JOURNAL OF CLINICAL MICROBIOLOGY, Jan. 2002, p. 284–286 Vol. 40, No. 1
0095-1137/02/$04.00+0 DOI: 10.1128/JCM.40.1.284–286.2002

Prevalence of the Pandemic Genotype of *Vibrio parahaemolyticus* in Dhaka, Bangladesh, and Significance of Its Distribution across Different Serotypes

N. A. Bhuiyan,[1] M. Ansaruzzaman,[1] M. Kamruzzaman,[1] Khorshed Alam,[1] N. R. Chowdhury,[2] M. Nishibuchi,[3] Shah M. Faruque,[1] David A. Sack,[1] Yoshifumi Takeda,[4] and G. Balakrish Nair[1,2]*

International Centre for Diarrhoeal Disease Research, Bangladesh, Dhaka-1212, Bangladesh[1]; National Institute of Cholera and Enteric Diseases, Calcutta-700 010, India[2]; and Center for Southeast Asian Studies, Kyoto University, Yoshida, Sakyo-ku, Kyoto,[3] and Faculty of Human Life Sciences, Jissen Women's University, Tokyo 191-8510,[4] Japan

Received 6 August 2001/Returned for modification 22 September 2001/Accepted 17 October 2001

Sixty-six strains of *Vibrio parahaemolyticus* belonging to 14 serotypes were isolated from hospitalized patients in Dhaka, Bangladesh, from January 1998 to December 2000. Among these, 48 strains belonging to four serotypes had the pandemic genotype and possessed the *tdh* gene. A marker (open reading frame ORF8) for a filamentous phage previously thought to correspond to the pandemic genotype was found to have a poor correlation with the pandemic genotype.

Vibrio parahaemolyticus, a seafood-borne pathogen, is the causative agent of gastroenteritis in humans. Unlike *Vibrio cholerae*, only two serogroups of which (serogroups O1 and O139) are involved in epidemic and pandemic diseases, *V. parahaemolyticus* gastroenteritis is a multiserogroup affliction, and as many as 75 different combinations of O and K serotypes of *V. parahaemolyticus* are recognized and known to be associated with gastroenteritis (7). Beginning in February 1996, a new clone of *V. parahaemolyticus* belonging to the O3:K6 serotype was responsible for a dramatic increase in the number of cases of diarrhea in Calcutta, India (1, 12). An increase in the incidence of *V. parahaemolyticus* food poisoning observed during 1997 and 1998 was also ascribed to the increased incidence of food poisoning caused by the O3:K6 clone in Japan (15). Evidence supporting the hypothesis that the O3:K6 clone has recently emerged and has pandemic potential was presented by Matsumoto and coworkers (9).

The *V. parahaemolyticus* pandemic has spread into at least eight countries, and the emergence of two other serotypes (serotypes O4:K68 and O1:KUT [UT indicates untypeable]) possessing pandemic potential has been documented (3, 9). Molecular biology-based analysis of these additional serotypes indicates that they may have diverged from the pandemic O3:K6 strains by alteration of the O:K antigens (4, 9). The present study was performed to understand the status of the pandemic genotype of *V. parahaemolyticus* among hospitalized patients in Dhaka, Bangladesh.

Stool specimens were collected from patients enrolled in the 2% systematic routine surveillance system at the Clinical Research and Service Centre of the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR, B). Stool specimens were plated directly, after enrichment in bile peptone broth, onto tauro-cholate-tellurite-gelatin agar (10). The colonies selected were screened for *V. parahaemolyticus* by a battery of biochemical tests elaborated previously (9). The commercially available *V. parahaemolyticus* antiserum kit manufactured by Toshiba Kagaku Kogyo Co., Ltd., Tokyo, Japan, was used for serological typing in accordance with the manufacturer's instructions.

PCR assays were performed to test for the presence of the species-specific *toxR* gene and also to test for the presence of the two known virulence genes, *tdh* and *trh* of *V. parahaemolyticus*, with primers for *toxR*, *tdh*, and *trh*, respectively, as described previously (8, 14). The group-specific PCR (GS-PCR) for determination of the presence of the pandemic genotype was performed as described previously (9). PCR for open reading frame (ORF) ORF8 was performed in 20-μl volumes containing 2.0 μl of 10× $MgCl_2$-free PCR amplification buffer, 1.6 μl of $MgCl_2$ (2.0 mM), 1.6 μl of deoxynucleoside triphosphates (dNTPs; 2.0 mM each dNTP), 0.5 μM (each) primers VP36RF8U and VP36RF8L (V. Laohaprertthisan, A. Chowdhury, U. Kongmuang, S. Kalnauwakul, M. Ishibashi, C. Matsumoto, and M. Nishibuchi, personal communication), 0.5 U of r*Taq* DNA polymerase (GIBCO), and 2.5 μl of the template solution (supernatant of the boiled culture diluted 1:10). The amplification conditions were set at one cycle of 96°C for 5 min, followed by 25 cycles at 94°C for 1 min, 53°C for 1 min, and 72°C for 1 min, with a final extension of 72°C for 7 min (Perkin-Elmer, Norwalk, Conn.). The PCR products were electrophoresed in a 1% agarose gel and were visualized under UV light following staining with ethidium bromide. Chromosomal DNA was extracted and arbitrarily primed PCR (AP-PCR) was done as described previously (4). Southern blot hybridization with an rRNA gene probe was performed as described previously (5).

During the 3-year study period, 66 strains of *V. parahaemolyticus* were isolated from that many patients (Table 1). All patients from whom specimens were collected were symptomatic and were hospitalized and had the characteristic symptoms

* Corresponding author. Mailing address: Laboratory Sciences Division, International Centre for Diarrhea Diseases, Bangladesh, GPO 128, Dhaka-1000, Bangladesh. Phone: 880-2-8812529. Fax: 880-2-8823116. E-mail: gbnair@icddrb.org.

TABLE 1. Serotypes of *V. parahaemolyticus* strains isolated from patients admitted to ICDDR,B Hospital, Dhaka, from January 1998 to December 2000

O:K serotype	Pandemic genotype[a]	No. of strains isolated in:		
		1998	1999	2000
O1:K25	GS^+ $ORF8^+$		1	2
O1:K38	GS^- $ORF8^-$	1		
O1:K56	GS^- $ORF8^-$			4
O1:KUT	GS^+ $ORF8^+$	2		2
O1:KUT	GS^- $ORF8^-$	2		
O2:K3	GS^- $ORF8^-$		1	
O3:K6	GS^+ $ORF8^+$	1	17	2
O3:K6	GS^+ $ORF8^-$	3	1	4
O3:K29	GS^- $ORF8^-$			1
O4:K10	GS^- $ORF8^-$		1	
O4:K11	GS^- $ORF8^-$			1
O4:K22	GS^- $ORF8^-$	1		
O4:K68	GS^+ $ORF8^+$	11		1
O4:K68	GS^+ $ORF8^-$	1		
O4:K55	GS^- $ORF8^-$			1
O5:KUT	GS^- $ORF8^-$		2	2
O8:K22	GS^- $ORF8^-$	1		
Total		23	23	20

[a] Abbreviations: GS^+, GS-PCR positive; GS^-, GS-PCR negative; $OR8^+$, ORF8 PCR positive; $ORF8^-$, ORF8 PCR negative.

TABLE 2. Genotypes of the various serotypes of *V. parahaemolyticus* isolated between 1998 and 2000 in Dhaka, Bangladesh

O:K serotype	No. of strains	Presence of the following gene:			Results of:	
		toxR	*tdh*	*trh*	GS-PCR	ORF8 PCR
O1:K25	3	+	+	−	+	+
O1:K38	1	+	+	−	−	−
O1:K56	1	+	−	+	−	−
O1:K56	3	+	+	−	−	−
O1:KUT	4	+	+	−	+	+
O1:KUT	1	+	+	−	−	−
O1:KUT	1	+	−	+	−	−
O2:K3	1	+	+	−	−	−
O3:K6	20	+	+	−	+	+
O3:K6	8	+	+	−	+	−
O3:K29	1	+	+	−	−	−
O4:K10	1	+	+	−	−	−
O4:K11	1	+	−	+	−	−
O4:K22	1	+	+	−	−	−
O4:K68	12	+	+	−	+	+
O4:K68	1	+	+	−	+	−
O4:K55	1	+	−	+	−	−
O5:KUT	2	+	+	−	−	−
O5:KUT	2	+	−	−	−	−
O8:K22	1	+	+	−	−	−

of *V. parahaemolyticus* infection. The total rate of isolation of *V. parahaemolyticus* during the 3 years of surveillance ranged from 0.8 to 1%, which is low compared to those for *V. cholerae* and diarrheagenic *Escherichia coli*, but the fact that the majority of the strains have acquired the pandemic genotype is ominous, in that isolation rates may increase in future, as has been seen in Calcutta (4, 12) and among travelers returning to Japan (15). All 66 strains of *V. parahaemolyticus* identified by a battery of biochemical tests were positive for the 368-bp specific *toxR* amplicon, thereby confirming the identities of the strains. Of the 66 strains, 60 strains were positive for the *tdh* gene but negative for *trh*, 4 strains carried only the *trh* gene, and 2 strains were negative for both the *tdh* gene and the *trh* gene but were isolated from hospitalized patients with acute diarrhea (Table 2).

Of the 75 different O:K serotype combinations of *V. parahaemolyticus* currently recognized, 14 different serotypes were isolated during the study period, with O3:K6 (42.4%) being the dominant serotype, followed by O4:K68 (19.7%), O1:KUT (9.1%), and O5:KUT (6.1%) (Table 1). On a yearly basis, the O4:K68 serotype dominated in 1998 (52.2%), while O3:K6 dominated in 1999 (78.3%) and 2000 (30%). Of the 66 strains of *V. parahaemolyticus* isolated in the 3 years, 48 strains were positive by the GS-PCR, indicating that these strains had the pandemic genotype (Table 2). The number of strains bearing the pandemic genotype (GS-PCR positive) in each year was 18 in 1998, 19 in 1999, and 11 in 2000, indicating a decline in the rate of isolation of such strains in Dhaka. During the 3-year study period, four serotypes (serotypes O3:K6, O4:K68, O1:KUT, and O1:K25) had the pandemic genotype, and all these strains were positive only for the *tdh* gene, which is the usual pattern (9). One serotype, serotype O1:K25, isolated in Bangladesh in 1999 and 2000, was recorded for the first time to carry the pandemic genotype.

A recent report has revealed that ORF8 is a specific marker of the filamentous phage, phage f237, exclusively associated with pandemic strains of serotype O3:K6 strains of *V. parahaemolyticus* (11). In the present study, we examined all strains for ORF8 of phage f237 using specific primers (Table 2). Of the 48 GS-PCR-positive strains, 39 were positive for the amplicon by the PCR for ORF8. Eight O3:K6 strains and one O4:K68 strain had positive results by the GS-PCR and negative results by PCR for ORF8. None of the GS-PCR-negative strains were positive for ORF8.

To understand if the strains which were ORF8 negative but GS-PCR positive belonged to the pandemic genotype, we examined a set of representative strains of O3:K6 and O4:K68 by AP-PCR and ribotyping. In the AP-PCR assay, seven O3:K6 strains (five GS-PCR-positive and ORF8-positive strains and two GS-PCR-positive and ORF8-negative strains) and four O4:K68 strains (three GS-PCR-positive and ORF8-positive strains and one GS-PCR positive and ORF8-negative strain) were analyzed with primers 1281 and 1283. All strains showed identical AP-PCR profiles. Similar results were obtained by ribotyping of these strains. All O3:K6 and O4:K68 strains had identical ribotype patterns, irrespective of the presence of ORF8. The reason for the identical AP-PCR and ribotype profiles of the GS-PCR-positive strains that were either ORF8 PCR positive or ORF8 PCR negative could relate to mutations in the primer annealing sequences. A recent study has shown that in ORF8 variants the entire phage genome is not lost since ORF10 was detected in these variants (Laohaprestthisan et al., personal communication).

Efforts to describe the bacterial factors of the pandemic O3:K6 strains led to the discovery that a related filamentous phage designated f237 is exclusively associated with the pandemic O3:K6 strains (11). Further analysis of the phage genome revealed that ORF8 was detected only in O3:K6 strains

isolated since 1996 and was not found in O3:K6 strains isolated before 1996, and therefore, the ORF8 gene was proposed as a useful genetic marker (11). Subsequently, ORF8 was also shown to be distributed in pandemic strains of O4:K68 and O1:KUT serovars (2, 6). The present study showed that the results of the ORF8 PCR poorly correlated with the results of the GS-PCR. The existence of such aberrant strains may be due to the loss of all or some part, including ORF8, of the phage f237 genome. Three different variants of the pandemic clone, the ORF8-negative variant, serovariants, and the AP-PCR profile variant, have recently been elucidated (Laohaprertthisan et al., personal communication). These results and the finding of ORF8-negative variants of O3:K6 and O4:K68 pandemic strains in the present study indicate that ORF8 is not always a stable marker for the pandemic clone.

According to Smith et al. (13), the repeated recovery of strains with identical or very closely related genotypes from patients with disease suggest that these changes are associated with increased fitness or, more specifically, transmissibility. The fact that the strains of *V. parahaemolyticus* belonging to O3:K6 isolated from 1996 onwards are more virulent than those isolated before 1996 (12) indicates that the O3:K6 serogroup is refurbishing its background genotype to a higher level of virulence. In this study, at least four different serotypes were seen to have the pandemic genotype. It thus appears overall that the enteropathogen is enriching its gene pool toward a higher level of virulence.

This research was funded by a special research grant from the government of Japan to ICDDR,B, and by a grant-in-aid for scientific research from the Ministry of Education, Science, Sports and Culture, Japan. ICDDR,B is supported by countries and agencies which share its concern for the health problems of developing countries.

REFERENCES

1. **Bag, P. K., S. Nandi, R. K. Bhadra, T. Ramamurthy, S. K. Bhattacharya, M. Nishibuchi, T. Hamabata, S. Yamasaki, Y. Takeda, and G. B. Nair.** 1999. Clonal diversity among the recently emerged strains of *Vibrio parahaemolyticus* O3:K6 associated with pandemic spread. J. Clin. Microbiol. **37:**2354–2357.
2. **Chang, B., S. Yoshida, H. Miyamoto, M. Ogawa, K. Horikawa, M. Ogata, M. Nishibuchi, and H. Taniguchi.** 2000. A unique and common restriction fragment pattern of the nucleotide sequences homologous to the genome of Vf33, a filamentous bacteriophage, in pandemic strains of *Vibrio parahaemolyticus* O3:K6, O4:K68 and O1:K untypable. FEMS Microbiol. Lett. **192:**231–236.
3. **Chowdhury, N. R, S. Chakraborty, B. Eampokalap, W. Chaicumpa, M. Chongsa-Nguan. P. Moolasart, R. Mitra, T. Ramamurthy, S. K. Bhattacharya, M. Nishibuchi, Y. Takeda, and G. B. Nair.** 2000. Clonal dissemination of *Vibrio parahaemolyticus* displaying similar DNA fingerprint but belonging to two different serovars (O3:K6 and O4:K68) in Thailand and India. Epidemiol. Infect. **125:**17–25.
4. **Chowdhury, N. R., S. Chakraborty, T. Ramamurthy, M. Nishibuchi, S. Yamasaki, Y. Takeda, and G. B. Nair.** 2000. Molecular evidence of clonal *Vibrio parahaemolyticus* pandemic strains. Emerg. Infect. Dis. **6:**631–636.
5. **Faruque, S. M., S. K. Roy, A. R. M. A. Alim, and M. J. Albert.** 1995. Molecular epidemiology of toxigenic *Vibrio cholerae* in Bangladesh studied by numerical analysis of rRNA gene restriction patterns. J. Clin. Microbiol. **33:**2833–2838.
6. **Iida, T., A. Hattori, K. Tagomori, H. Nasu, R. Naim, and T. Honda.** 2001. Filamentous phage associated with recent pandemic strains of *Vibrio parahaemolyticus*. Emerg. Infect. Dis. **7:**477–478.
7. **Ishibashi, M., K. Ohta, T. Shimada, T. Honda, J. Sugiyama, T. Miwatani, and H. Yokoo.** 2000. Current status of OK serotype combinations of *Vibrio parahaemolyticus* Nippon Saikingaku Zasshi **55:**539–541. (In Japanese.).
8. **Kim, B. K., J. Okuda, C. Matusmoto, N. Takahashi, S. Hashimoto, and M. Nishibuchi.** 1999. Identification of *Vibrio parahemolyticus* strains at the species level by PCR targeted to the *toxR* gene. J. Clin. Microbiol. **37:**1173–1177.
9. **Matsumoto C., J. Okuda, M. Ishibashi, M. Iwanaga, P. Garg, T. Ramamurthy, H. C. Wong, A. Depaola, Y. B. Kim, M. J. Albert, and M. Nishibuchi.** 2000. Pandemic spread of an O3:K6 clone of *Vibrio parahaemolyticus* and emergence of related strains evidenced by arbitrarily primed PCR and *toxRS* sequence analyses. J. Clin. Microbiol. **38:**578–585.
10. **Monsur, K. A.** 1961. A highly selective gelatin-taurocholate-tellurite medium for the isolation of *Vibrio cholerae*. Trans. R. Soc. Trop. Med. Hyg. **55:**440–442.
11. **Nasu, H., T. Iida, T. Sugahara, Y. Yamaichi, K. S. Park, K. Yokoyama, K. Makino, H. Shinagawa, and T. Honda.** 2000. A filamentous phage associated with recent pandemic *Vibrio parahaemolyticus* O3:K6 strains. J. Clin. Microbiol. **38:**2156–2161.
12. **Okuda, J., M. Ishibashi, E. Hayakawa, T. Nishino, Y. Takeda, A. K. Mukhopadhyay, S. Garg, S. K. Bhattacharya, G. B. Nair, and M. Nishibuchi.** 1997. 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. J. Clin. Microbiol. **35:**3150–3155.
13. **Smith, J. M., E. J. Feil, and N. H. Smith.** 2000. Population structure and evolutionary dynamics of pathogenic bacteria. Bioessays **22:**1115–1122.
14. **Suthienkul, O., M. Ishibashi, T. Iida, N. Nettip, S. Supavej, B. Eampokalam, M. Makino, and T. Honda.** 1995. Urease production correlates with possession of the *trh* gene in *Vibrio parahaemolyticus* strains isolated in Thailand. J. Infect. Dis. **172:**1405–1408.
15. **World Health Organization.** 1999. *Vibrio parahaemolyticus*, Japan, 1996–1998. Wkly. Epidemiol. Rec. **74:**357–364.