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Global Dissemination of *Vibrio parahaemolyticus* Serotype O3:K6 and Its Serovariants

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INTRODUCTION

Vibrio parahaemolyticus, a gram-negative halophilic bacterium, is recognized as a worldwide cause of food-borne gastroenteritis, particularly in the Far East, where seafood consumption is high. The halophile was first identified as a cause of food-borne illness in Japan in 1950, when 272 individuals became ill and 20 died after the consumption of semidried juvenile sardines (27). *V. parahaemolyticus* causes three major syndromes of clinical illness, i.e., gastroenteritis, wound infections, and septicemia. The most common syndrome is gastroenteritis; the symptoms include diarrhea with abdominal cramps, nausea, vomiting, headache, and low-grade fever (34). Sometimes the diarrhea is bloody, with stools described as "meat washed" since the stool is reddish watery stool (77) but unlike that seen in dysentery caused by *Shigella* species or in amebiasis. The mean incubation period for *V. parahaemolyticus* infection is 15 h (range, 4 to 96 h). The illness is self-limiting and of moderate severity and lasts an average of 3 days in immunocompetent patients.

V. parahaemolyticus strains that are isolated from diarrheal patients produce either the thermostable direct hemolysin (TDH), the TDH-related hemolysin (TRH), or both, while hardly any isolates from the environment have these properties (34, 83, 87). An isolate producing TDH is referred to as Kanagawa positive and can be identified by β hemolysis on a special agar known as Wagatsuma blood agar (87, 93). TDH has been shown to have hemolytic, enterotoxic, cardiotoxic, and cytotoxic activities (34, 68, 83, 93). A strong correlation between urease production (an unusual phenotype for *V. parahaemolyticus*) and the *trh* gene exists (38). Enteroinvasiveness of the bacterium has been reported for a rabbit model, in which the organism invaded, colonized, and produced inflammation in the small intestine (13). In patients in the acute stage of *V. parahaemolyticus* infection, the inflammatory response in the gut and in the circulation is less severe than that observed in patients with shigellosis but more severe than that seen in patients with cholera (77). It has been shown that gastroenteritis caused by *V. parahaemolyticus* results in strong systemic and mucosal B-cell responses to TDH and lipopolysaccharide; both antigens also induce an increase in the presence of immunoglobulin M antibody-secreting cells, which suggests that this is a primary response to the antigen (77). In a recent study, it was shown that irrespective of TDH production, *V. parahaemolyticus* profoundly disturbs epithelial barrier function in Caco-2 cells due to the involvement of another virulence factor(s) (53). The overall mechanism of pathogenesis by *V. parahaemolyticus*, however, remains unclear.

Most gram-negative pathogens disrupt the normal physiology of the intestinal mucosa by inducing cytoskeleton rearrangements, proinflammatory responses, and/or cell death. Many of these cellular events are caused by bacterial effector proteins, which are delivered into intestinal cells that directly modulate the activities of host cell proteins and are secreted and translocated into host cells through the bacterial type III secretion systems (TTSSs). The TTSS macromolecular assembly is a needle-like complex composed of more than 20 proteins, and its components are highly conserved among bacteria. Genes encoding the TTSS apparatus are generally found on the chromosomal pathogenicity islands or plasmids (35, 99). In several bacteria, the genes encoding TTSS-secreted proteins are located outside the gene clusters encoding the TTSS apparatus (35). The specific properties of the effectors and their

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symptomatic effects on the host vary widely (35). Based on the assembly and functions of different proteins, the TTSSs in animal pathogens are classified into the following three major groups: (i) the Ysc-plus-Psc system, (ii) the 1-plus-Mxi/Spe system of the *Salmonella* pathogenicity island, and (iii) the enterohemorrhagic *Escherichia coli* system (22).

In *V. parahaemolyticus*, two sets of genes, for TTSS1 and TTSS2, were identified on the large and small chromosomes, respectively (54, 76). *V. parahaemolyticus* TTSS1 is a Ysc-plus-Psc system and is similar to the TTSSs of *Yersinia* spp. and *Pseudomonas aeruginosa* in the number of genes, their order, and the identity of each protein (54, 61, 73). However, no homologue of the effector protein genes has been found in the TTSS1 region. Four new TTSS1-secreted proteins were identified in *V. parahaemolyticus*, and one was located in the small chromosome (73). TTSS2 is detected only in Kanagawa-positive *V. parahaemolyticus* strains and is not similar to any particular TTSS of other bacteria (76). The G+C contents of the DNA regions of TTSS1 and TTSS2 suggest that the former is intrinsic in *V. parahaemolyticus* while the latter has a feature of laterally transferred DNA (54). Both TTSSs in *V. parahaemolyticus* may act as effectors during infections, as mutational studies and an adenylate cyclase fusion assay with TTSS1 showed it to be involved in the cytotoxicity in HeLa cells, while TTSS2 has a role in enterotoxicity in a rabbit model (73, 76). It was assumed that both TTSSs might act synergistically in the pathogenesis of *V. parahaemolyticus* (73).

V. parahaemolyticus is widely disseminated in estuarine, marine, and coastal environments throughout the world (42) and has been detected as far north as Alaska (91). Water temperature, salinity, zooplankton blooms, tidal flushing, and dissolved oxygen play an important role in dictating its spatial and temporal distribution (45). This pathogen is typically not recovered from estuarine waters during winter months in temperate zones, when the water temperature is too low for its existence. Water temperatures have been shown to influence the growth of *V. parahaemolyticus* (44, 46, 47, 88), and the importance of water temperature in the epidemiology of infections is reflected by the fact that most outbreaks occur during the warmer months. In tropical countries, in contrast, the seasonality of *V. parahaemolyticus* is less defined, with infection occurring throughout the year. Studies in Calcutta have shown that both marine and freshwater fishes provide ideal substrates for the survival and proliferation of *V. parahaemolyticus*. The isolation of *V. parahaemolyticus* from market samples of freshwater fishes was attributed to cross-contamination due to mishandling at fishmongers' stalls (81). Most *V. parahaemolyticus* outbreaks that occurred between 1973 and 1998 in the United States occurred during the warmer months and were attributed to seafood, particularly oysters and other shellfish, and the median attack rate among persons who consumed the implicated seafood was 56% (63). Many investigations have shown that marine mollusks are associated with the spread of toxigenic *V. parahaemolyticus* (10, 15, 24, 32, 59).

The primary basis of classification of strains of *V. parahaemolyticus* is a serotyping scheme, which depends on the antigenic properties of the somatic (O) and capsular (K) antigens. The serotyping scheme for *V. parahaemolyticus* is a combination of O and K antigens, and serotyping is done using commercially available antisera that include 11 different O antigens and 71 different K types (37). The serotyping scheme was developed using strains of clinical origin. Early investigations carried out by Baross et al. (6) with 20 bacteriophages against *V. parahaemolyticus* showed no correlation between O and K serotypes in the lytic pattern, and hence they were assumed to have a wide host range. The first phage typing scheme for *V. parahaemolyticus* was formulated in 1992 with 46 phages belonging to morphological groups II, IV, and V (49). However, for these phages the specificities for the serotypes have also not been established. Libinzon et al. (51) tested 34 *V. parahaemolyticus* phages isolated from the Black Sea. Except for one, which was specific for O5:K15, the majority of the phages were also found to lyse *Vibrio alginolyticus* strains. Phage typing studies conducted during an O3:K6 outbreak in Vladivostok showed types 1, 2, 7, and 10 with *V. parahaemolyticus* strains and phage types 2, 4, 5, and 7 with *V. alginolyticus* strains (85). Due to the lack of specificity, the phage typing scheme for *V. parahaemolyticus* is not customary in international practice. In recent years, a variety of molecular typing techniques, such as ribotyping, pulsed-field gel electrophoresis (PFGE), and multilocus sequence typing (MLST) techniques, as alluded to later in this review, have been used successfully to study the molecular epidemiology of the organism.

THE MYSTERIOUS ORIGIN OF THE O3:K6 SEROTYPE

During active surveillance of diarrheal etiologies among hospitalized patients in Calcutta, a city in the northeastern part of India, an increase in hospital admissions of patients with *V. parahaemolyticus* gastroenteritis was observed beginning in February 1996. Analysis of the strains revealed that a unique serotype, O3:K6, which was not previously isolated during surveillance in Calcutta, accounted for 50 to 80% of infections in the following months (69). The O3:K6 isolates had identical genotypes (*tdh* positive, *trh* and urease negative) and nearly identical arbitrarily primed PCR (AP-PCR) profiles and shared similar antibiotic sensitivity patterns (69).

From the literature, it seems that the first O3:K6 isolate was isolated from a traveler returning from Indonesia to Japan in 1995 (69). Curiously, the seventh pandemic strain of *Vibrio cholerae* O1, which is of the El Tor biotype, also had its origin in the island of Sulawesi in Indonesia (43). However, based on hospital admissions, the first localized cluster of cases of O3:K6 occurred in Calcutta starting in February 1996. Thus, the epidemiological setting in Calcutta at that time was conducive to infecting a larger population. Food-borne outbreak statistics for Taiwan revealed that the O3:K6 serotype could have emerged as early as October 1995, when it was responsible for a single outbreak with three isolates (15). Four *tdh*-negative O3:K6 isolates obtained in Japan between 1983 and 1988 grouped with the unique O3:K6 cluster (>75% similarity) when recently examined by AP-PCR and showed *toxRS* sequences identical to that of an O3:K6 clone, leading Okura et al. (70) to speculate that the O3:K6 isolates might have originated from these nonpathogenic strains after acquisition of the *tdh* gene. Therefore, at this point it appears that the progenitors of the O3:K6 isolate might have originated in the environs of Japan.

The regional dominance of a specific serotype of *V. parahaemolyticus* has occasionally been reported. Early investigations in Calcutta revealed the dominance of serotype O1:K56

among diarrheal cases (12) and in index cases and carriers (75, 84). Along the western coasts of Mexico and the United States, *V. parahaemolyticus* O4:K12 was the dominant serotype causing infections (1). The O3:K6 clustering of cases, however, differs from the previously reported clustering of single *V. parahaemolyticus* serotypes in possessing additional attributes. One is the ability to rapidly increase hospitalizations in areas where it prevails and the other is to become the dominant serotype, supplanting other serotypes of *V. parahaemolyticus* in the given area. Both of these attributes were not observed before for *V. parahaemolyticus*. For example, in the Aichi Prefecture in Japan, the percentage of outbreaks by *V. parahaemolyticus* O3:K6 increased from 3% for the period 1988 to 1995 to 75% for the period 1996 to 2001 (104).

SEROVARIANTS OF THE O3:K6 SEROTYPE

The development of a specific method to facilitate the rapid identification of O3:K6 isolates (57) led to the serendipitous finding of other serotypes, such as O4:K68, O1:K25, and O1:KUT (untypeable), that had *toxRS* sequences, AP-PCR profiles, ribotypes, and PFGE profiles identical to those of the O3:K6 serotype (17, 18, 57). The discovery of the filamentous phage designated f237 in O3:K6 isolates (62) led to the development of another specific PCR method, which targeted ORF8 of f237, claimed to be a specific genetic marker of O3:K6 isolates. Later, it was found that other serotypes that were not like the O3:K6 isolates also carried ORF8 (39). In addition to O4:K68, O1:K25, and O1:KUT, another serotype, O6:K18, which shared high molecular identity with an O3:K6 isolate, was detected in Taiwan (102). Therefore, from a single O3:K6 serotype, other serotypes that had identical genotypes and molecular profiles to those of O3:K6 isolates emerged, and these were collectively referred to as "serovariants" of O3:K6 isolates (57). These serotypes appeared to have diverged from the O3:K6 isolates by alteration of the O:K antigens and were postulated to be clonal derivatives of the O3:K6 serotype. MLST data have further confirmed the finding that multiple serotypes occur in a single genetic lineage (18, 19, 57). The acquisition of additional serotypes of the pandemic strain may be a selected response to host immunological pressure (19). Eleven O:K serotypes were detected among the strains isolated during a survey of diarrhea patients in Khanh Hoa Province, Vietnam, and all were found to be closely related to O3:K6 (16). To date, 21 serotypes that are similar to the O3:K6 serotype have been identified (Table 1) by a variety of molecular typing techniques.

Table 2 shows the chronology of appearance of the various serotypes in Calcutta that are similar to the O3:K6 isolates recorded from February 1996. Clearly, the vigor with which the O3:K6 isolates appeared in the beginning of 1996 is on the wane. This matches the epidemiology of cholera, which generally settles into an endemic pattern of seasonal outbreaks separated by periods of quiescence after passage of a pandemic wave through a geographic region (62). The more recent serotypes, which have molecular traits similar to those of O3:K6, do not seem to have the propensity for elevating hospital admissions due to *V. parahaemolyticus* gastroenteritis observed with O3:K6 and the earlier serovariants of O3:K6. Some kind of decay in the epidemic process seems to be evident.

TABLE 1. Chronology of appearance of *Vibrio parahaemolyticus* O3:K6 and its serovariants in different countries

Serotype	Country (yr of isolation)	Reference(s)
O3:K6	India (1996)	17, 69
	Vietnam (1997)	16
	Laos (1997)	57
	Indonesia (1997)	69
	United States (1997–1998)	23, 25
	Korea (1997–1998)	57
	Chile (1998 and 2004)	21, 30
	Taiwan (1996–1999)	15, 101
	Bangladesh (1998–2000)	7
	Japan (1998)	103
	Thailand (1999)	17, 50
	Russia (2001)	85
	France (2004)	78
	Mozambique (2004)	3
O4:K68	India (1998)	18
	Thailand (1999)	17
	Bangladesh (1998 and 2000)	7
	Vietnam (1998)	16
	Mozambique (2004)	3
O1:K25	India (1998)	18, 57
	Thailand (1999)	50
	Vietnam (1998–1999)	16
	Bangladesh (1999–2000)	7
O1:KUT	India (1998)	18
	Bangladesh (1998 and 2000)	7, 57
O4:K12	Thailand (1998–1999)	50
	Vietnam (1998–1999)	16
	Chile (2004)	30
O1:K41	Thailand (1998–1999)	50
	Vietnam (1998–1999)	16
O1:K56	Vietnam (1998–1999)	16
O3:K75	Vietnam (1998–1999)	16
O4:K8	Vietnam (1998–1999)	16
O4:KUT	Vietnam (1998–1999)	16
O5:KUT	Vietnam (1998–1999)	16
	India (2004)	Dutta and Ramamurthy (unpublished data)
O5:K17	India (2002)	Dutta and Ramamurthy (unpublished data)
O5:K25	India (2002)	Dutta and Ramamurthy (unpublished data)
O1:K33	India (2002)	Dutta and Ramamurthy (unpublished data)
O2:K3	India (2002)	Dutta and Ramamurthy (unpublished data)
OUT:KUT	India (2003–2004)	Dutta and Ramamurthy (unpublished data)
O3:KUT	India (2003–2004)	Dutta and Ramamurthy (unpublished data)
O3:K5	India (2004)	Dutta and Ramamurthy (unpublished data)
O4:K4	India (2004)	Dutta and Ramamurthy (unpublished data)
O4:K10	India (2004)	Dutta and Ramamurthy (unpublished data)
O6:K18	Taiwan (2005)	96

IS THE SPREAD OF O3:K6 AND ITS SEROVARIANTS A PANDEMIC?

When the Calcutta O3:K6 isolates were compared with an archived collection of O3:K6 strains isolated between 1982 and 1996 from travelers returning to Japan, it was found that the Calcutta O3:K6 isolates were identical to the isolates obtained from travelers from 1995 onwards but differed from O3:K6 strains

TABLE 2. Emergence of O3:K6 serotype of *Vibrio parahaemolyticus* and its serovariants in Calcutta from January 1994 to December 2004[a]

Serotype	No. of isolates											Total no. of isolates
	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	
O3:K6			70	33	12	20	4	1	2	9	1	152
O1:KUT				6	5		3	4	2		3	23
O4:K68					7	5		1	2			15
O1:K25						1	22	1	6		18	48
O5:K25									1			1
O1:K33									1			1
O2:K3									1			1
OUT:KUT										1	1	2
O3:KUT										2	7	9
O3:K5											1	1
O4:K4											1	1
O4:K10											1	1
O5:KUT											2	2
O5:K17											1	1
Total			70	39	24	26	29	7	15	12	36	258

[a] Data in this table are from references 14, 15, 52, and 64 and from Dutta and Ramamurthy (unpublished data).

isolated prior to 1993. This comparison also fortuitously revealed that isolates of O3:K6 like those found in Calcutta were already prevalent in Indonesia, Thailand, and Singapore, since travelers returning from these countries were infected (69). Evidence supporting the hypothesis that the O3:K6 serotype emerged only recently was furnished by Matsumoto et al. (57), who showed that O3:K6 isolates from clinical sources in Taiwan, Laos, Japan, Thailand, Korea, and the United States in 1997 and 1998 were identical to the Calcutta O3:K6 strains. Based on this evidence, they concluded that the unique O3:K6 isolate and its serovariants were causing a pandemic. Such a widespread occurrence of one clonal type of *V. parahaemolyticus* was unprecedented and spurred great interest.

In retrospect and following the trend of the spread of O3:K6 isolates over the past decade, it seems that use of the word "pandemic" is somewhat misleading and might be a bit of a strong term in a true epidemiological sense. The Webster's dictionary definition of pandemic describes it as "occurring over wide geographic areas and afflicting an exceptionally high proportion of the population." Although the O3:K6 serotype of *V. parahaemolyticus* has been isolated over a wide geographic area, supplanting the other serotypes of *V. parahaemolyticus* in the process, it has not affected an exceptionally high proportion of the population, and it apparently has caused little, if any, mortality. In fact, compared to the seventh pandemic El Tor biotype *V. cholerae* O1 (80) or compared to *V. cholerae* O139 (65), *V. parahaemolyticus* is a pretty rare disease. In this review, we use the term "pandemic" only to allude to the description investigators have used in their publications, but as explained above, we are of the opinion that the spread of O3:K6 and its serovariants is not a pandemic in the real sense.

CLONALITY OF O3:K6 ISOLATES AND SEROVARIANTS

Starting with AP-PCR (17, 18, 57, 69), a variety of molecular techniques, including ribotyping and pulsed-field gel electrophoresis (18, 25, 101, 105), revealed that O3:K6 isolates from widely different geographic areas were genetically similar to each other and distinct from O3:K6 isolates obtained before 1995 and from non-O3:K6 serotypes. The O3:K6 isolates appearing after 1995 carried the *tdh* but not the *trh* gene, did not produce urease, and were defined by a positive group-specific PCR (GS-PCR) based on the mismatched nucleotides at seven base positions of the *toxRS* gene sequences and ORF8 from the f237 phage. Ribotyping revealed a certain degree of instability in the early Calcutta O3:K6 isolates, reflected as genomic reassortment during their initial period of existence (5). According to biochemical fingerprinting by a PhenePlate system (PhP-48; PhenePlate Microplate Techniques, Stockholm, Sweden) based on the kinetic measurement of the fermentation of selected reagents, the pandemic strains belonged to the same biochemical phenotype, whereas the nonpandemic strains were heterogeneous (79). Thirty-five isolates of *V. parahaemolyticus* from different countries and belonging to different serotypes (O3:K6, O4:K68, and O1:KUT) showed identical ribotypes and PFGE patterns, with some exceptions, including a Japanese *tdh*-negative O3:K6 strain and a U.S. clinical O3:K6 isolate (18). At least two different ribotype patterns were observed among O3:K6 isolates from the United States and Asia, and most of the strains from the 1998 Galveston Bay outbreak were different from those isolated in New York and parts of Asia (28). The 11 serotypes of *V. parahaemolyticus* obtained from surveillance in Vietnam were shown to be closely related, regardless of the ORF8 genotype, using AP-PCR and PFGE (16).

MLST provided strong molecular evidence for the clonal origin of *V. parahaemolyticus* O3:K6 and revealed that isolates within such a clonal group may acquire previously identified serotypes of *V. parahaemolyticus*. The MLST study also confirmed genetic diversity among the *V. parahaemolyticus* strains that prevailed before O3:K6 and genetic uniformity between O3:K6 and its serovariants in spite of their serotype diversity (16). All of the O3:K6 isolates collected from 1983 to 1993 from diverse countries around the Indian Ocean were related. However, the sequence types (STs) of all 11 other serotypes were distinct. In contrast, the four distinct serotypes O3:K6, O4:K68, O1:KUT, and O1:K25 were clonally related. Fifty-one of 54 isolates had the ST 1,1,1,1 (19).

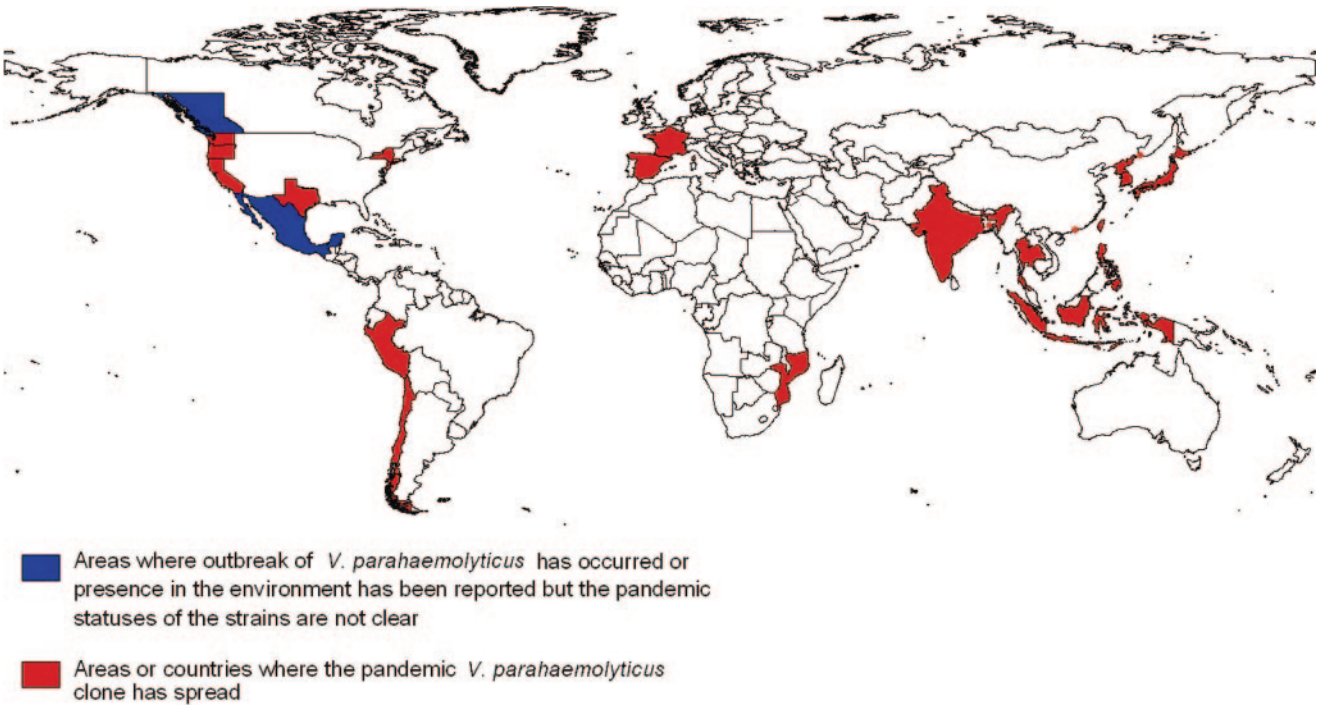

FIG. 1. Global dissemination of the unique O3:K6 isolate of *Vibrio parahaemolyticus* and its serovariants.

GLOBAL SPREAD

At about the same time as the Calcutta occurrence, information on steadily increasing numbers of *V. parahaemolyticus* infection in Japan was documented. The number of food-borne infections caused by this pathogen in 1998 in Japan doubled compared to that in 1997 and exceeded the number of infections by *Salmonella*, which previously was the dominant cause of food-borne infections in Japan (103). An increase in food-borne disease outbreaks was recorded in Taiwan in 1996, and this increase correlated with the high rate of isolation of *V. parahaemolyticus* O3:K6 (15, 100). During 1995 in Taiwan, the O3:K6 serotype accounted for only 0.6% of *V. parahaemolyticus* infections, and this level abruptly increased to 50.1% in 1996 and reached a peak of 83.8% in 1997 (15). From 1996 onwards, the O3:K6 serotype was isolated from diarrhea patients admitted to the ICDDR,B hospital in Dhaka, Bangladesh (7, 15, 57). Between May and June 1998, 416 persons in 13 states reported having gastroenteritis after eating oysters harvested from Galveston Bay, Tex. All 28 available stool samples yielded *V. parahaemolyticus* O3:K6 isolates which closely resembled the Asian O3:K6 isolates by PFGE (23). During July to September 1998, an outbreak of *V. parahaemolyticus* O3:K6 infections associated with the consumption of oysters and clams harvested from Long Island Sound occurred among residents of Connecticut, New Jersey, and New York (10, 11). Significantly, before this series of outbreaks in the United States, *V. parahaemolyticus* serotype O3:K6 was not reported in this country (23). Subsequently, O3:K6 isolates obtained in 1997 and 1998 from clinical sources in Taiwan, Laos, Japan, Thailand, Korea, and the United States were found to share nearly identical AP-PCR profiles (57).

Outbreaks caused by the O3:K6 serotype occurred in the northern city of Antofagasta, Chile, from November 1997 to March 1998, and other outbreaks occurred during the summer months of 2004 and 2005, mainly in Puerto Montt, a region with usually cold waters (21, 26, 30). Interestingly, a recent retrospective analysis of *V. parahaemolyticus* strains isolated from several places in Peru showed the prevalence of the O3:K6 serotype and other pandemic serovariants of *V. parahaemolyticus*, with the earliest O3:K6 isolate being recovered as early as 1996, the same year that the pandemic O3:K6 serotype was identified in Calcutta, India (29). The majority of the *V. parahaemolyticus* strains isolated during an outbreak of acute enteric disease in Vladivostok, Russia, in 1997 belonged to serotype O3:K6 (85). More recently, the O3:K6 serotype was isolated from hospitalized diarrhea patients in Mozambique, ushering its spread into the African continent (3). *V. parahaemolyticus* O3:K6 strains similar to the pandemic clone have been isolated from the coasts of Spain and France (55, 78). A diarrheal outbreak caused by serotype O3:K6 was reported from Calcutta, India (82). The incidence of *tdh*-positive strains of *V. parahaemolyticus* in patients in Hangzhou, China (107), has been reported, but the molecular traits of the isolates were not examined. Clearly, global dissemination of a specific clone of *V. parahaemolyticus* is apparent, and at the time of writing of this review, this clone has spread into Asia, America, Africa, and Europe (Fig. 1).

HOW ARE O3:K6 ISOLATES AND THEIR SEROVARIANTS DIFFERENT?

Since the discovery of the unique O3:K6 serotype of *V. parahaemolyticus*, efforts have been made to determine the factor that endows these isolates with the ability to rapidly

increase hospitalizations and to become the dominant serotype. The initial efforts were centered on whether the O3:K6 isolates produced more TDH than the other existing serotypes of *V. parahaemolyticus*. Such a difference in production of TDH was not evident (69, 101), but later studies revealed one amino acid polymorphism in the *tdh* open reading frame (ORF) that appeared to differentiate the O3:K6 isolates (Gly_{109}) from non-O3:K6 strains (Asp_{109}) (105). Interestingly, sequence polymorphisms were also observed in the putative *tdh* promoter region of these isolates (99), but the significance of these differences was not clear given that there was no discernible difference in the amounts of TDH produced by O3:K6 and non-O3:K6 strains of *V. parahaemolyticus* (69). These findings match well with an early report from Calcutta indicating that the clinical symptoms of patients infected by O3:K6 isolates did not differ significantly from those of patients infected by non-O3:K6 serotypes. O3:K6 isolates compared to non-O3:K6 strains that were *tdh* positive were shown to demonstrate an enhanced ability to swarm over agar surface plates, and the presence of magnesium appeared to further stimulate swarming (105). There were, however, no significant differences between survival rates under the same environmental stresses, such as extreme temperatures, low pH, and high salinity, for O3:K6 and non-O3:K6 strains of *V. parahaemolyticus* (69, 101).

The *V. parahaemolyticus* phage f237 is similar to the CTX filamentous phage of *V. cholerae* O1 (94), but instead of the *ctxAB* genes, f237 has ORF8, and therefore it was thought that ORF8 may play a significant role (like the profound role that cholera toxin has in the disease cholera) in increasing the virulence of O3:K6 isolates. Based on the similarity of the motifs of the predicted amino acid sequence of ORF8 to those of the *plx* gene of *Drosophila*, which encodes a novel adhesion molecule (106), Nasu et al. (67) speculated that ORF8 encodes an adherence protein and that strains possessing this gene could be more adhesive to host intestinal cells or to the surfaces of marine plankton. In vitro adherence and cytotoxicity studies with human epithelial cells showed that O3:K6 isolates exhibited statistically higher levels of adherence and cytotoxicity to host cells than did non-O3:K6 isolates (105). Studies in Bangladesh, however, showed that ORF8 could not be detected in several O3:K6 isolates from hospitalized diarrhea patients (7).

Recently, Okura et al. (71) identified, cloned, and sequenced a 930-bp AP-PCR fragment that was unique to the O3:K6 isolates and their serovariants; the sequence of this fragment was found to be 80% homologous to the Mn^{2+} and Fe^{2+} transporter of the NRAMP family of *Vibrio vulnificus*. Again, a specific function was not attributed to this fragment. In O3:K6 isolates of *V. parahaemolyticus*, a histone-like DNA-binding protein, HU-α, with a C-terminal amino acid sequence different from those in other strains of *V. parahaemolyticus*, has been identified (72, 98). Further study has revealed that the gene encoding this protein has a 16-kb insert at the 3′ terminus of the ORF, but the effect of this insertion sequence on the activity of the HU-α protein in relation to a change in pathogenicity is unknown at this time (72, 98). The functional role of a histone-like protein in streptococci was shown to be associated with tissue inflammation (86). The 16-kb insertion sequence might have evolved from a phage, as it includes a gene encoding a putative phage protein and has the insertion gene sequence TTCTTCAG at its 5′ and 3′ ends (54, 72). Bioinformatic study of the whole genome sequence of an O3:K6 isolate (RIMD2210633) revealed the exclusive presence of four genomic islands, termed *V. parahaemolyticus* islands (VPaIs), in the pandemic group, which may represent DNAs acquired by the pandemic group that increased its fitness either in the aquatic environment or in the ability to infect humans (36). The direct repeats present in VPaIs 1, 4, and 5 and those in VPaI 6 are located in the large and small chromosomes, respectively. Moreover, the phage-like integrases show that these VPaIs were acquired by horizontal gene transfer (36). Clearly, O3:K6 isolates seem to have acquired some attributes that are not seen in non-O3:K6 isolates, but so far the determining factor(s) remains elusive. A methyltransferase gene carried by a 23-kb novel pathogenicity island-like element was identified among pandemic *V. parahaemolyticus* strains (95). However, its specific advantages in virulence traits among pandemic strains have not yet been established.

ENVIRONMENTAL ISOLATION

The detection of virulent *V. parahaemolyticus* organisms against a background of numerically greater numbers of avirulent *V. parahaemolyticus* organisms has remained a daunting problem. Undifferentiated total *V. parahaemolyticus* counts are therefore used as an indicator for the control of food contamination and the prevention of infection. Clearly, this is inadequate, as reflected in the outbreaks caused in the United States, where despite bacteriologic monitoring at harvest sites and despite the number of *V. parahaemolyticus* organisms being lower than the permissible most probable number of 10,000, the outbreaks could not be prevented (23). Attempts to isolate O3:K6 from the environment and from seafood have not met with much success.

An immunomagnetic separation technique targeting different K antigens was established for food poisoning investigations of *V. parahaemolyticus* (89). Application of this technique, targeting the K6 antigen for the identification of O3:K6 isolates, was successfully demonstrated using clinical and environmental samples (31, 92). An extensive environmental study in Japan combining a *tdh*-specific PCR method, chromogenic agar medium, and the most-probable-number method showed that isolates similar to the Calcutta O3:K6 isolates were widely distributed throughout the Japanese coastal environment. Of the 19 strains examined, 14 were *tdh* and GS-PCR positive and showed the same AP-PCR profile as the reference O3:K6 isolates (32). Studies conducted at Kii Channel, Tokushima, Japan, further confirmed this finding (33). However, neither the O3:K6 serotype nor any of the ribogroups associated with the O3:K6 serotype was found among any of the environmental or food isolates examined, suggesting that the O3:K6 serotype has not become established in the United States (25). It was felt that the O3:K6 serotype did not have an environmental reservoir in the United States and that the origin and spread of this organism may have occurred via ship ballast water. Cargo ships entering the Gulf of Mexico were thought to be responsible for the introduction of the Latin American epidemic strain of *V. cholerae* O1 into Gulf Coast waters in 1991 (58). O3:K6 isolates have also been isolated

from the aquatic environs of Bangladesh (40) and from the east coast of India (24). O3:K6 and other serovariants seem to have established an ecological niche in Asia (4). It appears that, in some regions, aquatic birds act as reservoirs of *V. parahaemolyticus* during the winter (60). However, more information should be generated in identifying the pandemic strains from such nonhuman reservoirs. Environmental surveys have shown that the application of sensitive techniques is essential for isolating specific pathogenic strains, such as O3:K6 isolates, from environmental and seafood samples.

ECOLOGY AND EPIDEMIOLOGY

The origin and subsequent spread of the O3:K6 isolates of *V. parahaemolyticus* must be the consequence of coincidental events occurring at the right time and at the right place. Is the emergence of O3:K6 isolates and their serovariants the consequence of the effects of global warming? For several of the reported outbreaks, especially during the period 1996 to 1998, elevated environmental temperatures have been ascribed as a cause (23, 59). The year 1998 was part of the El Niño years, when elevated seawater temperatures were shown to influence the incidence of *V. cholerae* (20) and other diarrheal diseases (14). In 2005, serotype O6:K18 caused a diarrheal outbreak in Alaska (59); however, its genetic relatedness with the O3:K6 isolate was not established. The Alaskan outbreak was associated with warming of ocean waters (59). Among the foodborne disease outbreaks in 13 provinces of China during 2003, about 40% of the patients were infected with *V. parahaemolyticus* (52). Are environmental conditions becoming conducive for the proliferation of pathogens like *V. parahaemolyticus*? It would at least seem so because *V. parahaemolyticus* has been recognized since the 1950s, and raw oysters and seafood have been consumed from time immemorial. Yet 1996 to 1998 seemed to be particularly unpleasant years for humans, especially for those who had an inclination for oysters, which is the most important source of infection of *V. parahaemolyticus* in the United States and the Far East. We still do not understand the epidemiology of *V. parahaemolyticus* infections. In Vietnam, the incidence of *V. parahaemolyticus* stopped abruptly without meteorological changes or changes in water supply and sanitation, and the reasons for this abrupt interruption in transmission are not clear (90). In recent times, there have been occurrences of other extraordinary events in relation to pathogenic *Vibrio* species. In the summer of 1996, a major outbreak of systemic *V. vulnificus* infections started among Israeli fish market workers and fish consumers (8). Molecular analysis showed that this strain evolved by hybridization of the genomes of two existing nonpathogenic forms of *V. vulnificus*, which apparently led to the emergence of an epidemic caused by the newly evolved pathogenic variant (9). Similarly, the appearance of new hybrids between the classical and El Tor biotypes of *V. cholerae* O1 has been reported from Matlab, Bangladesh (66), and from Mozambique (2).

The transmission and epidemiology of *V. parahaemolyticus* infections in places such as Calcutta, India, and Bangladesh are entirely different because seafood is never eaten raw and freshwater fish is preferred over seawater fish by the local population. Contamination of freshwater fish by seawater fish at the fish market and secondary contamination of other foods in the kitchen by *V. parahaemolyticus*-contaminated fish brought from markets are thought to be the most likely routes of transmission in this setting (75). Early ecological studies have shown the occurrence of *V. parahaemolyticus* in freshwater plankton and in freshwater fishes (81). The survival of *V. parahaemolyticus* in freshwater ecosystems has been shown to be transient and dependent on a biological host (81).

METHODS FOR DETECTION

The emergence of the O3:K6 serotype and its serovariants and its widespread distribution have necessitated the development of specific methods to detect such strains. Serotyping enabled the detection of the O3:K6 isolates in Calcutta. Although serotyping is a relatively easy technique for identifying clusters of cases caused by a specific serotype and also for tracking their spread, the cost of antisera is prohibitive and therefore limits their availability and applicability. In the Calcutta episode, the identification of the clustering of the O3:K6 serotype did not occur in real time because a *V. parahaemolyticus* antiserum kit was not available at that time at the National Institute of Cholera and Enteric Diseases in Calcutta, and the strains were confirmed and serotyped later at the Osaka Prefecture Institute of Public Health, Osaka, Japan.

The API-20 E identification system is used in many laboratories for the identification of enteric bacteria. For the identification of *V. parahaemolyticus*, it appears that the use of specific concentrations of NaCl makes a considerable difference in the identification of clinical (0.85%) and environmental (2.0%) strains (56). Over the past few years, PCR-based detection techniques, including GS-PCR (52) and *orf8* PCR (57, 64, 67), have been developed to specifically detect the O3:K6 serotype and its serovariants. The development of GS-PCR was a milestone in simplifying the identification of the O3:K6 isolate and also for detecting other serotypes that share identical molecular traits (57). Inconsistencies between the results of the *toxRS* and ORF8 PCRs related to serotypes that were not like the O3:K6 isolates or their serovariants and that were *toxRS* negative but positive by ORF8 PCR were reported (74). Additionally, some of the O3:K6 isolates from Bangladesh isolated between 1998 and 2000 (7) and from Vietnam (16) were negative by *orf8* PCR, indicating that neither the *toxRS* nor the ORF8 sequence is a reliable gene marker for the definite identification of the pandemic group. A real-time PCR assay targeting the *orf8* gene was shown to be specific and sensitive for the detection of pandemic O3:K6 strains (97). However, this assay may not be useful for the detection of pandemic strains that are devoid of *orf8*. Pyrolysis metastable atom bombardment mass spectrometry was shown to identify various phenotypic characteristics of *V. parahaemolyticus* (96). By targeting specific phenotypic markers, this technique may differentiate pandemic and nonpandemic *V. parahaemolyticus* strains.

Okura et al. (70) developed a novel multiplex PCR assay specific for the O3:K6 isolates and their serovariants that successfully distinguished these stains from other *V. parahaemolyticus* strains by yielding two distinct PCR products, for *tdh* (263 bp) and the *toxRS*/new sequence (651 bp). A PCR-based assay was developed, employing an oligonucleotide primer pair derived from the group-specific sequence of an arbitrarily

primed PCR fragment which was located in the genome encoding a hypothetical protein. The assay distinguished the O3:K6 isolates and their serovariants from other *V. parahaemolyticus* strains by yielding a 235-bp specific amplicon (70). Khan et al. (48) reported that all of the O3:K6 strains isolated from the Texas and New York outbreaks yielded an 850-bp DNA fragment along with other amplicons in enterobacterial repetitive intergenic consensus PCR. The primers designed from this 850-bp gene sequence were specifically identified in O3:K6 isolates with a 327-bp amplicon, whose function and homology remain unknown. However, this PCR method was not validated. Using the detection of an insertion mutation in the HU-α ORF, a PCR technique was reported for the identification of not only the O3:K6 serotype but also other serovariants, such as O1:K25, O1:KUT, and O4:K68 (72, 98), which had molecular traits identical to those of the O3:K6 isolate. Genes located on VPaIs 4, 5, and 6 (36) can be targeted for the specific detection of pandemic strains by PCR. However, the specificity and sensitivity are not yet established for routine use in the laboratory. Use of microarray technology with specific amplification and oligonucleotides was shown to be useful for the detection and identification of pathogenic bacteria, including *V. parahaemolyticus* (41). Targeting specific genes in this microarray technique will differentiate pandemic and nonpandemic strains.

CONCLUSION

The emergence and spread of the O3:K6 isolate and its serovariants offer an invaluable opportunity to examine factors that abet and perpetuate events like this. Many aspects of O3:K6 and its serovariants are still unknown. For example, we do not know the factors that triggered the genesis of the O3:K6 serotype and the bacterial factors that are involved. We also do not know the mechanism of formation of serovariants and those extraneous factors that drive this event.

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