

Review

Epidemiology, Pathogenesis, and Prevention of Foodborne *Vibrio parahaemolyticus* Infections

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ABSTRACT

Since its discovery about 50 years ago, *Vibrio parahaemolyticus* has been implicated as a major cause of foodborne illness around the globe. *V. parahaemolyticus* is a natural inhabitant of marine waters. Human infections are most commonly associated with the consumption of raw, undercooked or contaminated shellfish. A few individual *V. parahaemolyticus* virulence factors, including the thermostable direct hemolysin (TDH) and TDH-related hemolysin (TRH), have been investigated in depth, yet a comprehensive understanding of this organism's ability to cause disease remains unclear. Since 1996, serotype O3:K6 strains have been associated with an increased incidence of gastroenteritis in India and in Southeast Asia, and with large-scale foodborne outbreaks in the United States (US). In light of the emerging status of pathogenic *V. parahaemolyticus*, the US Food and Drug Administration conducted a microbial risk assessment to characterize the risk of contracting *V. parahaemolyticus* infections from consuming raw oysters. This review summarizes epidemiological findings, discusses recognized and putative *V. parahaemolyticus* virulence factors and pathogenicity mechanisms, and describes strategies for preventing *V. parahaemolyticus* infections.

INTRODUCTION

Vibrio parahaemolyticus IS A GRAM-NEGATIVE, non-sporeforming, curved rod-shaped bacterium. Its high motility in liquid media is attributed to a polar flagellum. It also possesses lateral flagella, which enable the microorganism to migrate across semi-solid surfaces in a phenomenon called swarming (Baumann et al., 1984).

As a natural inhabitant of estuarine marine water, *V. parahaemolyticus* is widely distributed in inshore marine waters throughout the world. In the US, the organism was first demonstrated in seawater, sediments, and shellfish in the Puget Sound region (Baross and Liston, 1968; Baross and Liston, 1970) and has been isolated consistently from such environments. It

is also part of the natural flora of bivalve shellfish (Kueh and Chan, 1985).

Environmental factors affecting V. parahaemolyticus numbers

The optimal growth temperatures for *V. parahaemolyticus* are between 35°C and 39°C (Jackson, 1974). Under optimal conditions, the generation time of this organism is less than 20 min; in certain conditions, it may double in as little as 5 min (Barrow and Miller, 1974; Jackson, 1974). *V. parahaemolyticus* is most prevalent in a given environment during the warm summer season. As an illustration of the effect of season on *V. parahaemolyticus* numbers, Colwell et al. (1984) reported that *V. parahaemolyti-*

cus was not detected in Chesapeake Bay seawater during the winter months (November to March), although a small number of *V. parahaemolyticus* was isolated from marine sediment samples. *V. parahaemolyticus* is proposed to survive through the winter in marine sediments and then to multiply when the temperature rises, following re-introduction of the organism into the seawater.

Salinity is an absolute requirement for multiplication and survival of *V. parahaemolyticus*. Based on laboratory studies, *V. parahaemolyticus* can grow in sodium chloride concentrations ranging from 0.5% to 10%, with optimal levels between 1% and 3%. Salinity concentrations encountered by *V. parahaemolyticus* in marine environments typically range between 0.8% and 3% (DePaola et al., 2000). A survey following outbreaks in Washington, Texas, and New York in 1997 and 1998 found a slight, but significant, negative correlation between *V. parahaemolyticus* numbers and a sample site's salinity (DePaola et al., 2000). The relatively small salinity differences commonly encountered in nature appear to have a smaller relative influence on bacterial numbers in seawater than do seasonal differences in ambient temperature (FDA, 2001a; Cook et al., 2002).

The ability of *V. parahaemolyticus* to utilize or to tolerate the presence of various concentrations of metal ions may affect the ability of the organism to survive in marine environments. For example, *V. parahaemolyticus* isolates from highly polluted coastal waters in India were found to be resistant to 300 mM Mg^{2+} , a level that is toxic to many other microorganisms (Bhattacharya et al., 2000). An enhanced ability to utilize magnesium may improve *V. parahaemolyticus* survival under various conditions. Thermally treated, or otherwise injured, *V. parahaemolyticus* cells have an enhanced ability to take up magnesium, indicating a possible increased requirement for Mg^{2+} for membrane and/or ribosome stability and repair under these conditions (Heinis et al., 1978). Bhattacharya et al. (2000) localized the gene(s) responsible for resistance to high magnesium concentrations to a 5.5-kb plasmid. The ability to tolerate high concentrations of magnesium, or other metal ions, may provide *V. parahaemolyticus* with the ability to out-compete

other normal seawater flora for growth and survival in the presence of the ions.

Viable but non-culturable state

Under extreme conditions that do not support optimal physiological functions, *V. parahaemolyticus* has been reported to enter a viable but non-culturable (VBNC) state (Jiang and Chai, 1996; Mizunoe et al., 2000; Johnston and Brown, 2002). "Extreme conditions" include starvation and temperature stress. *V. parahaemolyticus* was reported to enter the VBNC state after 12 days of starvation at 4°C (Mizunoe et al., 2000). Direct microscopic observations supplemented with staining suggested that bacterial cells were viable, however, plating did not produce visible colonies on bacterial growth media. These so-called non-culturable *V. parahaemolyticus* cells were altered in shape (cocci or spheroid, rather than rod-shaped), yet appeared to have intact membranes and metabolic activity (Jiang and Chai, 1996; Johnston and Brown, 2002). VBNC may be a common phenomenon, both among marine microbes such as *V. cholerae*, *V. vulnificus*, and *Aeromonas hydrophila*, and among microbes not usually associated with the marine environment, such as *Escherichia coli* and *Listeria monocytogenes*. Oliver (1995) reports that as many as 30 bacterial species may enter the VBNC state.

EPIDEMIOLOGY OF FOODBORNE *VIBRIO PARAHAEMOLYTICUS* INFECTIONS

Incidence

V. parahaemolyticus was discovered after an outbreak of food poisoning in Japan, which affected 272 patients in 1950 (Fujino et al., 1974). Although not all strains are believed to cause disease, *V. parahaemolyticus* is one of the leading causative agents of foodborne disease in countries such as Japan and Taiwan (Pan et al., 1997; Wong et al., 2000), where consumption of raw seafood is not uncommon. Its impact in the US is also noteworthy. The first US outbreak due to *V. parahaemolyticus* occurred in Maryland in 1971, as a result of contaminated crab-

meat consumption (Molenda et al., 1972). Since then, an additional 42 outbreaks of *V. parahaemolyticus* infection have been documented (Fishbein et al., 1974; Daniels et al., 2000a; ProMED-mail 2002). Based on surveillance data collected from Florida, Alabama, Louisiana, and Texas, there were 345 sporadic cases of *V. parahaemolyticus* infection in these states between 1988 and 1997 (Daniels et al., 2000a). In addition to routine surveillance by the states participating in the Gulf Coast *Vibrio* Surveillance System, *V. parahaemolyticus* was also included in the Centers for Disease Control and Prevention's Foodborne Diseases Active Surveillance Network (CDC FoodNet) in 1996. Based on CDC FoodNet data, foodborne *V. parahaemolyticus* infection is estimated to be responsible for around 5000 illnesses annually in the US (1996: 2,700 cases; 1997: 9,800 cases; 1998: 5,600 cases) (FDA, 2001a).

Clinical manifestations

V. parahaemolyticus is the most frequent cause of foodborne *Vibrio*-associated gastroenteritis in the US (Daniels et al., 2000a). Typical symptoms include diarrhea, abdominal pain, nausea, vomiting, headache, fever and chills. Wound infection and septicemia due to exposure to *V. parahaemolyticus* have also been reported. Most cases of infection are self-limiting and can be treated by oral rehydration, alone. Occasionally, treatment with antibiotics such as doxycycline, ciprofloxacin, or erythromycin is necessary (Qadri et al., 2003). The infection can be fatal for immunocompromised patients or for those with a preexisting medical condition such as liver disease or diabetes.

Associated foods

Shellfish, which obtain food by filter feeding, can concentrate bacteria such as *V. parahaemolyticus* to levels higher than those in the surrounding water. Therefore, illnesses due to *V. parahaemolyticus* are usually associated with the consumption of contaminated raw or under-cooked molluscan shellfish (e.g., oysters, clams and mussels). Cooked crustaceans (e.g., shrimp, crab, and lobsters) have also been incriminated in *V. parahaemolyticus* infections (Blake et al., 1980). As *V. parahaemolyticus* is ex-

tremely sensitive to heat, its presence in cooked products results from either improper cooking or recontamination of the cooked product.

Risk groups

While all oyster-consuming populations appear equally susceptible to *V. parahaemolyticus* gastroenteritis, epidemiological investigations have identified a subpopulation that is more vulnerable to development of life-threatening septicemia as a result of *V. parahaemolyticus* infections (FDA, 2001a). This subpopulation includes patients with underlying medical conditions, such as cancer, liver disease, kidney disease, heart disease, recent gastric surgery, or antacid use. This subpopulation also represents a high-risk group for contracting severe illnesses caused by *V. vulnificus*, a bacterium also commonly found in shellfish.

Trends

V. parahaemolyticus is a mesophilic bacterium that is most prevalent during the summer months. As a consequence, foodborne outbreaks due to *V. parahaemolyticus* typically show a seasonal pattern, peaking in the warmer months (Daniels et al., 2000a; Lesmana et al., 2001). While levels of *V. parahaemolyticus* in freshly harvested seafood are generally below the predicted infectious dose of 10^7 to 10^8 organisms (Sanyal and Sen, 1974; Hoashi et al., 1990), the ability of this organism to multiply rapidly at ambient temperatures can result in the presence of sufficient bacteria in foods to cause disease. To illustrate, 10^2 to 10^3 *V. parahaemolyticus* organisms/g in live American oysters have been shown to increase 50-fold (1.7 log cfu/g) and 790-fold (2.9 log cfu/g) at 26°C by 10 and 24 h after harvest, respectively (Gooch et al., 2002). Therefore, failure to immediately reduce and maintain the temperature of freshly harvested shellfish at sufficiently low levels can allow this pathogen to rapidly multiply to infectious levels. Analyses of recent outbreaks, including the 1998 New York State outbreak, suggest that the numbers of *V. parahaemolyticus* present in oysters from the contaminated harvest sites were less than the FDA recommended maximum level of 10,000 organisms/g (FDA, 2001a). These results suggest

the possibility of the emergence of *V. parahaemolyticus* strains with enhanced virulence characteristics and hence, a lower infectious dose.

VIBRIO PARAHAEMOLYTICUS SEROTYPES AND THEIR ROLES IN HUMAN DISEASE

Emergence of V. parahaemolyticus serotype O3:K6

Strains of *V. parahaemolyticus* are typically serotyped on the basis of O and K antigens. As many as 13 O groups and 71 K types can be identified by commercial antisera. Until recently, the majority of *V. parahaemolyticus* infections worldwide have been sporadic in nature, characterized by causal associations with multiple, diverse serotypes. One exception is the predominant association of O4 strains with gastroenteritis cases on the US West Coast (Nolan et al., 1984; Abbott et al., 1989; DePaola et al., 2000). To illustrate this general lack of association between illness cases and specific serotypes, a 1994/95 surveillance study of the typical two to four *V. parahaemolyticus* isolations made each month among hospitalized patients in Calcutta, India identified multiple, diverse serovars from the various patients (Okuda et al., 1997a). In February 1996, however, the infection rate attributed to *V. parahaemolyticus* in Calcutta suddenly jumped to between 10 and 20 isolations each month. These cases marked the recognition of the emergence of a unique O3:K6 serotype which accounted for 50–80% of the strains isolated during this period. The O3:K6 clone was further characterized as being *tdh*-positive, *trh*-negative, and urease-negative. To assess the possibility that this O3:K6 strain was emerging on a more global scale, Okuda et al. (1997a) analyzed *V. parahaemolyticus* isolates from travelers arriving in Japan from countries in Southeast Asia. Strains of O3:K6 isolated in Japan in 1995 and 1996 were indistinguishable from the Calcutta O3:K6 strain with regard to recognized virulence gene profiles and arbitrarily primed PCR patterns. Interestingly, *V. parahaemolyticus* O3:K6 strains isolated prior to 1993 are distinct from the strains isolated in 1995 and later. These findings suggest that the *V. parahaemolyticus* O3:K6

serotype became more prevalent (“emerged”) as a pathogen in Calcutta, India and in other South-east Asian countries in approximately 1995.

V. parahaemolyticus O3:K6 strains with characteristics nearly identical to the strains identified in India and in Southeast Asia (*tdh*-positive, *trh*-negative, and urease-negative) also have been associated with two outbreaks of gastroenteritis in the US. The first outbreak occurred in Texas in July 1998 and involved 296 oyster consumers (Texas Department of Health, 1999; Daniels et al., 2000b). A September 1998 outbreak in New York State was linked to shellfish harvested from Oyster Bay on Long Island. Following both outbreaks, the implicated harvesting areas were closed and all shellfish originating from those areas were recalled (CDC, 1999). Clinical isolates from infected individuals in both the Galveston Bay (Texas) and the Oyster Bay outbreaks were identified as the O3:K6 Calcutta strain (CDC, 1999; Texas Department of Health, 1999; Daniels et al., 2000b). However, *V. parahaemolyticus* isolates from oysters obtained from these sites were not of the O3:K6 serotype (Daniels et al., 2000b). The inability to isolate and identify O3:K6 strains from oyster samples in affected harvesting bays suggests that (i) current methods for isolation of this serotype from foods may be inadequate; (ii) the presence of low numbers of this organism in seafood is masked by the presence of other strains of *V. parahaemolyticus*; and/or (iii) this strain may not have been present in the oyster beds by the time environmental sampling was initiated (Daniels et al., 2000b; DePaola et al., 2000).

Serotype O4:K68 and O1:KUT strains, which have been responsible for gastroenteritis in India and other Southeast Asian countries, had been reported as genetically similar to recent O3:K6 isolates, as they share similar arbitrarily primed PCR fingerprints, ribotype and pulsed-field gel electrophoresis patterns (Chowdhury et al., 2000; Matsumoto et al., 2000; Yeung et al., 2002). Based on their chronological order of appearance, these O4:K68 and O1:KUT strains are proposed to have originated from the pandemic O3:K6 strain. Recent studies suggest some strains bearing serotypes O1:K25, O1:K41 and O4:K12 also may be genetic variants of O3:K6 strains (Hara-Kudo et al., 2003).

Emergence of new pathogenic strains through transfer of genetic elements.

One mechanism through which virulence capability can be altered or "new" pathogenic strains can emerge is through the transfer of genetic elements, including virulence factors, both horizontally and vertically through bacterial populations (Waldor and Mekalanos, 1996). Bacterial virulence factors (e.g., genes encoding toxins) can be encoded by accessory genetic elements including plasmids, bacteriophages, transposons, and pathogenicity islands. To illustrate, the emergence of toxigenic strains of *Vibrio cholerae* producing the cholera toxin can result from horizontal transfer of the toxin gene on a filamentous bacteriophage designated CTX ϕ (Waldor and Mekalanos, 1996).

Several studies support the likelihood of horizontal gene transfer among *V. parahaemolyticus* strains. For example, *tdh*, which encodes a virulence-associated hemolysin, has been demonstrated to exist on plasmid DNA, chromosomal DNA (Nishibuchi and Kaper, 1990; Baba et al., 1991), and in *Vibrio* strains other than *parahaemolyticus* (Yoh et al., 1995; Nishibuchi et al., 1996). These data support the hypothesis that this hemolysin gene is mobile among bacterial populations. The presence of phage in *V. parahaemolyticus* is common. To illustrate, Taniguchi et al. (1984) isolated extrachromosomal elements, including two replicative forms of filamentous bacteriophages (Vf12 and Vf33), from nine of 37 *V. parahaemolyticus* strains. Nasu et al. (2000) also reported the presence of a filamentous phage f237 in *V. parahaemolyticus* O3:K6 strains isolated since 1995. Based on findings from Iida et al. (2002) and *V. parahaemolyticus* genomic sequence data (Makino et al., 2003), phage f237 integrates into a region located in the replication terminus called the *dif*-like site of the bacterial chromosome, which is also commonly used by other phages for chromosomal integration in other vibrios. *V. parahaemolyticus* O4:K68 strains also harbor a filamentous phage VfO4:K68, which is almost identical to phage f237 and is also able to infect the O3:K6 clone (Chan et al., 2002). The genome sequence of an O3:K6 strain reveals multiple putative phage proteins, and thus, multiple events of phage element transfer may have occurred in this strain (Makino et al.,

2003). Exchange of mobile genetic elements is very likely to contribute to the continuing emergence of "new" pathogenic *V. parahaemolyticus* strains (Chang et al., 1998).

VIRULENCE FACTORS OF *VIBRIO PARAHAEMOLYTICUS*

Although individual virulence factors have been established for *V. parahaemolyticus*, a comprehensive understanding of this organism's ability to cause disease remains elusive. In particular, specific mechanisms that contribute to the ability of *V. parahaemolyticus* strains lacking recognized virulence factors (e.g., *tdh* and *trh*) to mount an infection are unknown. A better understanding of *V. parahaemolyticus* virulence characteristics was identified in the FDA microbial risk assessment process (FDA, 2001a) as a knowledge gap that needs to be filled to enable more accurate characterization of disease risk. The factors described below have been either established or proposed to be associated with virulence in *V. parahaemolyticus*.

Thermostable direct hemolysin (TDH)

Early epidemiological studies identified an association between a *V. parahaemolyticus* strain's ability to cause beta-hemolysis on Wagatsuma blood agar, referred to as the Kanagawa phenomenon (KP), and its ability to cause gastroenteritis (Miyamoto et al., 1969). TDH is the hemolysin responsible for the KP (Takeda, 1983). TDH was demonstrated to be associated almost exclusively with clinical isolates, with only <5% of environmental isolates producing TDH (Miyamoto et al., 1969; Wong et al., 2000), suggesting TDH production as a *V. parahaemolyticus* virulence marker. In comparison to a *tdh*+ wild-type *V. parahaemolyticus*, a *tdh*-mutant showed no fluid accumulation in the ligated rabbit ileal loop model (Nishibishi et al., 1992), thus implicating TDH as an important virulence factor in *tdh*+ *V. parahaemolyticus* strains. TDH appears to act on cellular membranes as a pore-forming toxin that alters ion flux in intestinal cells, thereby leading to a secretory response and diarrhea (Honda et al., 1992; Fabbri et al., 1999; Raimondi et al., 2000; Takahashi et al., 2000a).

Relationship between TDH and pathogenicity

A variety of in vivo and in vitro studies have been performed to examine the relationship between TDH and the pathogenesis of *V. parahaemolyticus*. For example, Hoashi et al. (1990) compared TDH expression patterns of 14 *V. parahaemolyticus* strains with their abilities to cause disease in mice following intraperitoneal or oral-gastric inoculations. Strain lethality and pathologic effects developed similarly regardless of a strain's TDH-production capacity. Further, some strains bearing *tdh* were found to be KP-negative. These results suggest that TDH expression may vary among strains and that the mere presence of the *tdh* gene may not reflect a strain's ability to cause disease (Hoashi et al., 1990). Using 16 *V. parahaemolyticus* strains, Hackney et al. (1980) found that the intensity of *V. parahaemolyticus* adherence to human fetal epithelial cells was related more to a strain's ability to cause foodborne illness than to the KP. Iijima et al. (1981) also showed that *V. parahaemolyticus* adherence to two types of human epithelial cells was not related to the hemolysin-induced KP in 32 strains. A KP-negative strain isolated from a clinical source was shown to produce a non-TDH hemolysin-like substance that induced fluid accumulation (Honda et al., 1988). This hemolysin, which is further described below, was termed TDH-related hemolysin (TRH). In 1991, Honda et al. reported the presence of yet another *V. parahaemolyticus* hemolysin that also is similar to TDH, but is not identical to TRH. Taken together, these results suggest that (i) *V. parahaemolyticus* strains may produce several different, but related, hemolysins; (ii) TDH is not the sole contributor to *V. parahaemolyticus* pathogenicity; and therefore, (iii) the presence of *tdh*, alone, will not reliably indicate a virulent strain. Additional putative virulence factors, as described below, appear to also contribute to the pathogenesis of *V. parahaemolyticus*.

TDH-related hemolysin (TRH)

Sochard and Colwell (1977) demonstrated that a KP-negative strain isolated from food produced a toxin-like proteinaceous substance that was lethal in mice at high concentrations and diarrheic at lower concentrations. The au-

thors concluded that this toxin was unlikely to be TDH as the protein was heat labile. In 1985, an outbreak of gastroenteritis in the Maldives was linked to KP-negative isolates of *V. parahaemolyticus*, suggesting the presence of other virulence factors in addition to TDH. These strains were shown to produce a TRH rather than TDH (Honda et al., 1987, 1988). TDH and TRH are immunologically and biologically similar (Honda et al., 1988). The *trh* and *tdh* genes, encoding TRH and TDH, respectively, have been cloned and sequenced (Nishibuchi and Kaper, 1985; Nishibuchi et al., 1989), revealing approximately 70% nucleotide sequence identity (Nishibuchi et al., 1989; Kishishita et al., 1992). In contrast to TDH, TRH is labile to heat treatment at 60°C for 10 min.

The mechanism of TRH appears to be similar to that of TDH. TRH induces Ca²⁺-activated Cl⁻ channels which result in altered ion flux (Takahashi et al., 2000b). A KP-negative *V. parahaemolyticus* strain with a genetically modified (truncated) TRH showed reduced fluid accumulation in rabbit ileal loops (Xu et al., 1994), supporting the role of this hemolysin in *V. parahaemolyticus* pathogenesis.

V. parahaemolyticus gastroenteritis has been linked to the presence of either *tdh* or *trh* (Shirai et al., 1990; Kishishita et al., 1992); thus, both of these genes are considered virulence factors for this pathogen. Although few clinical isolates contain both *tdh* and *trh*, most environmental isolates possess neither hemolysin gene (Shirai et al., 1990; Baba et al., 1991; Kishishita et al., 1992).

Urea hydrolysis

Urea hydrolysis also has been proposed as an additional virulence marker for some pathogenic *V. parahaemolyticus* strains as a strong correlation has been demonstrated between the presence of the *trh* gene and urease production (Suthienkul et al., 1995; Osawa et al., 1996; Okuda et al., 1997b). In particular, urea hydrolysis has been shown to be a good marker for the O4 *V. parahaemolyticus* strains associated with sporadic illness in the Pacific Northwest (Kaysner et al., 1994). This correlation is likely due to the close proximity of the *trh* and *ure* genes on the chromosome of a subset of pathogenic *V. parahaemolyticus* strains (Iida et al.,

1998). Urease activity is not a universal marker for pathogenic *V. parahaemolyticus*, however, as urease negative strains, including the new O3:K6 strain, have been isolated from a number of patients. Furthermore, its role in *V. parahaemolyticus* pathogenesis is unclear.

*Vibrio*ferrin

Iron is essential for bacterial survival. Iron-storage proteins, such as the heme-containing bacterioferritins and the heme-free ferritins, are widespread in bacteria (Andrews, 1998). In order to acquire iron from their surroundings, many pathogens such as *E. coli* and *Klebsiella pneumoniae*, produce iron (particularly ferric iron) chelators called siderophores.

Under iron limiting conditions, *V. parahaemolyticus* produces a novel siderophore called vibrioferrin (Yamamoto et al., 1994). The vibrioferrin is produced when the medium contains a limited amount of iron (e.g., 0–1 μM FeCl_3), or when an iron chelator, EDDA, is added to iron rich (e.g., 5 μM) medium (Yamamoto et al., 1999). Yamamoto et al. (1999) showed that clinical isolates ($n = 44$) had higher vibrioferrin levels in spent supernatant than food ($n = 37$) and environmental ($n = 26$) isolates when they were grown in medium containing only 0.2 μM of FeCl_3 . An increased production of vibrioferrin may provide strains with a competitive survival advantage in iron-limiting environments such as in the human host.

The link between iron concentration present in bacterial growth media and the virulence of *V. parahaemolyticus* has been studied by Wong and Lee (1994) and Dai et al. (1992). Findings from both groups showed that *V. parahaemolyticus* cultures grown under iron-limited conditions demonstrated greater adherence, increased hemolytic activities and higher proliferation rates. Thus, the production of vibrioferrin may also contribute to the pathogenesis of *V. parahaemolyticus*.

Pathogenicity island and type III secretion system

As mentioned above, the emergence of "new" *V. parahaemolyticus* strains with enhanced virulence is likely the result of incorporation of new genetic elements among existing strains. Genomic sequence data identified

the presence of a pathogenicity island in a clinical KP+ *V. parahaemolyticus* O3:K6 strain (Makino et al., 2003). The sequence data also confirmed the existence of two circular chromosomes (3.3 and 1.9 Mbp) in this species (Tagomori et al., 2002). Both chromosomes contain genes essential for growth and viability, although the majority of these genes are located on the larger chromosome. The pathogenicity island, which is located on the smaller chromosome, has a GC content of 39.8%, in contrast with the average GC content of 45.4% for the remainder of the genome, suggesting that the island was acquired by horizontal transfer. This pathogenicity island bears two *tdh* genes as well as other genes that have been associated with virulence in other organisms, including those encoding homologues of the *E. coli* cytotoxic necrotizing factor and of the *Pseudomonas* exoenzyme T.

Among genes identified in the *V. parahaemolyticus* pathogenicity island are those encoding a predicted type III secretion system (TTSS). TTSS, which are found in various gram-negative organisms, are associated with the export of bacterial virulence factors directly to host cells. TTSS has been studied extensively in different microbes and various functional and evolutionary aspects of these systems have been the subject of many reviews (e.g., Nguyen et al., 2000; Ramamurthi and Schneewind, 2002; Blocker et al., 2003; Waterman and Holden, 2003). The genes encoding several TTSS components are usually located on pathogenicity islands. For example, the Locus of Enterocyte Effacement (LEE) region of enterohemorrhagic *E. coli* O157:H7 and enteropathogenic *E. coli* contains genes encoding TTSS and other genes involved in pathogenesis (Clarke et al., 2003). TTSS is triggered when a bacterium comes in close contact with host cells, and hence, this system is also called contact-dependent secretion. The TTSS may also be regulated by networks that respond to environmental conditions (Sperandio et al., 1999).

It is interesting to note that TTSS has not been found in *V. cholerae*. Although some clinical manifestations are similar between *V. cholerae* and *V. parahaemolyticus* infections (i.e., gastroenteritis), *V. parahaemolyticus* is generally considered to elicit inflammatory diarrhea, as

opposed to the non-inflammatory secretory diarrhoea caused by *V. cholerae* (Qadri et al., 2003). Furthermore, the main virulence factors of *V. parahaemolyticus* and *V. cholerae* (TDH and cholera toxin, respectively) have different modes of action. Therefore, it is tempting to speculate that a major difference in pathogenicity between these closely related organisms is, at least in part, due to the presence of TTSS in *V. parahaemolyticus*. It has yet to be established if the invasion capabilities of some *V. parahaemolyticus* strains can be attributed to the presence of TTSS. For other organisms, these systems have been shown to enable a pathogen to establish an intimate interaction with the host and enhance intracellular infection (e.g., *Burkholderia pseudomallei*—Stevens et al., 2002; *Salmonella typhimurium*—Steele-Mortimer et al., 2002; *Shigella flexneri*—Schuch et al., 1999).

In summary, epidemiological studies suggest that *tdh* and *trh* are important virulence factors in *V. parahaemolyticus*, and this conclusion is well supported by multiple laboratory studies. Evidence linking the presence of other virulence factors with *V. parahaemolyticus* pathogenesis is currently very limited. Thus, further research is necessary to gain an understanding of the role of these proposed factors in the ability of *V. parahaemolyticus* to cause human disease.

METHODS TO ASSESS *VIBRIO PARAHAEMOLYTICUS* STRAIN PATHOGENICITY

Phenotypic characterization is a critical step for assessing the pathogenic potential of a given microbe. This concept may have been most clearly illustrated by Gorden and Small (1993), who established a link between a food-borne pathogen's ability to survive exposure to acidic conditions such as those encountered in the human stomach and that organism's infectious dose.

V. parahaemolyticus growth and survival capabilities, parameters commonly measured to reflect bacterial fitness, have been examined under different experimental conditions, including various concentrations of iron, acid, and bile (Pace et al., 1997; Koga et al., 1999; Ya-

mamoto et al., 1999). The experimental conditions typically have been chosen to represent (i) conditions that may be experienced by *V. parahaemolyticus* in its natural habitat; (ii) conditions experienced during food processing or preservation treatment, and (iii) conditions experienced during infection of animal and human hosts.

Molecular studies of *V. parahaemolyticus* pathogenesis have primarily focused on creation of various targeted mutations in virulence genes. The resulting gene products have been either non-functional (deletion or insertion mutants) (Nishibuchi et al., 1992) or of modified function (i.e., as a consequence of amino acid sequence changes) (Tang et al., 1994; Iida et al., 1995). To provide quantitative assessments of the specific contributions of the targeted virulence determinants to the pathogenic potential of *V. parahaemolyticus*, the mutant strains are typically assessed relative to the wild type strain in phenotypic assays, as described above, as well as in tissue culture or animal models.

Status of animal and tissue culture models for virulence characterization of V. parahaemolyticus

Commonly applied strategies for in vitro analysis of bacterial virulence capabilities are measurement of: pathogen adherence to cultured cells (Hackney et al., 1980; Merrell et al., 1984; Baffone et al., 2000), cytotoxicity induction (Baffone et al., 2000; Raimondi et al., 2000), and invasion capabilities (Akeda et al., 1997, 2002). As production of TDH has been recognized as a virulence factor for some clinical *V. parahaemolyticus* isolates, the majority of virulence studies have focused on characterization of this hemolysin. Studies have been reported on the mode of hemolysin action using viable cells (Honda et al., 1991), culture filtrates (Nishibuchi et al., 1991), genetically modified bacterial cells harboring *tdh* (Iida et al., 1995), and purified TDH in various systems (Fabbri et al., 1999; Raimondi et al., 2000). Various host cell lines have been used for *V. parahaemolyticus* characterization, including rat small intestine cells (IEC-6) (Fabbri et al., 1999), human colon cells (Caco-2) (Raimondi et al., 2000; Takahashi et al., 2000a), and human epithelial cells (HeLa) (Iijima et al., 1981; Yeung et al.,

2002). Mice and rabbits have been used to determine *in vivo* lethal and diarrheal effects. Due to the typical self-limiting nature of the disease induced by *V. parahaemolyticus*, a relatively high bacterial dose (10^7 – 10^8) (Sanyal and Sen, 1974; Hoashi et al., 1990) or equivalent, such as a culture filtrate, has been used in lethality studies. Rabbit ligated ileal loops have been used to measure fluid accumulation activity (Sochard and Colwell, 1977; Honda et al., 1991; Nishibuchi et al., 1992; Raimondi et al., 1995). Further, measurement of electrical parameters and ion concentrations in and surrounding intestinal cells following exposure to *V. parahaemolyticus* have been coupled with tissue culture and animal models to explore bacterially induced secretion in mammalian tissues (Nishibuchi et al., 1992; Raimondi et al., 1995; Takahashi et al., 2000a).

A major limitation of *V. parahaemolyticus* growth, survival, and virulence studies conducted to date is that most studies have examined characteristics associated with only a single bacterial strain. Findings from a particular strain may not necessarily apply to *V. parahaemolyticus* as a whole due to strain-to-strain differences. It is, therefore, important to characterize multiple strains representing different serotypes. Furthermore, the application of identical experimental conditions among studies is highly encouraged to enable direct comparisons among *V. parahaemolyticus* strains.

MEASURES TO REDUCE FOODBORNE VIBRIO PARAHAEMOLYTICUS INFECTIONS

Relaying and depuration

Common approaches to reduce or remove bacterial contaminants in shellfish (e.g., oysters) include relaying and depuration (National Shellfish Sanitation Program, 2002). In the relaying process, contaminated shellfish are transferred from restricted areas to approved areas for natural biological purification. Depuration is similar to relaying, except that contaminated shellfish are transferred to a controlled aquatic environment instead of a natural ambient environment. Nonetheless, either method is

often inadequate to completely remove *V. parahaemolyticus* (FDA, 2001a; Croci et al., 2002).

Post-harvest handling

V. parahaemolyticus multiplies exponentially, with a doubling time of 1.8 h, in live oysters that are held at room temperature (Gooch et al., 2002). After storing live oysters at 26°C for 24 h, Kaufman et al. (2003) reported a 13–26-fold increase in *V. parahaemolyticus* numbers. Gooch et al. (2002) also reported as much as 790-fold increase in *V. parahaemolyticus* numbers under these conditions. Under approved guidelines, shellfish may remain unrefrigerated for as long as 10 h after harvest. Retail oysters that have been held at ambient temperatures for 10 h could harbor 10–100 times more *V. parahaemolyticus* than present at harvest (Gooch et al., 2001). Therefore, it is of paramount importance to immediately reduce and maintain the temperature of freshly harvested shellfish at sufficiently low levels to prevent this pathogen from rapidly multiplying to infectious levels.

Post-harvest processing treatments

V. parahaemolyticus is sensitive to heat. To illustrate, a >7-log reduction in viable cell count was reported when *V. parahaemolyticus* cells were heat treated at 55°C for 2 min (Nishikawa et al., 1993). The time required to cause a 1-log reduction in viable cell count (D-value) at 47°C is 2.03 min (Wong et al., 2002). Therefore, a mild heat treatment is effective in substantially reducing *V. parahaemolyticus* numbers. For example, a low temperature pasteurization treatment (e.g., 50°C for up to 15 min) can reduce the risk of vibrio infections from raw oyster consumption (Andrews et al., 2000; Johnston and Brown, 2002). *V. parahaemolyticus* also appears to be sensitive to high-pressure processing (or high-hydrostatic-pressure processing) and irradiation. A pressure of 300 MPa for 180 sec is sufficient to achieve a >5-log reduction in *V. parahaemolyticus* numbers, including *V. parahaemolyticus* O3:K6 strains, in oyster samples (Cook, 2003). While high doses of irradiation have the undesirable effect of killing oysters, lower, non-lethal doses (e.g., <3.0 kGy) can substantially reduce *V. parahaemolyticus* numbers (Jakabi et al., 2003) without signifi-

cantly altering the textural and sensory properties of oysters.

An important challenge for the successful application of the aforementioned treatments is the variability among strains in sensitivities to these treatments (Andrews et al., 2000; Cook, 2003). Therefore, optimization of each treatment will be necessary to ensure that a new method will be effective in reducing or eliminating the most resistant *V. parahaemolyticus* subtypes. Other critical considerations include the impact of these treatments on the appearance, organoleptic, and sensory qualities of the shellfish.

Detection

According to the FDA risk assessment document (2001a), the number of *V. parahaemolyticus* present in oysters is the most critical predictor of the risk of contracting *V. parahaemolyticus* infections. Given the ubiquitous presence of this organism in the marine environment, preventing contamination of shellfish with *V. parahaemolyticus* is virtually impossible. Therefore, to prevent *V. parahaemolyticus* infections and to determine the safety of reopening shellfish growing areas following an outbreak, regulatory agencies and the seafood industry need reliable methods for detecting pathogenic *V. parahaemolyticus* that may be present in a variety of matrices.

The standard method for *V. parahaemolyticus* detection is described in the FDA's Bacteriological Analytical Manual (FDA, 2001b). The standard method specifies a three-tube most-probable-number (MPN) enrichment in alkaline peptone water or alkaline peptone salt broth followed by isolation on thiosulfate-citrate-bile salts-sucrose agar. Colonies typical of *V. parahaemolyticus* are then subjected to a number of biochemical tests to distinguish *V. parahaemolyticus* from other marine vibrios. To further detect *V. parahaemolyticus* strains predicted to be pathogenic, TDH, encoded by *tdh*, is currently used as the virulence marker (Nishibuchi et al., 1985; Shirai et al., 1990). Specifically, *V. parahaemolyticus* isolates obtained as described by the standard method are then screened for the presence of *tdh* by colony hybridization utilizing gene probes and for the presence of TDH

by the KP test. If *tdh* is detected, isolates are usually serotyped.

The standard method for detection of pathogenic *V. parahaemolyticus* is fraught with drawbacks. First, detection strategies for pathogenic strains based solely on the presence of *tdh* will not detect *tdh*- strains with pathogenic potential. The existence of pathogenic *tdh*- *V. parahaemolyticus* strains illustrates the necessity to modify detection methods, possibly to screen for multiple virulence markers. Second, the standard methods for serotype determination are expensive, tedious and time consuming. Rapid methods are needed to facilitate timely decision-making on the need to close or ability to reopen shellfish growing areas. Third, routine growth media may be inadequate for resuscitating non-culturable *V. parahaemolyticus* (Mizunoe et al., 2000). Further, application of a single enrichment step, as currently specified, may fail to recover VBNC or otherwise injured cells. The addition of supplements or the application of a multi-step enrichment procedure (e.g., culturing samples in nutrient broth at least twice) may be necessary to resuscitate VBNC or injured cells. However, inclusion of a multi-step enrichment procedure would inevitably further prolong detection time. Detection strategies based on molecular techniques (e.g., detection of target genes from bacterial DNA extracted directly from food or water samples) ultimately may provide more rapid strategies for detecting the presence of potentially harmful microbes.

Education

Consumer behavior also represents an integral component affecting numbers of *V. parahaemolyticus* infections. As with many other foodborne illnesses, the risk of *V. parahaemolyticus* infections can be reduced through application of hygienic food handling techniques. Such practices include holding seafood at a sufficiently low temperature to prevent *V. parahaemolyticus* from multiplying rapidly, avoiding cross-contamination of ready-to-eat foods with uncooked seafood, and thorough cooking of raw seafood to destroy *V. parahaemolyticus*. These precautions are important at the consumer level, as mishandled raw or under-

cooked oysters can cause illness even if they had originally been harvested from approved sites (Daniels et al., 2000a,b). Last but not least, notices or warnings, such as those displayed conspicuously in food service establishments that serve raw oysters in some US states, may help educate consumers about the risks associated with consuming raw oysters.

CONCLUSION

Previously associated only with sporadic illnesses, *V. parahaemolyticus*, specifically, strains bearing the O3:K6 serotype, has recently caused large-scale foodborne outbreaks and is now considered an emerging foodborne pathogen. A comprehensive understanding of *V. parahaemolyticus* virulence mechanisms remains elusive. Two *V. parahaemolyticus* virulence factors have been established (TDH, TRH); others have been suggested, but remain to be verified. The recent availability of *V. parahaemolyticus* genomic sequence data is likely to promote rapid advances in identification and verification of additional contributors to virulence capabilities in this organism. Coordinated and complementary efforts by seafood producers, regulatory agencies, scientists and consumers will all be necessary to reduce the incidence of *V. parahaemolyticus* infections.

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