Emergence of a Unique O3:K6 Clone of *Vibrio parahaemolyticus* in Calcutta, India, and Isolation of Strains from the Same Clonal Group from Southeast Asian Travelers Arriving in Japan

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Active surveillance of *Vibrio parahaemolyticus* infection among hospitalized patients in Calcutta, India, was initiated in January 1994. The incidence of cases of *V. parahaemolyticus* infection suddenly increased in February 1996 and has remained high since then. One hundred thirty-four strains of *V. parahaemolyticus* isolated from January 1994 to August 1996 were examined for serovar, the presence of the thermostable direct hemolysin gene (*tdh*) and *tdh*-related hemolysin genes (*trh1* and *trh2*), production of urease, and antibiogram. Strains of the O3:K6 serovar appeared for the first time in February 1996. The O3:K6 serovar strains accounted for 50 to 80% of the strains isolated during the high-incidence period (February to August 1996). All of the serovar O3:K6 strains carried the *tdh* gene but not the *trh* genes and did not produce urease. All of the isolates except two were sensitive to all of the antibiotics tested. These and the results of analysis by an arbitrarily primed PCR method indicated that the O3:K6 serovar strains belong to a unique clone. When the O3:K6 serovar strains, isolated from travelers arriving in Japan from Southeast Asian countries, were compared by the arbitrarily primed PCR method, the strains isolated between 1982 and 1993 were distinct from Calcutta O3:K6 while the strains isolated in 1995 and 1996 were indistinguishable from the Calcutta O3:K6 strains. The results suggest that this unique O3:K6 clone may have become prevalent not only in Calcutta but also in Southeast Asian countries very recently. Not only the O3:K6 strains but also the non-O3:K6, *tdh*-bearing strains isolated in 1996 produced thermostable direct hemolysin at high levels, and thus the level of hemolysin produced does not appear to have influenced the high incidence of serovar O3:K6 strains.

Vibrio parahaemolyticus can cause seafood-borne gastroenteritis, but not all strains are considered pathogenic. Early studies demonstrated that the Kanagawa phenomenon, a beta-type hemolysis on Wagatsuma agar medium, was associated with most clinical strains but with very few environmental strains (16, 28). The hemolysis is due to production of thermostable direct hemolysin (TDH) and thus, TDH has long been considered a major virulence factor (33). A TDH-related hemolysin produced by a Kanagawa phenomenon-negative strain was discovered in the investigation of an outbreak of gastroenteritis in the Maldives Islands in 1985 (3, 4). The *tdh* and *trh* genes, encoding TDH and TDH-related hemolysin, respectively, share approximately 70% nucleotide sequence identity (22). The *trh* gene sequence varies considerably from strain to strain, but the *trh* sequences in various strains can be clustered into two subgroups represented by the *trh1* and *trh2* genes, which share 84% sequence identity (5). Molecular epidemiological studies revealed that not only strains carrying the *tdh* gene but strains carrying a *trh* gene or both genes are strongly associated with gastroenteritis (10, 29). Recently, proportion of *trh*-bearing strains among clinical strains has been

increasing and a very strong correlation exists between the presence of the *trh* gene and urease production, an unusual characteristic for *V. parahaemolyticus* (12, 24–26, 30–32). The urease-positive strains have been isolated in various parts of the world (1, 2, 5, 9, 13, 23).

In this report, we document the appearance of a new type of *tdh*-positive, *trh*-negative, and urease-negative *V. parahaemolyticus* that may have become prevalent in Calcutta, India, and in some countries in Southeast Asia.

MATERIALS AND METHODS

Hospital surveillance. The present study is part of the continuing surveillance program of the National Institute of Cholera and Enteric Disease on *Vibrio*-mediated enteric infections and surveillance for *V. parahaemolyticus* that was initiated in January 1994. Stool specimens or rectal swabs were obtained from patients with diarrhea who were admitted to the Infectious Diseases Hospital in Calcutta. The specimens were subjected to bacteriological examination within 1 h of collection.

Bacteriology. Stool samples were plated directly, while rectal swabs were plated after enrichment in alkaline peptone water onto thiosulfate-citrate-bile salts-sucrose agar (Eiken Chemical, Co., Ltd., Tokyo, Japan) as described previously (17). The sucrose-nonfermenting colonies appearing on the agar medium were screened by using a multitest medium for presumptive identification (8). Strains that yielded an alkaline slant and acid butt were examined by oxidase test, and the presumptive identification was subsequently confirmed by standard cultural and biochemical characteristics (7) and by the presence of the *V. parahaemolyticus* *toxR* gene, a species-specific regulatory gene (11, 19). *V. parahaemolyticus* strains other than those isolated in Calcutta were obtained from the Osaka and Kansai Airport Quarantine Stations in Japan, and their identities were

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FIG. 1. Monthly isolation of *V. parahaemolyticus* from diarrheal patients admitted to the Infectious Disease Hospital, Calcutta, India, from January 1994 to August 1996. The isolates are divided into O3:K6 strains, non-O3:K6 strains, and strains not tested because they became extinct during storage.

confirmed as described above. These strains were isolated from travelers arriving from Southeast Asian countries. Urease production of the test organism was examined by using the liquid urea medium described by Maslen (14), except that the NaCl concentration of the medium was adjusted to 1%. Urease production was recorded after a 20-h incubation at 37°C.

O:K serovar. The test strains were grown, and their O:K serovars were determined by using specific antisera as described previously (30).

Gene detection. The presence or absence of the *tdh*, *trh1*, *trh2*, and *toxR* genes of *V. parahaemolyticus* and the *ctx* (cholera enterotoxin) gene of *V. cholerae* in the test strain was determined by the DNA colony hybridization test using polynucleotide probes under high-stringency conditions as described previously (11, 15, 25).

MIC. MICs of the following antibiotics for the test strains were determined: ciprofloxacin hydrochloride (Bayer AG, Leverkusen, Germany), nalidixic acid (Daiichi Pharmaceutical Co., Tokyo, Japan), ampicillin (Sigma Chemical Co., St. Louis, Mo.), kanamycin sulfate (Banyu Pharmaceutical Co. Ltd., Tokyo, Japan), chloramphenicol (Sigma Chemical Co.), tetracycline (Lederle [Japan] Ltd., Tokyo), cefditrene (Meiji Seika Kaisha Ltd., Tokyo, Japan), and sulfamethoxazole and trimethoprim (Sigma Chemical Co.). The MICs were determined by the twofold agar dilution method recommended by the Japan Society of Chemotherapy. One loopful (ca. 10^6 CFU) of a diluted broth culture was inoculated onto test agar media. MICs were recorded after an 18-h incubation at 37°C.

TDH detection. The test strain was grown in a broth medium composed of 2% Bacto Peptone (Difco Laboratories, Detroit, Mich.), 0.5% D-mannitol, and 5% NaCl (pH 7.8) with shaking (50 rpm) at 37°C for 16 h. TDH liberated by *V. parahaemolyticus* into the spent culture medium was detected by the reversed-phase latex agglutination reaction with rabbit anti-TDH immunoglobulin G as described previously (25). The level of TDH produced was expressed as the titer of TDH that is the reciprocal of the highest dilution of the culture supernatant that gave a positive reaction. A titer of 64 or greater was defined as a high titer because most of the Kanagawa phenomenon-positive strains so far examined exhibited titers of 64 or more (6).

AP PCR. Cellular DNA was extracted from the test strain as described previously (21) and was used as the template for arbitrarily primed (AP) PCR. Two different oligonucleotide primers, 1 and 2, were used, and the amplification products were analyzed as described previously (25).

RESULTS

Surveillance data. The results of the hospital surveillance in Calcutta between January 1994 and August 1996 are presented in Fig. 1. The number of monthly isolations was four or less, except for August, and isolations occurred predominately in the warmer months in 1994 and 1995. However, the incidence

TABLE 1. Characteristics of *V. parahaemolyticus* strains isolated from patients with diarrhea at the Infectious Diseases Hospital in Calcutta, India

Yr of isolation and no. of strains	O:K serovar ^a	Presence of gene ^b :			Representative strain(s) (TDH titer)
		<i>tdh</i>	<i>trh1</i>	<i>trh2</i>	
1994					
4	1:38	+	—	—	
2	3:29	+	—	—	
2	4:8	+	—	—	
2	8:41	+	—	—	
1	1:41	+	+	—	
1	1:UT	+	—	+	
1	1:UT	—	—	+	
1	1:UT	—	—	—	
1	2:3	+	—	—	
1	3:7	—	—	—	
1	5:UT	+	—	—	
1	11:UT	+	—	—	
1995					
4	4:55	+	—	—	
2	1:38	+	—	—	
2	4:8	+	—	—	
2	8:21	+	—	—	
1	1:1	+	—	+	
1	1:33	—	—	+	
1	1:56	+	—	—	
1	2:3	+	—	—	
1	3:29	+	—	—	
1	4:13	—	—	—	
1	4:63	+	—	—	
1	5:UT	+	—	—	
1	8:20	+	—	—	
1	8:41	+	—	—	
1	8:UT	—	—	—	
1996					
61	3:6	+	—	—	VP47 (128), VP49 (128), VP58 (2,048), VP62 (64), VP71 (128), VP81 (256), VP92 (64), VP96 (128), VP98 (256), VP108 (128), VP123 (64), VP138 (64), VP121 (128), VP121 (128)
6	4:8	+	—	—	VP93 (128)
5	4:55	+	—	—	VP67 (512)
4	5:UT	+	—	—	
3	1:1	+	—	+	
3	8:22	+	—	—	VP78 (256), VP120 (1,024)
2	1:38	+	—	—	
2	8:41	+	—	—	VP122 (256)
2	12:61	+	—	—	VP144 (1,024)
1	1:56	+	—	—	VP107 (256)
1	1:UT	+	+	—	
1	2:3	+	—	—	
1	3:UT	—	—	—	
1	4:4	+	—	—	
1	8:21	+	—	—	
1	8:39	+	—	—	

^a UT, untypeable.

^b +, present; -, absent.

TABLE 2. Characteristics of *V. parahaemolyticus* O3:K6 strains isolated from travelers at the Osaka and Kansai Airport Quarantine Stations in Japan

Strain ^a	Yr of isolation	Origin of traveler	Urease ^b	Presence of gene: ^c			TDH titer
				<i>tdh</i>	<i>trh1</i>	<i>trh2</i>	
AQ3732	1982	Unknown	+	—	+	—	NT ^d
AQ3794	1983	Singapore	+	—	+	—	NT
AQ3810	1983	Singapore	—	+	—	—	NT
AQ4019	1985	Maldiv Islands	+	—	+	—	NT
AQ4037	1985	Maldiv Islands	+	—	—	+	NT
AQ4093	1986	Maldiv Islands	+	—	—	+	NT
AQ4133	1986	Hong Kong	+	—	—	+	NT
AQ4235	1987	Thailand	+	—	+	—	NT
AQ4299	1987	Thailand	+	—	+	—	NT
AQ4644	1991	Thailand	+	—	+	—	NT
AQ4733	1992	Singapore	+	—	—	+	NT
AQ4853	1993	Hong Kong	+	—	+	—	NT
AQ4901	1993	Thailand	+	—	+	—	NT
KX-V138	1995	Indonesia	—	+	—	—	NT
KX-V224	1996	Thailand	—	+	—	—	1,024
KX-V225	1996	Thailand	—	+	—	—	1,024
KX-V226	1996	Singapore	—	+	—	—	512
KX-V231	1996	Thailand	—	+	—	—	1,024

^a Strain designations starting with AQ and KX-V were isolated, respectively, at the Osaka and Kansai Airport Quarantine Stations.

^b +, produced; —, not produced.

^c +, present; —, absent.

^d NT, not tested.

suddenly increased in February 1996 and remained high during the rest of the surveillance period; the number of monthly isolations ranged between 10 and 21 from February to August 1996.

Characteristics of isolates. The strains isolated during the above surveillance period, except for those that became extinct during storage, were examined for the O:K serovar and the presence or absence of virulence genes (Table 1). The strains isolated until the end of 1995 belonged to diverse serovars. The strains belonging to the O3:K6 serovar suddenly appeared in February 1996 and dominated until the end of the surveillance period; the monthly isolation frequency of serovar O3:K6 strains ranged between 50 and 80% between February and August 1996 (Fig. 1). All strains of serovar O3:K6 had the *tdh* gene but had neither the *trh1* nor the *trh2* gene. Non-O3:K6 strains had the *tdh* gene alone (80.8%), both the *tdh* gene and the *trh1* or *trh2* gene (9.6%), the *trh* gene alone (2.7%), or neither the *tdh* gene nor a *trh* gene (6.8%). All *trh* gene-bearing strains produced urease, but none of the *trh*-negative strains produced urease (data not shown in Table 1). None of the strains listed in Table 1 had the *ctx* gene (data not shown in Table 1 but discussed below). The susceptibilities of the isolates to eight antibiotics were also examined. The antibiotics were chosen to represent commonly used antibiotics. For all but two strains, VP53 and VP111, the MICs indicated susceptibility to all of the antibiotics examined (data not shown in Table 1). The ranges of the MICs of ciprofloxacin, nalidixic acid, ampicillin, kanamycin, chloramphenicol, tetracycline, cefditrene, and sulfamethoxazole-trimethoprim were, respectively, ≤ 0.78 , ≤ 1.56 , 12.5 to 100, 6.25 to 25, 0.78 to 3.13, 0.39 to 3.13, ≤ 0.2 , and 0.78 to 3.13 $\mu\text{g/ml}$. The two exceptional strains were resistant to nalidixic acid (MIC, $>100 \mu\text{g/ml}$), kanamycin (MIC, $\geq 100 \mu\text{g/ml}$), and cefditrene (MIC, 100 $\mu\text{g/ml}$). Complete raw data on the characteristics of the isolates are available upon request.

Eighteen strains representing the serovar O3:K6 strains isolated between 1982 and 1996 from travelers arriving in Japan from Southeast Asian countries were examined for comparison

(Table 2). Most of the strains isolated between 1982 and 1995 had either the *trh1* or the *trh2* gene and produced urease, whereas four strains isolated in 1996 had the *tdh* gene and did not produce urease.

Clonality of serovar O3:K6 strains. To investigate whether the O3:K6 strains that appeared in Calcutta in 1996 are a new clone or not, the representative O3:K6 strains were compared with related strains by an AP PCR method. Thirteen and seven strains representing the O3:K6 and non-O3:K6 strains, respectively, isolated in 1996 in Calcutta were selected (Table 1). These strains possessed the *tdh* gene but no *trh* gene. Four *tdh*-positive and *trh*-negative strains of serovar O3:K6 that were isolated in 1996 from travelers coming from Thailand and Singapore to Japan (Table 2) were also included in the comparison. Two different primers were used, and there was no essential difference between the results obtained with the two primers (Fig. 2A1 and A2). The O3:K6 strains isolated in 1996 both in Calcutta and at a quarantine station in Japan showed identical or nearly identical AP PCR profiles (Fig. 2A1 and A2, lanes 3 to 16 and 19 to 21), whereas non-O3:K6 strains isolated in Calcutta exhibited various AP PCR profiles that were distinct from those of the O3:K6 strains (Fig. 2A1 and A2, lanes 22 to 28). Strain VP47, representing the O3:K6 strains isolated in 1996, was then compared with 14 O3:K6 strains isolated between 1982 and 1995 from travelers arriving in Japan from Southeast Asian countries by using the same AP PCR method. There was no essential difference between the results obtained with the two primers (Fig. 2B1 and B2). The O3:K6 strains isolated between 1982 and 1993 shared some of the amplicon bands with strain VP47, but their total AP PCR profiles were clearly different from that of strain VP47 (Fig. 2B1 and B2, lanes 2 to 15). The profile of strain KX-V138, isolated in 1995, was indistinguishable from that of strain VP47 (Fig. 2B1 and B2, lanes 2 and 16).

TDH production by representative strains. The representative O3:K6 and non-O3:K6 strains isolated in 1996 that were compared by the AP PCR method (Fig. 2A and B) were compared for the level of TDH production. The results are shown

FIG. 2. AP PCR assay results. The results obtained with primer 1 are presented in A1 and B1, and those obtained with primer 2 are presented in A2 and B2. The samples in A1 and A2 are selected O3:K6 strains (lanes 3 to 14 and 21) and non-O3:K6 strains (lanes 15, 16, 19, and 20). Lanes: 1 and 17, phage λ DNA digested with *Hind*III (used as molecular size markers); 2, 18, and 29, phage ϕ X174 DNA digested with *Hae*III (used as molecular weight markers); 3, VP47; 4, VP49; 5, VP58; 6, VP62; 7, VP71; 8, VP81; 9, VP92; 10, VP96; 11, VP98; 12, VP108; 13, VP123; 14, VP138; 15, KX-V224; 16, KX-V225; 19, KX-V226; 20, KX-V231; 21, VP121; 22, VP67; 23, VP78; 24, VP93; 25, VP107; 26, VP120; 27, VP122; 28, VP144. The samples in B1 and B2 are an O3:K6 strain isolated in 1996 in Calcutta (lane 2) and the O3:K6 strains isolated between 1982 and 1995 from travelers at the Osaka and Kansai Airport Quarantine Stations (lanes 3 to 16). Lanes: 1, mixture of phage λ DNA digested with *Hind*III and phage ϕ X174 DNA digested with *Hae*III (used as molecular weight markers); 2, VP47; 3, AQ3732; 4, AQ3794; 5, AQ3810; 6, AQ4019; 7, AQ4037; 8, AQ4093; 9, AQ4133; 10, AQ4235; 11, AQ4299; 12, AQ4644; 13, AQ4733; 14, AQ4853; 15, AQ4901; 16, KX-V138. The details of the test strains are presented in Tables 1 and 2.

in Tables 1 and 2. All of the O3:K6 strains isolated in Calcutta and from travelers in Japan produced TDH at high levels. The TDH titers ranged between 64 and 2,048, and the average titer was 422. The non-O3:K6 strains isolated in Calcutta also exhibited high titers (range, 128 to 1,024; average, 896).

DISCUSSION

Infections caused by *V. parahaemolyticus* are usually associated with diverse serovars. Such a pattern was observed in this surveillance in Calcutta in 1994 and 1995 (Table 1). There was a sudden increase in the incidence of *V. parahaemolyticus* infection in Calcutta in February 1996, and the incidence remained high during the rest of the surveillance period (Fig. 1).

Disproportional appearance of O3:K6 was evident during the high-incidence period; the monthly isolation frequency of O3:K6 strains ranged between 50 and 80% (Fig. 1). Interestingly, serovar O3:K6 was not isolated before February 1996 since the beginning of this surveillance and in earlier epidemiological studies in Calcutta (27). Therefore, the overall increase in the incidence of *V. parahaemolyticus* in Calcutta was clearly due to the spurt in the incidence of O3:K6 strains. Such a predilection for a single serovar to dominate among *V. parahaemolyticus* infections is very unusual. The only reported instance of a similar propensity for a single serovar of *V. parahaemolyticus* to be associated with the majority of infections was the occurrence of O4:K12 strains of urease-positive *V. parahaemolyticus* that were associated with cases of gastroen-

teritis domestically acquired on the western coasts of the United States and Mexico (1, 23).

Intense investigations of the O3:K6 strains isolated in Calcutta in 1996 revealed that all 61 strains shared identical traits, with only 2 strains showing a different antibiogram. In addition, the representative O3:K6 strains were shown to be genetically indistinguishable by AP-PCR analysis. Accordingly, we considered the Calcutta O3:K6 strains to belong to a single clone. The virulence gene and AP-PCR patterns of the O3:K6 strains isolated between 1982 and 1996 from travelers arriving in Japan from Southeast Asian countries were also compared with those of the Calcutta O3:K6 strains. The results indicated that strains indistinguishable from the Calcutta O3:K6 clone also appeared in or spread to some countries in Southeast Asia in 1995.

A basic question which needs to be addressed is why this unique clone has developed this propensity to be associated with infections in Calcutta more often than other serovars. An argument could be that this serovar is more potent than other serovars in inducing diarrhea or establishing infection. We examined some of the possibilities in this study. The O3:K6 strains had the *tdh* gene. The level of TDH produced by *tdh*-bearing strains is known to vary due to variation in the *tdh* gene (20). The differences in promoter strength and in the level of transcriptional modulation by ToxR seem to influence the level of *tdh* expression (11). Although the O3:K6 strains isolated in Calcutta produced TDH at high levels, the non-O3:K6 strains isolated in Calcutta during the same period also produced TDH at high levels. Thus, the level of TDH produced does not appear to have influenced the high incidence of O3:K6 strains. Another possibility is that O3:K6 strains produce an enterotoxin besides TDH. It must be mentioned that the O3:K6 strains had neither the *trh1* nor the *trh2* gene. We previously presented molecular genetic evidence which showed that the *tdh* gene could be transferred as a transposon-like unit among some *Vibrio* species, including *V. cholerae* non-O1 (34). The cholera enterotoxin (*ctx*) gene of *V. cholerae* is present in a transposon-like structure which is part of a bacteriophage (35). *V. cholerae* strains carrying the *ctx* gene are commonly isolated from diarrheal patients at the Infectious Diseases Hospital, Calcutta (17). This stimulated us to examine the possibility that the O3:K6 strains acquired the *ctx* gene from *V. cholerae* by a horizontal transfer mechanism. However, none of the O3:K6 strains or other strains of *V. parahaemolyticus* examined in this study possessed the *ctx* gene. We are now investigating if the O3:K6 strains produce an unknown toxin. Like the non-O3:K6 strains, almost all of the O3:K6 strains were susceptible to all of the representative antibiotics tested. Therefore, antibiotic resistance-associated spread of the O3:K6 strains seems unlikely.

The incidence of diarrhea due to O3:K6 strains remains high: 63% of the *V. parahaemolyticus* strains isolated from patients in Calcutta between September 1996 and April 1997 belonged to the O3:K6 serovar and possessed the *tdh* gene alone (18); O3:K6 strains continue to be isolated very frequently from travelers at a quarantine station in Japan (6). The spread of this new clone needs to be carefully charted, and efforts are being made to understand the factor(s) which favored the emergence of this clone.

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