994) demonstrated that when *Aeromonas hydrophila* suspended in broth was shifted from pH 7.2 to 5.0 (conditioned), the cells survived longer when exposed to a further pH downshift to 3.5. It appears that *A. hydrophila* exhibits an adaptive acid-tolerance response capable of protecting cells at pH values as low as 3.5. Wong and others (1998) found that *V. parahaemolyticus* was more acid tolerant in the broth model when first conditioned by a pH downshift from 7.5 to 5.0. Koga and others (1999) found acid-adapted *V. parahaemolyticus* cells had an increased resistance to heat (47 °C), crystal violet, bile, and deoxycholic acid, as compared to nonadapted cells. In addition, these investigators noticed a change in the composition of the outer membrane protein of acid-adapted cells. Koo and others (2000) reported strain-to-strain differences in acid tolerance for *V. vulnificus*, although there did appear to be a pH value (somewhere around pH 2.0) below which all strains were inactivated.

Bang and Drake (2004) reported that 3 strains of *V. vulnificus* had increased acid resistance in broth acidified with citric acid (pH 3.5) after prior adaptation at pH 5.5, regardless of strain and duration of adaptation time; the same phenomenon was not observed when acetic acid was used as the acidulant. These same investigators demonstrated that acid adaptation involves induction of specific proteins. Freeze-thaw resistance and cold storage survival were improved with prior exposure to citric acid (pH 5.0) for 10 h, but this effect was strain specific (Bang and Drake 2004). Most recently, Wong and Lui (2006) found that *V. vulnificus* cells adapted by exposure to acid (pH 4.4) or heat (41 °C) were not cross-protected when exposed to low salinity (0.04% NaCl) conditions.

Although we know that prolonged exposure to nutrient-depleted media and cold temperatures can induce the VBNC state, some investigators became interested in the response of *Vibrios* to so-called cold stress. Indeed, some organisms are able to adapt and persist at very low temperatures when previously conditioned by exposure to less-cold temperature. Bryan and others (1999) suggested that cold-adaptive or protective proteins produced by *V. vulnificus* may enhance survival and tolerance to cold and freezing temperatures. They also hypothesized that iron plays a role in adaptation at cold temperature, since the removal of iron from the growth medium prior to cold adaptation reduced viability by 2-$\log_{10}$ CFU/mL. It was demonstrated that 40 different proteins were synthesized at higher levels by *V. vulnificus* upon exposure to cold stress (McGovern and Oliver 1995). Lin and others (2004) found that when *V. parahaemolyticus* was cold-shocked at 20 or 15 °C for 2 or 4 h, the cells demonstrated better survival upon subsequent exposure to low temperature of 5 or −18 °C, or to crystal violet, but were more susceptible to high temperature (47 °C), hydrogen peroxide, and lactic and acetic acids, when compared to unconditioned cells. Bryan and others (1999) showed that a culture of *V. vulnificus* demonstrated better survival during frozen storage (−78 °C) when freezing was preceded by cold shock. Bang and Drake (2002) also found improved survival of *V. vulnificus* under cold temperature storage when cells underwent a cold temperature preconditioning step.

Techniques to eliminate *Vibrio* species from oysters

Currently, shellfish harvesting waters are classified using the coliform or fecal coliform index. Unfortunately, since *Vibrio* species are ubiquitous to the marine environment, the levels of the traditional fecal indicators do not correlate with the presence or levels of the environmental vibrios, and hence the fecal coliform index is not useful for controlling these organisms (Tamplin and others 1982). This was confirmed by O'Neil and others (1992) who found no correlation between fecal coliform and *V. vulnificus* levels. However, Watkins and Cabelli (1985) found an indirect relationship between *V. parahaemolyticus* levels and pollution in Naraganssett Bay and hypothesized that this was a result of nutrient stimulation. Ruple and Cook (1992) observed correlation between the fecal coliform level and *V. vulnificus* during the warmer months (May to September), but this relationship did not hold up during the cooler months of the year.

Commercial heat shock. Currently, the commercial heat shock process is used as a processing aid, primarily in North and South Carolina, to facilitate the shucking of shellstock oysters (Hesselman and others 1999). This process involves submerging about 70 chilled oysters in wire baskets into a heat-shock tank containing approximately 850 L of potable water at a temperature of 67 °C for about 5 min, depending on oyster size and relative oyster condition. After heat-shocking, the oysters are cooled by spraying for 1 min with potable water prior to shucking and washing. Hesselman and others (1999) found that this commercial heat-shock process reduces *V. vulnificus* levels by 2- to 4-$\log_{10}$. No reduction in *V. vulnificus* levels were observed in oysters that were merely washed. Ruple and Cook (1992) showed that while commercial heat-shock processing of oysters did not reduce the levels of *V. vulnificus*, immediate storage on ice did reduce the levels by 1- to 2-$\log_{10}$ CFU/g.

Cook and Ruple (1992) demonstrated that low-temperature pasteurization (50 °C) for 10 min reduced *V. vulnificus* and *V. parahaemolyticus* counts from 10^5 MPN/g to nondetectable levels in inoculated shellstock oysters; this was confirmed by Andrews and others (2000). In a later report, Andrews and others (2003a) demonstrated that a combined hot-water/cold-shock "pasteurization" process with a temperature of 50 to 52 °C reduced *V. parahaemolyticus* 03:K6 (10^6 CFU/g of oyster) in shellstock oysters to nondetectable levels within 22 min, without changing the sensory properties of the product.

Depuration and relaying. Depuration is the process of controlled purification whereby shellfish are placed in disinfected, recirculating or flow-through seawater and allowed to actively filter-feed, typically for 24 to 48 h. The use of this practice is quite limited in the United States but extensive in Europe. Disinfectants commonly used in depuration waters are chlorine, ozone, and ultraviolet light. Groubert and Oliver (1994) used a *V. vulnificus* strain (CVD713), which was genetically transformed to carry a stable TnphoA transposon encoding kanamycin resistance and alkaline phosphatase activity, to demonstrate that oysters allowed to filter-feed in artificially contaminated waters were able to reduce, to nondetectable levels, accumulated *V. vulnificus* within 48 h of the onset of depuration. Interestingly, however, the level of naturally occurring *V. vulnificus* in these oysters was not reduced by depuration. Eyles and Davey (1984) also reported that depuration did not produce a substantial reduction in *V. parahaemolyticus* levels in shellfish, but Nordstrom and others (2004) were able to achieve better reduction in *V. parahaemolyticus* levels after overnight tidal submersion, compared to intertidal exposure, in Hood Canal, Wash. While this is technically not depuration, it does suggest that, under certain conditions, *V. parahaemolyticus* can be eliminated. Tamplin and Capers (1992) found that recirculation of depuration waters through UV light at above 23 °C was an ineffective control because *V. vulnificus* was able to multiply in oyster tissues under these conditions. However, when the seawater was maintained at 15 °C, *V. vulnificus* could not be detected in seawater, nor did multiplication of *V. vulnificus* occur in the oyster.

Relaying is another purification method that involves moving shellfish from a restricted harvesting area to an open area where natural cleansing can occur. Cook and Ellender (1986) found that the temperature and the microbiological quality of the relaying water had an impact on the length of time needed to reduce fecal coliform levels in oysters. Additionally, oysters that were physiologically stressed took longer to cleanse than did unstressed oysters, presumably due to slower metabolic activity. While pathogens such as *Salmonella* can be eliminated within 5 d by relaying (Cook and Ellender 1986), Motes and DePaola (1996) demonstrated that longer relaying periods (17 to 49 d) and high salinity (> 30 ppt) were required to decrease *V. vulnificus* levels from 10^3 CFU/g to <10 MPN/g. As with depuration, relaying cannot be relied upon to completely eliminate *V. vulnificus* from shellfish.

GRAS compounds. As a possible aid in controlling *Vibrio* contamination in shellfish, investigators have examined certain preservatives that are generally recognized as safe (GRAS) by the U.S. Food and Drug Administration. Sun and others (1994) were able to achieve a 2-$\log_{10}$ reduction in the levels of naturally occurring *V. vulnificus* in oysters treated with diacetyl at a concentration of 0.05%, while lactic acid and butylated hydroxyanisole (BHA) compounds at a concentration of 0.05% did not have an

effect on *V. vulnificus* levels. Diacetyl appears to affect the permeability of cell membranes and accumulates in the membrane lipid bilayer (Johnson and Steele 2001).

There are also naturally occurring compounds in oysters that may promote the inactivation of *V. vulnificus* and *V. parahaemolyticus.* For example, oysters contain hemocytes, which entrap bacteria within phagosomes, after which an enzymatic degradation process begins (Cheng 1975). Although theoretically this process may be lethal to the vibrios, Genthner and others (1999) reported that oyster hemocytes did not have a significant lethal effect on either opaque or translucent strains of *V. vulnificus*; for *V. parahaemolyticus*, the opaque stains were more resistant to the effect of hemocytes than were the translucent strains. Unfortunately, most oysters found in mid-Atlantic and Gulf Coast waters are infected with *Perkinsus marinus*, an oyster pathogen which produces a serine protease capable of digesting oyster connective tissues. Tall and others (1999) found that oyster hemocytes treated with the serine protease produced by *P. marinus* were less efficient in controlling the levels of naturally occurring *V. vulnificus* when compared to untreated hemocytes, suggesting that *P. marinus,* may actually suppress the natural ability of oyster hemocytes to eliminate *V. vulnificus.*

Ionizing irradiation. Gamma irradiation can eliminate *Vibrio* species from shellstock and shucked oysters. *Vibrio* species are among the most radiation-sensitive bacteria; *V. cholerae* and *V. vulnificus* can be eliminated when exposed to doses less than 0.1 kGy (Mallett and others 1991). Novak and others (1966) found that a 0.2 Mrad (2 kGy) dose of gamma radiation could be applied for pasteurization of oyster meat without causing changes in organoleptic quality. After this treatment, total bacterial counts decreased by 99%. Matches and Liston (1971) found that, in most cases, *V. parahaemolyticus* was reduced 4- to 6-$\log_{10}$ using a dose of 30 to 40 krad (0.3 to 0.4 kGy). Andrews and others (2003b) showed that ionizing irradiation doses of 1.0 kGy reduced *V. vulnificus* at initial inoculum of 10^7 CFU/g to nondetectable levels as applied to whole shell oysters. Oysters inoculated with *V. parahaemolyticus* 03:K6 (10^4 CFU/g) reached nondetectable levels after treatment with 1.5 kGy. Most oysters survived the treatment and sensory data showed that consumers could not tell a difference between irradiated and nonirradiated oysters. Recently, the FDA approved irradiation as a food additive for seafood, including oysters.

Temperature control and refrigeration. Refrigeration controls the multiplication of *V. vulnificus* and *V. parahaemolyticus* in oysters. Cook and Ruple (1989) investigated the effects of various storage temperatures (10, 22, 30 °C) on oysters and found that members of the *Vibrionaceae* family increased in shellstock oysters stored at 22 and 30 °C, while10 °C storage prevented growth. Cook (1994) also observed that *V. vulnificus* did not multiply in oysters stored at below 13 °C and growth at 18 °C was significantly slower than at ambient air temperature (23 to 34 °C).

Prolonged refrigeration may actually reduce the levels of the pathogenic vibrios. For instance, Cook and Ruple (1992) observed that within 14 to 21 d of refrigerated storage, *V. vulnificus* in shellfish could be reduced to nondetectable levels (< 3 MPN/g). Later Cook and others (2002) estimated that *V. vulnificus* levels declined by 0.041-log unit/d during refrigeration of retail oysters. However, Kaysner and others (1989) demonstrated that, in artificially contaminated shellstock and shucked oysters, *V. vulnificus* survived for 14 d at 2° C. It is generally recognized that, while levels may decline over time, prolonged refrigeration cannot be relied upon to eliminate *V. vulnificus* or *V. parahaemolyticus* from contaminated shellstock.

If the temperature of shellstock is not immediately controlled, growth of vibrios can occur quite rapidly. For example, Cook (1997) observed that the levels of *V. vulnificus* in freshly harvested shellstock oysters held without refrigeration for 3.5, 7, 10.5, and 14 h increased 0.75-, 1.30-, 1.74-, and 1.94-log units, respectively. For this reason, the U.S. National Shellfish Sanitation Program stipulated (in 1993) the 1st refrigeration guidelines for raw molluscan shellfish. These were made more stringent in 1995, with a requirement that shellstock be placed under temperature control within 12 to 14 h of harvest, depending on the average monthly maximum water temperature (Cook 1997). More recently, regulations state that commercial shellfish must be refrigerated within 10 h after harvest when water temperature exceeds 27 °C (U.S. Department of Health and Human Services 1999). In 1995, the Interstate Shellfish Sanitation Conference (ISSC) adopted an additional control plan for states that had been confirmed as the originating site of shellstock products associated with two or more *V. vulnificus* illnesses. In this case, if water temperature was between 18 and 23 °C, shellstock was required to be placed under temperature control within 14 h; if greater than 23 °C and less than 28 °C, the time limit was less than 12 h; and if the water temperature was greater than 28 °C, the time limit was less than 6 h (Associated Press 1996). Once placed under temperature control, shellstock must be iced, or the storage area or conveyance otherwise continuously maintained at 7.2 °C or below, until final sale to the consumer.

Not only is time unrefrigerated on boat docks an issue, but commercial cooling of oyster sacks has been estimated to take an average of 5.5 h (CFSAN/FDA 2005), during which time *Vibrio* growth can still occur, albeit more slowly. This is contrasted to die-off that occurs during extended refrigerated storage. Taken together, the growth of *V. vulnificus* and *V. parahaemolyticus* that occurs before oysters reach the target refrigeration temperatures of 13° C (*V. vulnificus*) and 10° C (*V. parahaemolyticus*) results in higher levels of these organisms at consumption relative to the levels at harvest. For example, Wright and others (1996) and Motes and others (1998) reported that the levels of *V. vulnificus* in Gulf of Mexico and Chesapeake Bay oysters at harvest are typically 1-$\log_{10}$ lower than they are at retail. Likewise, Cook and others (2002) observed that *V. vulnificus* and *V. parahaemolyticus* levels in retail oysters originating from the Gulf of Mexico were 1- to 2-$\log_{10}$ greater than at harvest.

Freezing and frozen storage. Cook and Ruple (1992) reported that freezing reduces the levels of *Vibrio* spp. in shellfish, although it does not eliminate the organism, even after frozen storage for up to 12 wk. A temperature of −20 °C was more effective for inactivating *V. vulnificus* than was 0 °C. At −80 °C, *V. vulnificus* and *V. parahaemolyticus* cell numbers in brain heart infusion broth supplemented with 3% NaCl dropped by 1-$\log_{10}$ CFU/g during the freezing process and remained stable thereafter for 35 d (Boutin and others 1985). Johnston and Brown (2002) showed that the total cell numbers were the same for freshly cultured *V. vulnificus*, *V. cholerae*, and *V. parahaemolyticus* both before and after freezing (−20 °C); similar results were obtained for VBNC cells. In a study conducted by Parker and others (1994), the combination of vacuum packaging and freezing decreased *V. vulnificus* levels in oysters by 3- to 4-$\log_{10}$ CFU/g within 7 d postfreezing, and levels continued to drop throughout frozen storage up to day 70, although complete elimination was never achieved. The combination of vacuum packaging and freezing controlled *V. vulnificus* levels more effectively than did freezing with conventional packaging (Parker and others 1994). ISSC has adopted freezing combined with frozen storage as an acceptable means for postharvest treatment to control *V. vulnificus* and *V. parahaemolyticus*. A number of firms now use this process, which must be validated and HACCP compliant (21 CFR 123).

High hydrostatic pressure. Most microorganisms are baroduric, meaning they can survive under high pressures but normally grow best at atmospheric pressure. High-pressure application is

a promising emerging technology to control pathogens in certain foods. When using pressure to inactivate microorganisms, the treatment depends on the intensity of the pressure and the length of exposure (Hoover and others 1989). In general, *Vibrio* spp. are extremely sensitive to pressure. Styles and others (1991) demonstrated that *V. parahaemolyticus* is rapidly reduced to nondetectable levels at pressures higher than 1700 atm when suspended in clam juice. More recent research has used the international system of units (SI) conversion and the megaPascal (MPa) unit in place of atmospheres (atm) (the relationship between the 2 is 10:1, atm:Mpa). Berlin and others (1999) reported that treatment with hydrostatic pressure of 250 MPa for 10 min at 25 °C reduced *V. vulnificus* in pure culture to nondetectable levels without triggering the VBNC state. However, *V. vulnificus* cells in the VBNC state appear to be more resistant to the lethal effects of high hydrostatic pressure (Berlin and others 1999). Cook (2003) found that *V. vulnificus* strains suspended in PBS were the most sensitive to high pressure (200 MPa), whereas *V. cholerae* strains were more resistant. Furthermore, strains of the pandemic O3:K6 serotype of *V. parahaemolyticus* were more resistant to pressure than were strains of other serotypes or *Vibrio* spp. For instance, in order to obtain a better than 5-$\log_{10}$ CFU/g reduction of *V. vulnificus* in oysters, a treatment of 250 MPa for 120 s was required, while a treatment of 300 MPa for 180 s was required to obtain a similar reduction in pandemic *V. parahaemolyticus* serotype O3:K6 (Cook 2003). Most recently, Koo and others (2006) found that at 241 MPa, it took 11 and 5 min (including a 3-min pressure come-up time) to achieve a 6-$\log_{10}$ reduction of pandemic *V. parahaemolyticus* O3:K6 and *V. vulnificus*, respectively, in PBS. Both *V. parahaemolyticus* and *V. vulnificus* reached nondetectable levels in PBS and oysters at 586 MPa after 8 and 7 min, respectively. Some companies have obtained ISSC approval to use this method for postharvest processing (A. DePaola, personal communication 2007).

Heat treatment. Heat is a very effective means to eliminate cells of *Vibrio* species and was approved as a postharvest process by the ISSC in 2003. *V. vulnificus* cells are rapidly and exponentially inactivated at 50 °C or higher (Ama and others 1994). Cook and Ruple (1992) demonstrated that *V. vulnificus* (4.3×10^3 CFU/g) in naturally contaminated shellfish could be reduced to nondetectable levels by exposing oysters to a temperature of 50 °C for 10 min. Cultures of *V. vulnificus*, *V. cholerae*, and *V. parahaemolyticus* showed D-values of 12 s, 22.5 s, and 1.75 min, respectively, at 55 °C, and all 3 organisms, when suspended in broth, were reduced by more than 7-$\log_{10}$ CFU/mL when treated at 70 °C for 2 min (Johnston and Brown 2002). In broth, *V. parahaemolyticus* was more resistant to heat inactivation at 47 °C when preceded by a heat shock at 42 °C for 30 min; unconditioned *V. parahaemolyticus* cells were readily inactivated at 47 °C (Wong and others 2002). *V. vulnificus* was more resistant to heating when suspended in oyster homogenate than in buffer, presumably due to the protective effects of the suspending matrix (Ama and others 1994). According to Kim and others (1997), *V. vulnificus* morphotype influences thermal death times; opaque strains have higher D and z_D values than do translucent strains, suggesting that the former have increased heat resistance. The D-values for opaque colonies range from 3.44 to 3.66 min and those for translucent colonies range from 3.18 to 3.38 min at 47 °C; the range of z_D-values for opaque colonies is 2.45 to 2.51 °C while the range for translucent colonies is 1.89 to 2.07 °C.

Risk assessment

Recently, the CFSAN-FDA (2005) conducted a quantitative risk assessment for *V. parahaemolyticus* (VPRA) in raw oysters in the United States. Since water temperature was considered the major factor affecting *V. parahaemolyticus* density at harvest, different models were constructed for seasons (winter, spring, summer, and fall), regions (Gulf Coast of Louisiana because oyster boats are on the water longer before refrigerating, Gulf Coast excluding Louisiana, mid-Atlantic, Northeast Atlantic, Pacific Northwest), and by harvesting practice (dredging and intertidal for the Pacific Northwest). The VPRA model predicted the highest levels of total and pathogenic *V. parahaemolyticus* at harvest in the Gulf Coast region due to warmer temperatures. The total levels of *V. parahaemolyticus* at harvest were predicted to be 2.1×10^3, 2.2×10^2, 5.2×10^1, and 9.4×10^2 cells/g oyster for summer, fall, winter, and spring, respectively, for the Gulf Coast region. Although the Pacific Northwest has the coolest water temperature, when harvesting by the intertidal method, the VPRA predicted that it has the 2nd-highest levels of pathogenic *V. parahaemolyticus*. This was due to warm air exposure during intertidal harvesting, along with the fact that the ratio of pathogenic to total *V. parahaemolyticus* is higher in this region than in others.

Air temperature was considered second to water temperature in terms of factors influencing the density of *V. parahaemolyticus* in oysters after harvest. This is because the organism's growth rate is temperature dependent and it continues to multiply after harvest unless shellstock are refrigerated rapidly. During the summer months in the Louisiana Gulf Coast region, the VPRA model predicted pathogenic *V. parahaemolyticus* levels of 720 cells/serving at harvest; at consumption, the levels reached 21000 cells/serving. In fact, the levels of pathogenic *V. parahaemolyticus* from harvest to consumption increased for all 6 harvest regions/practices in the United States, suggesting the need to provide better control of *V. parahaemolyticus* multiplication immediately after harvest.

Human challenge data in conjunction with the Beta–Poisson model were used to estimate the dose-response relationship. The VPRA suggested that there was a low risk (<0.001%) of gastroenteritis following the consumption of 10^4 cells of *tdh*+ *V. parahaemolyticus*/serving, and a high (50%) risk when 10^8 cells/serving were consumed. The model was calibrated to the CDC's estimate of 2800 oyster-associated *V. parahaemolyticus* cases annually in the United States. However, the Alaskan outbreak investigation suggested that the infectious dose of the Alaska strains may be thousands of times lower (McLaughin and others 2005). The risk assessment predicted the mean annual number of illnesses to be the highest in the Gulf Coast (Louisiana) region with 1406, 132, 7, and 505 cases occurring in the summer, fall, winter, spring seasons, respectively. The other regions, in descending order of total annual illnesses, were as follows: the Gulf Coast (non-Louisiana) (546 cases), Pacific Northwest (intertidal) (192 cases), Northeast Atlantic (19 cases), mid-Atlantic (15 cases), and Pacific Northwest (dredging) (4 cases). The Pacific Northwest (intertidal) region had relatively high predictions of illness due to the fact that oysters harvested in intertidal areas are normally exposed to higher temperatures before refrigeration.

The VPRA clearly demonstrated that the use of postharvest treatments (PHT) will reduce the number of illnesses caused by this organism. For example, the model predicted that if a 4.5-$\log_{10}$ reduction of *V. parahaemolyticus* were obtained, the probability of illness would decrease to less than 1.0 case/y in all regions of the United States. Using this same benchmark for inactivation, PHT such as heat, pressure, and freezing were predicted to reduce the number of cases by > 99.99%. Rapid postharvest chilling of oysters could reduce theoretically reduce *V. parahaemolyticus* illness by 90% to 99%.

Using a framework and parameters similar to those of the VPRA, the World Health Organization (WHO) and Food and Agriculture Organization (FAO-WHO 2005) conducted a quantitative risk assessment for *V. vulnificus* in raw oysters from the U.S. Gulf

Coast (VVRA). Consistent with the previous *V. parahaemolyticus* work, the levels of *V. vulnificus* in oysters at harvest were most influenced by water temperature; they were, however, also influenced by salinity. The highest estimated levels of *V. vulnificus* at harvest were 5.6×10^3 CFU/g during the summer, and the lowest were 8.0×10^1 CFU/gin the winter, for oysters harvested from waters with a salinity of below 30 ppt. The risk assessment model predicted that *V. vulnificus* levels increased substantially during postharvest storage, with predicted mean levels of 5.7×10^4 and 8.0×10^1 *V. vulnificus*/g in the summer and winter, respectively. A serving size of approximately 196 g of oyster meat would provide an ingested dose of *V. vulnificus* of 1.1×10^7 and 1.6×10^4 in the summer and winter, respectively.

For hazard characterization, the Beta–Poisson dose-response model was used in conjunction with human clinical data. Under current harvest and postharvest conditions, *V. vulnificus* illnesses were estimated at 0.5, 11.7, 12.2, and 8.0 for winter, spring, summer, and autumn, respectively. If alternative processes were used to reduce *V. vulnificus* levels to 300, 30, and 3 CFU/g, the annual number of cases was estimated at 7.7, 1.2, and 0.16, respectively.

Summary

V. vulnificus and *V. parahaemolyticus* infections occur worldwide and are associated with significant morbidity and mortality. Although *V. vulnificus* is more abundant than *V. parahaemolyticus* in the Gulf of Mexico during the warmer months, *V. parahaemolyticus* has a greater seasonal and geographic range than does *V. vulnificus*, and it is generally more abundant year round. Because of their association with seafood, these agents are a significant concern to the shellfish industry and public health agencies. Much research has been conducted regarding the effects of environmental factors, such as water temperature and salinity, on the prevalence and levels of *V. parahaemolyticus* and *V. vulnificus* in water and shellfish. However, less is known about the levels of pathogenic strains of *V. parahaemolyticus* and *V. vulnificus* in oysters and waters, particularly with respect to environmental and seasonal effects.

There are numerous culture-based and molecular methods for the detection of *V. parahaemolyticus* and *V. vulnificus*. These methods have become more efficient over the past 20 y. *V. parahaemolyticus* and *V. vulnificus* can readily be detected and enumerated, but differentiating pathogenic strains from nonpathogenic strains remains a challenge. Most *V. parahaemolyticus* and *V. vulnificus* strains have been shown to be genetically heterogeneous, with the exception of the pandemic strains of *V. parahaemolyticus*. Overall, no precise conclusions can be drawn about pathogenic strains as compared to nonpathogenic strains and questions remain about pathogenicity and the role of recognized and purported virulence factors. With the ability to sequence the entire genomes of *V. parahaemolyticus* and *V. vulnificus,* we will soon be able to explain how these organisms evolved to survive the changing aquatic environment and to better characterize genes associated with virulence and survivability. This will lead to improved understanding of risk, and hopefully, new and more effective control measures.

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